JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

January 1967 volume 56, number 1

Review Article

Radiopharmaceuticals

By WALTER WOLF and MANUEL TUBIS*

TWO MAJOR developments occurred in 1940–1946 that revolutionized the methodology of research involving biological systems, by lowering the amounts of materials detectable to the microgram and submicrogram level. They were the chromatographic methods of separation and the availability of radioactive nuclides for the labeling and tracing of molecules and submolecular fragments. These tools were rapidly applied in all areas of biological research, including the diagnosis of disease. The use of radionuclides in the diagnosis and therapy of disease created the new specialty of nuclear medicine, and the specific drugs used in this specialty are called radiopharmaceuticals. The birth of nuclear medicine, and the concomitant development of radiopharmaceuticals, predate the creation in 1946 of the United States Atomic Energy Commission.

The first use of radioactive nuclides in man is to be credited to Blumgart and Yens (1), who in 1927 measured circulation (in man), after injection of a saline solution that had been exposed to radon, using a cloud chamber as the radiation detection device. The isotopes available at the time were only those belonging to the natural uranium, thorium, and actinium series, which limited their use in clinical work. The discovery, by Joliot and Curie (2), that other nuclides could be produced artificially, and the subsequent syntheses, by nuclear transmutation, of hundreds of artificial radioactive nuclides (3, 4), provided the tools for their biological and clinical use.

The use of ^{128}I by Hertz, Roberts, and Evans (5) in 1938, for the study of thyroid function, marks the real beginning of the systematic clinical use of radionuclides. Developments since then have been astronomical, and they have been thoroughly reviewed by Christian in 1961 in *J. Pharm. Sci.* (6) and by many others elsewhere since (7–51). Of special interest are the proceedings, published in April 1966, of the November 1965 Oak Ridge Conference on radiopharmaceuticals (30).

The development of a new science can usually be divided into several phases. First, there is discovery, and there are hopes that the possibilities of the new tool will be nearly unlimited. In the second phase, problems begin to be better appreciated and the initial enthusiasm is somewhat abated. And in a third phase, the new field achieves maturity, becomes fully aware of its potential and limitations, and achieves considerable sophistication.

Radiopharmaceuticals have entered this third phase. Thus, while originally tests were devised to make use of available radionuclides, now new

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This investigation was supported in part by grant GM-10721 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and grant GY-327 from the National Science Foundation, Washington, D. C.

The technical assistance of D. Fox, B. Goldstein, and C. Hays is gratefully acknowledged.

nuclides are being produced to meet the specific requirements of half-life, emission characteristics, and chemical properties tailored to a specific test. Pinajian has stated that most of the over 1000 nuclides known can now be prepared by reactor (52), cyclotron (53), or related processes (54). We will first discuss the radionuclides that have proven useful in nuclear medicine, and the radiopharmaceuticals that contain them.

Radioactive nuclides are measured by the radiation they emit, and considerable progress has been achieved in the instrumentation used in nuclear medicine. Solid-state circuitry has often decreased the size and weight of instruments to a fraction of the older tube models, and increased their accuracy and reliability. Special tools have been developed to measure specific radiopharmaceuticals in specific diagnostic tests. Thus, highlights of new instrumentation and their relevance to radiopharmaceuticals will be considered next. Following will be a discussion of the application of radiopharmaceuticals in clinical medicine, production, and use in ancillary areas.

If we define a pharmaceutical agent as a compound used in the diagnosis, mitigation, or therapy of a disease, then a number of chemical entities containing a radioactive nuclide meet these requirements. Such compounds are called "radiopharmaceuticals," and our present discussion is to be limited to recent advances in the nature, preparation, and detection of such labeled materials. We will thus not cover many other very closely related (and sometimes overlapping) areas, such as clinical use of radiation (55) (except when radiopharmaceuticals are used as therapeutic agents), activation analysis of biological materials (56–61), radiochemistry (62–65), radiobiology (66–69), Mossbauer effects (70–71), radiological health (72–75), and others (76–83).

While radiopharmaceuticals differ from other pharmaceuticals only in that they contain a radioactive nuclide, they have generally not been handled by pharmacists. This is due partly to historical reasons (radiopharmaceuticals are usually handled and dispensed by, or under the supervision of, the radiologist or the radioisotope department) and partly to lack of training. However, the number of schools of pharmacy that have radioisotope training in their curricula is increasing and it is hoped that a greater number of pharmacists will become more actively involved in dispensing radiopharmaceuticals (32) once they have adequate training.

Radiopharmaceuticals offer the pharmacist an unusual opportunity for returning to the type of work that was his own years ago, and for which he is still being trained—namely, that of preparing and compounding a pharmaceutical, not just repackaging a completely finished preparation. This is especially true if we consider that there are definite advantages in preparing radiopharmaceuticals at the place of use, particularly those containing many of the "short-lived" radionuclides (see below). The possibility that radiopharmaceuticals may be handled at the retail level (32) is remote, as labeled products cannot be properly transported, stored, and handled except in restricted areas.

DEFINITIONS, TERMS, AND NOTATIONS

While it is assumed that the reader has a working knowledge of radioactivity (62), certain terms and symbols pertinent to this field require some clarification. For more detailed glossaries and dictionaries, the reader is referred to *References 84–87.*

A *nuclide* is a unique atomic species, characterized by its atomic number Z, mass number A, and energy.

Nuclides can be stable or can suffer radioactive decay. Thus, hydrogen has a stable isotope, the nuclide ^{2}H (or D), deuterium, and a radioactive isotope, the nuclide tritium ^{3}H (T). As the term *isotope* has been used in a broad sense, and leads to some confusion when such terms as "isotopic purity," etc., are used, the more precise term nuclide is now being generally adopted.

Atomic species having the same atomic number but different mass numbers are *isotopes;* and atomic species having the same mass *and* atomic numbers, but differing in the energy content of the observable state, are called *isomers* (*e.g.*, ^{99m}Tc and ^{99}Tc, where m denotes metastable).

Radioactive decay is characterized by four parameters. (*a*) The half-life of the radioactive nuclide, *e.g.*, the time necessary for 50% of the material present to decay ($T_{1/2}$). (*b*) The nature of the radiation, *e.g.*, α, β^- (negatron), β^+ (positron), and γ radiation. Of interest also are such phenomena as EC (electron capture) and IT (isomeric transition). (*c*) The energy of the radiation emanating from the nuclide upon decay. (*d*) The nature of the nuclide remaining *after* the radioactive decay process (daughter element).

From a clinical point of view, it is desirable to use labeled molecules *in vivo* for only such time as is necessary and no longer, so as to minimize radiation exposure. For this reason, the terms "long-lived" and "short-lived" are very much used, but they have different meanings according to the interests of the user. Furthermore, in *in vivo* work, we do not have to consider only the physical half-life, T_p, but also the biological half-

life, T_b. This leads to the "effective" half-life T_e (19). We propose the following definitions of effective half-lives for radiopharmaceutical work.

"Very short-lived," T_e less than the duration of the clinical procedure.
"Short-lived," T_e equal to and up to 3 times the duration of the clinical procedure.
"Long-lived," T_e greater than 5 times the duration of the clinical procedure.

These definitions differ from those generally used chemically, where only the physical $T_{1/2}$ is relevant, and where nuclides of less than 2–3 months are considered "short-lived," and where "long-lived" applies to ^{14}C ($T_{1/2}$ = 5720 years) and similar nuclides (88).

Problems raised by the lack of precision in the terminology of radionuclides were carefully analyzed at the Oak Ridge Conference (30) especially by Cohen (89). Thus, while the International Union of Pure and Applied Chemistry adopted in 1952 and 1958 (90) the convention that the mass number is to be written as the *left superscript*, (*e.g.*, ^{14}C, *not* C^{14}), many authors still continue to use in 1967 the older (and incorrect) symbol. Much confusion also arises in the nomenclature of labeled molecules used as radiopharmaceuticals. Thus, U.L. stands for "uniformly labeled," meaning molecules usually obtained by a biological procedure where all the atoms of a given element are labeled with the same specific activity (88, 91). This is not to be confused with random labeling, *e.g.*, when a product is exposed to tritium by the Wilzbach technique (92–94), where large variations can occur in the specific activity of different chemical functions. Several lists of U. S. adopted names (USAN) have been published in the *Journal of the American Medical Association* (95).

Another source of confusion is the term "carrier free." A stable nuclide, *e.g.*, ^{127}I, is often added as a "carrier" to trace amounts of the radioactive nuclide ^{131}I. Sometimes this dilution is intentional, and sometimes it is inherent in the procedure of isolation and purification of the radionuclide (88, 96, 97). Thus, "carrier free" has been used alternatively to mean "no carrier added," and "no carrier present." To avoid confusion, when it is meant that no carrier has been added, it should be so stated (98). Furthermore, the nuclidic composition should be given. For a discussion of radiochemical, chemical, and radioisotopical purity, see under *Manufacture of Radiopharmaceuticals*. Another important designation is the *specific activity* of the radiopharmaceutical, defined as activity [in curies (c.) or fractions thereof] per unit weight or volume. Specific activities are often expressed as mc./m*M*, μc./mg., or μc./ml. A high turnover and a highly exchangeable biological pool of the material under study requires materials of high specific activity, especially in the newly developing field of radioimmunoassay (99, 100). The importance of a careful consideration of biochemical relationships, such as turnover, exchangeable pools, etc., has been carefully discussed by Bayly (101). The chemical stability or exchangeability of the radionuclide of the tagged radiopharmaceutical is also important to ensure the reliability of the chemical diagnosis (102, 103). Thus, many tagged products have a short shelf-life, and should be freshly analyzed before usage (104).

RECENTLY INTRODUCED RADIOISOTOPES AND THEIR COMPOUNDS FOR USE IN NUCLEAR MEDICINE AND PHARMACY

The use of radiopharmaceuticals in diagnostic nuclear medicine and therapy falls into several, not too rigidly defined, categories as shown in Table I. These illustrate the diversity of the uses and recent developments in radiopharmaceuticals.

Of the 102 elements and about 1,000 nuclides of these, only nine nuclides of six elements comprise 70% of the medical uses. These are ^{131}I, ^{51}Cr, ^{125}I, ^{99m}Tc, ^{132}I, ^{22}Na, ^{85}Kr, ^{197}Hg, and ^{203}Hg (31).

TABLE I.—SCOPE OF RADIOACTIVE TRACER METHODOLOGY IN BIOLOGY AND MEDICINE

Isotope Dilution	Diffusion and Flow	Metabolic Studies
Body compn., *e.g.*, total body K and H_2O.	Spatial distribution as in organ and whole body scanning.	Metabolism of amino acids *e.g.*, ^{75}Se-methionine.
Body spaces, *i.e.*, volumes of distribution, *e.g.*, chloride and hormone spaces.	Positive and negative concentration in organs and tissues by space-occupying structures.	Metabolism of carbohydrates, drugs, hormones, lipids, minerals, and proteins.
Body fluid volumes, *e.g.*, blood and plasma volumes.	Cardiac output.	
Exchangeable body elements, *e.g.*, Na and K.	Cell membrane absorption and permeability.	
	Regional circulation, *e.g.*, coronary, hepatic, and renal.	

A radiopharmaceutical or substance used in nuclear medicine is characterized by four major parameters: (*a*) its sojourn or time-course within the organ or lesion under study, relative to other structures as in tumor or organ scanning, (*b*) the type and rate of radioactive decay, (*c*) its detection characteristics, and (*d*) its production and preparation (31).

In view of the above parameters, it is readily understandable why the development of new radiopharmaceuticals has been in the direction of the use of compounds containing short-lived nuclides, preferentially those which produce only γ or positron radiation. These radionuclides provide radiation adequate for scanning with little or no useless and undesirable β radiation, and have only a short physical half-life, thereby yielding minimal total body or regional radiation.

Presently there are 32 γ or positron emitting radionuclides used in nuclear medicine, either as their inorganic salts, or as organic compounds. These nuclides vary in physical half-life from 1 hr. for ^{68}Ga to 10.3 years for ^{85}Kr (31). A major drawback in the development of nuclear medicine has been the limited ability to synthesize appropriate radiopharmaceuticals with definite biochemical behavior incorporating these short-lived radionuclides.

However, as an example of the successful preparation of such compounds, ^{99m}Tc with a physical half-life of 6 hr. and emitting only desirable γ rays for scanning, has been incorporated extemporaneously into several compounds with desirable biological behavior. These compounds are ^{99m}Tc-labeled human serum albumin as a sol (105) for placental scanning (106); as a macroaggregate of several particle sizes for distribution and scanning the liver and spleen and lung (107).

Several other ^{99m}Tc compounds have been prepared: colloidal ^{99m}Tc-sulfide has been used for liver scanning (108); ^{99m}Tc-thiocyanate dispersed in an injectable fat emulsion (109, 110) has also been used as a liver scanning agent. The pertechnetate, $^{99m}TcO_4^-$, has found wide acceptance as an agent for brain tumor scanning (110), for determining the trapping index of the thyroid (111), and for scanning the thyroid (112).

Technetium-99*m* is prepared as sodium pertechnetate by eluting or "milking" the generator or "cow" in which it is formed by the decay of ^{99}Mo which has a half-life of 2.8 days. Thus, the ^{99}Mo can be prepared at a reactor site or cyclotron, separated, and shipped as a "cow" and the ^{99m}Tc prepared extemporaneously.

Similarly, other generators are available for the production of other potentially desirable and useful short-lived radionuclides as radiopharmaceuticals. Six other generators are presently available—namely, ^{132}Te yielding ^{132}I (96); ^{87}Y yielding ^{87m}Sr (96); ^{137}Cs yielding ^{137}Ba (113); ^{68}Ge yielding ^{68}Ga (96); ^{113}Sn yielding ^{113m}In (54); and ^{103}Pd yielding ^{103m}Rh (54).

Iodine-132 is produced by decay of the parent ^{132}Te which has a $T_{1/2}$ of 3.2 days. Iodine-132, $T_{1/2}$ of 2.33 hr., has been used as the iodide for thyroid scanning and uptakes, especially in children and pregnant women in order to minimize the radiation dosage to the thyroid gland (114).

Other radioiodine isotopes have been proposed (115) for studies of thyroid physiology, measurement of uptake, and scanning based on short physical half-life and lack of undesirable radiation, but ^{131}I continues to be used most generally (114, 115).

In consideration of the parameters stated above for a radiopharmaceutical and the principle of minimum radiation dosage to the patient, the development of ^{57}Co-labeled cyanocobalamin (vitamin B_{12}) is exemplary.

Schilling (116) in 1953 devised a test for pernicious anemia using cyanocobalamin containing ^{60}Co. The liver is the repository of any unexcreted cyanocobalamin Co 60 and thus becomes the critical organ of exposure, especially since the biological half-life was shown to be from 315 to 450 days (117). In order to reduce the radiation received by the liver other radioisotopes of cobalt were sought which would reduce the radiation, and ^{57}Co appears best (118). By the substitution of ^{57}Co, the radiation dose to the liver is reduced to $^1/_{29}$th of that delivered by cyanocobalamin Co 60, a very desirable result.

In an attempt to reduce the radiation delivered to the kidneys during scanning of these organs (119) and also as a result of brain scanning using radioactive mercury-labeled chlormerodrin, ^{197}Hg has been substituted for ^{203}Hg in this compound (120). Since the $T_{1/2}$ of ^{197}Hg is 2.7 days *versus* a $T_{1/2}$ of 47 days for ^{203}Hg, the radiation dose is markedly reduced (28).

Recently, 1-mercuri-2-hyroxypropane labeled with ^{197}Hg has been proposed for scanning the spleen (121). This substance binds tightly to the red cells and damages them, so that now these damaged red cells are sequestered by the spleen, providing a tool for scanning that organ.

The spleen can also be scanned successfully by the use of red cells which were previously treated with sodium chromate Cr 51 and subsequently heat-damaged, resulting in their sequestration by that organ (122).

An example of the development of a new agent for scanning the pancreas for inflammation, car-

cinoma, and morphological abnormalities is that of ^{75}Se-L-methionine. This selenium analog of methionine is incorporated rapidly into pancreatic tissue with as much as 7% being incorporated in this organ and with a pancreas to liver ratio of concentration of 8:1 (123). Selenium-75 has a $T_{1/2}$ of 128 days and a principal γ ray energy of 0.27 Mev., quite suitable for scanning.

More recently ^{75}Se-L-methionine has been proposed as an agent for scanning the parathyroid gland (124). It was found that after injection, the activity in the parathyroids was 2 to 3 times higher than that found in thyroid, muscle, and blood. Adenomata were successfully located preoperatively, but this method awaits further clinical establishment.

In recent years attempts have been made to develop new radiopharmaceuticals for assessing the biochemical functions, or for delineating morphological structures, based on biochemical or physiological principles. The use of ^{131}I-labeled iodohippuric acid for kidney function testing and scanning was based on the well known conjugation and elimination of benzoic acid in the form of hippuric acid, and the established rapid renal tubular secretion of iodohippuric acid (125). Sodium Iodohippurate I 131[1] has become the agent of choice for renal function testing and has found wide use as an agent for measuring differential renal blood flow, coronary blood flow, and for many other dynamic fluid physiological studies. This compound is included in the U.S.P. XVII (126).

When no suitable radioisotope of an element is available, as is the case with chlorine, it has been possible to substitute one chemical element for an analog. This is exemplified by the use of ^{82}Br, as the bromide, instead of the chloride ion. Bromine-82 with a $T_{1/2}$ of 36 hr. has essentially the same body distribution as chloride and is used as an acceptable and reliable measure of the extracellular distribution of the latter (127).

Similarly, ^{86}Rb has been used to measure exchangeable potassium and suitable clinical methods have been developed (128).

In line with the thinking that minimal radiation to the patient is optimal, recent developments are the *in vitro* tests, as, for example, those used for thyroid function. In these tests, no radioiodine is administered to the patient; rather, the test is performed on the patient's serum, permitting even serial tests of children and pregnant females. These tests use L-iodothyronine I 131 and anion-exchange resins; more recently, the resin has been incorporated in a polyurethane sponge, thus simplifying the test and allowing greater reproducibility (129). Other techniques using anion-exchange resins to replace the red cells, as proposed by Hamolsky *et al.*, are also available (130).

A promising field of development likely to be expanded in the future is the preparation of chelates of many short-lived radionuclides. As an example, ^{197}Hg has been chelated with calcium and ethylenediaminetetraacetic acid for use in kidney scanning (131). It is selectively secreted by the renal cortex and the radiation dose to the patient is somewhat less than that from a much smaller dose of chlormerodrin Hg 203.

Heretofore, studies of magnesium, a biologically important element, have been hampered. Recently, however, ^{28}Mg with a half-life of 21.3 hr. became available with high specific activity. Extensive investigations of magnesium metabolism, such as exchangeable Mg in animals and in man, have been made possible (132, 133).

Recent advances in scanning techniques of great importance in nuclear medicine were coincident with the development of new radiopharmaceuticals and new forms of previously used radiopharmaceuticals. In "scanning," the delineation of the size, shape, location, or morphological characteristics of an organ or structure are indicated by a diagrammatic or photographic (light or dark) rendition of the localization of the radionuclide or its compound. It is obvious that radiopharmaceuticals, trophic to these structures by virtue of their biochemical or physiological characteristics, are desired.

Recent developments have occurred with the introduction of macroaggregates of human serum albumin containing radioiodine for scanning the liver, lung, and spleen (106, 107) and several compounds of ^{99m}Tc (108–111) for similar purposes; diuretics containing radioactive mercury for brain and kidney scanning; labeled amino acids for incorporation into organs rapidly synthesizing proteins, as for example, the use of ^{75}Se-L-methionine in pancreas scanning; and the labeling of erythrocytes with ^{51}Cr or 1-mercuri-2-hydroxypropane-^{203}Hg or MHP-^{197}Hg for spleen scanning (121).

The development of therapeutic radiopharmaceuticals is paralleling the development of diagnostic agents. As an example, ethiodized oil[2] I 131, which consists of the ethyl esters of the iodized fatty acids of poppy seed oil, has been prepared containing approximately 5 mc. ^{131}I

[1] Marketed as Hippuran I 131 by Volk Radiochemical Co.

[2] Marketed as Ethiodol by E. Fougera & Co., Inc., Hicksville, L. I., N. Y.

per ml. Following intralymphatic infiltration this material delivers therapeutic irradiation to pathological lymph nodes and can be used alone or in conjunction with irradiation for the destruction of malignant lymphatic tissue (134, 135).

Recent additions to the armamentarium of radioisotope therapy are the radioactive colloids. Yttrium-90, an almost pure β emitter with a half-life of 65 hr., is "milked" from a ^{90}Sr generator. The ^{90}Y is converted to the chloride which hydrolyzes at a pH of 7.0 to 7.8 and behaves like a colloid. It has been used to treat malignant serous effusions (136). Colloidal yttrium fluoride and hydroxide have been proposed also for use in yttrium therapy.

Recent investigations of the use of ^{131}I combined with colloidal size ion-exchange resins have been made for use in localized treatment of some types of cancer (136).

Radioisotopes for diagnostic and therapeutic uses have been prepared in a variety of dosage forms. (See Tables II and III.)

Capsules containing radioisotopes for diagnostic and therapeutic purposes were designed as a safe and effective method of administration (137) and are presently widely used for several labeled materials.

Radioiodinated human serum albumin is available in standardized doses in plastic disposable syringes from several suppliers. These can be used in conjunction with digital computing equipment for rapid blood volume determinations based on the principle of isotope dilution (138).

Most radioactive chemicals for use in nuclear medicine have been chosen on the basis of the characteristics of the radioisotope contained or its chemical or physical form, rather than its biological behavior or sojourn.

Only four commonly used radiopharmaceuticals can be classed as "biochemicals" and are used on the basis of their biochemical behavior (101). They are: labeled cyanocobalamin, thyroxine, triiodothyronine, and ^{75}Se-labeled selenomethionine.

According to the authors, a similar justifiable inclusion would be the labeled ions of iodide, phosphate, calcium, and those of some trace metals.

Obviously, there is need for the development of radiopharmaceuticals resembling natural biochemicals, which could be incorporated in tissues and biological structures. Such radiopharmaceuticals should have desirable characteristics for their detection in nuclear medical procedures. With the development of newer and simpler techniques for assaying ^{14}C and ^{3}H compounds, labeled analogs of natural biochemicals will find ever-increasing use in nuclear medicine.

METHODS OF DETECTION

When radionuclides decay they emit one or more types of radiation: α (helium nuclei), β^- (negatrons), β^+ (positrons), γ or X-rays. These radiations are able to ionize matter and produce ion-pairs. Thus, one method of detection of radioactive decay is based on the collection and measurement of the ions produced by the ionizing radiation. Such an instrument is the Geiger-Mueller (G.M.) tube. A second method of detection is based on the fact that certain molecules will absorb energy and not become ionized, but instead undergo a transition to an excited state. The decay of that excited molecule to the ground state is accompanied by the emission of electromagnetic energy, usually in the ultraviolet or visible range of the spectrum. This light pulse is converted by photomultipliers to an electrical pulse which is then measured. These techniques are called scintillation detection methods, and are the most widely used in the measurement of radionuclides of clinical interest. New methods of detection using semiconductor or solid-state detectors are being developed (139), but they are not yet used clinically to any great extent.

A fourth method of detection is based on chemical reactivity, with, for example, silver halides in photographic emulsion. Photographic methods of detection such as autoradiography are extensively used in pharmacology.

For a more detailed discussion of detectors, the reader is referred to Price (139). Recent progress in solid-state detection, which may become very useful for measurement of low energy γ emitters such as ^{125}I and ^{197}Hg, has recently been reviewed (140, 141).

Perhaps the most important development in the last 10 years has been the introduction of solid state electronics. Thus, large and bulky tube models have been replaced by more efficient, reliable, and compact solid-state electronic systems. For a full discussion of these topics, see Yanof (142) and Malmstadt (143).

Most radionuclides of biological interest are either β^-, γ, or β^+ emitters; practically no radionuclide of clinical interest is an α emitter. β^- Radiation is very easily absorbed by tissue, and does not lend itself easily to dynamic clinical studies of deep-seated organs. Best suited are radionuclides emitting γ radiation, either by direct decay, as a result of β^+ annihilation (the $T_{1/2}$ of the positron is 1.5×10^{-10} sec.) or X-rays emitted after electron capture. γ Radiation is

best detected by scintillation type detectors, and most instrumentation used in clinical work utilizes them.

The radiopharmaceuticals most widely used are discussed below. They are used in the visualization of organs for space-occupying defects, to assess their function, and to determine dynamic changes in body constituents.

Scanning was first utilized to visualize the thyroid gland (144) and has since developed into a most important procedure of nuclear medicine. Several comprehensive reviews cover the subject (145–147).

When a labeled compound localizes preferentially in an organ, *e.g.*, I^- in the thyroid gland, the intensity of the radiation emanating from each part of the organ under study will be proportional to its physiological state. Thus, a nonfunctional nodule will appear to concentrate less radioactivity, whereas a hyperfunctional area will concentrate a larger amount of the radiopharmaceutical.

A scanner is a scintillation detector, capable of moving mechanically over the area to be studied. A scanning system is composed of: (*a*) a detector, (*b*) a collimator and shield, (*c*) a mechanical movement device, (*d*) data presentation and display.

The original thyroid scanning detector used a $^1/_4$-in. crystal (144); the efficiency of such a system was low, and presently crystals of 2 × 2 in. and 3 × 2 in. are used routinely, with an increase in efficiency of over 100-fold. Larger detectors need larger shielding and efficient collimation, and much effort has been devoted to improving the quality of focusing collimators (148). Furthermore, the increase in shielding and in collimation increases the weight of the scanning device, creating severe mechanical problems. Further details on scanning devices can be obtained from several major manufacturers of such equipment.

A major effort has been devoted to improvement of data presentation. The original dot scan, giving series of black dots on white paper, has been considerably improved for greater ease of interpretation of the results obtained. Thus, Kakehi *et al.* (149) developed a device whereby the impulses from the spectrometer were fed into a strobo-tube, with different colors representing increasing intensities. A similar device is the photorecorder of Adams and Jaffe (150).

While scanning devices are still extremely important, they have several disadvantages, such as the long time needed to complete a scan and the high dose of radionuclide needed. A new approach is that of the scintillation cameras, first developed by Anger (151). The large crystal detector views the whole organ, and the scintillations are transmitted to a battery of small phototubes. The whole organ is thus seen in its entirety, reducing the time necessary for a single scan, allowing a dynamic observation of the organ, the use of nuclides of shorter half-life, etc. (152).

Several models of such cameras have been developed. The original Anger camera (151) consists of a single large crystal. A modification of the scintillation camera is the Autofluoroscope of Bender and Blau (153), consisting of a mosaic of several hundred scintillators. A third instrument has been described by Ter-Pogossian (154) which consists of a fluorescent screen and a specially designed image amplifier tube.

More recently, much interest has been raised by spark-chamber types of devices, such as the Spintharicon (155), the spark-chamber of Kellershohn (156) and the cross-wire spark-chamber (157), usable for both β and γ counting.

Decay by positron emission is followed in 1.5×10^{-10} sec. by positron annihilation, producing 2 photons of 0.51 Mev. each, that are emitted at an angle of 180°. The use of two scanners, 180° apart, or of two scintillation cameras, also 180° from each other, has been used for brain tumor localization (158), although conventional γ scanning appears to be equally effective.

Certain nuclides, such as ^{40}K, are distributed throughout the body. Their level can best be measured by whole body counters. These can be either of the NaI type, with one or more detectors (159–161), or of the liquid scintillation type first described by Anderson *et al.* (162). More recently, a system has been described with 18 organic plastic detectors employing 72 photomultiplier tubes, able to determine whole body counts as well as localization in organs (163).

Thus, a highly sophisticated array of instrumentation exists and is being developed for the measurement of radionuclides *in vivo*. As will be seen below, the next great advance in this field will come by the use of other radionuclides, which according to Stang can be "tailored to will" (164), and by a thorough understanding and application of biochemical and pharmacological principles applied to radiopharmaceutical drug design (31, 101).

Also, the development of clinical tests using ^{14}C and ^{3}H-labeled compounds has been made possible by improvements in counting techniques such as the liquid scintillation counters (139, 165, 166).

CLINICAL APPLICATIONS

The clinical applications of radiopharmaceuticals are divided into two main categories: (*a*) those used in diagnostic procedures and (*b*) those used for therapy. An extensive survey of these uses is beyond the scope of this review and the reader is therefore referred to some very excellent recent source material (14, 29, 31, 38, 167–169). However, the general lines of research and development will be presented here.

The improvement and simplification of counting techniques and equipment for weak β emitters has resulted in the use of ^{14}C and ^{3}H-labeled compounds in medical and biomedical techniques. Tritiated water is now widely used in the determination of total body water (170). Uniformly labeled ^{14}C-glucose has been used to study glucose kinetics in normal, diabetic, and acromegalic subjects (171). In this study the quantities of expired CO_2 and $^{14}CO_2$ in the mixture were monitored (172) and deviations from the normal range for the diabetic and the acromegalic subjects were observed.

Studies of fat absorption, using ^{131}I-labeled triolein, are open to serious question because the iodinated triolein is not a single compound, and frequently contains impurities. These have also been labeled, but they do not behave biologically like triolein (173). It is absolutely necessary to separate the labeled triglyceride from the other materials using techniques such as thin-layer chromatography and gas–liquid chromatography, both in their analytical and preparative aspects. It is incumbent upon the investigator to prove the purity of his materials so as to eliminate any uncertainty in the clinical test. Some investigators using the commercially available ^{131}I-labeled triolein in an empirical manner have obtained consistent results suitable for diagnostic purposes.

It has been argued that an iodinated, or an iodinated and chlorinated, fat or fatty acid no longer behaves biologically like the original fat or fatty acid, and only ^{14}C-labeled lipids prepared by biosynthetic methods truly resemble the unlabeled fat. This subject was recently reviewed (174).

Tables II and III illustrate the diversity of composition of some radiopharmaceuticals used in nuclear medicine. The doses given are variable, depend on the equipment and technique used, and are thus only approximate. The references

TABLE II.—DIAGNOSTIC USES OF RADIONUCLIDES

Radiopharmaceutical[a]	Diagnostic Use	Dose, μc.	Ref.
Sodium Iodide I 131	Thyroid uptake and excretion studies	1–20	(114)
	scanning thyroid gland	50–300	(175)
Liothyronine I 131 or I 125 (L-Triiodothyronine I 131 or I 125)	*In vitro* studies of thyroid function (resin and red cell uptake tests)	1 or less	(129, 176)
Iodinated Human Serum Albumin I 131 or I 125	Blood and plasma volumes	3–20	(177)
Macroaggregate Iodinated Human Serum Albumin I 131 or I 125	Lung, liver, and spleen scanning	200–300	(178)
Sodium Iodohippurate I 131 or I 125	Kidney function	10–50	(125, 179)
Rose Bengal I 131 or I 125	Liver function	10–25	(180)
	Liver scanning	100–150	(181)
Oleic Acid I 131	Fat absorption studies	25–50	(182)
Triolein I 131	Fat absorption studies	25–50	(183)
Tritiated Water	Total body water	500–2000	(170)
Sodium Phosphate P 32	Detection of eye tumors	250–500	(184)
Calcium Chloride Ca 47 or Ca 45	Distribution and metabolism	1 μc./Kg.	(185)
Sodium Chromate Cr 51	Red cell mass and volume	10–25	(186)
	Red cell survival studies and spleen scanning	75–150	(187)
		150–300	(122)
Cyanocobalamin Co 57 Cyanocobalamin Co 58 Cyanocobalamin Co 60	Pernicious anemia and malabsorption syndromes	0.25–1	(188)
Gold Au 198	Liver tumor localization	300	(189)
Ferrous Citrate Fe 59	Oral iron absorption	5–10	(190)
	Plasma iron turnover and disappearance	5–10	(191)
	Iron utilization and turnover	5–10	(192)
^{99m}Tc Sodium Pertechnetate	Brain tumor scanning	10,000	(110)
	Thyroid uptake	1000	(111)
^{99m}Tc-Labeled Human Serum Albumin	Placental scanning	1000	(106)
^{99m}Tc-Technetium Sulfide	Scanning liver and spleen	2000	(108)
Macroaggregates of ^{99m}Tc-Labeled Human Serum Albumin	Scanning liver and spleen	3000	(193)
	and lung	1500	(194)

[a] USAN names used where applicable.

TABLE III.—THERAPEUTIC USES OF RADIONUCLIDES

Radiopharmaceutical[a]	Therapeutic Use	Dose, mc.	Ref.
Sodium Iodide I 131	Hyperthyroidism	2–10	(195)
	Cardiac and pulmonary disease	25–50 divided doses	(196)
	Thyroid cancer	100–150	(197)
Sodium Phosphate P 32	Polycythemia vera	3–8	(198)
	Chronic leukemia (lymphatic or myeloid)	1–2 per week	(199)
	Metastatic bone cancer	10–15 divided doses	(200)
Chromic Phosphate P 32	Treatment of peritoneal effusions	10–15	(201)
	Treatment of pleural effusions	8–12	(201)
Gold Au 198	Treatment of pleural effusions	75–200	(202)
Gold Au 198 seeds or wires	Interstitial implantation into tumors	Variable	(203)
Cobalt 60 needles, seeds or wires	Interstitial implantation into tumors	Variable	(204)
	Treatment of tumors in body cavities	50–75	(204)
Strontium 90 applicators	Treatment of ophthalmological lesions	Variable	(205)
Iridium 192 seeds	Interstitial tumor irradiation	Variable	(206)
Tantalum 182 needles	Bladder tumors	Variable	(207)
Ethiodized Oil I 131	Intralymphatic radioisotope therapy		(134)

[a] USAN names.

are representative and not exhaustive. Names follow the USAN nomenclature (95) where available; otherwise common names are used. They are arranged very generally in the order of frequency of use. The reader is also referred to a more extensive table compiled by Silver (169).

MANUFACTURE OF RADIOPHARMACEUTICALS

Radioisotopes used in the preparation of radiopharmaceuticals and labeled compounds are prepared by prime producers who use reactors or cyclotrons. The products are then processed, purified, prepared in pharmaceutically acceptable forms, and assayed. They may be purified to meet the official requirements of the U.S.P. XVII (126) if standards have been established for such materials. These standards are minimal and many manufacturers have set their own more rigid specifications for identity and purity.

The provisions of the Federal Food, Drug and Cosmetic Act of 1938, as amended, apply equally to radiopharmaceuticals as to all other kinds of drugs (208).

Standards of purity, for example, as applied to usual pharmaceuticals are wholly inadequate with reference to radiopharmaceuticals. Some consideration of the necessary criteria for radiopharmaceuticals and their significance will be discussed here. *Chemical purity* is used in the customary sense to denote the presence or absence of chemical substances other than the desired single or multiple species. *Radiochemical purity* might be defined as the fraction of the stated radionuclide in the stated chemical form, as for example, sodium *o*-radioiodohippurate I 131 should not contain I 131 in the form of radioiodide or *o*-radioiodobenzoate. *Radionuclidic purity* refers to the proportion of the total activity in terms of the designated radionuclide. For example, chlormerodrin Hg 197 ($T_{1/2}$ = 65 hr.) prepared from irradiated naturally occurring mercury might contain ^{203}Hg amounting to 4% at the time of receipt. This amount of long-lived ^{203}Hg ($T_{1/2}$ = 46.9 days) is acceptable for immediate use, though undesirable. However, this level will have risen to 21% of the total activity at the end of 1 week and this is not acceptable. The use of enriched ^{196}Hg for the preparation of the ^{197}Hg used in formulating radiomercury diagnostic agents is an important development (209). The use of ^{197}Hg with its $T_{1/2}$ of 65 hr. is desirable for reducing the radiation delivered to the kidneys.

In addition to the criteria of purity, injectable solutions must meet the requirements of sterility, nonpyrogenicity, and all the other standards for parenteral products. While these are the responsibility of the original manufacturer, they apply equally to extemporaneous dilutions and subdoses as prepared in the hospital or nuclear medical clinic. Terminal sterilization in the final container or sterilization by filtration are most acceptable for radiopharmaceutical preparations.

Radiopharmaceuticals suffer radiolytic disruption and change, especially during storage, due to the radiant energy contained. Therefore, the nature and content of the products must be known. Inasmuch as the biological behavior of the split products may be entirely different from that of the desired material, clinical or scientific deductions would be erroneous. Radiopharmaceutical manufacturers therefore subject their products to analysis at the time of preparation, and after appropriate storage, to determine

"shelf-life" (210). Methods employed are column and paper chromatography and autoradiography, electrophoresis, γ spectrometry for radionuclidic purity and contamination, spectrography, chemical separations by the use of carriers, and others (89). Instability associated with radiolytic breakdown is dependent upon the energy of decay of the radionuclide, the specific activity, and the usual physical factors of light, temperature, solvent, oxygen, etc.

The reader is referred to an excellent review of quality control concerning the preparation, formulation, sterility procedures, and all other operations involved in production of radiopharmaceuticals and extemporaneous dilutions for a moderately large installation (104). Many of the procedures are equally applicable to small hospital and nuclear medical clinics (211).

In the manufacture of radiopharmaceuticals, antioxidants, buffers, antibacterial preservatives, stabilizers, and solvents are all required. The composition and reactivity of these materials are of prime importance.

The maintenance of sterility in radiopharmaceutical solutions is achieved by the addition of antimicrobial agents such as phenol, the methyl and propyl esters of *p*-hydroxybenzoic acid, and, most frequently benzyl alcohol. The choice of the bacteriostat is of utmost importance. It has been reported that benzyl alcohol, used in high activity solutions of radioiodide, reacted with the products of the radiolysis of water to form iodinated products, which showed a lower thyroid uptake and urinary excretion in rats after 4 hr., compared to a solution not so preserved (212). However, similar studies in humans have not been reported.

β-Naphthol used as a bacteriostatic agent in gelatin capsules for ^{131}I led to an iodinated β-naphthol as a radiochemical impurity, which made this pharmaceutical unsuitable for determining the function of the thyroid gland (89).

Antioxidants such as ascorbic acid, sodium ascorbate, cysteine hydrochloride, sodium sulfite, and thiosulfate are used to maintain radionuclides in the reduced form and at the proper valence state. Examples are ferrous citrate Fe 59 and sodium iodide I 131. In the case of the latter, solutions not properly protected against oxidation led to erroneous thyroid uptakes (213).

Buffers and stabilizers are added to radiopharmaceuticals to prevent the precipitation and reactions of the pharmaceutical with contaminants in the solution and from the glass containers. Sodium citrate has been used by the manufacturers to prevent calcium precipitation in some solutions. Great care must be exercised in the choice of additives to radiopharmaceuticals, so as to prevent precipitation and loss or removal of the minute quantity of the radionuclide present, or of inducing chemical changes in the nature of the tagged material.

Sterility is of prime importance in injectable radiopharmaceuticals. The problem is compounded by the short half-life of the radionuclide and the time required for an adequate insurance of sterility and nonpyrogenicity. The U.S.P. XVII (126) prescribes the methods to be used. The manufacturers have been permitted to release parenteral radiopharmaceuticals before the completion of the usual sterility test. Extensive testing, as well as the use of additional techniques, and a backlog of experience in the preparation of injectables, have insured an excellent record for the manufacturers. Many lots of radiopharmaceuticals, where the half-life and other factors do not preclude the preparation in advance of the required demand, allow for adequate sterility and pyrogenicity testing. The experience of one manufacturer, which is typical, is of interest (214).

The large-scale producers and manufacturers have been quick to translate the research findings of the radiopharmaceutical chemists and clinicians into practical production. Although they have not themselves developed new radiopharmaceuticals, they have made an enormous contribution in furthering the development, assisting research, providing materials for clinical trials, and conducting research on product improvement, dosage forms, and availability. The radiopharmaceutical manufacturers have offered "custom" services in the preparation of new materials such as iodination and tritiation as well as the preparation of specifically labeled compounds and intermediates and providing commercially unavailable radionuclides. Product improvement has taken the form of providing radiopharmaceuticals which yield less radiation such as in the substitution of the radionuclides ^{57}Co or ^{58}Co in place of ^{60}Co in preparations of radiocyanocobalamin, and of ^{197}Hg in place of ^{203}Hg in chlormerodrin.

The record of the manufacturers in making ^{99m}Tc available as "cows" and as rapidly delivered sterile solutions is impressive. Much credit is to be given to Brookhaven National Laboratories for developing and making available "cows" or generators for short-lived radionuclides before they were available from other sources (96).

The presentation of dosage forms of radiopharmaceuticals by the manufacturers has facilitated their use. Capsules, predated to contain definite doses at some specified time, single

and multiple dose vials, and unitary packages of sterile precalibrated doses in disposable syringes, are some outstanding examples.

Research and development has resulted in safe and convenient containers designed to minimize the radiation hazard to those engaged in handling and dispensing them. One manufacturer ships the products in outer containers made of polyethylene and lead which prevents shattering if dropped and also provides for complete drainage while held in such a container. Another major manufacturer provides bottles coated with a thick plastic covering to prevent shattering if the bottle is dropped or subjected to stress. Ease of opening both inner and outer containers has been provided by the manufacturers. Containers are adequately shielded for shipment and storage (167).

In 1946, 38 licenses were issued by the U.S.A.E.C. to medical institutions and hospitals to use radioisotopes and in 1955 this grew to 1149, while in September 1965, the number issued to medical institutions and physicians increased to 3600, in addition to about 1900 government laboratories and 500 colleges and universities, many of which were using radiopharmaceuticals and labeled compounds (215). From these figures the growth of the radiopharmaceutical industry is readily understandable. One large company has recently opened a new plant reputed to double its output of radiopharmaceuticals. It has been reported that the radiopharmaceutical industry has grown about 20% per year (216) and current sales are about $8 million per year (217).

There are several large American pharmaceutical companies and a number of smaller ones providing a wide range of radiopharmaceuticals, radionuclides of varying quality, and labeled compounds as well as special services. Much desirable reference material is available without charge from these companies, and the reader is urged to consult these sources.

Attempts have been made to prepare labeled drugs by exposure to a neutron flux, so as to induce the formation of radionuclides from some element present. As an example, probenecid [*p*-(dipropylsulfamyl)benzoic acid] was irradiated with fast and slow neutrons giving rise to ^{35}S and ^{32}P, both β emitters (218). There was much decomposition but pure material was isolated with a specific activity of about 12 μc./m*M* as ^{35}S. A trace of ^{32}P was formed and not removable. The distribution was studied in rats and found to accumulate mostly in the kidney. 5-Acetamido-1,3,4-thiadiazole-2-sulfonamide[3] was also irradiated by these same workers and a very pure product was obtained in a lower yield containing ^{35}S.

In the U. S. another group has reported the results of preliminary investigations of neutron irradiation of many compounds such as ethanol, nicotinic, phenylacetic, and salicylic acids, glucose, reserpine, and tetracycline. The instrumentation and techniques as well as the advantages and disadvantages are described (219). In these techniques radioisotopic yields are small and extensive purification of the products is required.

However, while recoil labeling has considerable limitations, it may be a very useful tool for the tagging of radiopharmaceuticals with radionuclides of very short half-life, such as ^{11}C ($T_{1/2}$ = 20.3 min.), ^{18}F ($T_{1/2}$ = 1.7 hr.) and others (220). As an example of at the site production of radiopharmaceuticals we can cite the work of Matthews *et al.* (221) on radioactive gases, used for respiration and lung function. They used ^{15}O ($T_{1/2}$ = 2 min.), ^{13}N($T_{1/2}$ = 10 min.), and ^{11}C.

RADIOPHARMACEUTICALS AND PHARMACOLOGY

"The use of isotopes in experimental pharmacology has facilitated investigations that without their use would have been cumbersome or difficult, and in other cases impossible to solve," states Roth (222). The pharmacologist has thus been able to make good use of the extraordinary tool provided by labeled compounds (drugs). It might also be safe to say that practically all new pharmaceuticals that are being studied today are labeled at some stage of preclinical testing, to study their biotransformation and other parameters of biological significance. A recent symposium has been devoted to Isotopes in Experimental Pharmacology (223), and this specific topic was reviewed in *J. Pharm. Sci.* a few years ago (224) as well as elsewhere (225–228).

Perhaps the greatest use of labeled compounds is in the study of drug metabolism and biotransformation. The information that can thus be obtained is: (*a*) rate and route of elimination; (*b*) biotransformation: number, nature, and rate of elimination of metabolites; (*c*) localization and penetration of a drug and its metabolites in organs and on specific subcellular structures; (*d*) identification of any "active" metabolite, and evaluation of its pharmacological action.

It is impossible to list here all metabolic studies of drugs elucidated with the aid of radionuclides; the following examples will serve to illustrate the potentials of such techniques.

[3] Marketed as Diamox by Lederle Laboratories, Pearl River, N. Y.

Phenobarbital is excreted either unchanged or as conjugated derivatives, as established after a study of the fate of 2-^{14}C-phenobarbital. When, however, it was suggested that the opening of the pyrimidine ring also occurred, labeling in the 5 position (5-^{14}C-phenobarbital) gave products identical to those obtained from the 2-^{14}C radioisomer, precluding any significant contribution of other metabolic routes (229). Thus, not only should that position be labeled that is easiest from the synthetic point of view, but so should any others that will provide significant information.

An elegant technique has been developed by Lissitzky *et al.* (230) for the study of thyroid function and the action of several pharmacological agents on thyroid processes. Pregnant rats were fed on a ^{127}I free diet, (*e.g.*, Remington), and given ^{125}I as the single iodine source. After birth, the young rats were kept on this same diet, so that their thyroid had only ^{125}I. When dynamic studies were conducted, ^{131}I was used, allowing a clearer understanding of the pharmacological action of goitrogenic agents. Such studies illustrate the potential of the simultaneous use of several isotopes of the same element (115).

Another aspect is illustrated by the work of Buyske and co-workers (231) on the agents affecting the activity of tetracycline. Tritium-labeled tetracycline was used, by an isotope dilution technique, for the determination of tetracycline levels in blood. Other labeled materials could be rapidly screened as synergistic agents, and this led to an understanding of the interaction of calcium salts with tetracycline.

Body distribution of drugs is of the utmost importance. Several techniques have been developed recently, based on autoradiographic methods, for determining drug distribution and localization. They involved administration of the labeled drug to an animal, which is then sacrificed by freezing at $-80°$, and sections are prepared at $-10°$ (232) or at $-60°$ (233) followed by autoradiography of the dried sections. The results of such methods of cellular localization are only now beginning to emerge, and will certainly constitute an important tool in the determination of the mechanisms of drug action.

Roth *et al.* (234) have studied the entry of drugs into the brain in an attempt to understand the factors that influence and control the barrier function that restricts the entry of substances into the central nervous system. Among the drugs studied were acetazolamide, thiopental, diphenylhydantoin, and pyridine 2-aldoxime methiodide (2-PAM). These authors observed that the blood-brain barrier could be altered by the percentage of CO_2 in plasma, convulsions, and sensory stimulation, thus suggesting that proper manipulation of such factors may allow a directive effect in brain localization.

An intriguing tool is that of the deuterium isotope effect. Deuterium is an isotope of hydrogen with a mass of 2. Differences in reaction rates have been observed between H- and D-labeled compounds. When there is a transfer of the isotopic atom from the reactant to an acceptor, a primary isotope effect ($k_H/k_D = 1.5$ to 10.0) can be observed. A further isotope effect (secondary) is observed when no transfer occurs, but the isotope is strongly involved in the interaction with the active site of the enzyme ($k_H/k_D = 1.05$ to 1.30). The significance of such isotope effects has been studied by several authors. Thus, the flavoprotein nature of the monoamine oxidase system was demonstrated by Belleau (235), while the nature of the interaction between morphine and its receptor site was studied with the aid of *N*-deuteromethyl morphine by Elison *et al.* (236).

A variety of methods have been developed specifically for the synthesis of labeled radiopharmaceuticals. Stepka and Larson (237) isolated nicotine, digitoxin, and ephedrine, among others, from higher plants grown in an atmosphere enriched with $^{14}CO_2$. Chemical syntheses have been widely reviewed, and the reader is especially referred to excellent papers by Rothchild (238) and Bayly (101, 239) as well as to reviews previously mentioned (88, 91, 102, 103).

While both pharmacologists and biochemists have been making excellent use of radionuclides in the understanding of physiological, pharmacological, and pathological processes, the same cannot be said for those in nuclear medicine. As Bayly points out (101), only a few radiopharmaceuticals currently in use as diagnostic agents are true "biochemicals," while all others are either inorganic salts or tagged polymers. The need for designing new radiopharmaceuticals, based on the principles so successfully applied to experimental pharmacology and biochemistry, is great, and Wagner (240) has analyzed the characteristics of an ideal radiopharmaceutical, which should guide research in the development of new agents of this type.

USE OF RADIONUCLIDES AND RADIATION PROCEDURES IN THE PHARMACEUTICAL INDUSTRY

Since the last excellent review of the applications of radionuclides and radiation in the pharmaceutical industry (241) only a limited number

of reports have appeared concerning application of radioisotope techniques to the pharmaceutical industry.

This review will present and correlate these reports and will show some diversified applications. It is sincerely hoped that pharmaceutical scientists will acquire proficiency in the techniques of radioisotope methodology and adapt this unique tool to the pharmaceutical industry.

The system of quality control, pyrogen testing, and sterility control in operation in a large clinical center laboratory for the preparation of radiopharmaceuticals is well described by Briner (104, 211). The recommendations he makes are equally applicable to smaller nuclear-medical and radiopharmaceutical installations.

A patent was issued for a capsule for radioactive pharmaceuticals consisting of a nontoxic water-soluble material and containing a solid material that melts at body temperature. The material is compounded from a pharmaceutically acceptable oil and contains the radioactive product (242). The thickness of the wall of hard gelatin capsules is determined by measuring through the capsule wall the attenuation of the β radiation of a ^{36}Cl source on the tip of the steel dipping pin. A change of 0.0001 in. in thickness was detectable by a change in the counting rate (243).

The moisture content of rubber stoppers and aluminum ferrules used for packaging biological products was determined by measuring retained tritiated water (244).

The determination of moisture content of calcium carbonate, which should be equally applicable to other pharmaceutical materials, was determined by measuring the transmission of β particles from a ^{90}Sr source (245).

A radioisotopic method for evaluating dispersed systems such as liquid petrolatum emulsions for uniformity has been developed (246). The radioisotopic method was much faster for determining the phase uniformity of emulsions and for expressing ratios of creaming, than any other method used.

The determination of rates of settling of suspensions was determined by measuring the characteristic irradiation from a sealed β source. The source was immersed and the attentuation of the beam which passed through a vertical column of the suspension was measured. This method was suggested for determining the sedimentation rate of pharmaceutical suspensions containing elements of high atomic number (247).

Several contributions have referred to the use of activation analysis for the quantitation of trace quantities of metals and other materials used in pharmaceuticals (56–61).

The status of radiation sterilization of pharmaceuticals and medical supplies has not altered significantly since the last general reviews were presented in 1960 (7, 241).

These processes of sterilization are based on the lethal effects of high energy ionizing radiation on living organisms. The radiation may be very high energy electron beams produced in a linear accelerator, or γ radiation produced by a large ^{60}Co source.

The advantages of this type of sterilization are: (*a*) the product may be prepackaged in nonsterile materials in a nonsterile atmosphere and then the product and packaging sterilized; (*b*) it permits greater latitude in the choice of packaging materials; (*c*) bulk materials and products difficult to sterilize by either moist or dry heat can be sterilized; and (*d*) the processes can be made continuous, requiring only small numbers of personnel.

Certain disadvantages are: (*a*) the cost of sterilization is higher than that of heat sterilization and the initial cost of the installation is very high, although this might be offset somewhat by the savings of the cost of operation; (*b*) many materials are damaged by the radiation, suffering changes in color, decomposition, loss of potency, and in some cases, complete impairment of their therapeutic properties.

However, research in the methodology continues, and the following reports have appeared in the recent literature.

Antibiotics and hormones were irradiated in sealed containers by exposure to 10^6 reps without physical damage or loss of biological activity (248). Toxins and enzymes were sterilized with 5 million reps, based on a lethal dose for bacteria of 1 to 4 million reps (248). The rep, now an obsolete unit of absorbed ionizing radiation, was defined as an absorption of 93 ergs of radiation per gram of matter. It has been replaced by the rad, which is equivalent to the absorption of 100 ergs of radiation per gram of matter.

It is, however, generally believed that the lethal dose for bacteria varies from 0.1 to 2.5 megarads (1 megarad = 1 million rads) (249), and the latter figure is presently generally accepted as being safe. In Poland, drugs in ampuls were sterilized by exposure to rays from a ^{60}Co source. The enzyme cocarboxylase hydrochloride in dry form, contaminated with spores of *C. welchii*, *C. septicum*, and *E. coli* was sterilized with 4×10^5 reps from ^{60}Co. The material was sterile and showed no signs of decomposition. In aqueous solutions nonsporulating *E. coli* required 8×10^5

reps, while *C. welchii* and *C. septicum* required 1.6×10^6 reps (250).

Ophthalmic ointments containing oxytetracycline, chlortetracycline, tetracycline, and polymyxin in petrolatum and lanolin were sterilized and could be treated so in aluminum tubes and packaged. The dose was 2 million rads (2 Mrad) from a ^{60}Co source (251). The pH decreased slightly but the chemical composition and biological activity were unchanged, and the ointment was stable at room temperature for 14 months.

Ophthalmic ointments containing penicillin and fluorescein were sterilized by a dose of 2.5 Mrad from a γ ray source in their final containers and packages (252).

Antibiotics, anticoagulants, hormones, plasma, multivitamin preparations, and steroids were sterilized with 2 Mrads. The importance of preconditioning the containers was emphasized (253).

A report of 7 years' experience with radiation sterilization was presented (254). The types of products, the materials used, as well as certain aspects of processing and control and a comparison of irradiators, were given.

Recent developments in Sweden were reported (255). The following radiation sources have been used: β radiation, γ radiation from ^{60}Co and from spent fuel elements from reactors, megavoltage from Van De Graaff generators, and from linear accelators. The dose of 2.5 Mrad is equivalent to autoclaving at 120°/20 min. Unfortunately, many pharmaceuticals discolor and develop off-odors and decrease in potency. Glass is also discolored. γ Radiation does not seem too favorable. The area of greatest application is for disposable medical supplies such as adhesive, dressings, catheters, and especially plastic syringes and other items for single use. However, the cost is high. A novel suggestion is the addition of radionuclides of short half-life to injectables for self-sterilization, an idea fraught with legal complications.

A recent monograph reviews sterilization and disinfection as applied in medical, industrial, and laboratory practice, using ionizing and nonionizing radiation (256). A related application of γ radiation is its use in the preparation of noninfective antigens for the laboratory diagnosis of virus diseases such as influenza, mumps, herpes, and small pox (249). These antigens are commonly prepared in a stable, noninfective form using formaldehyde, but the infectivity of soluble antigens cannot be destroyed in this manner. γ Radiation offers an alternative way and appears to be superior for the preparation of the herpes simplex antigens.

The preparation of vaccines using γ radiation instead of formaldehyde is under investigation, and this technique shows promise (249).

Cold sterilization using linear accelerators has been used successfully for surgical sutures for many years.

Active research and development will undoubtedly show that many pharmaceuticals and medical supplies can be sterilized by radiation suitably and economically to compete with present day methods.

CONCLUSIONS

The authors have attempted to present here an overview of the present status of radiopharmaceuticals, the methods for detecting them, some of the advances in new short-lived radionuclides, and some directly related subjects.

If we would like to characterize developments in recent years, one single term would suffice to describe it: sophistication. Thus, newer, better, complex, and very reliable instrumentation has been developed for the detection both *in vitro* and *in vivo* of radionuclides, thanks in part to the notable developments in solid-state electronics. The ability of the A.E.C. sponsored national laboratories (Brookhaven, Argonne, Oak Ridge) to produce any desired radionuclide and of the nuclear industry to make these available at reasonable cost to investigators in the health sciences, has further widened the horizons of radiopharmaceuticals. The example of ^{99m}Te, a radionuclide with a half-life of 6 hr., is indicative of the present trend toward radionuclides with a shorter half-life involving much less exposure of the patient to radiation. *In vitro* testing of biological functions such as the various methods available for protein bound thyroid hormone based on the original work by Hamolsky in 1957 illustrates another trend: that of using radionuclides in the clinical lab without any exposure to the patient. A third development, only beginning, is that of the use of tritium and ^{14}C-labeled biochemicals for measurement of specific metabolic disorders.

The rationale for present and future developments is given by work in biochemistry and in experimental pharmacology where radionuclides constitute a self-evident and most significative tool.

The authors feel that radiopharmaceuticals are a rapidly growing field with a tremendous potential which has not been fully explored. Especially, new radiopharmaceuticals are required, more

directly based on metabolic functions and designed by applying biochemical and pharmacological principles. This challenge stands before the pharmaceutical sciences.

REFERENCES

(1) Blumgart, H. L., and Yens, O. C., *J. Clin. Invest.*, **4**, 1(1927).
(2) Joliot, F., and Curie, I., *Nature*, **133**, 201(1934).
(3) Sommerville, J. L., "The Isotope Index, 1963–1964," Scientific Equipment Co., Indianapolis, Ind., 1963.
(4) IAEA, "International Directory of Isotopes," 3rd ed., IAEA, Vienna, Austria, 1964.
(5) Hertz, S., Roberts, A., and Evans, R. D., *Proc. Soc. Exptl. Biol. Med.*, **38**, 510(1938).
(6) Christian, J. E., *J. Pharm. Sci.*, **50**, 1(1961).
(7) "Radioactivity for Pharmaceutical and Allied Research Laboratories," Edelman, A., ed., Academic Press Inc., New York, N. Y., 1960.
(8) "Radioactive Isotopes in the Clinic and in Research," vol. 4 (in German), Fellinger, D., and Hoefer, R., eds., Urban and Schwarzenberg, Munich-Berlin, Germany, 1960.
(9) *Ibid.*, vol. 5, 1963.
(10) *Ibid.*, vol. 6, 1965.
(11) Von Hevesy, G., Schwiegt, H., and Turba, F., in "Radioactive Isotopes in Physiology, Diagnostics and Therapy," vol. 1, Springer-Verlag, Berlin, Germany, 1961, p. 536.
(12) "Clinical Use of Radioisotopes: A Manual of Technique," Fields, T., and Seed, L., eds., Year Book Medical Publishers, Inc., Chicago, Ill., 1961.
(13) Fields, T., and Grant, E. H., *ibid.*, 1962.
(14) "The New Year Book of Nuclear Medicine," Quinn, J. L., III, ed., Year Book Medical Publishers, Inc., Chicago, Ill., 1966.
(15) "Radioactivity in Man," Meneely, J. R., ed., Charles C Thomas, Springfield, Ill., 1961.
(16) Cohen, Y., *Prod. Pharm.*, **17**, 1(1962).
(17) Lawrence, J. H., "Use of Radioisotopes in Animal Biology and the Medical Sciences," vol. 1, Academic Press Inc., New York, N. Y., 1962, pp. 1–22.
(18) Copp, D. H., *ibid.*, p. 23.
(19) Quimby, E. H., and Feitelberg, S., "Radioactive Isotopes in Medicine and Biology. Basic Physics and Instrumentation," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1963.
(20) Silver, S., "Radioactive Isotopes in Medicine and Biology· Medicine," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1962.
(21) Wagner, H. N., Jr., *Clin. Pharmacol. Therap.*, **4**, 351(1963).
(22) Goette, H., *Euratom Bull.*, **1963**, 26.
(23) Spaander, P., *Pharm. Weekblad*, **98**, 1008(1963).
(24) Greenlaw, R. H., and Strain, W. H., *Med. Radiography Phot.*, **40**, 82 (Suppl.)(1964).
(25) Kautz, H. D., *et al.*, *Am. J. Nursing*, **64**, 124(1964).
(26) Schulte, K. E., *Arzneimittel-Forsch.*, **7**, 59(1964).
(27) Anon., *Brit. J. Clin. Pract.*, **19**, 597(1965).
(28) Blahd, W. H., "Nuclear Medicine," McGraw-Hill Book Co., New York, N. Y., 1965.
(29) Silver, S., *New. Engl. J. Med.*, **272**, 466, 515, 569 (1965).
(30) "Radioactive Pharmaceuticals," Andrews, G., Kniseley, R. M., and Wagner, H. N., Jr., eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966.
(31) Wagner, H. N., Jr., *Radiology*, **86**, 601(1966).
(32) Eisen, J., *New Jersey J. Pharm.*, **January 1965**, 6.
(33) Von Hevesy, G., "Adventures in Radioisotope Research," Pergamon Press, New York, N. Y., 1962.
(34) Schwiegt, H., and Turba, F., "Artificial Radioisotopes in Physiology, Diagnosis and Therapy," (in German), Springer-Verlag, Berlin, Germany, 1962.
(35) Heim, W., Schumacher, W., and Frost, D., "Radioactive Isotopes in Surgery," Walter de Gruyter and Co., Berlin, Germany, 1961.
(36) Lajtha, L. G., "The Use of Radioisotopes in Hematology," Charles C Thomas, Springfield, Ill., 1961.
(37) Lawrence, J. H., Manowitz, B., and Loeb, B., "Radioisotopes and Radiation: Recent Advances in Medicine, Agriculture and Industry," McGraw-Hill Book Co., New York, N. Y., 1964.
(38) "Progress in Atomic Medicine," Lawrence, J. H., ed., Grune and Stratton, New York, N. Y., 1965.
(39) IAEA Report, "Radioisotopes in Tropical Medicine," IAEA, Vienna, Austria, 1962.
(40) IAEA Report, "Use of Radioisotopes in Animal Biology and the Medical Sciences," Academic Press Inc., New York, N. Y., 1962.
(41) Breeling, J., *Today's Health*. **43**, 54(1965).
(42) Ingraham, S. C., II, *et al.*, *Mod. Hospital*, **105**, 112 (1965).
(43) Belcher, E. H., *Sci. Basis Med. Ann. Rev.* (*London*), **1964**, 250.
(44) Loken, M. K., *Lancet*, **85**, 431(1965).
(45) Nahum, L. H., *Conn. Med.*, **29**, 849(1965).
(46) McCasker, E. N., *J. Kansas Med. Soc.*, **67**, 2(1966).
(47) Soremark, R., *Adv. Biol. Med. Phys.*, **10**, 91(1965).
(48) Fowler, J. F., *Chem. Ind.*, **31**, 1353(1965).
(49) Steinberg, J., *J. Nucl. Med.*, **6**, 691(1965).
(50) "Radioisotopes and Circulation," Sevelius, G., ed. Little, Brown and Co., Boston, Mass., 1965.
(51) Charlton, J. C., *Pharm. J.*, **195**, 37(1965).
(52) Baker, P. S., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 140.
(53) Pinajian, J. J., *ibid.*, p. 143.
(54) Richards, P., *ibid.*, p. 155.
(55) Clocher, R., and Macherauch, E., "X-Ray and Nuclear Physics in Medicine," G. Thieme Ver., Stuttgart, Germany, 1965.
(56) Bowen, H. J. M., and Gibbons, D., "Radioactivation Analysis," Clarendon Press, Oxford, England, 1963.
(57) Koch, R. C., "Activation Analysis Handbook," Academic Press Inc., New York, N. Y., 1960.
(58) Lyon, W. S., Jr., "Guide to Activation Analysis," D. Van Nostrand and Co., Princeton, N. J., 1964.
(59) Taylor, D., "Neutron Irradiation and Activation Analysis," D. Van Nostrand and Co., Princeton, N. J., 1964.
(60) Guinn, V. P., *Encycl. Ind. Chem. Anal.*, **1**, 52(1966).
(61) Leniham, J. M. A., *Nucleonics*, **23**, 65(February 1965).
(62) Friedlander, D., Kennedy, J. W., and Miller, J. M., "Nuclear and Radiochemistry," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1964.
(63) Overman, R. T., and Clark, H. M., "Radioisotope Techniques," McGraw-Hill Book Co., New York, N. Y., 1960.
(64) "Advances in Tracer Methodology," vol. 1., Rothchild, S., ed., New England Nuclear Corp., New York, N. Y., 1962; *ibid.*, vol. 2, 1965.
(65) Haissinsky, M., and Adloff, J. P., "Radiochemical Survey of the Elements," Elsevier Publishing Co., Amsterdam, The Netherlands, 1965.
(66) Bacq, Z. M., and Alexander, P., "Fundamentals of Radiobiology," 2nd ed., Pergamon Press, Oxford, England, 1961.
(67) Errera, M., and Forssberg, A., "Mechanism in Radiobiology," Academic Press Inc., New York, N. Y., 1961.
(68) Wolf, G., "Isotopes in Biology," Academic Press Inc., New York, N. Y., 1964.
(69) "Advances in Radiation Biology," Augenstein, L. G., *et al.*, eds., Academic Press Inc., New York, N. Y., 1964.
(70) Wertheim, G. K., "Mossbauer Effect: Principles and Applications," Academic Press Inc., New York, N. Y., 1964.
(71) "Mossbauer Effects Symposium," Gruverman, E., ed., Plenum Press, New York, N. Y., 1966.
(72) Eaves, G., "Principles of Radiation Protection," Iliffe Books, London, England, 1964.
(73) Abbott, J. D., Lakey, J. R. A., and Mathias, D. J., "Protection Against Radiation," Charles C Thomas, Springfield, Ill., 1961.
(74) Handloses, J. S., "Health Physics Instrumentation," Pergamon Press, London, New York, 1959.
(75) Braestrup, C. B., and Wyckoff, H. O., "Radiation Protection," Charles C Thomas, Springfield, Ill., 1958.
(76) "Workshop on Radionuclides in Foods and Agricultural Products," Haghiri, F., and Johnson, R. R., eds., Ohio Agricultural Station, Columbus, Ohio, 1963.
(77) Eisenbud, M., "Environmental Radioactivity," McGraw-Hill Book Co., New York, N. Y., 1964.
(78) "Radionuclides in Foods," National Academy of Sciences–National Research Council, Washington, D. C. 1962.
(79) IAEA Report, "Radioactive Substances in the Biosphere," IAEA, Vienna, Austria, 1961.
(80) Farmer, F. R., "The Packaging, Transport, and Related Handling of Radioactive Materials," IAEA, Vienna, Austria, 1961.
(81) Amphlett, C. B., "Treatment and Disposal of Radioactive Waste," Pergamon Press, New York, N. Y., 1961.
(82) Mawson, C. A., "Processing of Radioactive Wastes," IAEA, Vienna, Austria, 1961.
(83) Straub, C. P., "Low Level Radioactive Wastes: Their Handling, Treatment, and Disposal," U. S. Atomic Energy Commission, Washington, D. C., 1964.
(84) Chase, G. D., and Rabinowitz, J. L., "Principles of Radioisotope Methodology," 2nd ed., Burgess Publishing Co., Minneapolis, Minn., 1962.
(85) Hogerton, J. F., "The Atomic Energy Desk Book," Reinhold Publishing Corp., New York, N. Y., 1963.
(86) Schilling, C. W., "The Atomic Energy Encyclopedia in the Life Sciences," W. B. Saunders Co., Philadelphia, Pa., 1964.
(87) "Radiological Health Handbook," Kinsman, S., *et al.*, eds., U. S. Department of Health, Education and Welfare, Washington, D. C., 1960.
(88) Murray, A., and Williams, E. L., "Organic Synthesis with Isotopes," 2 vols., Interscience Publishers, New York, N. Y., 1958.
(89) Cohen, Y., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 67.
(90) International Union of Pure and Applied Chemistry, Report, *J. Am. Chem. Soc.*, **82**, 5517, 5526(1960); *J. Am. Med. Assoc.*, **190**, 160(1964).
(91) Catch, J. R., "Carbon-14 Compounds," Butterworth Scientific Publications, London, England, 1961.

(92) Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013 (1957).
(93) Whisman, M. L., and Eccleston, B. H., *Nucleonics*, **20**, 98(June 1962).
(94) "Tritium in the Biological and Physical Sciences," 2 vols., IAEA, Vienna, Austria, 1962.
(95) USAN Report, *J. Am. Med. Assoc.*, **184**, 228(1963); **186**, 1034(1963); **187**, 943(1964); **189**, 629(1964).
(96) "Manual of Isotope Production Processes in Use at Brookhaven National Laboratory," Stang, L. G., Jr., ed., BNL-864, Brookhaven National Laboratory, New York, N. Y., 1964.
(97) Case, F. N., *et al.*, "ORNL Radioisotopes Procedures Manual," ORNL-3633, Oak Ridge National Laboratory, Oak Ridge, Tenn., 1964.
(98) Aebersold, P. C., *Isotop. Rad. Technol.*, **2**, 201(1965).
(99) Hughes, W. L., *et al.*, eds., *J. Lab. Comp.*, **1**, 311 (1965).
(100) Yalow, S., and Berson, J. A., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 265.
(101) Bayly, R. J., *ibid.*, p. 51.
(102) "Methods of Preparing and Storing Marked Molecules," Sirchis, J., ed., Euratom, Brussels, Belgium, 1964.
(103) "Preparation and Biomedical Application of Labelled Molecules," Sirchis, J., ed., Euratom, Brussels, Belgium, 1964.
(104) Briner, W. H., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 93.
(105) Stern, H. S., *et al.*, *Intern. J. Appl. Radiation Isotopes*, **16**, 283(1965).
(106) McAfee, J. G., *et al.*, *J. Nucl. Med.*, **5**, 936(1964).
(107) Taplin, G. V., *et al.*, in "Medical Radioisotope Scanning," vol. 2, IAEA, Vienna, Austria, 1964: Taplin, G. V., *et al.*, *Health Phys.*, 10, 1219(1964).
(108) Harper, P. V., *et al.*, *J. Nucl. Med.*, **5**, 382(1964).
(109) *Ibid.*, **4**, 189(1963).
(110) Harper, P. V., *et al.*, in "Medical Radioisotope Scanning," vol. 2, IAEA, Vienna, Austria, 1964.
(111) Harper, P. V., *et al.*, *Nucleonics*, **22**, 50(1964).
(112) Sanders, T. P., and Kuhl, D. E., *J. Nucl. Med.*, **5**, 341(1966).
(113) Carr, E. A., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 619.
(114) Koplowitz, J. M., and Solomon, D. H., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 114.
(115) Myers, W. G., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 217.
(116) Schilling, R. F., *J. Lab. Clin. Med.*, **42**, 860(1953).
(117) Reizenstein, P. G., *Acta Med. Scand.*, **165**, Suppl. 347(1959).
(118) Rosenblum, C., *Am. J. Clin. Nutr.*, **8**, 276(1960).
(119) Reba, R. C., *et al.*, *Medicine*, **42**, 269(1963).
(120) Rhoton, A. L., Jr., *et al.*, *Arch. Neurol.*, **10**, 369 (1964).
(121) Wagner, H. N., Jr., *et al.*, *Arch. Internal Med.*, **113**, 696(1964).
(122) Winkelman, J. W., *et al.*, *Radiology*, **75**, 465(1960).
(123) Blau, M., and Bender, M. A., *ibid.*, **78**, 974(1962).
(124) DiGiulio, W., *et al.*, *Clin. Res.*, **11**, 297(1963).
(125) Tubis, M., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **103**, 497(1960).
(126) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(127) Howe, C. T., and Eakins, R. P., *J. Nucl. Med.*, **4**, 469(1963).
(128) Bauer, F. K., *et al.*, *ibid.*, **1**, 199(1960).
(129) Mitchell, M. L., *et al.*, *J. Clin. Endocrinol. Metab.*, **20**, 1474(1960).
(130) Sterling, K., and Tabachnik, M., *ibid.*, **21**, 456 (1961).
(131) Krasznai, I., *et al.*, *Nucl. Med.*, **5**, 122(1966).
(132) Silver, S., *et al.*, *J. Clin. Invest.*, **39**, 420(1960).
(133) Avioli, L., *Clin. Res.*, **11**, 40(1963).
(134) Chiappa, S., *et al.*, *Am. J. Roentgenol.*, **92**, 134 (1964).
(135) Halaby, F. A., *et al.*, *Surg. Gynecol. Obstet.*, **118**, 52 (1964).
(136) Muller, J. H., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965.
(137) Libby, R. L., and Tubis, M., *Nucleonics*, **10**, 63 (1952).
(138) Williams, J. A., and Fine, J., *New Engl. J. Med.*, **264**, 842(1961).
(139) Price, W. J., "Nuclear Radiation Detection," McGraw-Hill Book Co., New York, N. Y., 1966.
(140) Heath, R. L., *et al.*, *Nucleonics*, **24**, 52(May 1966).
(141) Glos, M. B., *ibid.*, 24, 44(May 1966).
(142) Yanof, H. M., "Biomedical Electronics," F. A. Davis Co., Philadelphia, Pa., 1965.
(143) Malmstadt, H. V., and Enke, C. G., "Electronics for Scientists," W. A. Benjamin, Inc., New York, N. Y., 1963.
(144) Allen, H. C., Jr., Libby, R. L., and Cassen, B., *J. Clin. Endocrinol.*, **11**, 492(1951).
(145) Knisely, R. M., Andrews, G. A., and Harris, C. C., eds., "Progress in Medical Radioisotope Scanning," U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1963.
(146) "Dynamic Clinical Studies with Radioisotopes," Kniseley, R. M., and Tauxe, W. N., eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1964.
(147) "Scintillation Scanning in Clinical Medicine," Quinn, J. L., III, ed., W. B. Saunders Co., Philadelphia, Pa., 1964.
(148) Harris, C. C., *et al.*, in "Progress in Medical Radioisotope Scanning," Kniseley, R. M., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1963, p. 25.
(149) Kakehi, H., *et al.*, *ibid.*, p. 111.
(150) Jaffe, H. L., Adams, R., and Krohn, L., *J. Nucl. Med.*, **6**, 364(1965).
(151) Anger, H. O., *Rev. Sci. Instr.*, **29**, 27(1958).
(152) Beierwalter, W. H., in "Scintillation Scanning in Clinical Medicine," Quinn, J. L., III, ed., W. B. Saunders Co., Philadelphia, Pa., 1964, p. 258.
(153) Bender, M. A., and Blau, M., *J. Nucl. Med.*, **1**, 105 (1960).
(154) Ter-Pogossian, M. M., *et al.*, *Radiology*, **86**, 463 (1966).
(155) Horwitz, N. H., *et al.*, *J. Nucl. Med.*, **6**, 724(1965).
(156) Lansiart, A. J., and Kellershohn, C., *Nucleonics*, **24**, 56(March 1966).
(157) Pullan, B. R., *et al.*, *ibid.*, **24**, 72(June 1966).
(158) Anger, H. O., and Rosenthal, D. J., in "Medical Radioisotope Scanning," IAEA, Vienna, Austria, 1959, p. 59.
(159) Miller, C. E., and Remenchik, A. P., *Ann. N. Y. Acad. Sci.*, **110**, 175(1963).
(160) Morris, A. C., *J. Nucl. Med.*, **6**, 481(1965).
(161) Anger, H. O., *ibid.*, **7**, 331(1966).
(162) Anderson, E. C., *et al.*, *Nucleonics*, **14**, 26(January 1956).
(163) Andrews, H. L., *et al.*, *J. Nucl. Med.*, **6**, 667(1965).
(164) Stang, L. G., Jr., *Nucleonics*, **22**, 46(January 1964).
(165) "Liquid Scintillation Counting," Bell, C. G., and Hayes, F. N., eds., Pergamon Press, New York, N. Y., 1958.
(166) Birks, J. B., "The Theory and Practice of Scintillation Counting," Pergamon Press, London, England, 1964.
(167) Tubis, M., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 145.
(168) "Recent Advances in Nuclear Medicine," Croll, M. N., and Brady, L. W., eds., Appleton-Century-Crofts, New York, N. Y., 1966.
(169) Silver, S., *Nucleonics*, **23**, 106(1965).
(170) Vaughan, B. E., and Boling, E. A., *J. Lab. Clin. Med.*, **57**, 159(1961).
(171) Manougian, E., *et al.*, *J. Nucl. Med.*, **5**, 763(1964).
(172) Tolbert, B. M., *et al.*, *UCRL*, **1957**, 3920.
(173) Rothfeld, B., and Rabinowitz, J. L., *Am. J. Digest. Diseases*, **9**, 263(1964).
(174) Snyder, F., and Piantadosi, A., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 553.
(175) Blahd, W. H., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 259.
(176) Hamolsky, M. W., *et al.*, *J. Clin. Endrocrinol. Metab.*, **17**, 33(1957).
(177) Crispell, K. R., *et al.*, *J. Clin. Invest.*, **29**, 25(1953).
(178) Taplin, G. V., *et al.*, *J. Nucl. Med.*, **5**, 264(1964).
(179) Nordyke, R. A., *et al.*, *J. Lab. Clin. Med.*, **56**, 438 (1960).
(180) Taplin, G. V., *et al.*, *ibid.*, **45**, 655(1955).
(181) Wagner, H. N., *et al.*, *Arch. Internal Med.*, **107**, 324 (1961).
(182) Isley, J. K., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **94**, 807(1957).
(183) Kaplan, E., *et al.*, *Gastroenterology*, **34**, 901(1958).
(184) Goldberg, B., *et al.*, *Arch. Ophthalmol.*, **65**, 196 (1961).
(185) Bauer, G. C. H., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 461.
(186) Sterling, K., and Gray, S. J., *J. Clin. Invest.*, **29**, 1614 (1950).
(187) Necheles, T. F., *J. Lab. Clin. Med.*, **42**, 358(1953).
(188) Schilling, R. F., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 338.
(189) Bender, M. A., *Sciences*, **125**, 443(1957).
(190) Weinstein, I. M., *Blood*, **14**, 950(1959).
(191) Pollycove, M., and Mortimer, R., *J. Clin. Invest.*, **40**, 753(1961).
(192) Silver, S., "Radioactive Isotopes in Medicine and Biology· Medicine," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1962, p. 186.
(193) Stern, H. S., *et al.*, in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 374.
(194) Harper, P. V., *et al.*, *ibid.*, p. 335.
(195) Chapman, E. M., in "Nuclear Medicine," Blahd, W. H., ed., McGraw-Hill Book Co., New York, N. Y., 1965, p. 617.
(196) Silver, S., "Radioactive Isotopes in Medicine and Biology· Medicine," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1962. p. 149.
(197) Catz, B., *et al.*, *Cancer*, **12**, 371(1959).
(198) Lawrence, J. H., "Polycythemia, Physiology, Diagnosis and Treatment," Grune and Stratton, New York, N. Y., 1955.
(199) Osgood, E. E., *Blood*, **16**, 1104(1960).
(200) Maxfield, J. R., Jr., *et al.*, *Southern Med. J.*, **51**, 320 (1958).
(201) Card, R. Y., *et al.*, *J. Nucl. Med.*, **1**, 195(1960).
(202) Kligerman, M. M., and Habif, D. V., *Am. J. Roentgenol.*, **74**, 651(1955).

(203) Henschke, U. K., *et al.*, *Nucleonics*, **11**, 46(May 1953).
(204) Myers, W. G., *Science*, **107**, 621(1948).
(205) Friedell, H. S., *et al.*, *Am. J. Roentgenol.*, **65**, 232(1951).
(206) Henschke, U. K., *Proc. Intern. Conf. Peaceful Uses At. Energy*, **10**, 48(1956).
(207) Cohen, L., *Brit. J. Radiol.*, **28**, 338(1955).
(208) Hauser, J., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 689.
(209) Charlton, J. C., *ibid.*, p. 33.
(210) Glenn, H. J., and Kidwell, R. E., *ibid.*, 1965, p. 165.
(211) Briner, W. H., *Am. J. Hosp. Pharm.*, **20**, 553(1963).
(212) Peng, C. T., *J. Pharm. Sci.*, **50**, 88(1961).
(213) Fawcett, D. M., *et al.*, *Can. Med. Assoc. J.*, **86**, 965 (1962).
(214) Numerof, P., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 181.
(215) Fowler, E. E., Atomic Industrial Forum, Twelfth Annual Conference, Washington, D. C., November 14, 1965.
(216) Wagner, H. N., Jr., *Nucleonics*, **24**, (No. 7) 62(1966).
(217) Editorial, *ibid.*, **24**, (No. 4) 20(1966).
(218) Schniewind, H., *et al.*, *Arch. Exptl. Pathol. Pharmakol.*, **241**, 527(1961).
(219) Ogborn, R. E., *et al.*, *Radiology*, **78**, 619(1962).
(220) Dworkin, H. J., and LaFleur, P. D., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 635.
(221) Matthews, C. M. E., *et al.*, *ibid.*, p. 567.
(222) Roth, L. J., *ibid.*, p. 119.
(223) "Isotopes in Experimental Pharmacology," Roth, L. J., ed., University of Chicago Press, Chicago, Ill., 1965.
(224) Bousquet, W. F., *J. Pharm. Sci.*, **51**, 297(1962).
(225) Rescigno, A., and Segre, G., "Drug and Tracer Kinetics," Blaisdell, New York, N. Y., 1966.
(226) Paoletti, R., and Poggi, M., in "Methods of Preparing and Storing Labelled Molecules," Sirchis, J., ed., Euratom, Brussels, Belgium, 1964, p. 1137.
(227) Sung, C. Y., *K'o Hsueh T'ung Pao*, **7**, 607(1964); transl. *Nucl. Sci. Abstr.*, **19**, 2010(1965).
(228) Snell, J. F., *Adv. Trac. Meth.*, **1**, 234(1963).
(229) Benakis, A., and Glasson, B., in "Preparation and Biomedical Applications of Labeled Molecules," Sirchis, J., ed., Euratom, Brussels, Belgium, 1964, p. 178.
(230) Lissitzky, S., *et al.*, *Biochim. Biophys. Acta*, **93**, 494(1964).
(231) Buyske, D. A., in "Isotopes in Experimental Pharmacology," Roth, L. J., ed., University of Chicago Press, Chicago, Ill., 1965, p. 275.
(232) Ullberg, S., *ibid.*, p. 63.
(233) Stumpf, W. E., and Roth, L. J., *J. Histochem. Cytochem.*, **14**, 3(1965).
(234) Roth, L. J., and Barlow, C. F., in "Isotopes in Experimental Pharmacology," Roth, L. J., ed., University of Chicago Press, Chicago, Ill., 1965, p. 49.
(235) Belleau, B., *ibid.*, p. 469.
(236) Elison, C., *et al.*, *J. Med. Pharm. Chem.*, **6**, 237 (1963).
(237) Stepka, W., and Larson, P. S., in "Isotopes in Experimental Pharmacology," Roth, L. J., ed., University of Chicago Press, Chicago, Ill., 1965, p. 7.
(238) Rothchild, S., *ibid.*, p. 15.
(239) Bayly, R. J., *Nucleonics*, **24**, 46(June 1966).
(240) Wagner, H. N., Jr., in "Radioactive Pharmaceuticals," Andrews, G. A., *et al.*, eds., U. S. Atomic Energy Commission, Oak Ridge, Tenn., 1966, p. 1.
(241) Christian, J. E., and Bousquet, W. F., "Radioisotopes and Nuclear Techniques in the Pharmaceutical and Allied Industries," U. S. Atomic Energy Commission, Washington, D.C., 1960.
(242) Brit. pat. 938,828.
(243) Peck, G. E., Christian, J. E., and Banker, G. S., *J. Pharm. Sci.*, **53**, 607(1964).
(244) Clark, G. S., and Swartz, H. A., *ibid.*, **52**, 999 (1963).
(245) Schneiter, M. C., Kessler, W. V., and Christian, J. E., *ibid.*, **51**, 1005(1962).
(246) Appino, J. B., Christian, J. E., and Banker, G. S., *ibid.*, **51**, 254(1962).
(247) Benedict, J. K., Banker, G. S., and Kessler, W. V., *ibid.*, **54**, 1659(1965).
(248) Smith, D. M., *Ciencia (Mex.)*, **19**, 262(1960); through *Nucl. Sci. Abstr.*, **16**, 24052(1962).
(249) The AECL Radioisotope Application Handbook, Technical Bulletin RAP-1, Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada, pp. 19–22.
(250) Krowcynski, L., *et al.*, *Acta Polon. Pharm.*, **18**, 125(1961); through *Nucl. Sci. Abstr.*, **16**, 23424(1962).
(251) Pochapinskii, V. I., *Med. Prom. SSSR*, **16**, (No. 7) 34(1962); through *Chem. Abstr.*, **58**, 3272*b*(1963).
(252) Ogg, A. J., *Lancet*, **2**, 96(1963).
(253) Bonet-Maury, P., *Ann. Pharm. Franc.*, **21**, 151 (1963).
(254) Artandi, C., *Bull. Parenteral Drug. Assoc.*, **18**, 2 (1964).
(255) Ehrlen, I. R., *Svensk. Farm., Tidskr.*, **68**, 818(1964).
(256) Rubbo, S. D., and Gardener, J. F., "A Review of Sterilization and Disinfection as Applied to Medical, Industrial and Laboratory Practice," The Year Book Medical Publishers, Chicago, Ill., 1965.

Aggregation of Certain Medicinal Amines in Aqueous Solutions of Their Salts

By BAHRAM FARHADIEH*, NATHAN A. HALL, and E. R. HAMMARLUND

The osmotic coefficient and the equivalent conductance of aqueous solutions of certain medicinal amine salts were determined in order to examine their aggregating properties. Procaine HCl and tripelennamine HCl formed small ionic micelles. Partially ionized micelles of approximately constant size were the main constituent of solutions of dibucaine HCl and bromodiphenhydramine HCl. Tetracaine HCl and diphenhydramine HCl formed aggregates of different sizes in their solutions. No definite conclusion could be drawn about the types of aggregates that were present in solutions of pyrilamine maleate. An apparent critical micelle concentration (CMC) was obtained only for dibucaine HCl and bromodiphenhydramine HCl. From the CMC values at 0° and 25° it was concluded that the standard enthalpy change associated with formation of micelles in solutions of these two salts was practically zero. This finding together with a negative change in the standard free energy of micellization indicated that the process of micelle formation was mainly an entropy effect.

AQUEOUS SOLUTIONS of many long chain amine salts, sulfonates, sulfates, etc., exhibit an abrupt change in physical properties over a relatively short concentration range (1). This phenomenon which is common to aqueous solutions of association colloids has been attributed to the formation of aggregates, and the concentration at which it occurs has been termed "the critical concentration for the formation of micelles" (CMC). The nature of the transition taking place at the critical concentration is as yet unknown and a controversy still exists concerning the possible existence of aggregates at lower concentrations (2).

The freezing point depression, ΔT_f, of aqueous solutions of many substituted amine salts of pharmaceutical interest has been measured by Hammarlund and co-workers (3). It was shown that in dilute solutions these amine salts behave as simple electrolytes. At higher concentrations, however, a number of them appear to form molecular aggregates as evidenced by a sharp change in slope when ΔT_f is plotted against the salt concentration. Recently Johnson *et al.* (4) measured the vapor pressure of aqueous solutions of several of these amine salts using a vapor pressure osmometer of their own design. They also concluded that these salts tend to form molecular aggregates above a certain concentration.

Generally, measurements of several physicochemical properties of a solution are needed in order to ascertain the formation of aggregates by a solute. The work presented here was designed to investigate the aggregating tendency of several amine salts by determining the molal osmotic coefficient as well as the equivalent conductance of their aqueous solutions.

EXPERIMENTAL

Materials.[1]—The amine salts used in this study were procaine HCl, tetracaine HCl, dibucaine HCl, tripelennamine HCl, pyrilamine maleate, diphenhydramine HCl, and bromodiphenhydramine HCl. Since they were of medicinal grade, no further purification was attempted. Only tetracaine HCl and dibucaine HCl lost weight upon drying in vacuum and in the presence of phosphorus pentoxide. These were dried to constant weight before use.

Preparation of Solutions.—Solutions employed for the determination of the osmotic coefficients were prepared on the molal basis in double distilled

Received July 1, 1966, from the College of Pharmacy, University of Washington, Seattle.
Accepted for publication September 20, 1966.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
Abstracted from a thesis submitted by Bahram Farhadieh to the University of Washington, Seattle, in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported by Initiative Measure 171 for Biological and Medical Research, University of Washington, Seattle.
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[1] The authors express sincere appreciation to Winthrop Laboratories, New York, N. Y., for tetracaine HCl; Ciba Pharmaceutical Co., Summit, N. J., for samples of dibucaine HCl and tripelennamine HCl; Merck, Sharp and Dohme Co., Rahway, N. J., for pyrilamine maleate; Parke, Davis and Co., Detroit, Mich., for samples of diphenhydramine HCl and bromodiphenhydramine HCl.

TABLE I.—MOLAL OSMOTIC COEFFICIENT OF AQUEOUS SOLUTIONS OF POTASSIUM CHLORIDE AND AMINE SALTS

Concn., Molal	Compd.[a] 1	2	3	4	5	6	7	8
0.01	0.965	0.943	0.943	0.942	0.952	0.952	0.933	0.924
	0.967[b]							
0.03	0.950	0.918	0.896	0.839	0.918	0.911	0.867	0.816
0.05	0.941		0.850	0.774			0.789	
	0.941[b]							
0.06		0.884			0.868	0.864	0.543[c]	0.724
0.100	0.929	0.835	0.754	0.611	0.816	0.798		0.625
	0.930[b]							
0.150	0.920		0.645	0.452	0.752	0.728		0.505
0.160		0.780					0.361	
0.200	0.915	0.754	0.554	0.367	0.697	0.650	0.306	0.448
	0.915[b]							
0.250		0.722	0.483	0.317	0.648	0.579	0.275	
0.260								0.381
0.300		0.687	0.428	0.282	0.599	0.522	0.250	0.349
0.350			0.389			0.472		0.315
0.360							0.230	

[a] The numbers represent: *1*, potassium chloride; *2*, procaine HCl; *3*, tetracaine HCl; *4*, dibucaine HCl; *5*, tripelennamine HCl; *6*, diphenhydramine HCl; *7*, bromodiphenhydramine HCl; *8*, pyrilamine maleate. [b] Data obtained from literature. [c] 0.09 molal solution.

water. For the study of electrical conductivity solutions were made on molar basis in conductivity water (specific conductance, 1–1.5 $\times$ 10^{-6} ohms^{-1} cm.$^{-1}$).

Apparatus and Measurement Procedures.—A Mechrolab[2] vapor pressure osmometer was used to determine the molal osmotic coefficients of the solutions. Since preliminary experiments with potassium chloride solutions showed stable readings within 6 min., all readings were taken 6 min. after the solution drop was placed on the sample thermistor bead. All measurements were made at 25.000 $\pm$ 0.001°.

A Dike-Jones conductivity bridge[3] was used to determine the electrical resistance of the solutions. The frequency of the bridge current was 1000 c.p.s. and the null point was detected by a cathode ray oscilloscope. Three different conductivity cells were employed. The cell constants were *A*, 1.0449; *B*, 1.0241; and *C*, 13.0078. Cell *A* had unplatinized electrodes and was used for the determination of resistance of the very dilute solutions. The polarization resistance introduced by the bright platinum electrodes of this cell amounted to less than 0.10% of the total resistance of the solutions at 1000 c.p.s. Therefore, the error due to polarization was considered to be negligible and no attempt was made to correct the electrical resistance of the very dilute solutions for this effect. The measurements were obtained at 25.00° in an oil bath which was maintained at 25.00° $\pm$ 0.02°. Usually readings were taken 45 min. after the cells containing the solutions were immersed in the oil bath.

The molal osmotic coefficients of the aqueous solutions of the samples were calculated from the following relationship (5):

$$\Delta R = \nu\alpha\phi m \qquad \text{(Eq. 1)}$$

where ΔR is the difference in the resistance of the sample and the solvent thermistor beads; ν is the theoretical number of ions per molecule of the solute; ϕ is the molal osmotic coefficient of the solvent; m is the molality of the solute; and α is the calibration constant of the thermoelectric vapor pressure osmometer.

Aqueous solutions of mannitol ranging in concentration from 0.04 to 0.250 molal were used for the calibration of the instrument. Mannitol was chosen because the molal osmotic coefficient of its aqueous solution is very nearly unity up to 1 molal (5). The calibration constant was obtained from the slope of a plot of the measured ΔR *versus* concentration. The plot gave a straight line, the slope of which, as calculated by the method of least squares, was 52.60 ohms/mole.

The accuracy of the method as well as the reliability of the vapor pressure osmometer were determined by calculating the molal osmotic coefficient of aqueous KCl solutions and comparing them with the corresponding reported values (6). The agreement between the two sets of data was excellent.

The amine salts used in this study have pKa's of about 8–9 and the pH's of their aqueous solutions is about 5–6. In this pH range the dissociation of the protonated amines to their corresponding free base is negligible. Thus, in calculation of the molal osmotic coefficients, the samples were treated as simple 1:1 electrolytes. Each ΔR used in either the determination of the calibration constant or the calculation of ϕ is a mean of four separate readings with a standard error of less than $\pm$0.04 ohms.

RESULTS

The osmotic coefficient of aqueous solutions of KCl and the amine salts are summarized in Table I and are plotted *versus* the square root of concentration in Figs. 1 and 2. In these figures are also included the lines representing the Debye-Huckel expression (6) which at 25° is:

$$\phi = 1.0 - 0.392\sqrt{m} \qquad \text{(Eq. 2)}$$

[2] Mechrolab Inc., Mountain View, Calif.
[3] Leeds and Northrup Co., Philadelphia, Pa.

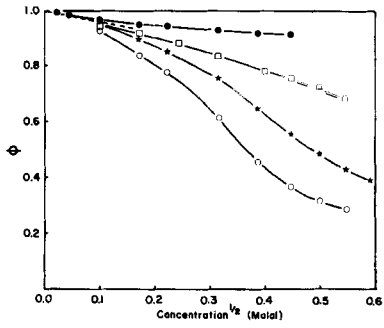

Fig. 1.—Molal osmotic coefficient of aqueous solutions. Key: ●, potassium chloride; □, procaine HCl; ★, tetracaine HCl; ○, dibucaine HCl; ----, Eq. 2.

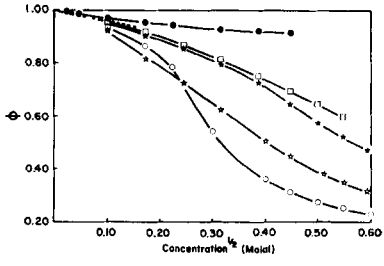

Fig. 2.—Molal osmotic coefficient of aqueous solutions. Key: ●, potassium chloride; □, tripelennamine HCl; ★, diphenhydramine HCl; ○, bromodiphenhydramine HCl; ☆, pyrilamine maleate; ----, Eq. 2.

Equation 2 holds true for extremely dilute aqueous solutions of a simple 1:1 electrolyte. Due to experimental limitations molal osmotic coefficients could not be measured for concentrations less than 0.01 molal. However, Figs. 1 and 2 indicate that at extremely dilute solutions the osmotic coefficient of the solutions should approach that given by the Debye-Huckel expression.

The equivalent conductance of solutions of NaCl and the amine salts used in this investigation are presented in Table II and are plotted *versus* the square root of concentration in Figs. 3 and 4. Each Λ is the average of two separate readings. According to Onsager (7), in extremely dilute solutions and at a temperature of 25° the equivalent conductance of any strong 1:1 salt is given by:

$$\Lambda = \Lambda_0 \left(1.0 - 0.22724 \sqrt{c}\right) - 59.730 \sqrt{c} \quad \text{(Eq. 3)}$$

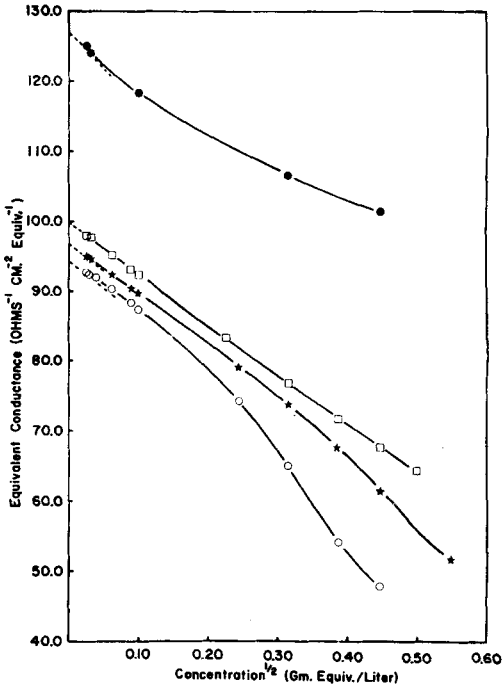

Fig. 3.—Equivalent conductance of aqueous solutions. Key: ●, NaCl; □, procaine HCl; ★, tetracaine HCl; ○, dibucaine HCl; ----, Eq. 3.

Table II.—Equivalent Conductance of Aqueous Solutions of Sodium Chloride and Amine Salts

Concn., *M*	Compd.[a] *1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
0.0007	124.30	98.09	95.03	92.70	97.90		96.00	71.80
0.0008			94.80	92.60	97.80	96.90	95.80	71.30
0.0009		97.99		92.50	97.60	96.60	95.70	70.90
0.0010	123.90	97.70	94.50		97.30	96.50	95.5	70.50
	123.74[b]							
0.0015				92.20		96.00	95.10	69.10
0.0040		95.40	92.40	90.50	95.0	93.90	92.5	65.60
0.0080		93.30	90.50	88.50	92.80	92.10	91.20	62.50
0.0100	118.40	92.50	89.70	87.50	91.70	91.10	90.00	61.40
	118.51[b]							
0.0500		83.40			83.20	82.60	80.50	47.80
0.0600			79.20	74.40				
0.1000	106.85	76.80	73.70	65.00	76.70	76.00	64.10	36.60
	106.74[b]							
0.1500		71.90	67.60	54.20	71.70	70.80	56.10	32.60
0.2000	101.66	67.80	61.46	47.90	67.60	66.20	51.50	28.30
0.2500		64.30				62.00	48.40	25.10
0.3000			51.90			58.42		

[a] The numbers represent: *1*, NaCl; *2*, procaine HCl; *3*, tetracaine HCl; *4*, dibucaine HCl; *5*, tripelennamine HCl; *6*, diphenhydramine HCl; *7*, bromodiphenhydramine HCl; *8*, pyrilamine maleate. [b] Data obtained from literature.

where Λ_0 is the limiting equivalent conductance and c is the concentration of the solute expressed in gram-equivalents per liter of solution. Broken lines in Figs. 3 and 4 represent Eq. 3. The value of Λ_0 for each salt was obtained by extrapolating an expanded plot of Λ *versus* $c^{1/2}$ to zero concentration of the salt solution. In the calculation of the equivalent conductance the salts were treated as 1:1 electrolytes and the contribution by the free bases was neglected.

In Fig. 5 the quantity ϕm, the product of molal osmotic coefficient and the molality of bromodiphenhydramine HCl, is plotted *versus* molality. Figure 6 represents the plot of the specific conductance *versus* concentration for the same salt. Each plot consists of two straight lines with different slopes which intersect each other at the CMC of the salt (8, 9). In Fig. 5 the slope of the straight line segment below the CMC of bromodiphenhydramine HCl coincides with that of an ideal solution of a strong 1:1 electrolyte, *i.e.*, KCl. The CMC's of dibucaine HCl and bromodiphenhydramine HCl as obtained by these two methods were 0.061 and 0.052 M, respectively.

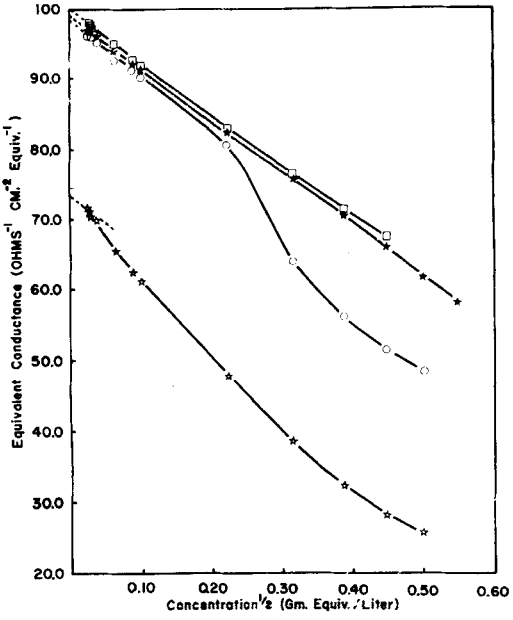

Fig. 4.—Equivalent conductance of aqueous solutions. Key: □, tripelennamine HCl; ★, diphenhydramine HCl; ○, bromodiphenhydramine HCl; ☆, pyrilamine maleate; ----, Eq. 3.

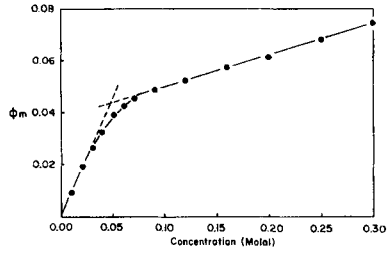

Fig. 5.—Apparent molality of aqueous bromodiphenhydramine HCl solutions.

DISCUSSION

Examination of the plots of Λ *versus* $c^{1/2}$ for the amine salts (Figs. 3 and 4) show that in extremely dilute solutions, where the Onsager expression is accepted to be valid, the experimental points coincide with those obtained from the Onsager equation. This agreement of the experimental and the theoretical data indicates that in these dilute solutions, the salts are totally dissociated into their constituent ions and, therefore, can be considered to be strong electrolytes.

In the concentration range shown in Fig. 1 the values of the molal osmotic coefficients for aqueous solutions of procaine HCl are less than the values predicted by the Debye-Huckel theory. Although this theory does not apply at such high concentrations, all deviations expected and found for a strong 1:1 electrolyte, KCl, are in the opposite direction to that of procaine HCl. These deviations indicate the presence of aggregates in solutions of this amine salt. Since the behavior of the equivalent conductance of procaine HCl solutions (Fig. 3) is similar to that of a strong 1:1 electrolyte, NaCl, the aggregates formed may be of the small ionic type suggested by McBain (10) as an explanation of the similar behavior observed in dilute solutions of colloidal electrolytes. Due to their increased charge, when compared to simple ions, these small aggregates should enhance the electrical conductance of the solutions provided that the decreased mobility of the ions in aggregates is overcome by the increased number of charges carried by the aggregates. The similarity in the behavior of the molal osmotic coefficients and the equivalent conductances of tripelennamine HCl solutions (Figs. 2 and 4) to those of procaine HCl suggests the presence of small ionic aggregates in the moderately concentrated solutions of this salt also.

Assuming that normality approximates molality in the concentration range studied, Figs. 2 and 4 show that up to 0.04 molal the osmotic coefficient as well as the conductance of aqueous bromodiphenhydramine HCl solutions are similar to that of procaine HCl. At concentrations above 0.04

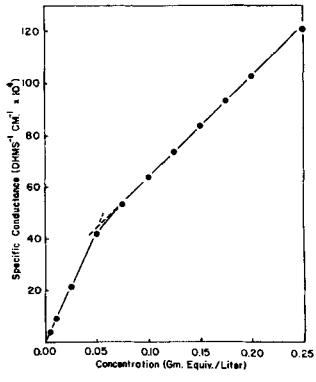

Fig. 6.—Specific conductance of aqueous bromodiphenhydramine HCl solutions.

molal the magnitude of the molal osmotic coefficient and the equivalent conductance decreases sharply over a narrow concentration region. At still higher concentrations the values of these two properties approach asymptotically a constant value. According to theories of micelle formation (2, 11, 12), the behavior of the physicochemical properties of bromodiphenhydramine HCl solutions are in accord with the formation of partially ionized aggregates of approximately constant size which are in equilibrium with the simple ions.

Figures 1 and 3 indicate that the behavior of dibucaine HCl solutions is similar to those of bromodiphenhydramine HCl. The abruptness of change in the physicochemical properties at the CMC is dependent on the micellar size and the ease with which the association equilibrium is established (2). The many polar groups that are present in dibucaine HCl molecules tend to reduce the ease of molecular association. Due to this fact the change in the physicochemical properties of dibucaine HCl, at the CMC, is not as abrupt as that of bromodiphenhydramine HCl.

The behavior of the osmotic coefficient and the equivalent conductance of dilute solutions of tetracaine HCl is similar to those of procaine HCl solutions. At higher concentrations these properties decrease slowly over an extended concentration region as seen in Figs. 1 and 3. These behaviors indicate the weak aggregating tendency of the tetracaine ions which should result in slow multiple equilibria between aggregates of different sizes. Due to the same reason no CMC value could be assigned to this salt. The slight drop in the molal osmotic coefficients together with a nearly linear decrease in the equivalent conductance of diphenhydramine HCl solutions with increasing concentrations also suggests the presence of aggregates of different sizes in moderately concentrated solutions of this salt.

The molal osmotic coefficient and the equivalent conductance of aqueous pyrilamine maleate solutions decreased without any noticeable change of slope at all concentrations studied (Figs. 2 and 4). It has been previously demonstrated by Stokes (13) that maleate ions tend to form ion pairs in aqueous solutions of the sodium salt. It is, therefore, possible that at the pH of the solution pyrilamine maleate ions form ion pairs.

According to Brady and co-workers (14) the amount of gegenions bound per mole of micellar ion is given by:

$$\frac{dm^B}{dm} = 1 - 2\,\frac{d\phi m}{dm} \qquad \text{(Eq. 4)}$$

where m^B is the concentration of the bound gegenions; m is the total concentration of the solute; ϕ is the molal osmotic coefficient of the solution; and $d\phi m/dm$ is the slope of ϕm *versus* m curve at concentrations above the CMC. For dibucaine HCl, this slope is 0.115 while for bromodiphenhydramine HCl (Fig. 5) it is 0.126. Consequently, dm^B/dm for the micelles of these two salts is 0.77 and 0.75, respectively. The excessive counter ion binding indicated by these data is in the expected range. The nonionic polar groups that are present in the molecules of dibucaine HCl and bromodiphenhydramine HCl tend to decrease the aggregation properties of these salts. Consequently, if aggregation is to occur, it is to be expected that the repulsive coulombic forces which come into play when the micelle-forming ions approach each other have to be practically neutralized by the gegenions.

Assuming that micelles are monodispersed and that their formation is governed by the law of mass action, Phillips (15) has shown that the standard free energy change, $\Delta F_m°$, and the standard enthalpy change, $\Delta H_m°$, of this process are given by the following equations:

$$\Delta F_m° = RT\left(\frac{\ln 3 + 2\ln N}{N} + \frac{2N - P - 1}{N}\ln \mathrm{CMC}\right) \qquad \text{(Eq. 5)}$$

and

$$-\frac{\Delta H_m°}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) = \left(2 - \frac{P}{N} - \frac{1}{N}\right)\ln\frac{\mathrm{CMC}_1}{\mathrm{CMC}_2} \qquad \text{(Eq. 6)}$$

where R is the gas constant, N is the aggregation number, P is the charge of the micelle, and CMC_1 and CMC_2 are the critical micelle concentrations at absolute temperature T_1 and T_2. The standard state chosen is mole fraction of unity, and, therefore, the CMC's should be expressed in mole fraction units. The freezing point depression of aqueous solutions of dibucaine HCl and aqueous bromodiphenhydramine HCl have been determined by Hammarlund and co-workers (3, 16). From their data the CMC of these two salts at the freezing points of their solutions were found to be 0.060 and 0.05 M, respectively. These CMC values, when compared with those obtained at 25° indicate that in the temperature range 0–25° the CMC of dibucaine HCl and bromodiphenhydramine HCl are not appreciably affected by the increase in temperature. According to Eq. 6, therefore, the $\Delta H_m°$ of micellization for both salts is practically zero. This finding would imply that $-\Delta F_m°/T = \Delta S_m°$. Since $N > 1$ and $P > 0$ for the micelles formed by dibucaine HCl and bromodiphenhydramine HCl, it follows that their $\Delta F_m° < 0$ and $\Delta S_m° > 0$. It can, therefore, be concluded that the formation of micelles in the solutions of these two salts is practically an entropy effect. A portion of the observed entropy increase should be due to the loss of ordered water that exists around the hydrocarbon moiety of both dibucaine HCl and bromodiphenhydramine HCl ions during micellization process (17, 18). Since the solvated polar groups present in the dissolved molecules of these two salts are practically desolvated during the course of aggregation, it should be expected that this desolvation will also contribute to the total entropy change (19).

REFERENCES

(1) Ralston, A. W., and Hoerr, C. W., *J. Am. Chem. Soc.*, **64**, 772(1942).
(2) Pethica, B. A., *3rd Intern. Congr. Surface Activity, Cologne*, **1**, 212(1960).
(3) Hammarlund, E. R., and Pedersen-Bjergaard, K., *Dansk Tidsskr. Farm.*, **Suppl. 2**, 107(1956).
(4) Johnson, R. D., Goyan, F. M., and Tuck, L. D., *J. Pharm. Sci.*, **54**, 1176(1965).
(5) Burge, D. E., *J. Phys. Chem.*, **67**, 2590(1963).
(6) Lewis, G. N., and Randall, M., "Thermodynamics," 2nd ed., revised by Pitzer, K. S., and Brewer, L., McGraw-Hill Book Co., Inc., New York, N. Y., 1961.

(7) Hughes, E. A. M., "Physical Chemistry," 2nd ed., The Macmillan Co., New York, N. Y., 1961, p. 909.
(8) Fineman, M. N., and McBain, J. W., *J. Phys. Colloid. Chem.*, **52**, 881(1948).
(9) Wright, K. A., Abbott, A. D., Sivertz, V., and Tartar, H. V., *J. Am. Chem. Soc.*, **61**, 549(1939).
(10) McBain, J. W., and Johnson, S. A., *Proc. Roy. Soc., Ser. A*, **181**, 119(1942).
(11) Hutchinson, E., Ineba, A., and Bailey, L. G., *Zeit. Physik Chem., N.F.*, **5**, 344(1955).
(12) Danielson, I., *Finska Kemists Samfundets Medd.*, **72**, 90(1963).
(13) Stokes, R. H., *Trans. Faraday Soc.*, **44**, 295(1948).
(14) Brady, A. P., and Salley, D. J., *J. Am. Chem. Soc.*, **70**, 914(1948).
(15) Phillips, J. N., *Trans. Faraday Soc.*, **51**, 561(1955).
(16) Hammarlund, E. R., and Pedersen-Bjergaard, K., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 107(1958).
(17) Frank, H. S., and Evans, M. W., *J. Chem. Phys.*, **13**, 507(1945).
(18) White, P., and Benson, G. C., *J. Phys. Chem.*, **64** 559(1960).
(19) Shick, M. J., *ibid.*, **66**, 3585(1964).

Catharanthus Alkaloids XIII

Antineoplastic and Hypotensive Activity of Alkaloid Fractions and Certain Alkaloids from *Catharanthus lanceus*

By NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, and J. P. BUCKLEY

Hypotensive evaluation of 14 alkaloid fractions derived from *C. lanceus* roots and leaves in anesthetized, normotensive rats and dogs revealed that eight of the fractions reduced blood pressure from 22–73 per cent for periods ranging from 38 to more than 378 min., at doses of 8–40 mg./Kg. Yohimbine, a potent α-adrenergic blocking agent, was isolated from three of the eight active fractions. However, removal of the yohimbine from one of these fractions did not result in a loss of hypotensive activity, thus indicating that other hypotensive agents may be present. Leurosine, perivine, and pericyclivine elicited only transient hypotensive activity, whereas vindoline, tetrahydroalstonine, ajmalicine, lochnerinine, and periformyline failed to induce a hypotensive response at several dose levels. The same crude alkaloid fractions were evaluated for antineoplastic activity against the P-1534 leukemia with only one of the 14 fractions being active against this neoplasm. Leurosine, isolated from the active fraction, was shown to be a potent antineoplastic alkaloid with a high degree of cytotoxicity. Lochnerinine, although devoid of activity against the P-1534 leukemia, exhibited reproducible cytotoxicity against the 9 KB cell culture. Vindoline, catharanthine, desacetylvindoline, perivine, perivinol, periformyline, pericyclivine, pericalline, catharine, ajmalicine, tetrahydroalstonine, and yohimbine were devoid of P-1534 leukemia activity, as well as cytotoxicity. Monitoring of column chromatographic cuts of the active fraction with the P-1534 leukemia has shown that at least one additional alkaloid, active against this neoplasm, is present.

THE MADAGASCAN periwinkle, *Catharanthus roseus* (*Vinca rosea*, *Lochnera rosea*), has yielded at least 66 alkaloids as a result of recent intensive phytochemical investigations. For the most part, these alkaloids have been discovered in the search for new antineoplastic agents, and many of them were obtained in only trace quantities. In certain instances, the small quantities available precluded any determination of their biological effects. However, the authors do know of the antineoplastic activity of vincaleukoblastine, leurocristine, leurosine, and leurosidine (1); of the pronounced oral hypoglycemic effects of vindolinine (hydrochloride), leurosine (sulfate), lochnerine, vindoline, desacetylvindoline, catharanthine (hydrochloride), and tetrahydroalstonine (2); and of the diuretic effect of catharanthine (hydrochloride) and vindolinine (hydrochloride) (3, 4); as well as the antidiuretic action of ajmalicine, lochnerine, and sitsirikine (sulfate) (3, 4). In addition, the antihypertensive and sedative properties of reserpine, also reported as present in *C. roseus*, are well known.

Because of interest in the isolation of biologically active compounds from plants, investigations were initiated on species of *Catharanthus* other than *C. roseus*. The antineoplastic activity of *C. lanceus* alkaloids (5, 6) and the isolation of leurosine, an alkaloid exhibiting a high order of activity against the P-1534 leukemia in DBA/2 mice (5, 6) were previously reported. Also, it has been reported that certain *C. pusillus* alkaloid fractions elicit marked hypotensive activity in anesthetized, normotensive rats (7).

Received May 11, 1966, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pa. 15213.
Accepted for publication August 12, 1966.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
This investigation was supported by research grants H-06162, HE-03475, and CA-08228 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and the Thaw Fund, University of Pittsburgh, Pittsburgh, Pa.
Previous paper: Farnsworth, N. R., Fong, H. H. S., and Blomster, R. N., *Lloydia*, to be published.

(7) Hughes, E. A. M., "Physical Chemistry," 2nd ed., The Macmillan Co., New York, N. Y., 1961, p. 909.
(8) Fineman, M. N., and McBain, J. W., *J. Phys. Colloid. Chem.*, **52**, 881(1948).
(9) Wright, K. A., Abbott, A. D., Sivertz, V., and Tartar, H. V., *J. Am. Chem. Soc.*, **61**, 549(1939).
(10) McBain, J. W., and Johnson, S. A., *Proc. Roy. Soc., Ser. A*, **181**, 119(1942).
(11) Hutchinson, E., Ineba, A., and Bailey, L. G., *Zeit. Physik Chem., N.F.*, **5**, 344(1955).
(12) Danielson, I., *Finska Kemists Samfundets Medd.*, **72**, 90(1963).
(13) Stokes, R. H., *Trans. Faraday Soc.*, **44**, 295(1948).
(14) Brady, A. P., and Salley, D. J., *J. Am. Chem. Soc.*, **70**, 914(1948).
(15) Phillips, J. N., *Trans. Faraday Soc.*, **51**, 561(1955).
(16) Hammarlund, E. R., and Pedersen-Bjergaard, K., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 107(1958).
(17) Frank, H. S., and Evans, M. W., *J. Chem. Phys.*, **13**, 507(1945).
(18) White, P., and Benson, G. C., *J. Phys. Chem.*, **64** 559(1960).
(19) Shick, M. J., *ibid.*, **66**, 3585(1964).

Catharanthus Alkaloids XIII

Antineoplastic and Hypotensive Activity of Alkaloid Fractions and Certain Alkaloids from *Catharanthus lanceus*

By NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, and J. P. BUCKLEY

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No reports have appeared in the literature concerned with the biological activity of *C. trichophyllus*, *C. longifolius*, or *C. scitulus*, the remaining three species of the genus *Catharanthus*.

At this time the antineoplastic and hypotensive activity of alkaloid fractions derived from the roots and leaves of *C. lanceus* is reported, in addition to certain alkaloids isolated in our laboratories from this plant.

EXPERIMENTAL

Preparation of Fractions and Isolation of Alkaloids.—Seven alkaloid fractions were prepared according to the method of Svoboda (8) from *C. lanceus* root (9) and seven from leaf material (6) of Madagascan origin. Following column chromatography of certain of these fractions, a number of alkaloids were isolated by direct crystallization. The preparation of these alkaloids has previously been reported and include leurosine (6), perivine (6), yohimbine (6, 9), vindoline (6), pericyclivine (10), ajmalicine (9), perimivine (9), cathalanceine (9), catharanthine (11), pericalline (9), vincoline (11), vinosidine (12), lanceine (12), catharine (13), periformyline (14, 15), tetrahydroalstonine (14), and lochnerinine (14). Prior to these studies, Janot *et al.* had reported on the isolation of ajmalicine, tetrahydroalstonine, yohimbine, and lanceine from the roots of this plant (16–18).

Preparation of Alkaloid Fractions and Alkaloids for Biological Evaluation.—All alkaloid fractions were prepared for use in the hypotensive evaluations by first being dissolved in an appropriate organic solvent, followed by the addition of 0.1 *M* citric acid solution, and removal of the organic solvent by *in vacuo* steam distillation. After filtration, the acidic extract was adjusted to as near neutrality as possible with dilute ammonia solution without precipitation of free alkaloid bases, and then adjusted with distilled water to a known volume. Control solutions were prepared with 0.1 *M* citric acid, and ammonia solution added to a pH identical with the extract to be injected. The details for this phase of the study have been reported previously (7).

Alkaloids were administered either as their salts which were dissolved in distilled water, or as the free base treated in the manner described for the crude fractions, or as the free base dissolved in dimethylacetamide (DMAC) diluted with water.

For the antineoplastic and cytotoxicity evaluations, the extracts and alkaloids were suspended in normal saline solution for the P-1534 leukemia evaluations and either dissolved or suspended in propylene glycol for the 9 KB cell culture assays.

Hypotensive Evaluation of the Alkaloid Fractions and Alkaloids.—The prepared test solutions were administered to albino Wistar, normotensive, anesthetized rats (urethan, 1.25 Gm./Kg. i.p.), and evaluated for hypotensive activity as described by Bickerton *et al.* (19). Initially the dose was 1.0 mg./Kg. i.v., and subsequent doses adjusted accordingly in order to estimate that dose producing approximately a 50% decrease in mean arterial blood pressure. The approximate ED_{50} of each active fraction was administered to a minimum of four rats and the mean time for the blood pressure to return to the predrug levels determined. Each animal received only one dose of extract or alkaloid. Equivalent volumes of control solvent were administered i.v. to determine the effect of the pH and solvent on arterial blood pressure of anesthetized rats.

Certain of the fractions and alkaloids were further investigated for hypotensive activity in dogs anesthetized with sodium pentobarbital, 35 mg./Kg. i.v. Blood pressure was recorded from a femoral artery onto a slow moving kymograph. Pressor responses to a 10-sec. bilateral carotid occlusion (BCO), epinephrine, 1–2 mcg./Kg., and angiotensin II, 1 mcg./Kg., were obtained prior to and after administration of each fraction or alkaloid into an exposed femoral vein.

Antineoplastic Evaluation of Alkaloid Fractions and Alkaloids.—Alkaloid fractions from *C. lanceus* leaves and roots, as well as certain of the alkaloids isolated from this plant, were evaluated for activity against the P-1534 leukemia in DBA/2 mice and for

TABLE I.—HYPOTENSIVE EVALUATION OF *C. lanceus* ALKALOID FRACTIONS[a]

Fraction	Dose, mg./Kg.	Animals, No.	Drop in B.P. % (Range)	Duration of B.P. Drop (min.) (Range)	Alkaloids Isolated from Fraction
Root (A)	20–40	4	33–44	203–310+	Ajmalicine, perimivine, cathalanceine, pericalline, ammocalline, lanceine, vinosidine
Root (A_1)	20	6	28–44	28–67	Ajmalicine
Root (B)	10	2	47–56	350+	Ajmalicine, yohimbine, pericalline[b]
Root (B_1)	20–40	5	32–55	38–147+	Ajmalicine[b]
Root (C)	40	2	27–37	70–120+	Ajmalicine[b]
Root (D)	20	4	13–53	8–70	...
Root (E)	10–20	4	[c]	[c]	Ajmalicine[b]
Leaf (A)	10	6	34–52	147–305+	Ajmalicine,[b] leurosine, yohimbine, perivine, pericyclivine
Leaf (A_1)	5–20	6	[c]	[c]	Ajmalicine,[b] lochnerinine, catharine, periformyline, tetrahydroalstonine
Leaf (B)	8	6	42–73	260–378	Yohimbine, perivine
Leaf (B_1)	8–16	6	22–36	56–222+	...
Leaf (C)	20–40	5	24–36	5–7	...
Leaf (D)	20	6	45–53	112–287+	Perivine
Leaf (E)	10–20	4	0–50	2–3	Vindoline, lochnerinine[b]

[a] In normotensive rats, anesthetized with urethan (1.25 Gm./Kg. i.p.). Extracts were administered intravenously. [b] Unpublished data. [c] No hypotensive activity.

TABLE II.—EFFECTS OF CERTAIN ALKALOID FRACTIONS AND ALKALOIDS FROM *C. lanceus* ON THE BLOOD PRESSURE AND SEVERAL PRESSOR RESPONSES IN THE ANESTHETIZED DOG

Fraction	Animals, No.	Dose, mg./Kg.	Blood Pressure Drop, %	Time to Return to Predrug Levels, min.	% of Control Responses BCO[a]	Epi[b]	Ang[c]
Leaf (A)	1	5.0	[d]	[d]	62	*R*[e]	77
Leaf (B)	1	4.0	42	62	25	*R*[e]	129
Leaf (B)	2	8.0	65	196+	*B*[f]	*R*[e]	81
Leaf (B_1)	1	20.0	[d]	[d]	*B*[f]	No change	No change
Leaf (C)	1	10.0	[d]	[d]	..	...	...
Leaf (D)	1	20.0	70	133+	50	*R*[e]	No change
Root (B)	1	5.0	87	200+	*B*[f]	35	116
Leurosine sulfate	1	2.5	[d]	[d]	*B*[f]	No change	No change
Yohimbine hydrochloride	1	1.0	[d]	[d]	*B*[f]	*R*[e]	80
	2	2.5	30	85	*B*[f]	*R*[e]	67–125

[a] BCO, bilateral carotid occlusion. [b] Epi, epinephrine. [c] Ang, angiotensin II. [d] No effect on blood pressure. [e] *R*, epinephrine reversal, prolonged depressor effect. [f] *B*, response blocked.

TABLE III.—HYPOTENSIVE EVALUATION OF CERTAIN *C. lanceus* ALKALOIDS[a]

Alkaloid	Dose, mg./Kg.	Animals, No.	Drop in B.P., %	Duration of B.P. Drop, min.	Solvent
Yohimbine (HCl)	2.5	6	32–56	217–315	Water
Leurosine (H_2SO_4)	5–10	6	40–60	7–28	Water
Perivine (H_2SO_4)	20–40	6	22–54	21–56	Water
Pericyclivine (citrate)	0.25–2.0	6	11–40	1–2	Water
Pericalline	2.5–5.0	6	[b]	[c]	20% DMAC[d]
Vindoline (citrate)	1.0–20	12	[c]	[c]	Water
Tetrahydroalstonine	25–50	6	[c]	[c]	20% DMAC
Ajmalicine	25–50	6	[c]	[c]	20% DMAC
Lochnerinine (HCl)	10–25	6	[c]	[c]	Water
Periformyline	2.5–20	8	[c]	[c]	50% DMAC

[a] In normotensive rats, anesthetized with urethan (1.25 Gm./Kg. i.p.). Alkaloid solutions were administered intravenously. [b] Analeptic effect was noted. [c] No hypotensive activity. [d] Dimethylacetamide–water.

cytotoxicity against Eagle's human carcinoma of the nasopharynx (9KB) in cell culture (20). These tests were performed through the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

RESULTS AND DISCUSSION

Hypotensive Activity.—Initial screening of the 14 *C. lanceus* alkaloid fractions in normotensive, anesthetized rats served to indicate that the root (A), (B), (B_1), and (C), as well as the leaf (A), (B), (B_1), and (D) fractions, elicited moderate to marked hypotensive effects (Table I). This activity was confirmed for the leaf (B) and (D) and the root (B) fractions in the anesthetized normotensive dog (Table II).

Of 10 alkaloids evaluated, only yohimbine was shown to elicit marked hypotensive activity of long duration. Leurosine, perivine, and pericyclivine induced only a transient hypotensive effect; whereas, vindoline, tetrahydroalstonine, lochnerinine, periformyline, ajmalicine, and pericalline were inactive as hypotensive agents. Pericalline also produced marked analeptic effects. (Table III.)

Since yohimbine was found to be present in the leaf (A) and (B) fractions, as well as the root (B) fraction, it most likely contributed to the hypotensive effect of these three fractions. The following experiment was conducted to determine the role of yohimbine in the over-all hypotensive activity of one of the four active crude alkaloid fractions.

The leaf (B) fraction, from which yohimbine was isolated (6) and which elicited marked hypotensive activity of long duration (Table I), was subjected to a column chromatographic separation (6). A total of 270 one-liter fractions were eluted from the column and yohimbine was isolated from cuts 115–164. Examination of the pre-115 and the post-164 cuts by thin-layer chromatography, and use of the ceric ammonium sulfate alkaloid detecting reagent (21), revealed that yohimbine was also present in

TABLE IV.—EFFECT OF YOHIMBINE-FREE *C. lanceus* LEAF ALKALOID (B) FRACTION ON THE BLOOD PRESSURE OF ANESTHETIZED NORMOTENSIVE RATS

Sex	Wt., Gm.	Dose, mg./Kg.	Normal Blood Pressure, mm. Hg	% Change	Time to Return to Normal, min.
M	294	10	80	62	180+[a]
M	265	10	82	58	179+[b]
M	410	10	92	47	166+[c]
F	199	20	84	57	203+[b]
F	165	20	112	33	209
F	153	20	106	39	192+[d]

[a] Blood pressure at termination of experiment was 60 mm. Hg. [b] Blood pressure at termination of experiment was 70 mm. Hg. [c] Blood pressure at termination of experiment was 78 mm. Hg. [d] Blood pressure at termination of experiment was 88 mm. Hg.

cuts 97–114 and 165–192. On the basis of this information, cuts 1–96 and 193–270 were combined and taken to dryness to yield a yohimbine-free alkaloid mixture. The mixture was then prepared for hypotensive evaluation as previously described.

In the normotensive, anesthetized rat, at doses of 10 and 20 mg./Kg., this yohimbine-free alkaloid (B) fraction retained its marked and prolonged hypotensive effects (Table IV). Equivalent activity of shorter duration was observed with the yohimbine-free fraction in the anesthetized dog at 8 mg./Kg. A 33% decrease in arterial blood pressure, which persisted for 32 min., decreasing the bilateral carotid occlusion response by 32%, reversing the epinephrine response (epinephrine produced a depressor effect after administration of the yohimbine-free fraction), was elicited by the fraction. The mechanism of action for the hypotensive alkaloid(s) in this fraction also appeared to be that of α-adrenergic blockade.

Although yohimbine was present in three of the active hypotensive fractions, and it must be considered to be one of the major alkaloids of *C. lanceus*, evidence has been presented suggesting that other hypotensive alkaloid(s) are present in this plant.

Antineoplastic Activity.—The data presented in Table V show that the total antineoplastic activity in *C. lanceus* resides in the leaf (A) alkaloid fraction. Leurosine, previously reported only from *C. roseus* by Svoboda (22, 23), was isolated in our studies

TABLE V.—ACTIVITY OF *C. lanceus* ALKALOID FRACTIONS AGAINST THE P-1534 LEUKEMIA

Source	Fraction	Dose, mg./Kg.	Survivors, ( ) of ( )	Animal Wt. Diff. (T/C)	Survival (Days) (T/C)	% (T/C)[a]
Leaf	A	25.0	6/6	0.5	18.0/14.0	128
		12.5	6/6	−0.5	24.0/14.0	171
		6.25	6/6	0.2	21.0/14.0	150
		3.13	6/6	0.7	20.0/14.0	142
		3.13	6/6	−0.9	17.0/13.0	130
		1.56	6/6	−0.7	15.0/13.0	115
		0.78	6/6	0.1	14.0/13.0	107
		0.39	6/6	−0.9	13.5/13.0	103
Leaf	A_1	Inactive at doses of 3.13–100.0 mg./Kg.				
Leaf	B	Inactive at doses of 3.13–50.0 mg./Kg.				
Leaf	B_1	Inactive at doses of 25.0–200.0 mg./Kg.				
Leaf	C	Inactive at doses of 6.25–50.0 mg./Kg.				
Leaf	D	Inactive at doses of 1.56–50.0 mg./Kg.				
Leaf	E	Inactive at doses of 25.0–200.0 mg./Kg.				
Root	A	Inactive at doses of 3.13–50.0 mg./Kg.				
Root	A_1	Inactive at doses of 3.13–100.0 mg./Kg.				
Root	B	Inactive at doses of 25.0–50.0 mg./Kg.				
Root	B_1	Inactive at doses of 3.13–100.0 mg./Kg.				
Root	C	Inactive at doses of 6.35–50.0 mg./Kg.				
Root	D	Inactive at doses of 3.13–50.0 mg./Kg.				
Root	E	Inactive at doses of 25.0–100.0 mg./Kg.				

[a] Evaluation of assay results by CCNSC is such that a material is considered active if it causes a prolongation of life in excess of 125%.

TABLE VI.—ACTIVITY OF *C. lanceus* ALKALOIDS AND RELATED COMPOUNDS AGAINST THE P-1534 LEUKEMIA AND 9KB CELL CULTURE

	P-1534 Activity					9KB Cell Culture
Alkaloid	Dose, mg./Kg.	Survivors, ( ) of ( )	Animal Wt. Diff. (T/C)	Survival (Days) (T/C)	% (T/C)[a]	ED_{50},[b] mcg./ml.
Leurosine sulfate	3.0	5/6	−1.0	36.5/14.0	260	$<1.0 \times 10^{-5}$
	1.5	6/6	−0.1	25.5/14.0	182	
	0.75	6/6	−0.9	20.5/14.0	146	
	0.75	6/6	−0.5	21.5/15.0	143	
	0.375	6/6	−1.0	18.5/15.0	123	
	0.188	6/6	−0.1	15.5/15.0	103	
	0.094	6/6	−0.4	14.5/15.0		
Lochnerinine	Inactive at doses of 3.75–30.0 mg./Kg.					$<1.0 \times 10^{-2}$
Vindoline	Inactive at doses of 7.5–60.0 mg./Kg.					5.9×10^{1}
Desacetylvindoline[c]	Inactive at doses of 3.75–30.0 mg./Kg.					NT[e]
Perivine (H_2SO_4)	Inactive at doses of 12.5–100.0 mg./Kg.					NT[e]
Perivinol[d]	Inactive at doses of 7.5–60.0 mg./Kg.					2.6×10^{1}
Periformyline	Inactive at doses of 3.13–25.0 mg./Kg.					$>1.0 \times 10^{2}$
Pericyclivine	Inactive at doses of 7.5–60.0 mg./Kg.					3.6×10^{1}
Pericalline	Inactive at doses of 0.13–1.0 mg./Kg.					NT[e]
Catharine	Inactive at doses of 50 mg./Kg.					2.7×10^{1}
Ajmalicine	Inactive at doses of 20.0–320.0 mg./Kg.					$>1.0 \times 10^{2}$
Tetrahydroalstonine	Inactive at doses of 100 mg./Kg.					$>1.0 \times 10^{2}$
Yohimbine (HCl)	Inactive at doses of 0.31–40.0 mg./Kg.					2.7×10^{1}
Catharanthine (HCl)	Inactive at doses of 2.5–20.0 mg./Kg.					2.3×10^{1}

[a] A rating of 125% or more is considered active. [b] A rating of ED_{50} = 4.0 mcg./ml. or less is considered active. [c] Prepared from vindoline. [d] Prepared from perivine. [e] Not tested.

TABLE VII.—ACTIVITY OF CHROMATOGRAPHIC CUTS FROM *C. lanceus* LEAF (A) ALKALOIDS AGAINST THE P-1534 LEUKEMIA IN MICE

Fraction[a]	Dose, mg./Kg.	Survivors, ( ) of ( )	Animal Wt. Diff. (T/C)	Survival (Days) (T/C)	% (T/C)[b]	Alkaloids Isolated from Fraction
1–179	NT[c]	...	...	...	...	Ajmalicine
180–355	30.0	5/6	−1.4	14.0/14.0	100	Pericyclivine
356–629	30.0	6/6	−0.8	37.5/15.0	250	Leurosine, perivine yohimbine
	15.0	6/6	−0.7	21.5/15.0	143	
	7.5	6/6	−0.0	22.0/15.0	153	
	3.75	6/6	−0.4	17.0/15.0	113	
630–740	30.0	6/6	−2.0	20.0/15.0	133	None
	15.0	6/6	−1.0	17.0/15.0	113	
	7.5	6/6	−0.6	16.0/15.0	106	
741–824	30.0	6/6	−0.8	33.0/15.0	220	None
	15.0	6/6	−0.5	24.0/15.0	160	
	7.5	6/6	0.4	17.5/15.0	116	
825–915	30.0	6/6	−0.1	14.0/15.0	93	None
	15.0	6/6	−0.1	15.0/15.0	100	
	7.5	6/6	0.4	13.0/15.0	86	

[a] One hundred grams of (A) fraction was chromatographed on 3 Kg. of partially deactivated Alcoa F-20 alumina as previously described (6). Eluate was collected in 1-L. fractions which were combined on the basis of thin-layer chromatographic patterns. [b] Evaluation of assay results by CCNSC is such that a material is considered active if it causes a prolongation of life in excess of 125%. [c] Not tested. Quantity insufficient for proper evaluation.

from this (A) fraction by means of column chromatography and has been shown to have a high order of activity against the P-1534 leukemia in mice (Table VI). In order to establish whether additional active alkaloids might be present in the leaf (A) fraction, selected grouped cuts from a chromatographic separation were monitored for activity against the P-1534 leukemia (Table VII). These results appear to support the conclusion that at least one additional alkaloid is present in cuts 741–824 from the column.

To date, 18 alkaloids have been isolated from *C. lanceus* in our laboratories. Twelve of these have been evaluated for antineoplastic activity against the P-1534 leukemia in mice and only leurosine is considered to be active (Table VI). These alkaloids have also been evaluated for cytotoxicity against Eagle's carcinoma of the nasopharynx (9KB cell culture), and lochnerinine, in addition to leurosine, is considered to be active, although the former alkaloid was found to be inactive against the P-1534 leukemia (Table VI).

SUMMARY

This investigation has been concerned with the evaluation of 14 crude alkaloid fractions derived from *C. lanceus* leaves and roots for hypotensive and antineoplastic activity. Several alkaloids isolated in our laboratories from this plant were similarly evaluated. From these studies, the following conclusions were made.

1. Eight of 14 crude alkaloid fractions elicited moderate to marked hypotensive activity in normotensive, anesthesized rats. Three of the fractions exhibited a similar activity in the anesthetized dog.

2. Of 10 alkaloids evaluated, yohimbine was found to be the most active as a hypotensive agent. Leurosine, perivine, and pericyclivine gave only a transient hypotensive effect, whereas vindoline, tetrahydroalstonine, ajmalicine, lochnerinine, and periformyline were inactive at the dose levels utilized.

3. Evidence has been presented that other highly active hypotensive alkaloids are present in crude alkaloid extracts from this plant. The identity of these alkaloids is presently unknown.

4. Only one of 14 alkaloid fractions from *C. lanceus* was active against the P-1534 leukemia in mice and leurosine was shown to be one of the alkaloids responsible for the activity of the fraction. Monitoring of chromatographic cuts of the active fraction against the P-1534 leukemia has shown that at least one additional active alkaloid remains in this plant.

5. Leurosine and lochnerinine were shown to have reproducible cytotoxic activity against Eagle's 9 KB carcinoma of the nasopharynx.

6. Perivine, periformyline, pericyclivine, vindoline, ajmalicine, tetrahydroalstonine, catharine, catharanthine, yohimbine, pericalline, desacetylvindoline, and perivinol were shown to be devoid of antineoplastic as well as cytotoxic activity.

REFERENCES

(1) Johnson, I. S., *et al.*, *Cancer Res.*, **23**, 1390(1963).
(2) Svoboda, G. H., Gorman, M., and Root, M. A., *Lloydia*, **27**, 361(1964).
(3) Svoboda, G. H., Gorman, M., and Tust, R. H., *ibid.*, **27**, 203(1964).
(4) Gorman, M., *et al.*, *ibid.*, **27**, 214(1964).
(5) Farnsworth, N. R., Loub, W. D., and Blomster, R. N., *J. Pharm. Sci.*, **52**, 111(1963).
(6) Loub, W. D., *et al.*, *Lloydia*, **27**, 470(1964).
(7) Fylypiw, W. M., *et al.*, *ibid.*, **28**, 354(1965).
(8) Svoboda, G. H., Neuss, N., and Gorman, M., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 659(1959).
(9) Blomster, R. N., *et al.*, *Lloydia*, **27**, 480(1964).
(10) Farnsworth, N. R., Loub, W. D., Blomster, R. N., and Gorman, M., *J. Pharm. Sci.*, **53**, 1558(1964).
(11) Farnsworth, N. R., *et al.*, *Lloydia*, to be published.
(12) Blomster, R. N., Farnsworth, N. R., and Abraham, D. J., *J. Pharm. Sci.*, to be published.
(13) Abraham, D. J., Farnsworth, N. R., and Blomster, R. N., *ibid.*, to be published.
(14) Maloney, E. M., *et al.*, *ibid.*, **54**, 1166(1965).
(15) Abraham, D. J., *et al.*, *Tetrahedron Letters*, **1965**, 317.
(16) Janot, M.-M., and LeMen, J., *Compt. Rend.*, **239**, 1311(1954).
(17) Janot, M.-M., LeMen, J., and Hammouda, Y., *Ann. Pharm. Franc.*, **14**, 341(1956).
(18) Janot, M.-H., LeMen, J., and Gabbai, Y., *ibid.*, **15**, 474(1957).
(19) Bickerton, R. K., Jacquert, M. L., Kinnard, W. J., Jr., Biamculli, J. A., and Buckley, J. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 183(1960).
(20) *Cancer Chemotherapy Rept.*, **25**, 1(1962).
(21) Farnsworth, N. R., *et al.*, *Lloydia*, **27**, 302(1964).
(22) Svoboda, G. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 834(1958).
(23) Svoboda, G. H., Johnson, I. S., Gorman, M., and Neuss, N., *J. Pharm. Sci.*, **51**, 707(1962).

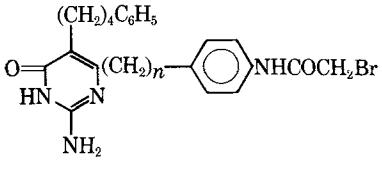
Irreversible Enzyme Inhibitors LXX

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase IV. Substituted 1-Phenyl-1,2-dihydro-*s*-triazines

By B. R. BAKER and BENG-THONG HO

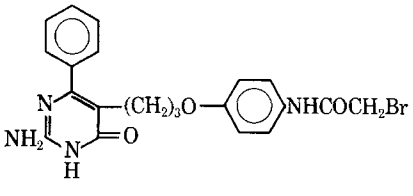
4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazines substituted on the phenyl ring by *p*-chloroacetyl (IV*a*), *m*-chloroacetyl (IV*c*), and 4-chloro-3-butanone-1-yl (IV*b*) groups were synthesized from the appropriate aromatic amine hydrochlorides, cyanoguanidine, and acetone. All three compounds were good reversible inhibitors of dihydrofolic reductase. However, none of the three compounds inactivated dihydrofolic reductase when incubated with the enzyme; this lack of active-site-directed irreversible inhibition by compounds of type IV has been attributed to the complexing of the phenyl group to the hydrophobic bonding region on dihydrofolic reductase—a region not apt to have attackable nucleophilic groups present.

SEVERAL active-site-directed irreversible inhibitors (1–3) of dihydrofolic reductase were successfully designed once the strong hydrophobic bonding area on the enzyme was discovered (4) and its effect on the mode of pyrimidine binding was explored (1, 5–10). When a hydrocarbon-type 5-side-chain was allowed to complex to the hydrophobic bonding region on the enzyme, the



I, $n = 2$

II, $n = 4$



III

6-side-chain of I and II could project to a hydrophilic area on the enzyme and form a covalent bond within the enzyme–inhibitor complex (11); similarly, the 6-phenyl group of III could complex to the hydrophobic region of dihydrofolic reductase with the result that the 5-side-chain projected into the hydrophilic area and formed a covalent bond with the enzyme (12).

Received August 5, 1966, from the Department of Chemistry, University of California, Santa Barbara, 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo 14214.

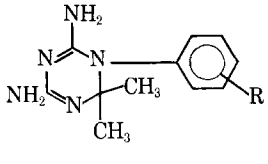
Accepted for publication September 27, 1966.

This investigation was supported by grants CA-05867, CA-06624, and CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

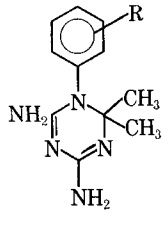
Previous paper: Baker, B. R., and Santi, D. V., *J. Pharm. Sci.*, unpublished data.

DISCUSSION

Prior to the synthesis and enzymic evaluation of I–III, over 30 candidate irreversible inhibitors for dihydrofolic reductase failed to inactivate the enzyme; most of these failures can now be rationalized on the basis that these heterocycles with a single large side chain bearing a covalent-forming group had their side chain complexed with the hydrophobic region of the enzyme where there was not apt to be a nucleophilic group on the enzyme that could be covalently linked with the inhibitor. One such unsuccessful class was derived from the 1-phenyl-1, 2-dihydro-*s*-triazine (IV) where the covalent forming group, R, was placed on the phenyl ring; these candidates failed since the dihydro-*s*-triazines presumably assumed a different conformation, IV*B*, rather than IV when complexed to the enzyme, due to the strong hydrophobic bonding of the phenyl group being determinate (10).



IV



IV*B*

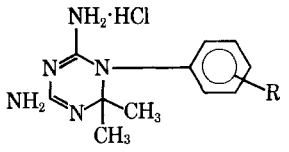
a, R = *p*-$COCH_2Cl$

b, R = *p*-$(CH_2)_2COCH_2Cl$

c, R = *m*-$COCH_2Cl$

The enzymic evaluations of IV*a–c* are summarized in Table I; a successful active-site-directed irreversible inhibitor [II (11)] is included for comparison. Note that IV*a* and IV*c* are still excellent reversible inhibitors of dihydrofolic reductase even though they are $^1/_{12}$ and $^1/_{17}$ as effective as the unsubstituted 1-phenyl-*s*-triazine (V). This decrease in binding by IV*a* and IV*c* can now be rationalized from a study on the mode of phenyl binding (6) subsequent to the evaluation of IV*a* and *c*. The phenyl group is most probably complexed to a hydrophobic region on the enzyme; it was previously

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Reversible[a] μM Concn. for 50% Inhibition	Reversible[a] Estimated $K_i \times 10^6$ M[c]	Irreversible[b] μM Concn.	Irreversible[b] % EI[d]	Irreversible[b] Time, min.	Irreversible[b] Inactivation, %
IV*a*	*p*-$COCH_2Cl$	1.2	0.2	1	83	60	0
IV*b*	*p*-$(CH_2)_2COCH_2Cl$	0.025	0.004	0.020	83	60	0
IV*c*	*m*-$COCH_2Cl$	1.7	0.3	1	77	60	0
V	H	0.11[e]	0.02				
VI	*p*-C_4H_9-*n*	0.064[f]	0.01				
VII[g]	*m*-CH_2OH	0.43	0.07				
II[h]		240	40	40	50	18	50
				10	20	25	43

[a] The dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol, as previously described (13). [b] Dihydrofolic reductase was incubated in the absence of TPNH with the inhibitor at 37° in 0.05 M Tris buffer (pH 7.4) containing *no* mercaptoethanol as previously described (14); in each case an enzyme control was run that showed 0–4% thermal inactivation. Similar results with IV*a–c* were obtained in the presence of 12 μM TPNH. [c] Estimated from $K_i = I \times K_m/S$, where I = inhibitor concentration giving 50% inhibition (4, 15); this equation is valid since $S = 6\ K_m > 4\ K_m$. [d] Calculated from $[EI] = [E_t]/[1 + \frac{K_i}{I}]$, where $[E_t]$ = the concentration of total active enzyme, $[EI]$ = the fraction of E_t reversibly complexed by I (1, 16), and I = the inhibitor concentration. [e] Data from *Reference 17*. [f] Data from *Reference 6*. [g] Hydrobromide salt. [h] Data from *Reference 11*.

noted that polar groups on the *m*- or *p*-position of the phenyl (IV) such as *m*-hydroxymethyl (VII, Table I), cyano, carboxylate, carbethoxy, and aminomethyl cause a decrease in binding, presumably by repulsion of these polar groups on the inhibitor from the hydrophobic region of the enzyme (6). Since the carbonyl group of IV*a* and *c* is highly polar, it too could be repulsed; the relative polarity of the C=O group can be calculated to have a Hansch π-constant of −1.1 from the constants for $CH_3C{=}O$ ($\pi = -0.55$) and CH_3 ($\pi = +0.56$).[1] The 4-chloro-3-butanone-1-yl side chain of IV*b* was not repulsed and actually gave additional binding; note that IV*b* is about twice as good a reversible inhibitor than is the *p*-(*n*-butyl) phenyl-*s*-triazine (VI).

When IV*a–c* were incubated at 37° with dihydrofolic reductase at concentrations sufficient to reversibly complex 77–83% of the total enzyme, no inactivation occurred (Table I). In contrast, the irreversible inhibitor (II) gave 50% inactivation in about 18 min. when 50% of the enzyme was reversibly complexed (11). Furthermore, at a concentration of 10 μM, where II reversibly complexes 20% of the total enzyme, 43% inactivation in 25 min. was seen; the 10 μM concentration of II is only 10 times the incubation concentrations of IV*a* and *c*.

CHEMISTRY

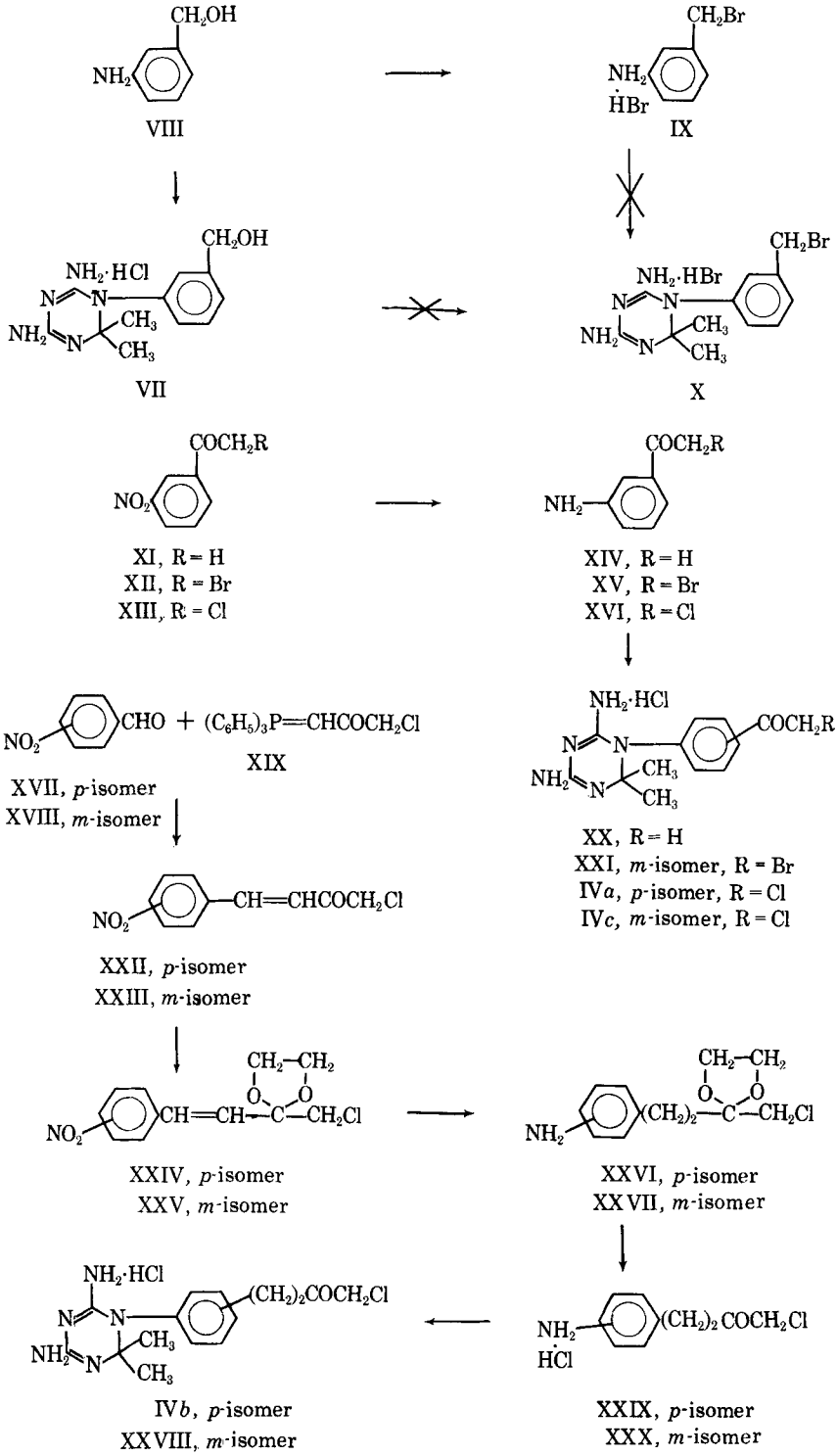
Methods

The first candidate active-site-directed irreversible inhibitor for dihydrofolic reductase chosen in this series was the *m*-bromomethylphenyl-*s*-triazine (X). *m*-Aminobenzyl alcohol (VIII) (19) was smoothly converted to the corresponding benzyl bromide, isolated as its hydrobromide salt (IX) in 76% yield, with hot 10% anhydrous hydrogen bromide in acetic acid. When attempts were made to convert IX to a dihydro-*s*-triazine (X) by the three component method of Modest using cyanoguanidine and acetone (20), a gummy, intractable (polymeric?) mass rapidly formed; apparently these conditions were not compatible with the high chemical reactivity of the benzylic bromide. The benzyl alcohol (VIII) was readily convertible to the *m*-hydroxymethylphenyl-*s*-triazine (VII) by the three component method (20) in 78% yield; unfortunately, treatment of VII with 10% anhydrous hydrogen bromide in acetic acid to give X—the type of conditions used for the conversion of VIII to IX—cleaved the triazine ring as shown by changes in the ultraviolet spectrum. Treatment of VII with anhydrous hydrogen bromide in acetic acid or 48% aqueous hydrobromic acid, both at 25°, gave oily mixtures.

Attention was therefore directed to the synthesis of the *m*-bromoacetylphenyl-*s*-triazine (XXI). Reduction of the nitro group of *m*-nitro-α-bromoacetophenone (XII) with copper powder in sulfuric acid also caused reduction of the halogen and led to *m*-aminoacetophenone (XIV) rather than the desired *m*-amino-α-bromoacetophenone (XV); these conditions had been used for the conversion of the corresponding α-chloro ketone (XIII) to *m*-amino-α-chloroacetophenone (21). The desired *m*-amino-α-bromoacetophenone (XV) was prepared by bromination of *m*-aminoacetophenone (XIV) in glacial acetic acid containing an excess of ethanesulfonic acid which protonated the amine and, in turn, stabilized the ring against halogenation; the product (XV) was isolated as the hydrobromide salt but

[1] The Hansch π-constant (18) is a measure of hydrophobic character on a log scale, where H is assigned $\pi = 0$.

direct bromination of XIV in acetic acid containing hydrogen bromide failed because of the insolubility of the hydrobromide salt of XIV. Attempts to convert XV to the dihydro-*s*-triazine, XXI, by the three component method (20) rapidly formed a brownish gummy (polymeric?) mass.



Scheme I

Since the attempts to prepare the dihydro-*s*-triazines (X and XXI) containing an active bromine appeared to fail because of the excessive chemical reactivity of this halogen, attention was directed toward the less reactive chloroacetyl-*s*-triazines (IV*a* and *c*). Commercially available *p*-amino-α-chloroacetophenone readily formed the requisite *p*-chloroacetylphenyldihydro-*s*-triazine (IV*a*) by the three component method (20) in 88% yield. Similarly, *m*-amino-α-chloroacetophenone (21) formed the *m*-chloroacetylphenyldihydro-*s*-triazine (IV*c*) in 42% yield. Although the *m*-acetylphenyldihydro-*s*-triazine (XX) was readily prepared from *m*-aminoacetophenone, bromination experiments to convert XX to XXI were discontinued when IV*a* and *c* were successfully synthesized.

4 - (*p* - Aminophenyl) - 1 - chloro - 2 - butanone hydrochloride (XXIX) was prepared in crystalline form by hydrolysis of the previously prepared dioxolane (XXVI) (22). Condensation of XIX with cyanoguanidine and acetone by the three component method (20) afforded the chlorobutanonyl-*s*-triazine (IV*b*) in 67% yield. The corresponding *m*-aminophenyl dioxolane (XXVII) was prepared *via* XXIII and XXV. The hydrochloride salt of XXX formed after acid hydrolysis of XXVII could not be purified; furthermore, the *s*-triazine (XXVIII) obtained from impure XXX by the three component method (20) also could not be purified. (Scheme I.)

Synthesis

Melting points were taken on a Fisher-Johns apparatus and those below 230° were corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 recording spectrophotometer.

m-Aminobenzyl Bromide Hydrobromide (IX).—A solution of 0.550 Gm. (4.47 mmoles) of VIII (19) in 6 ml. of 10% anhydrous hydrogen bromide in glacial acetic acid was refluxed for 8 hr. The solution was cooled to about 20°; the product was collected on a filter and washed with a small amount of glacial acetic acid, then ether; yield, 0.91 Gm. (76%) of an off-white solid, m.p. 244° dec., with browning at 230°; $\nu_{max.}$ 2950, 2650 (NH, NH^+); 1600, 1575, 1550, 1525 (NH, NH^+, C=C); 885, 783 $cm.^{-1}$ (*m*-C_6H_4).

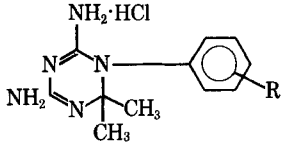
Anal.—Calcd. for $C_7H_8BrN \cdot HBr$: C, 31.5; H, 3.40; N, 5.25. Found: C, 31.6; H, 3.50; N, 5.40.

α-Chloro-m-nitroacetophenone (XIII).—To a solution of 1.65 Gm. (10 mmoles) of XI in 15 ml. of chloroform was added 2 ml. of sulfuryl chloride. After 24 hr. protected from moisture, the solution was spin-evaporated *in vacuo;* the crystalline residue was triturated with ether; yield, 1.34 Gm. (67%), m.p. 97–99°. Recrystallization from petroleum ether (b.p. 60–110°) with only slight loss gave light yellow crystals, m.p. 101–103°.

This procedure is considerably simpler than the direct treatment of molten XI with chlorine gas (23). [Lit. (23) m.p. 103°.]

m - Amino - α - bromoacetophenone (XV) Hydrobromide.—A mixture of 540 mg. (7 mmoles) of XIV, 5 ml. of glacial acetic acid, and 0.65 (8 mmoles) of ethanesulfonic acid was warmed to complete solution, then cooled to room temperature. After the addition of 0.21 ml. of bromine, the solution was magnetically stirred for 12 hr. with ultraviolet light irradiation. Some of the hydrobromide salt separated during this time. The mixture was treated with 3 ml. of 30% anhydrous hydrogen bromide in acetic acid, then diluted with about 5 vol.

TABLE II.—PHYSICAL PROPERTIES OF



Compd.[a]	R	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
IV*a*	*p*-$COCH_2Cl$	88	197–199[b]	C, 44.8[c] H, 5.50 N, 20.1	C, 44.7 H, 5.47 N, 19.9
					C, 44.8 H, 5.51 N, 20.3
IV*b*	*p*-$(CH_2)_2COCH_2Cl$	81	204–206[b]	C, 50.3 H, 5.91 N, 19.5	C, 49.9 H, 5.89 N, 19.2
IV*c*	*m*-$COCH_2Cl$	43[d]	174–176	C, 47.3 H, 5.19 N, 21.2	C, 47.0 H, 5.21 N, 21.0
VII	*m*-CH_2OH	78	191–192[e,f]	C, 43.9[f] H, 5.53 N, 21.3	C, 43.7 H, 5.51 N, 21.4
XX	*m*-$COCH_3$	41	195–197[f,g]	C, 46.0 H, 5.34 N, 20.6	C, 46.4 H, 5.10 N, 20.4

[a] All compounds had ultraviolet and infrared spectra in agreement with their assigned structures. [b] Recrystallized from methanol–ether. [c] Monohydrate. [d] Yield after crystallization of the amorphous product from methanol–ether. [e] Recrystallized from absolute ethanol–ether. [f] Hydrobromide salt; 4 mmoles of aromatic amine was reacted with cyanoguanidine in acetone containing 4 mmoles 48% aqueous hydrobromic acid. [g] Recrystallized from glacial acetic acid–ether.

of ether. The product was collected and washed with ether; yield, 608 mg. (52%); the compound gradually decomposes above 190° without definite melting. Recrystallization of 100 mg. from absolute ethanol–ether gave 50 mg. of beige crystals with unchanged melting behavior; $\lambda_{max.}$ 2950–2850, 2600 (NH, NH^+); 1700 (C=O); 1625, 1600, 1550, 1500 (NH^+, C=C); 798 $cm.^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_8H_8BrNO \cdot HBr$: C, 32.6; H, 3.08; N, 4.75. Found: C, 32.3; H, 2.96; N, 4.61.

Chloromethyl m-Nitrostyryl Ketone (XXIII).—A mixture of 0.705 Gm. (2 mmoles) of XIX (24) and 0.378 Gm. (2.5 mmoles) of *m*-nitrobenzaldehyde in 18 ml. of benzene was refluxed 18 hr., then spin evaporated *in vacuo*. The residue was triturated with petroleum ether, then recrystallized from absolute alcohol to give 0.333 Gm. (74%) of light yellow crystals, m.p. 120–121°; $\nu_{max.}$ 1700 (C=O); 1625, 1575 (C=C); 1525, 1340 $cm.^{-1}$ (NO_2).

Anal.—Calcd. for $C_{10}H_8ClNO_3$: C, 53.2; H, 3.57; N, 6.21. Found: C, 53.3; H, 3.70; N, 6.00.

2 - Chloromethyl - 2 - (m - nitrostyryl) - 1,3-dioxolane (XXV).—A mixture of 4.52 Gm. (20 mmoles) of XXIII, 35 ml. of benzene, 10 ml. of ethylene glycol, and 40 mg. of *p*-toluenesulfonic acid was refluxed under a Dean-Stark trap for 18 hr. The solution was washed with water, then spin evaporated *in vacuo*. Trituration of the residue with ether gave 5.2 Gm. (97%) of a pale yellow solid, m.p. 87–89°. Recrystallization from absolute ethanol gave 5.07 Gm. (94%) of pale yellow crystals, m.p. 90–91°; $\nu_{max.}$ 1530, 1350 (NO_2); 1040 (C—O—C); no C=O near 1700 $cm.^{-1}$.

Anal.—Calcd. for $C_{12}H_{12}ClNO_4$: C, 53.4; H, 4.49; N, 5.19. Found: C, 53.7; H, 4.81; N, 5.15.

Catalytic reduction of XXV as previously described for XXIV (22) proceeded smoothly to XXVII which was obtained as an oil that moved as a single spot on thin-layer chromatography (22); its ultraviolet spectrum showed that the double bond had been reduced.

4 - (p - Aminophenyl) - 1 - chloro - 2 - butanone Hydrochloride (XXIX).—A solution of 600 mg. (2.5 mmoles) of XXVI (22) in 6 ml. of ethanol and 6 ml. of 1 *N* aqueous hydrochloric acid was refluxed for 2 hr., then spin evaporated *in vacuo*. The solid residue was triturated with acetone, then collected on a filter and washed with ether; yield, 290 mg. (50%) of a beige-white solid, m.p. 160–162°; $\nu_{max.}$ 3450 (NH), 2850, 1950, 1570 (NH^+); 1730 (C=O); 1615, 1500 (C=C); 840, 810 $cm.^{-1}$ (*p*-C_6H_4). No suitable solvents for recrystallization could be found.

A similar hydrolysis of XXVII gave a gummy product that showed a C=O bond at 1730 $cm.^{-1}$, but could not be crystallized.

1 - (p - Chloroacetylphenyl) - 4,6 - diamino - 1,2-dihydro - 2,2 - dimethyl - s - triazine Hydrochloride (IVa).—A mixture of 848 mg. (5 mmoles) of *p*-amino-α-chloroacetophenone (Eastman), 450 mg. (5.35 mmoles) of cyanoguanidine, 3 ml. of acetone, and 0.42 ml. (5 mmoles) of 1 *N* aqueous hydrochloric acid was refluxed with magnetic stirring. Solution was complete when the b.p. was reached, then within 10 min. the product began to separate. An additional 5 ml. of acetone was added, then the mixture was refluxed for 17 hr. After 1 hr. at 0°, the mixture was filtered and the product was washed with acetone; yield, 1.46 Gm. (88%), m.p. 200–202°. Recrystallization from methanol–ether gave 587 mg. (36%) of white crystals, m.p. 198–199°; $\nu_{max.}$ 3450, 3350, 3200 (NH); 1700 (C=O); 1670, 1640, 1600, 1550, 1540, 1520, 1500 (NH, C=NH^+, C=N, C=C); 830 $cm.^{-1}$ (*p*-C_6H_4); $\lambda_{max.}$ (H_2O): 247 mμ (ϵ 17,200). See Table II for analytical data and additional compounds prepared by this method (20), except the second addition of acetone was omitted.

REFERENCES

(1) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site," John Wiley & Sons, Inc., New York, N. Y., to be published.
(2) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(3) Baker, B. R., *Cancer Chemotherapy Rept.*, **4**, 1(1959).
(4) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(5) Baker, B. R., and Jordaan, J. H., *ibid.*, **54**, 1740(1965).
(6) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(7) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(8) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
(9) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).
(11) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417(1966).
(12) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422(1966).
(13) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(14) Baker, B. R., and Jordaan, J. H., *ibid.*, **2**, 21(1965).
(15) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(16) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(17) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **53**, 1137(1964).
(18) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(19) Phillips, A. P., and Maggiolo, A., *J. Org. Chem.*, **15**, 659(1950).
(20) Modest, E. J., *ibid.*, **21**, 1(1956).
(21) Catch, J. R., *et al.*, *J. Chem. Soc.*, **1949**, 552.
(22) Baker, B. R., and Jordaan, J. H., *J. Med. Chem.*, **8**, 35(1965).
(23) Baker, J. W., *J. Chem. Soc.*, **1931**, 2416.
(24) Hudson, R. F., and Chopard, P. A., *J. Org. Chem.*, **28**, 2446(1963).

Irreversible Enzyme Inhibitors LXXI

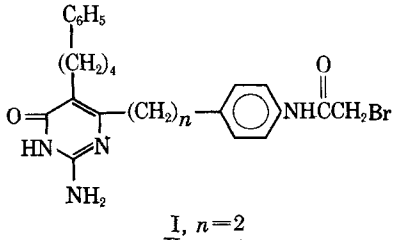
Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase V. Derivatives of 6-Phenylpyrimidine

By B. R. BAKER and HOWARD S. SHAPIRO

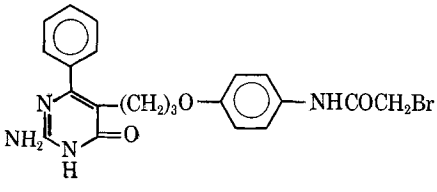
A series of 2-amino-5-anilinopropyl-6-phenyl-4-pyrimidinols bearing *p*-chloroacetyl (V*a*), *p*-(4-chloro-3-butanon-1-yl) (V*b*), *p*-(4-chloro-1-buten-3-one-1-yl) (V*c*), and *p*-bromoacetamidoethyl (IV*d*) groups on the anilino moiety were synthesized as candidate active-site-directed irreversible inhibitors of dihydrofolic reductase. Only V*b* inactivated dihydrofolic reductase when they were incubated together at 37°; the half-life was about 60 min. when sufficient V*b* was utilized to convert 45 per cent of the enzyme to a reversible complex. That this inactivation by V*b* proceeded through a reversible enzyme–inhibitor complex and not by a random bimolecular mechanism was indicated by the lack of inactivation of dihydrofolic reductase by chloroacetone at 2.5 times the concentration of V*b*. The failure of IV*d* and V*e* to inactivate the enzyme at a concentration sufficient to convert 50–60 per cent of the enzyme to the respective reversible complexes indicated that the alkylating function of IV*d* and V*e* could not bridge to a nucleophilic site on the enzyme within the enzyme–inhibitor complex.

IN ORDER for a compound to be an active-site-directed irreversible enzyme inhibitor the following requirements must be met (1, 2): (*a*) a reversible complex between the compound and the enzyme must form; (*b*) the proper positioning of a leaving group on the inhibitor must be attained that does not interfere with reversible complex formation; (*c*) the leaving group on the inhibitor—when complexed to the enzyme—must be able to bridge to a nucleophilic group on the enzyme surface with resultant covalent bond formation *via* a neighboring group reaction.

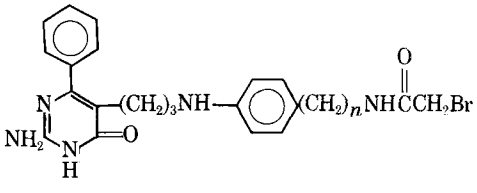
An unusual amount of difficulty was encountered for the successful design of an active-site-directed irreversible inhibitor of dihydrofolic reductase; although studies on requirements (*a*) and (*b*) appeared to follow the normal course of the *modus operandi* for the design of these irreversible inhibitors (1, 2), factor (*c*) was particularly difficult until the strong hydrophobic bonding region of dihydrofolic reductase was discovered (3–8) and utilized properly (9, 10).



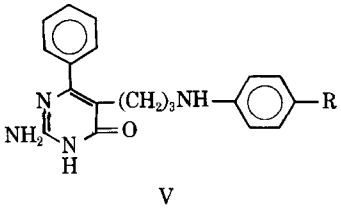
I, $n=2$
II, $n=4$



III



IV
series *d*, $n=2$



V

a, R = —$COCH_2Cl$
b, R = —$(CH_2)_2COCH_2Cl$
c, R = —$CH=CH-COCH_2Cl$

DISCUSSION

Successful candidates for active-site-directed irreversible inhibitors were achieved by complexing the hydrophobic region of the enzyme by the 5-phenylbutyl group of I and II (9) or the 6-phenyl group of III (10) which then positioned their bromoacetamido groups in a nucleophilic area of the enzyme. In order for I–III to irreversibly

Received August 5, 1966, from the Department of Chemistry, University of California, Santa Barbara, 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo 14214.

Accepted for publication September 19, 1966.

This work was generously supported by grants CA-05865, CA-06624, and CA-08695, from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

Previous paper: Baker, B. R., and Ho B.-T., *J. Pharm. Sci.*, **56**, 28(1967).

inhibit dihydrofolic reductase, it was apparent that the leaving bromo group was properly juxtaposed to a nucleophilic site on the enzyme, thus forming a covalent bond within the enzyme–inhibitor complex. That this bridge distance (1, 2, 11, 12) between the binding locus for the 6-phenylpyrimidine and the leaving group on the inhibitor was an important factor in active-site-directed irreversible inhibition of dihydrofolic reductase was noted with compounds of types IV and V; three of the four compounds of type IV and V were at least as good reversible inhibitors as III, but only one inactivated the enzyme *via* a neighboring group reaction within the enzyme–inhibitor complex. Of the compounds in series IV, only the synthesis of $n = 2$ was completed—for reasons that will be discussed later. Three compounds of series V were synthesized.

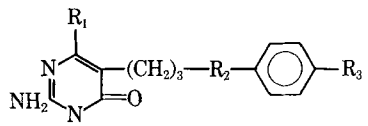
The enzymic evaluation of these compounds are summarized in Table I. Compounds IV*d* and V*c*, at a concentration sufficient to convert 50–60% of the enzyme to a reversible complex, failed to show inactivation of dihydrofolic reductase in 60 min. at 37°; in contrast, III showed 50% inactivation in 45 min. at 37° with sufficient inhibitor to convert only 25% of the enzyme to the reversible complex (10). The chloromethyl ketones have about the same chemical reactivity toward thiosulfate as bromoacetamides and iodoacetamides (14); furthermore, the chloromethyl ketones can attack the same nucleophilic groups on a protein that are attacked by bromoacetamides. Therefore, the failure of IV*d* and V*c* to inactivate the enzyme is most probably due to the lack of juxtaposition of their respective leaving group with an enzymic nucleophilic group within the enzyme–inhibitor reversible complex. Stated another way, the respective leaving group of the inhibitors apparently cannot bridge to an enzymic nucleophilic group within the reversible complex (1, 2, 11, 12, 18).

Due to lack of solubility, the amount of reversible inhibition by the chloroketone (V*a*) could not be determined; nevertheless, V*a* was investigated at its limit of solubility (20 μM) as an irreversible inhibitor, but with negative results that could be due to either a lack of reversible complex formation or a lack of proper bridging.

Only one of the 6-phenyl-4-pyrimidinols in Table I showed inactivation of the enzyme in addition to the previously examined III (10); the chlorobutanone derivative (V*b*), when incubated at 37° with dihydrofolic reductase at a concentration to convert 45% of the enzyme to the reversible complex, showed inactivation of the enzyme with a half-life of about 60 min. This result was first attributed to a random bimolecular inactivation of the enzyme without complex formation (2, 16), since 1-chloro-4-phenyl-3-*p*-tolylsulfonamido-2-butanone (VII, Table I) (19, 20) showed no reversible inhibition of the enzyme, but could inactivate the enzyme even more rapidly than V*b*. The fallacy of this experiment became apparent when the lack of reversible inhibition by VII ($K_i > 13 \times 10^{-5} M$) was converted to the maximum amount of reversible complex that could be formed at a concentration of 40 μM; note that as much as 25% of the enzyme could have been reversibly complexed with VII.

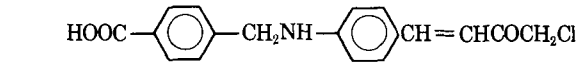
In a later study on inhibitors that might complex only with the hydrophobic region of dihydrofolic reductase then bridge to and alkylate a nucleophilic group on the enzyme, the benzoic acid derivative

TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_1	R_2	R_3	Reversible[a] $[I/S]_{0.5}$[c]	Reversible[a] $K_i \times 10^5$ M[d]	Irreversible[b] μM Concn.	Irreversible[b] EI, %[e]	Irreversible[b] Time, min.	Irreversible[b] Inactivation, %[f]
III[g]	C_6H_5	O	$-NHCOCH_2Br$	130	13	40	25	45	50
IV*d*	C_6H_5	NH	$-(CH_2)_2NHCOCH_2Br$	20	2.0	30	60	120	0
V*a*	C_6H_5	NH	$-COCH_2Cl$	>13	>1.3	20	<60	60	0
V*c*	C_6H_5	NH	$-CH=CHCOCH_2Cl$	200[h]	20	200	50	60	0
V*b*	C_6H_5	NH	$-(CH_2)_2COCH_2Cl$	50	5.0	40	45	60	50
VI	CH_3	NH	$-(CH_2)_2COCH_2Cl$	20[i]	2.0	50	71	120	<10
VII	$C_6H_5CH_2CHCOCH_2Cl$			>130[j]	>13	40	<25	35	50
	$NHSO_2C_6H_4CH_3-p$								
VIII	Chloroacetone					100		120	0

[a] Dihydrofolic reductase was a 45–90% ammonium sulfate fraction isolated from pigeon liver; the reversible inhibition studies were run with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 10% N,N-dimethylformamide as previously described (13). [b] The dihydrofolic reductase was incubated at 37° with the candidate irreversible inhibitor in 0.05 M Tris buffer (pH 7.4) containing *no* mercaptoethanol and *no* TPNH, but diluted with 10% N,N-dimethylformamide as previously described (14). [c] Ratio of concentration of inhibitor to 6 μM dihydrofolate giving 50% inhibition. [d] Calculated from $K_i = K_m \times [I/S]_{0.5}$ where $K_m = 1 \times 10^{-6} M$; this equation is valid if $S > 4\ K_m$ (3, 15). [e] Calculated from $[EI] = \frac{[E_t]}{1 + K_i + I}$, where $[EI]$ = the fraction of $[E_t]$ in a reversible enzyme–inhibitor complex and E_t = the total amount of active enzyme (2, 16); the rate of active-site-directed inactivation is dependent upon the fraction of $[E_t]$ in the complex, $[EI]$ (2, 16). [f] In all cases an enzyme control with no inhibitor was run as a control and either II or III was run as a standard simultaneously; 0–4% thermal inactivation of the enzyme control occurred and the per cent inactivation by the inhibitor was corrected for thermal inactivation. [g] Data from *Reference 10*. [h] Estimated from 27% inhibition at 0.4 mM, the maximum concentration allowing full light transmission. [i] Data from *Reference 17*. [j] No inhibition was observed at 0.2 mM, the maximum solubility; since 20% inhibition is readily detectable, the concentration for 50% inhibition is >0.8 mM (13).



IX

(IX) was investigated (21); at a concentration of IX sufficient to reversibly complex 50% of the dihydrofolic reductase, IX inactivated the enzyme, but some related compounds did not inactivate the enzyme. Therefore, chloroacetone was investigated for inactivation; at a concentration of 100 μM, chloroacetone showed no inactivation of dihydrofolic reductase in 120 min. at 37°, in contrast to V*b* and VII which showed 50% inactivation at 40 μM in 50 and 35 min., respectively. If either had inactivated the enzyme by the bimolecular mechanism, then chloroacetone should have also; *ergo*, neither V*b* nor VII inactivate by the bimolecular mechanism, but presumably inactivate through a complex. For example, if the K_i of the tosylamido ketone (VII) were 20×10^{-5} M—which is greater than 13×10^{-5} M [Table I (VII)]—the amount of reversible complex with 40 μM of VII would be 16% of the total enzyme; this could still give a reasonable rate of inactivation *via* the reversible complex.

Although the K_i of VII could not be determined due to its lack of solubility, it should be possible to determine K_i by observing the effect of concentration of inhibitor on the rate of inactivation (2, 22); as a first approximation, one should also see a "rate-saturation" effect (2, 16) if the inactivation proceeds through a complex. If the tosylamido ketone (VII) is weakly complexed to dihydrofolic reductase, it is most likely through hydrophobic bonding as seen with IX. Even though such studies with VII could be of scientific interest, they were off the main stream of the research goals of this laboratory (1, 2), and were, therefore, not pursued further. Thus, the main evidence that the chlorobutanone (V*b*) inactivates dihydrofolic reductase by the active-site-directed mechanism (1, 2) is the fact that chloroacetone at over twice the concentration of V*b* shows no inactivation. Furthermore, the corresponding chlorobutanone attached to a 6-methylpyrimidinol (VI) (17) shows barely detectable inactivation at a concentration sufficient to convert 71% of the enzyme to a reversible complex. This result further supports the concept (2, 8–10) that the 6-phenyl group of V*b* is complexed to the hydrophobic region of dihydrofolic reductase which then projects the 5-side-chain bearing the alkylating function into a polar nucleophilic region of the enzyme; in contrast, the 6-methylpyrimidine (VI) probably has its 5-side-chain bearing the alkylating function complexed to the hydrophobic region of the enzyme which is not apt to have polar nucleophilic groups present. The results clearly eliminate the possibility of a random bimolecular mechanism (1, 2) for inactivation and give strong support to the concept that 6-phenylpyrimidines and 6-methylpyrimidines complex to the enzyme in different rotomeric configurations (2, 8, 23)—else both V*b* and VI should have inactivated the enzyme at the same rate for the same percentage of reversible E···I complex.

Similar to the effect of the phenylbutyl pyrimidine (II) as an irreversible inhibitor (9), the rate of inactivation by the chlorobutanone (V*b*) and the tosylamidoketone (VII) was slowed by the presence of TPNH; whether or not I, II, III, V*b*, and VII all attack the same amino acid—presumably in the TPNH binding region of the enzyme—must await sequence experiments after inactivation of pure enzyme, a problem for specialists in protein sequence research.

CHEMISTRY

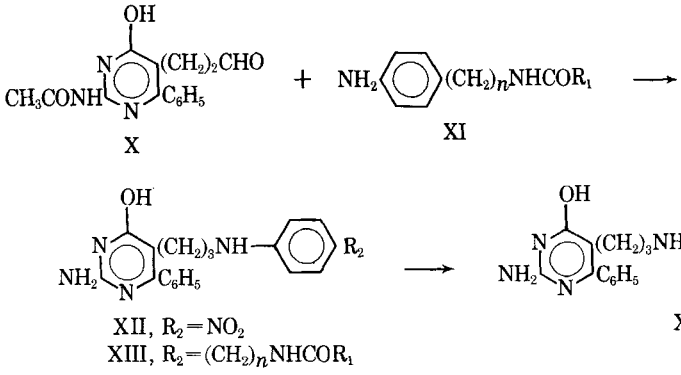
Methods.—Two general routes to 2-amino-6-R-4-pyrimidinols bearing a *p*-substituted anilinopropyl side chain at the 5-position such as IV and V have been developed (24–29). The first route involved reductive condensation of a substituted aniline with a pyrimidine-5-propionaldehyde such as X where the substituted anilino moiety is introduced last (24–26); the second route introduced the substituted anilinopropyl group at the first step (27–29). The first route was chosen for compounds such as IV and V since it is superior if a number of *p*-substituted anilino moieties are required.

Reductive condensation of X with *p*-aminoacetanilide with sodium borohydride in a mixture of *N,N*-dimethylformamide and methanol gave XIII*a* in 21% yield of what appeared to be pure material; although XIII*a* had the proper spectral characteristics and was uniform on thin-layer chromatography, it gave erratic results on combustion analyses. Furthermore, attempts to acid hydrolyze XIII*a* to XIV*a* gave intractable, unstable materials that could not be purified due in part to the air sensitivity of the *p*-phenylenediamine moiety. The alternate route (27–29) was not investigated since catalytic reduction of XII or its 6-methyl analog (27) could also lead to unstable, intractable materials. This problem was ultimately solved by using the more stable 5-(*p*-aminophenoxypropyl) side chain (30) which subsequently resulted in the synthesis and enzymic evaluation of the active-site-directed irreversible inhibitor (III) (10).

The higher homologs of XIII having more parafinic character proved to be easier to purify and gave satisfactory combustion results. Reductive condensation of X with *N*-(*p*-aminobenzyl) acetamide (XI*b*) (32) using the sodium borohydride procedure gave a 55% yield of XIII*b*. Unfortunately, attempts to remove the *N*-acetyl blocking group of XIII*b* with acid or base under a variety of conditions failed to give any isolatable *p*-aminomethylanilinopropyl pyrimidinol (XIV*b*); the *p*-aminomethylanilino system was unstable to such extended treatment and appeared to undergo a reverse Mannich reaction with formation of ammonia (or ammonium chloride) and formaldehyde which led to polymerization (Scheme I).

Reductive condensation of benzyl *N*-(*p*-aminobenzyl)urethan (XI*c*) with X using sodium borohydride in methanol did proceed, but the carbobenzoxy group of XIII*c* was transesterified to carbomethoxy; whether such a condensation could be performed in neutral or slightly acidic medium to avoid transesterification was not ascertained. An intermediate such as XIII*c* could well lead to the desired XIV*c* by cleavage of the carbobenzoxy group with hydrogen bromide in acetic acid.[1]

[1] By this time the synthesis and enzymic evaluation of III had led to an active-site-directed irreversible inhibitor (10); therefore, this sequence was not pursued further.



X XI

XII, $R_2 = NO_2$
XIII, $R_2 = (CH_2)_nNHCOR_1$

XIV

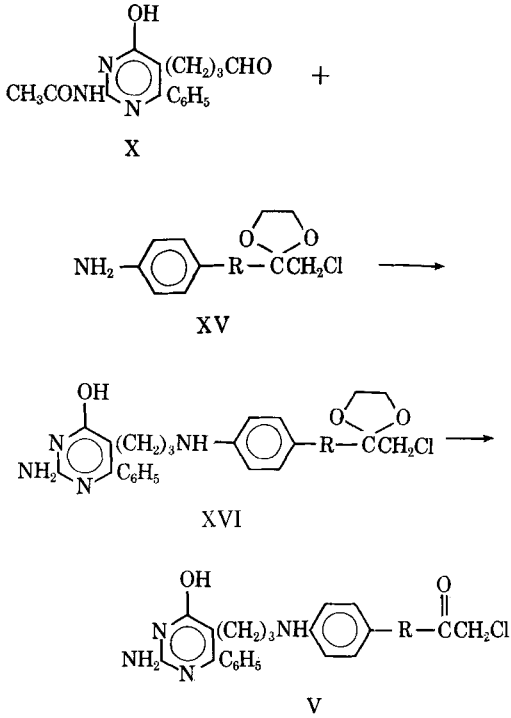
IV
series *a*, $n=0$, $R_1=CH_3$
b, $n=1$, $R_1=CH_3$
c, $n=1$, $R_1=OCH_2C_6H_5$
d, $n=2$, $R_1=CH_3$
e, $n=4$, $R_1=C_6H_5$

Scheme I

The reductive condensation of *N* - (*p* - aminophenethyl)acetamide (XI*d*) (32) with X using sodium borohydride proceeded smoothly to an 83% yield of XIII*d*. Hydrolysis of XIII*d* with hot 10% sodium hydroxide gave a 70% yield of the *p*-aminoethylanilinopropyl-4-pyrimidinol (XIV*d*), isolated as its monohydrochloride. The bromoacetylation of XIV*d* to the candidate irreversible inhibitor (IV*d*) with bromoacetic anhydride has been described previously in a paper on selective methods for bromoacylation of polyfunctional amides (31).

Reductive condensation of *N*-(*p*-aminophenylbutyl)benzamide (XI*e*) (33) with X also proceeded satisfactorily, an 85% yield of XIII*e* being obtained.[1]

Three 6-phenylpyrimidinols (V) bearing a chloromethyl ketone group in the *p*-position of 5-anilinopropyl moiety were synthesized by the route used for the corresponding 6-methyl pyrimidines (17). Condensation of the appropriate *p*-substituted aniline bearing a *p*-chloromethylketone function blocked as a dioxolane (XV) were used to reductively aminate the pyrimidine-5-propionaldehyde (X); the blocked derivatives (XVI) were obtained in 47–80% yields. Hydrolysis of the dioxolane with hot 0.25 *N* 25% aqueous alcoholic hydrochloric acid gave the candidate active-site-directed irreversible inhibitors V*a–c* in 49–76% yields of pure material (Scheme II).



X

XV

XVI

V

series *a*, R = —
b, R = —$(CH_2)_2$—
c, R = —CH=CH—

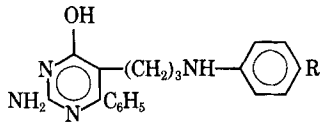
Scheme II

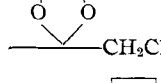
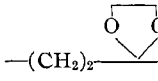
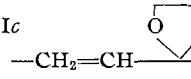
EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in a KBr pellet with a Perkin-Elmer 137B recording spectrophotometer. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 recording spectrophotometer. Thin - layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by visual examination under ultraviolet light.

2 - Amino - 5 - (p - acetamidoethylanilinopropyl) - 6 - phenyl - 4 - pyrimidinol (XIIId).—*Method A*.—A solution of 100 mg. (0.351 mmole) of X (25) and 264 mg. (1.40 mmoles) of XI*d* (32) in 1 ml. of *N*,*N*-dimethylformamide was magnetically stirred for 20 min., then diluted with 10 ml. of methanol and treated portionwise with 150 mg. of sodium borohydride over a period of about 15 min. (24–26). After being stirred for about 18 hr., the mixture was spin evaporated *in vacuo* to near dryness. The residue was dissolved in 10 ml.

TABLE II.—PHYSICAL AND ANALYTICAL DATA OF



Compd.	R	Method	M.p., °C.	Yield, %[b]	Anal. Calcd.	Anal. Found	Ultraviolet Spectra[a] $\lambda_{max.}$ (mμ)	$\epsilon \times 10^{-3}$	pH
XIII*a*	$NHCOCH_3$	*A*[c]	>275	21[d-f]			239	24.5	1
							275	11.7	1
					N, 18.6[e]	N, 18.5	267	17.8	13
XIII*b*	$-CH_2NHCOCH_3$	*A*[g]	243–246	55[h]	C, 67.5	C, 67.7	276	10.3	1
					H, 6.39	H, 6.50	238[i]	21.8	13
					N, 17.9	N, 17.9	289	8.3	13
XIII*d*	$-(CH_2)_2NHCOCH_3$	*A*[j]	243–246	83[h]	C, 68.2	C, 68.0	277	8.5	1
					H, 6.67	H, 6.62	293	6.8	13
					N, 17.3	N, 17.5			1
XIII*e*	$-(CH_2)_4NHCOC_6H_5$	*A*[g]	223–226	85[h]	C, 72.7	C, 72.6	275	8.5	1
					H, 6.72	H, 6.59	292	7.6	13
					N, 14.1	N, 14.2			
XIV*d*	$-(CH_2)_2NH_2 \cdot HCl$[l]	*B*[m]	285–288 dec.	70[d]	C, 63.1	C, 63.0			
					H, 6.51	H, 6.61			
					N, 17.5[k]	N, 17.4			
XVI*a*		*A*[g]	189–191	51[h]	C, 62.6	C, 62.3	280	13.7	1
					H, 5.67	H, 5.75	250	21.0	13
					N, 12.7	N, 12.2	285[i]	8.7	13
XVI*b*		*A*[g]	222–224 dec.	80[n]	C, 61.7[o]	C, 62.0	275	10.1	1
					H, 6.37	H, 6.20	290	10.1	13
					N, 11.5	N, 11.4			
XVI*c*		*A*[g]	218–221 dec.	47[d]	C, 63.1[p]	C, 63.0	257	21.1	1
					H, 5.88	H, 5.51	280[i]	14.8	1
					N, 11.7	N, 11.4	291	24.7	13
V*a*	$-COCH_2Cl$	*C*	220 dec.	76[d]	C, 63.6	C, 63.8	282	19.6	0
					H, 5.29	H, 5.61	295	11.1	13
					O, 8.07[q]	O, 8.10	352	23.8	13
V*b*	$-(CH_2)_2COCH_2Cl$	*C*	201–203 dec.	49[d]	C, 65.0	C, 65.3	280	9.5	1
					H, 5.89	H, 5.83	245	19.0	13
					O, 7.54[q]	O, 7.79[q]	293	9.1	13
V*c*	$-CH{=}CHCOCH_2Cl$	*C*	220–225 dec.	60[h]	C, 65.3	C, 65.3	285	21.6	1
					H, 5.44	H, 5.78	287	11.4	13
					O, 7.57[q]	O, 7.76[q]	412	16.5	13

[a] In 10% ethanol. [b] Yield of analytically pure material; all compounds had infrared spectra compatible with their structures. [c] Ratio of XI:X = 5. [d] After recrystallization from aqueous 2-methoxyethanol. [e] Erratic CH values were obtained presumably due to difficulty in combustion. [f] The compound moved as a single spot on TLC in 3:1 benzene–methanol. [g] Ratio of XI:X = 1. [h] After recrystallization from aqueous ethanol. [i] Inflection. [j] Ratio of XI:X = 4. [k] Cl, Calcd.: 8.89. Found: 8.75. [l] For conversion to IV*d* see *Reference 31*. [m] See under *Experimental*. [n] Crystallized directly from reaction mixture; collected and washed with methanol. [o] Calcd. for monohydrate. [p] Calcd. for hemihydrate. [q] Oxygen, the nitrogen could not be completely combusted.

of water, then adjusted to about pH 8 with 3 *N* aqueous hydrochloric acid. After being cooled for several hours at 0°, the mixture was filtered and the white solid was washed with water. Recrystallization from aqueous ethanol gave 118 mg. (83%) of white crystals, m.p. 243–246°; $\lambda_{max.}$ 2.89, 3.00 (NH); 6.05, 6.29, 6.60 (NH, C=C, C=N, C=O); 12.25 (*p*-C_6H_4); 13.20, 14.3 μ (C_6H_5). For analytical data see Table II.

2 - Amino - 5 - (p - aminoethylanilinopropyl)-6 - phenyl - 4 - pyrimidinol Hydrochloride (XIVd).—*Method B.*—A solution of 1.00 Gm. (2.50 mmoles) of XIII*d* in 25 ml. of 10% aqueous sodium hydroxide was heated on a steam bath for 3 hr. The hot solution was clarified by filtration, then cooled in an ice-bath and adjusted to about pH 7 with 3 *N* hydrochloric acid. The white precipitate was collected on a filter, washed with water, then recrystallized from aqueous 2-methoxyethanol; yield, 0.636 Gm. (70%) of white crystals, m.p. 285–288° dec. $\lambda_{max.}$ 2.96 (NH); 6.01, 6.10, 6.30, 6.60 (NH, C=C, C=N, C=O); 12.24 (*p*-C_6H_4); 14.33 μ (C_6H_5). See Table II for analytical data.

2 - Amino - 5 - [p - (4 - chloro - 3 - oxo - 1 - buten-1 - yl)anilinopropyl] - 6 - phenyl - 4 - pyrimidinol (Vc).—*Method C.*—A solution of 200 mg. (0.428 mmole) of XVI*c* in 16 ml. of ethanol and 5.5 ml. of 1 *N* aqueous hydrochloric acid was refluxed for 1 hr., then diluted with 21 ml. of water. While being kept below 10° in an ice-bath, the stirred solution was made slightly alkaline by the dropwise addition of 10% aqueous sodium hydroxide. The product was collected on a filter and washed with water; yield, 162 mg. (90%) of orange solid, m.p. 220–225° dec. Recrystallization from warm ethanol by addition of water gave 108 mg. (62%) of orange crystals of unchanged m.p. $\lambda_{max.}$ 2.90 2.98 (NH); 6.05, 6.10, 6.30, 6.40, 6.59 (NH, C=O, C=C, C=N); 12.33 (*p*-C_6H_4); 14.3 μ (C_6H_5). See Table II for analytical data.

REFERENCES

(1) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(2) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N. Y., to be published.
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(8) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).
(9) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417(1966).
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(11) Baker, B. R., *J. Med. Chem.*, **5**, 654(1962).
(12) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).
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(16) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
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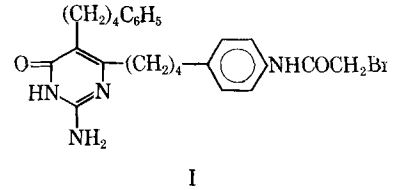
Irreversible Enzyme Inhibitors LXXII

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase VI. Derivatives of Hydrophobically Bonded *p*-Alkyl and *p*-Aralkyl Benzoic Acids

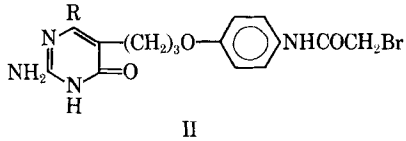
By B. R. BAKER, THOMAS J. SCHWAN, and BENG-THONG HO

Since *p*-(bromobutyl)benzoic acid (IV), *N*-(*m*-aminobenzyl)-*p*-aminobenzoic acid (V), and *p*-benzylbenzoic acid (VI) showed good hydrophobic bonding to dihydrofolic reductase (6), four candidate active-site-directed irreversible inhibitors related to structures IV–VI were synthesized that contained a terminal alkylating function. Two of the candidates, *p*-(4-bromoacetamidobutyl)benzoic acid (IX) and *N*-(*m*-bromoacetamidobenzyl)-*p*-aminobenzoic acid (X), lost their ability to form a reversible complex with the enzyme. In contrast, α-(*p*-chloroacetylanilino)-*p*-toluic acid (XI) and α-[*p*-(4-chloro-1-buten-3-one-1-yl)anilino]-*p*-toluic acid (XII) formed reasonably good reversible complexes with dihydrofolic reductase and could inactivate the enzyme; chloroacetone under comparable conditions showed no inactivation, thus affording strong evidence that XI and XII inactivated the enzyme *via* a neighboring-group reaction within a reversible complex with the enzyme.

THE DISCOVERY of hydrophobic bonding to dihydrofolic reductase (1) with its conformational implications on the mode of binding of pyrimidines (2–8) soon led to the proper design of active-site-directed irreversible inhibitors of this enzyme (9–11). These successful candidates, such as I (9) and II*a* (10), were designed on the principle that the 5-phenylbutyl group of I could complex to the hydrophobic region on dihydrofolic reductase which then projected the 6-side-



I



II

a, R = C_6H_5
b, R = CH_3

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Accepted for publication September 27, 1966.
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REFERENCES

(1) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
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Irreversible Enzyme Inhibitors LXXII

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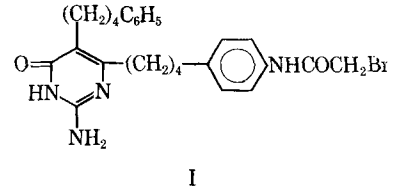
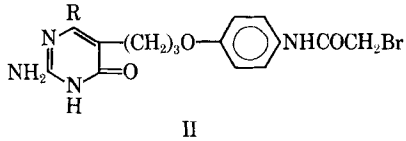


I



II

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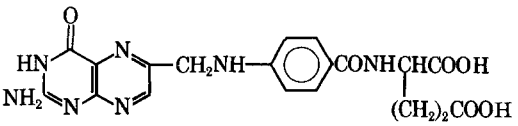
chain into a hydrophilic area on the enzyme, then formed a covalent bond (9); similarly, the 6-phenyl group of II*a* could complex with the hydrophobic region, thus projecting the 5-side-chain bearing an alkylating group into a nucleophilic region of the enzyme (10). In contrast, the candidate irreversible inhibitor (II*b*) with a 6-methyl group failed to inactivate the enzyme (12), since the 5-side-chain bearing the alkylating group was probably complexed to the hydrophobic region of the enzyme where nucleophilic groups are not apt to be present (2, 8, 11). Thus, over 30 candidate irreversible inhibitors failed to inactivate dihydrofolic reductase for the same reasons that III failed; some of these unsuccessful attempts—prior to knowledge of hydrophobic bonding to dihydrofolic reductase—have been described (11–15).

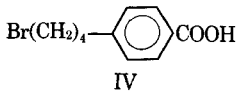
DISCUSSION

During the time that synthetic methods for structures such as I (16–19) and II*a* (10, 19) were being devised, a parallel investigation of candidate irreversible inhibitors of dihydrofolic reductase was embarked upon that utilized hydrophobic bonding in a different way. One of the studies on dihydrophobic bonding (6) sought to answer the question of where the hydrophobic bonding region on the enzyme was located with respect to the binding area for the substrate, dihydrofolic acid, or its inhibitor, folic acid (III). The results of this study suggested that the hydrophobic bonding region was near either the 4-position or 8-position of folic acid when it was complexed to the enzyme; furthermore, the hydrophobic region was not in between the pyrimidyl and *p*-aminobenzoyl-L-

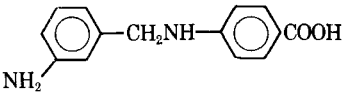


III

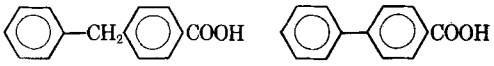
glutamate moieties when folic acid (III) was complexed to the enzyme. As part of the evidence for these deductions, it was observed that *p*-alkyl, *p*-aralkyl, or *p*-aryl groups on benzoic acid, such as IV–VII, could hydrophobically bind to dihydrofolic reductase. It was therefore reasoned that if a



IV



V

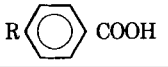


VI VII

terminal chloromethyl ketone or bromoacetamido group were attached to molecules such as IV–VII these alkylating functions might project into a hydrophilic area on the enzyme when the inhibitors were complexed to the hydrophobic region of the dihydrofolic reductase. In Table I are summarized the enzyme data on inhibitors of this type.

Since the *p*-bromobutyl group of IV showed hydrophobic bonding, the corresponding *p*-(4-bromoacetamidobutyl)benzoic acid (IX) was synthesized as a candidate irreversible inhibitor. Unfortunately, IX showed no reversible inhibition,

TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Reversible[a] I_{50}[c]	Reversible[a] Estimated[d] $K_i \times 10^4$ *M*	Irreversible[b] m*M* Concn.	Irreversible[b] *EI*,[e] %	Irreversible[b] Time, min.	Irreversible[b] Inactivation, %
VIII	H	>300[f]	>500				
IV	$Br(CH_2)_4$	6.2[f]	10				
IX	$BrCH_2CONH(CH_2)_4$—	>24	>40				
V	*m*-$NH_2C_6H_4CH_2NH$—	0.76[f]	1.3				
VI	*p*-$C_6H_5CH_2$—	10[f]	17				
VII	*p*-C_6H_5	6.7[f]	11				
X	*m*-$BrCH_2CONHC_6H_4CH_2NH$—	>20	>33				
XI	*p*-$ClCH_2COC_6H_4NHCH_2$—	0.5[g]	0.83	0.20	70	120	35
XII	*p*-$ClCH_2COCH{=}CHC_6H_4NHCH_2$—	1.2[h]	2.0	0.20	50	120[i]	95
XIII	Chloroacetone			0.10		60	0

[a] The dihydrofolic reductase was a 45–90% ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described (20). [b] Dihydrofolic reductase was incubated at 37° with the inhibitor in Tris buffer (pH 7.4) as previously described; in each case an enzyme control showed 0–4% inactivation. [c] I_{50} = millimolar concentration necessary for 50% inhibition. [d] Estimated from $K_i = K_m\,(I/S)$ where $K_m = 1 \times 10^{-6}$ *M*, *I* = inhibitor concentration, and *S* = dihydrofolate concentration; this equation is usually valid when $S > 4\ K_m$, since $S = 6\ K_m$ (1, 21). [e] Calculated from $[EI] = [E_t]/(1 + K_i/[I])$ where E_t = total enzyme, $[EI]$ = fraction of enzyme reversibly complexed, and *I* = inhibitor concentration (2, 22). [f] Data from *Reference 6*. [g] Estimated from 17% inhibition observed at 0.1 m*M*, the highest concentration allowing full light transmission. [h] Estimated from 34% inhibition observed at 0.6 m*M*, the highest concentration allowing full light transmission. [i] Intermediate times were not run to determine if 95% inactivation was reached sooner.

greater than fourfold loss in reversible binding occurring compared to IV; therefore, IX was not further investigated. One of the best benzoic acid derivations showing hydrophobic bonding was the *m*-aminobenzylamino derivative, V (6); when V was converted to its bromoacetyl derivative (X), reversible binding was again lost. Apparently the planar amide group of IX and X are not tolerated within the enzyme–inhibitor complex; furthermore, the amide group is quite polar and may still be in the vicinity of the hydrophobic region of dihydrofolic reductase. Therefore, the chloromethyl ketones (XI and XII) were synthesized and evaluated.

The α-chloroacetophenone derivative (XI) was one of the best reversible inhibitors of the *p*-substituted benzoic acid type with $K_i = 8 \times 10^{-5}$ *M*. When 0.2 m*M* of XI was incubated with dihydrofolic reductase at 37°, 35% inactivation occurred in 2 hr.; this concentration of XI is sufficient to convert 70% of the dihydrofolic reductase to a reversible complex.

Although XII with its longer *p*-group was not so good a reversible inhibitor as XI, XII was a better irreversible inhibitor; at a concentration of 0.2 m*M*, XII converts 50% of the available enzyme to a reversible complex, and then showed 95% inactivation in 2 hr. at 37°. Note that chloroacetone at 0.1 m*M* failed to show any inactivation in 1 hr. at 37°; if XI and XII had inactivated dihydrofolic reductase by a random bimolecular mechanism (2, 22), then XI, XII, and chloroacetone should have inactivated the enzyme at near the same rate; these differences in rates are taken as strong evidence that the inactivation of XI and XII occurs through a complex by the active-site-directed mechanism (2, 22).

Whether XI and XII alkylate the same amino acid on dihydrofolic reductase that is attacked by the pyrimidines (I and II*a*) must await experiments on a purified enzyme—a problem for protein structure chemists.

CHEMISTRY

Methods

When *p*-bromopropylbenzoic acid (XIV) (6, 23) was treated with 2.5 mole ratio of sodium cyanide in *N*,*N*-dimethylformamide, considerable polymeric material was found; presumably, the carboxylate anion of XIV displaced the bromine atom by intermolecular attack. When the ratio of sodium cyanide was increased to 6:1, the reaction proceeded more smoothly to give *p*-cyanobutylbenzoic acid (XV) in 60% yield with considerably less formation of polymer. Hydrogenation of the cyano group in alcohol containing a 2:1 ratio of hydrochloric acid (24) in the presence of platinum oxide proceeded smoothly to the hydrochloride of XVI in quantitative yield which was suitable for further transformation, but was characterized as the free base. Reaction of XVI hydrochloride as its anion in aqueous acetone containing two equivalents of sodium hydroxide with *p*-nitrophenyl bromoacetate (19) gave the candidate irreversible inhibitor (IX) in 38% yield; the purified IX moved as a single spot on TLC and gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (19).

In order to synthesize X, a number of blocking groups for the secondary amine group of XVII—such as tosyl—were investigated. Since these blocked derivatives gave a number of unexpected problems, the selective bromoacylation of XVII was investigated; a primary amino group should be more reactive than a secondary amino group and in addition this secondary amino group of XVII should be further deactivated by the electron-withdrawing *p*-carboxyl group (19). Reaction of XVII (6) with one equivalent of bromoacetic anhydride in acetone at 0° (19) gave the desired candidate irreversible inhibitor, X, in 71% yield; further purification gave a compound that moved as a single spot on TLC and gave a negative Bratton-Marshall test for primary aromatic amines, but gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (19)—thus showing that the bromoacetyl group had reacted with the primary amino group of XVII to give X.

Since the aminophenyl dioxolanes (XVIII and XIX) were readily available from another study (12), their reductive condensation with *p*-formylbenzoic acid was investigated. Reductive condensation of XVIII in methanol with the aid of sodium borohydride afforded XX in 69% yield; similarly, the styryl dioxolane (XIX) afforded XXI in 48% yield. Hydrolysis of the dioxolane blocking group of XX with boiling 65% ethanol containing 0.05 *N* hydrochloric acid afforded the active-site-directed irreversible inhibitor (XI) in 67% yield; similarly, hydrolysis of XXI gave XII in 34% yield (Scheme I).

Synthesis

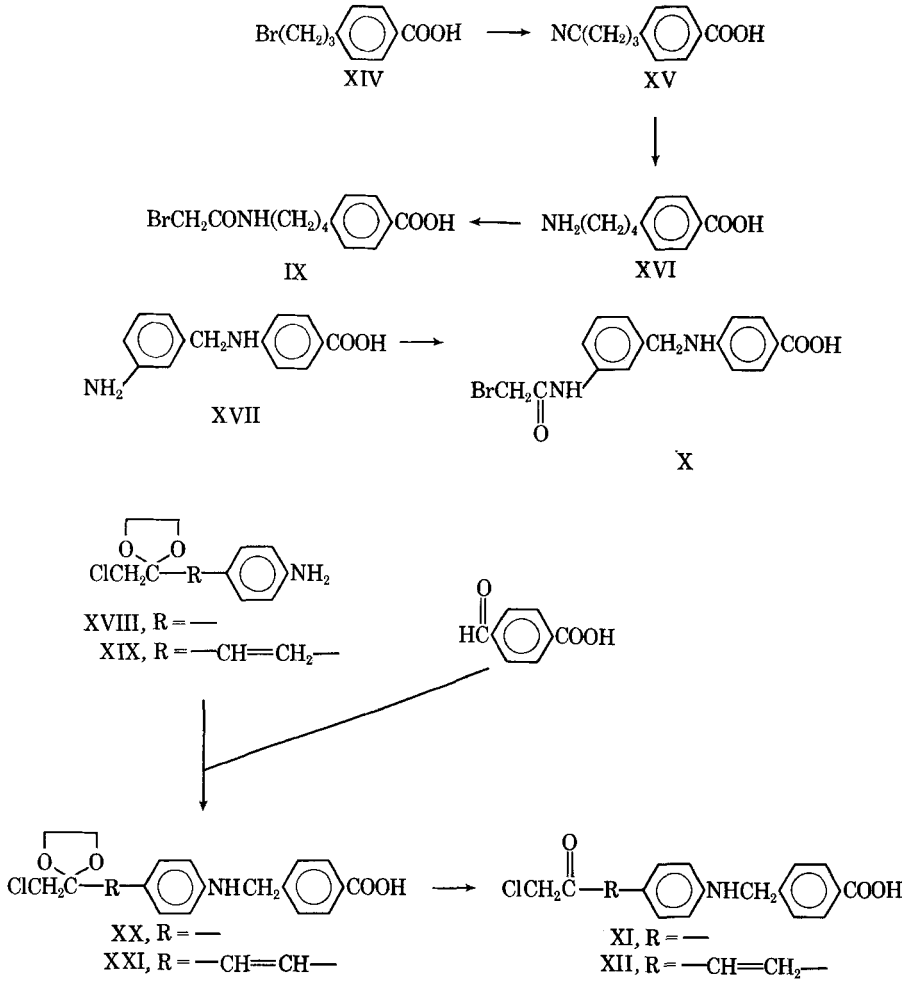
Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in Nujol mull, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in 10% alcohol with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by visual examination under ultraviolet light and by the 4-(*p*-nitrobenzyl)pyridine spray for active halogen (19).

p-Cyanopropylbenzoic Acid (XV).—A magnetically stirred mixture of 972 mg. (4 mmoles) of XIV (6, 23), 1.17 Gm. (23.9 mmoles) of sodium cyanide, and 40 ml. of *N*,*N*-dimethylformamide was heated in a bath at 100–110° for 18 hr. The cooled mixture was poured into 200 Gm. of ice water and the pH was adjusted to about 1 with dilute hydrochloric acid. The polymeric material was separated by filtration and had $\lambda_{max.}$ 5.88 (ester C=O); 5.95 (acid C=O); 9.08 μ (aromatic ester C—O—C).

The filtrate was extracted with chloroform (5 × 50 ml.). The combined, dried extracts were spin evaporated *in vacuo*. Recrystallization of the residue from benzene–petroleum ether (b.p. 60–110°) gave 451 mg. (60%) of product, m.p. 150–157°, suitable for further transformation. Recrystallization from the same solvent pair gave white crystals, m.p. 158–160°; $\lambda_{max.}$ (pH 1): 244; (pH 7): 235 mμ; $\lambda_{max.}$ 4.45 (C≡N); 5.94 (C=O); 6.24 μ (C=C).

Anal.—Calcd. for $C_{11}H_{11}NO_2$: C, 69.8; H, 5.86; N, 7.40. Found: C, 70.1; H, 5.83; N, 7.70.

p-(4-Aminobutyl)benzoic Acid (XVI).—A solution of 859 mg. (4.55 mmoles) of XV in 100 ml. of ethanol and 0.75 ml. of 12 *N* aqueous hydrochloric



Scheme I

acid was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide until 3 mole equivalents of hydrogen were absorbed. The filtered solution was spin evaporated to dryness *in vacuo* to give 1086 mg. (>100%) of crude XVI hydrochloride, m.p. 230–240° dec., that was suitable for the next step; this material contained some ammonium chloride after recrystallization from methanol–ether, as shown by combustion analysis.

A portion of the hydrochloride was dissolved in 10 ml. of water, then the solution was clarified with decolorizing carbon. The solution was made basic with ammonia water, then spin evaporated *in vacuo*. Recrystallization from the minimum of hot water gave white crystals of XVI as a zwitterion, m.p. 259–262°; $\lambda_{max.}^{H_2O}$ (pH 1): 244; (pH 7): 234; (pH 13): 239 mμ; $\lambda_{max.}$ 2.90, 3.10 (NH); 4.74 (NH^+); 6.32 (C=C); 6.55–6.65 (COO^-); 11.80 μ (*p*-C_6H_4).

Anal.—Calcd. for $C_{11}H_{15}NO_2$: C, 68.4; H, 7.82; N, 7.25. Found: C, 68.1; H, 7.98; N, 7.36.

p - [4 - (Bromoacetamido)butyl]benzoic Acid (IX).—To a solution of 230 mg. (1 mmole) of XVI hydrochloride in 30 ml. of water was added 0.67 ml. (2 mmoles) of 3 *N* aqueous sodium hydroxide followed by 5 ml. of acetone. After addition of 291 mg. (1.1 mmoles) of *p*-nitrophenyl bromoacetate, the mixture was magnetically stirred at ambient temperature for about 1 hr. The solution was spin evaporated *in vacuo* to about 20 ml. when an oil separated. The supernatant liquid was decanted and the oil rinsed with two 10-ml. portions of water. The combined aqueous solutions were acidified to about pH 1 with dilute hydrochloric acid. The product was collected on a filter and washed with water; yield, 120 mg. (38%), m.p. 160–168°. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°), then toluene gave white crystals, m.p. 175–176°; $\lambda_{max.}$ (pH 1): 247; (pH 7): 238 mμ; $\lambda_{max.}$ 3.04 (NH); 5.98 (carboxyl C=O); 6.09 (amide I); 6.24 (C=C); 6.50 μ (amide II). Thin-layer chromatography with benzene–methanol (3:1) showed a single spot under ultraviolet light; this spot gave a positive 4-(*p*-nitrobenzyl)pyridine test (19) for active halogen. Even though TLC indicated only one component, combustion analysis indicated the compound was only 90% pure.

Anal.—Calcd. for $C_{13}H_{16}BrNO_3$: C, 49.7; H,

5.12; N, 25.4. Found: C, 50.2; H, 5.10, Br, 23.1, 23.4.

N - (m - Bromoacetamidobenzyl) - p - aminobenzoic Acid (X).—To a solution of 484 mg. (2 mmoles) of XVII (6) in 4 ml. of acetone in an ice bath was added 520 mg. (2 mmoles) of bromoacetic anhydride (19). During 30 min. at 0°, the solution deposited tan crystals; 5 ml. of ether was added and the product was collected by centrifugation and washed with ether (5 × 8 ml.); yield, 518 mg. (71%), m.p. 192–195° dec. Recrystallization from aqueous ethanol with the aid of decolorizing carbon gave 355 mg. (49%) of nearly white crystals, m.p. 183–186°; $\lambda_{max.}^{KBr}$ 2.94, 3.03 (NH); 3.78–3.96 (broad, acidic OH); 6.05, 6.25, 6.43, 6.76 μ (C=O, NH, C=C). The compound gave a negative Bratton-Marshall test for primary aromatic amines (19) and a positive test for active halogen with 4-(*p*-nitrobenzyl)pyridine (19); it moved as a single spot on TLC in benzene–dioxane–glacial acetic acid (90/25/4).

Anal.—Calcd. for $C_{16}H_{15}BrN_2O_3$: C, 52.9; H, 4.16; Br, 22.0. Found: C, 53.1; H, 4.28; Br, 21.8.

2 - {p - [N - (p - Carboxybenzyl)amino]phenyl} - 2 - chloromethyl - 1,3 - dioxolane (XX).—To a magnetically stirred mixture of 1.285 Gm. (6 mmoles) of XVIII (12) and 0.903 Gm. (6 mmoles) of *p*-formylbenzoic acid in 150 ml. of methanol was added 3.0 Gm. of sodium borohydride in portions over about 30 min. with cooling in a water bath; at the end of the addition, the mixture was homogeneous. After being stirred for 18 hr. at ambient temperature, the solution was spin evaporated *in vacuo*. The residue was leached with 50 ml. of boiling benzene to remove any unreacted XVIII. The residue was dissolved in water, a little insoluble material was removed by filtration, then the filtrate was acidified to about pH 1 with dilute aqueous hydrochloric acid with cooling. After the addition of 2 Gm. of Celite, the mixture was filtered and the filter cake washed with water, then dried. The cake was extracted with boiling acetone (3 × 50 ml.). The combined, filtered extracts were spin evaporated *in vacuo*. The residue was triturated with petroleum ether; yield, 1.446 Gm. (69%), m.p. 206–208°. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°) gave white crystals, m.p. 212–213°; $\lambda_{max.}$ (pH 1): 236; (pH 7): 259; (pH 13): 254 mμ; $\lambda_{max.}$ 2.95 (NH); 5.98 (carboxyl C=O); 6.21 (C=C); 9.65, 9.81 (ether C—O—C); 12.16 μ (*p*-C_6H_4).

Anal.—Calcd. for $C_{18}H_{18}ClNO_4$: C, 62.2; H, 5.22; N, 4.03. Found: C, 62.0; H, 5.26; N, 3.94.

2 - {p - [N - (p - Carboxybenzyl)amino]styryl} - 2 - chloromethyl - 1,3 - dioxolane (XXI).—This was prepared from 482 mg. (2 mmoles) of XIX as described for the preparation of XX. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°) gave 358 mg. (48%) of product, m.p. 181–183°. Recrystallization from the same solvent pair gave nearly white crystals, m.p. 183–185°; $\lambda_{max.}$ (pH 1): 251; (pH 7): 232, 299; (pH 13): 291 mμ; $\lambda_{max.}$ 2.99 (NH); 6.00 (carboxyl C=O); 6.25 (C=C); 9.73, 9.85 (ether C—O—C); 12.25 μ (*p*-C_6H_4).

Anal.—Calcd. for $C_{20}H_{20}ClNO_4$: C, 64.3; H, 5.39; Cl, 9.48. Found: C, 64.0; H, 5.51; N, 9.62.

α - (p - Chloroacetylanilino) - p - toluic Acid (XI).—A solution of 363 mg. (1.04 mmoles) of XX in 54 ml. of absolute ethanol and 18 ml. of 0.2 *N* hydrochloric acid was refluxed for 30 min., then cooled overnight at 3°. The product (283 mg.) was collected on a filter and recrystallized from absolute ethanol to give 212 mg. (67%) of yellow crystals, m.p. 240° dec.; $\lambda_{max.}$ (pH 1): 242, 343; (pH 7): 238, 339; (pH 13): 235 (inflection), 343 mμ; $\lambda_{max.}$ 2.99 (NH); 5.82 (sh, ketone C=O); 6.03 (carboxyl C=O); 6.31 (C=C); 12.31 (*p*-C_6H_4); no C—O—C bands near 9–10 μ. The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test (19) for active halogen and moved as a single spot on TLC in benzene–methanol (3:1).

Anal.—Calcd. for $C_{16}H_{14}ClNO_3$: C, 63.3; H, 4.65; Cl, 11.7. Found: C, 63.1; H, 4.79; Cl, 11.8.

α - [p - (4 - Chloro - 1 - butene - 3 - one - 1 - yl) anilino] - p - toluic Acid (XII).—This was prepared from XXI as described for XI. Recrystallization from absolute ethanol gave 88 mg. (34%) of yellow-orange crystals which blackened at 200° and did not melt to 250°; $\lambda_{max.}$ (pH 1): 292; (pH 7, 13): 240, 410 mμ; $\lambda_{max.}$ 3.00 (NH); 5.90 (sh, ketone C=O); 6.00 (carboxyl C=O); 6.38 (C=C); 12.45 (*p*-C_6H_4); no C—O—C bands near 9.5 μ. The product moved as a single spot on TLC in benzene–methanol (3:1) and gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (19).

Anal.—Calcd. for $C_{18}H_{16}ClNO_3$: C, 65.6; H, 4.89; Cl, 10.8. Found: C, 65.4; H, 4.82; Cl, 11.0.

REFERENCES

(1) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(2) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site", John Wiley & Sons, Inc., New York, N. Y., to be published.
(3) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
(4) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(5) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(6) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
(7) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(8) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).
(9) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417 (1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422 (1966).
(11) *Ibid.*, **56**, 33(1967).
(12) Baker, B. R., and Jordaan, J. H., *J. Med. Chem.*, **8**, 35(1965).
(13) Baker, B. R., and Santi, D. V., *J. Pharm. Sci.*, unpublished data.
(14) Baker, B. R., and Ho, B.-T., *ibid.*, **56**, 28(1967).
(15) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(16) *Ibid.*, **3**, 315(1966).
(17) *Ibid.*, **3**, 319(1966).
(18) *Ibid.*, **3**, 324(1966).
(19) Baker, B. R., Santi, D. V., Coward, J.K., Shapiro, H. S., and Jordaan, J. H., *ibid.*, **3**, December(1966).
(20) Baker, B. R., Ho, B.-T., and Neilson, T., *ibid.*, **1**, 79(1964).
(21) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(22) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(23) Blicke, F. F., and Lilienfeld, W. M., *J. Am. Chem. Soc.*, **65**, 2281(1943).
(24) Baker, B. R., and Coward, J. K., *J. Pharm. Sci.*, **54**, 714(1965).

Determination of Acetylsalicylic Acid and Barbiturate Combinations in Dosage Forms

By SONG-LING LIN* and MARTIN I. BLAKE

A nonaqueous differentiating titrimetric procedure is presented for determining dosage forms containing acetylsalicylic acid and barbiturate combinations. Preliminary extraction of the active components is avoided. The titrant is sodium methoxide in benzene–methanol and the titration solvent is methyl isobutyl ketone. Titrations are effected potentiometrically. An additive procedure is suggested when the ratio of acetylsalicylic acid to barbiturate is greater than 10 to 1. The effect of additives and other active constituents is considered.

THE A.O.A.C. method for the analysis of acetylsalicylic acid and phenobarbital tablets is a spectrophotometric procedure involving preliminary separation of the components on a column of diatomaceous earth[1] which has been treated with dipotassium phosphate. It is based on a procedure developed by Banes (1) for determining phenobarbital in the presence of salicylates, and modified by Byers (2). Although satisfactory results are obtainable by this procedure, it is tedious and time-consuming.

In a previous paper (3) synthetic mixtures of acetylsalicylic acid and a variety of barbiturates were analyzed by a differentiating nonaqueous titration procedure. Titration was effected with a Fisher titrimeter equipped with a glass-calomel or antimony-calomel electrode system. The titration solvent was methyl isobutyl ketone, and the titrant was sodium methoxide in benzene–methanol (10:1). In the present study the method is applied to numerous dosage forms which contain acetylsalicylic acid and barbiturates in addition to additives, excipients, and other active constituents. The dosage form is titrated differentially without preliminary extraction of the active constituents. The effect of interfering substances on the sensitivity of the procedure is considered.

EXPERIMENTAL

Apparatus.—All titrations were performed potentiometrically with a Fisher titrimeter, model 35, equipped with a sleeve-type calomel (Beckman No. 41240) and a glass (Beckman No. 40495) electrode system.

Reagents.—(*a*) Acetylsalicylic acid and all barbiturates were the best quality available from commercial sources and were purified as described in an earlier paper (3). (*b*) Tenth normal sodium methoxide in benzene–methanol (10:1) was prepared and standardized as described by Fritz and Lisicki (4). (*c*) Other chemicals and all solvents used in this study were reagent grade and were employed without further purification. (*d*) Dosage forms were obtained from commercial sources.

General Procedure.—Appropriate amounts of acetylsalicylic acid and phenobarbital, accurately weighed, were transferred to a 150-ml. beaker and dissolved in 70 ml. of methyl isobutyl ketone with the aid of magnetic stirring. The solution was titrated potentiometrically with 0.1 *N* sodium methoxide in benzene–methanol (10:1), using a glass-calomel electrode system. The end points were determined from the inflections of the titration curve obtained by plotting millivolts (mv.) *versus* volume (ml.) of titrant added. During the titration process, the titration beaker was covered with a rubber plate having holes for the passage of the electrodes and the buret tip.

Effect of Other Ingredients on Differentiating Titration.—Approximately 0.60 meq. of acetylsalicylic acid, 0.40 meq. of phenobarbital, and an amount of other active constituents as indicated in Table II, accurately weighed, were dissolved in 70 ml. of methyl isobutyl ketone. The titration and end point detection were carried out as previously described.

Application of Additive Method.—Twenty tablets were weighed and powdered. An aliquot of the powder mass equivalent to about 0.90 meq. of acetylsalicylic acid was accurately weighed and transferred to a 150-ml. beaker. Amounts of pure phenobarbital, accurately weighed, were added to aliquots of the powder mass to bring the phenobarbital content from 0.07 meq. to a maximum of 0.35 meq. The samples were then dissolved in 70 ml. of methyl isobutyl ketone, and the solutions were titrated as described previously.

Analysis of Dosage Forms.—Seven dosage forms containing acetylsalicylic acid, a barbiturate, and other active ingredients were analyzed for their content of both acetylsalicylic acid and barbiturate by differentiating nonaqueous titration. Twenty tablets were weighed and powdered, or 20 capsules were emptied as completely as possible and the

Received August 19, 1966, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago 60612.

Accepted for publication September 30, 1966.

The authors acknowledge the following companies for supplying samples of pure barbiturates and dosage forms used in this study: Burroughs Wellcome and Co., Eli Lilly and Co., A. H. Robins Co., Sandoz Pharmaceuticals, Smith Kline & French Laboratories, and the U. S. Vitamin and Pharmaceutical Co.

The authors express their appreciation to Mr. Jeffrey S. Rudolph for technical assistance in certain aspects of this study.

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[1] Marketed as Celite 545 by the Johns-Manville Corp., New York, N. Y.

contents weighed. A sample of the powder mass equivalent to about 0.85 meq. of acetylsalicylic acid was accurately weighed and transferred to a 150-ml. beaker. The sample was dissolved in 70 ml. of methyl isobutyl ketone. When the additive method was employed, sufficient pure barbiturate powder was accurately weighed and added to the powder mass to make the ratio of acetylsalicylic acid-to-barbiturate more favorable for differentiating titration. The sample was then dissolved in 70 ml. of methyl isobutyl ketone. The direct titration procedure and the additive modification were applied to each dosage form. End points were determined as described previously.

Table I.—Differentiating Titration of Acetylsalicylic Acid and Phenobarbital

Amt. Weighed, Gm.		Recovery, %	
Acetylsalicylic Acid	Phenobarbital	Acetylsalicylic Acid	Phenobarbital
0.1942	0.2488	100.72	98.79
0.1908	0.2038	100.09	99.10
0.1832	0.2149	100.02	99.75
0.1810	0.1936	100.54	100.12
0.1777	0.2008	98.32	100.58
0.1546	0.1832	99.65	99.30
0.1433	0.1992	100.42	100.05
0.1268	0.1662	99.89	100.00
0.1092	0.1190	99.90	100.38
0.0901	0.1224	100.06	98.96
	Av.	99.96	99.70
	S.D.	±0.67	±0.63

RESULTS AND DISCUSSION

Differentiating nonaqueous titrimetry has provided a simple and useful technique for determining mixtures of acids (or bases) in dosage forms. The acids (or bases) in the mixture must differ significantly in their ionization constants, interfering substances must be absent, and the proper selection of titrant, titration solvent, and electrode system must be made. When these conditions have been met, complex dosage forms can be assayed by a single titration and preliminary treatment of the sample is unnecessary. Time-consuming and involved extraction procedures are avoided.

In an earlier paper (5) the differentiating titration of the weak bases acetophenetidin and caffeine in APC dosage forms was reported. The titrant was perchloric acid in acetic acid–acetic anhydride (1:1) and the titration solvent was acetic anhydride–chloroform–benzene (1:1:9). The differentiating

Table II.—Effect of Other Active Constituents on Differentiating Titration of Acetylsalicylic Acid and Phenobarbital

Active Constituent Added[a]	Amt. Weighed, mg.	Recovery, %	
		Acetylsalicylic Acid	Phenobarbital
Acetophenetidin	90	99.85 ± 0.63[b]	100.06 ± 0.49
Caffeine	50	99.67 ± 0.50	98.65 ± 0.62
Acetophenetidin	90	100.46 ± 0.48	100.16 ± 0.72
Caffeine	45		
Dextroamphetamine sulfate	10	99.62 ± 0.56	99.41 ± 0.84
Dextroamphetamine sulfate	40	99.76 ± 0.67	99.68 ± 0.52
Acetophenetidin	90		
Dextroamphetamine sulfate	10	100.16 ± 0.82	99.98 ± 0.60
Hyoscyamine sulfate	10	99.60 ± 0.70	99.00 ± 0.50
Hyoscyamine sulfate	30	100.06 ± 0.82	98.86 ± 0.76
Acetophenetidin	90		
Hyoscyamine sulfate	5	100.64 ± 0.65	100.82 ± 0.86

[a] Active constituents added in addition to acetylsalicylic acid and phenobarbital. [b] Standard deviation is based on at least 4 determinations.

Table III.—Application of Additive Method to the Determination of a Dosage Form Containing Acetylsalicylic Acid and Phenobarbital

Curve Ref.[a]	Aliquot of Tablet Mass Weighed, meq.		Pure Phenobarbital Added, meq.	% Label Claim Found	
	Acetylsalicylic Acid	Phenobarbital		Acetylsalicylic Acid	Phenobarbital
A	0.90	0.07	0.00	One End Point[b]	
B	0.90	0.07	0.07	98.92 ± 0.62[c]	100.06 ± 0.50
C	0.90	0.07	0.14	100.41 ± 0.51	99.69 ± 0.43
D	0.90	0.07	0.21	100.27 ± 0.35	99.77 ± 0.28
E	0.90	0.07	0.28	100.34 ± 0.46	100.04 ± 0.38

[a] The letter corresponds to those in Fig. 1. [b] Corresponds to acetylsalicylic acid plus phenobarbital. [c] Standard deviation is based on at least 4 determinations.

TABLE IV.—COMPARATIVE STUDY OF DIRECT AND ADDITIVE PROCEDURES FOR THE ANALYSIS OF DOSAGE FORMS CONTAINING ACETYLSALICYLIC ACID AND BARBITURATE

Dosage Form	Active Ingredients Labeled Amt.[a]	% Label Claim Found—Direct Titration: Acetylsalicylic Acid	Direct Titration: Barbiturate	Additive Method: Acetylsalicylic Acid	Additive Method: Barbiturate
Capsule A	Acetylsalicylic acid, 5 gr. Amobarbital, 3/4 gr.	100.80 ± 0.47[b]	98.63 ± 0.65	101.38 ± 0.36	99.88 ± 0.48
Capsule B	Acetylsalicylic acid, 3 gr. Allylisobutylbarbituric acid, 3/4 gr. Acetophenetidin, 2 gr. Caffeine, 2/3 gr.	101.68 ± 0.39	99.09 ± 0.72	98.32 ± 0.52	100.48 ± 0.81
Tablet A	As in capsule B	100.39 ± 0.45	98.87 ± 0.62	99.93 ± 0.48	98.72 ± 0.57
Yellow Tablet B	Acetylsalicylic acid, 2.5 gr. Amobarbital, 1/2 gr. Acetophenetidin, 2.5 gr. Dextroamphetamine sulfate, 5 mg.	98.53 ± 0.81	100.71 ± 0.91	99.31 ± 0.67	99.82 ± 0.51
Yellow Tablet C	Acetylsalicylic acid, 3.5 gr. Phenobarbital, 1/4 gr. Acetophenetidin, 2.5 gr.	One end point[c]		98.27 ± 0.85	99.37 ± 0.38
Gray Tablet D	Acetylsalicylic acid, 2.5 gr. Phenobarbital, 1/4 gr. Acetophenetidin, 3 gr. Hyoscyamine sulfate, 0.031 mg.	One end point		98.86 ± 0.76	100.39 ± 0.60
Capsule C	As in gray tablet D	One end point		100.42 ± 0.28	98.83 ± 0.46

[a] Per unit dosage form. [b] Standard deviation is based on at least 4 determinations. [c] Corresponds to aspirin plus barbiturate involved.

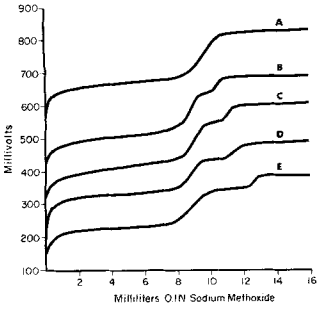

Fig. 1.—Titration curves for differentiating titration of acetylsalicylic acid and phenobarbital in dosage forms by additive method. Data are shown in Table III.

titration of synthetic mixtures of acetylsalicylic acid and barbiturates has been reported (3). The titration solvent was methyl isobutyl ketone and sodium methoxide in benzene–methanol was the titrant. In the present study this procedure has been applied to complex dosage forms containing acetylsalicylic acid and a variety of barbiturates in addition to certain additives, excipients, and other active constituents.

A series of 10 samples of a synthetic mixture of acetylsalicylic acid and phenobarbital was analyzed by the proposed method. The data in Table I indicate the accuracy and precision obtainable by the procedure.

Since acetylsalicylic acid and barbiturates are frequently combined with other therapeutic agents, the effect of these agents on the differentiating titration was studied. The constituents listed in Table II were added individually to a mixture of acetylsalicylic acid and phenobarbital and the combination was titrated by a differentiating titration. The content of the added active component was roughly that which is found in commercial dosage forms. It is apparent from the data in Table II that the added constituents do not interfere in the proposed assay pressured. In general, basic compounds and salts will not interfere. Acids comparable in strength to acetylsalicylic acid or phenobarbital will interfere with the titration. One of the commercial dosage forms procured for this study contained ascorbic acid in addition to acetylsalicylic acid and phenobarbital. A differentiating titration was not successful in this case.

The effect of disproportionate concentrations of acetylsalicylic acid to barbiturate on the differentiating titration was reported in an earlier paper (3). In synthetic mixtures when the acetylsalicylic acid-to-phenobarbital ratio was as high as 10:1, differentiation was not possible. Only one end point corresponding to the total acid present was obtained. An additive method is therefore proposed in which an amount of pure barbiturate is added to the powder mass to assure a ratio more favorable for differentiation. A similar technique (5) was shown to be useful in the differentiation of acetophenetidin and caffeine in APC combinations where dosage forms usually contain the components in the ratio of about 5:1. Dosage forms customarily (Table IV) contain a significantly greater proportion of acetylsalicylic acid than barbiturate. The applicability of the additive procedure was therefore tested on a typical dosage form. The data are reported in Table III. The ratio of the components in the dosage form was 13:1. By the direct titration method (no added phenobarbital) only one end point, corresponding to total acid present, was obtained. With the additive method the ratio of components was varied from 6.4:1 to 2.6:1. In each case differentiation was realized. Typical titration curves are shown in Fig. 1. Curves B through D show two distinct inflections, the first corresponding to the acetylsalicylic acid, and the second representing the barbiturate end point. Quantitative recoveries for both components are recorded in Table III.

Seven commercially available dosage forms were analyzed by the proposed assay using both the direct method and the additive modification. The results of the analyses are reported in Table IV. When the acetylsalicylic acid-to-barbiturate ratio is below 10:1, the direct method is suitable. For those dosage forms having a ratio of 10 or greater, the additive method must be applied.

The weak bases, caffeine and acetophenetidin, and the salt, amphetamine sulfate, did not interfere in the proposed assay. The excipients, coloring agents, diluents, and fillers employed in the formulation of the dosage forms apparently do not interfere. While these agents may suggest a possible source of difficulty in the titrimetric analysis of dosage forms, it has been shown by Chatten and Mainville (6) that in fact these agents do not represent an important source of interference even when the agents themselves are titratable.

REFERENCES

(1) Banes, D., *J. Assoc. Offic. Agr. Chemists*, **34**, 566(1951).
(2) Byers, T. E., *ibid.*, **38**, 635(1955).
(3) Lin, S. L., and Blake, M. I., *J. Pharm. Sci.*, **55**, 781 (1966).
(4) Fritz, J. S., and Lisicki, N. M., *Anal. Chem.*, **23**, 589 (1951).
(5) Lin, S. L., and Blake, M. I., *ibid.*, **38**, 549(1966).
(6) Chatten, L. G., and Mainville, C. A., *J. Pharm. Sci.* **52**, 146(1963).

Neutralization of Aluminum Hydroxide Dried Gel

Citrate and Tartrate Inhibition

By MILO GIBALDI and DANIEL MUFSON

Studies were conducted on the neutralization rate of aluminum hydroxide dried gel (AHDG) with HCl as a function of rate of agitation and amount of AHDG. On the basis of these studies it is suggested that the AHDG–HCl neutralization reaction is chemically controlled. Sodium citrate and tartrate were tested as inhibitors of AHDG. Their effect on the neutralization rate was investigated by neutralization, sedimentation, and complexation studies. It was concluded that a dual mechanism is operative to produce inhibition; the AHDG reacts with the salt to produce protons, and this effect, coupled with flocculation, causes a retardation in the rate of change of pH with time.

DESAI *et al.* (1), in 1963, employing the "buffering capacity" procedure, found extensive inhibition of the antacid activity of a large number of commercial antacid products when polypeptides were added to artificial gastric juice. The polypeptides had no effect on the time required to terminate the procedure, but greatly reduced the maximum pH attained. Products containing AHDG were most sensitive to this inhibitory effect.

Gibaldi and co-workers (2) found that the effect of polypeptides on "buffering capacity" could be ascribed to an inhibitory influence on the rate of neutralization. They, therefore, proposed that neutralization rate studies would be as indicative of the activity of a given antacid under different experimental conditions as the more elaborate "buffering capacity" experiments. These workers found that the neutralization rate is strongly dependent on the amount of aluminum hydroxide dried gel (AHDG) employed as well as the amount of inhibiting agent. Inhibition of activity was noted with various proteins, polypeptides, and amino acids. In addition, inhibition was found in the presence of acetate and citrate. Neither of these materials had a perceptible effect on the initial pH of the test solution or on the equilibrium pH. Two possible explanations for the observed inhibition effects, *viz.*, the formation of an *insoluble* AHDG–inhibitor complex and the adsorption of the inhibitor on the surface of the dispersed AHDG particles, were ruled out by the results of their investigations. The authors suggested the formation of a *soluble* complex between AHDG and the inhibitors tested.

In 1933, Tartar *et al.* (3) studied the influence of adsorbed ions on the dissolution of colloidal aluminum hydroxide in HCl. Aluminum hydroxide was allowed to dissolve in HCl in the presence of various salts. The solubility of aluminum hydroxide was determined at the end of 24 hr. at 25°. The data indicated that the greater the concentration of electrolyte, such as arsenate, phosphate, or sulfate, the greater the amount of aluminum hydroxide dissolved.

These authors noted that the "speed" (24-hr. solubility) of the reaction between colloidal aluminum hydoxide and 0.2 *N* hydrochloric acid is increased several-fold by the presence of electrolytes yielding anions of higher valence. It should be pointed out, however, that these workers did not clearly show that the test electrolytes increased the velocity of dissolution since only apparent equilibrium solubility was measured. They proposed that their findings were attributable to the ability of the anions of higher valence to modify the speed of the reaction by influencing the electrical potential at the solid-liquid interface. Tarter (3) also presented evidence which indicated that the change in the "speed" of the reaction was not due to an increase in the surface

Received July 22, 1966, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo 14214.
Accepted for publication September 20, 1966.
Based on a thesis submitted by Daniel Mufson to the College of Pharmaceutical Sciences, Columbia University, New York, N. Y., in partial fulfillment of Master of Science degree requirements.
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REFERENCES

(1) Banes, D., *J. Assoc. Offic. Agr. Chemists*, **34**, 566(1951).
(2) Byers, T. E., *ibid.*, **38**, 635(1955).
(3) Lin, S. L., and Blake, M. I., *J. Pharm. Sci.*, **55**, 781 (1966).
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area of the colloid. He notes, in fact, that the reverse is true; phosphate, arsenate, and sulfate ions exert a flocculating effect with an accompanying decrease in surface area.

Thus, a paradoxical situation appears to exist. On the one hand, the anions increase the solubility of the AHDG which should be attended by an increase in the reaction rate. On the other hand, these anions exert a flocculating effect and thereby decrease the effective surface area of the gel. The latter effect would tend to decrease the reaction rate and is in agreement with the recent findings of Desai (1) and Gibaldi (2).

It was the purpose of the present investigation to study the neutralization reaction of AHDG in HCl and to elucidate further the nature of the inhibition of AHDG by various anions. Of interest was the determination of whether the neutralization reaction is diffusion or chemically controlled, as well as the consideration of the role of flocculation and complex formation in the inhibition mechanism.

THEORETICAL CONSIDERATIONS

Neutralization.—Heterogeneous reactions are regulated by chemical effects and by physical phenomena occurring at the interface. When the chemical reaction rate is high, the physical effects become rate-limiting. Three separate steps are necessary for a heterogeneous reaction to proceed: (*a*) the reacting species must diffuse to the interface; (*b*) the chemical reaction occurs at the interface; and (*c*) the reaction products diffuse out into the bulk solution. The slowest of these three steps becomes the rate-limiting step.

Van Name and Hill (4) separated heterogeneous reactions into three classes: (*a*) the chemical reaction proceeds much faster than the diffusion, and the observed reaction rate will be determined by the rate of diffusion; (*b*) the chemical reaction is much slower than the diffusion and is, therefore, the controlling factor; and (*c*) chemical reaction rate and diffusion rate are both of the same order of magnitude, and the observed rate is a function of both.

Rate of agitation has a significant effect on the rate of a diffusion controlled reaction. Wurster and Taylor (5), in a recent review of dissolution rate theory presented the empirical equation,

$$DR = a(AR)^b \qquad \text{(Eq. 1)}$$

relating the agitation rate (AR) and the dissolution rate (DR), where a and b are constants. The value of b for a purely diffusion controlled reaction approximates unity. Its value for a chemically controlled reaction should approach zero.

Davion (6) presented an equation which describes the dissolution of a solid by a diffusion controlled process,

$$\frac{dW}{dt} = \frac{DA}{h}(C_s - C) \qquad \text{(Eq. 2)}$$

where dW/dt is the change in weight of the dissolving solid with time, D is the diffusion coefficient of the solute, h is the thickness of the diffusion layer, A is the area of the dissolving surface, C_s is the equilibrium solubility of the compound, and C is the concentration present in solution at time t. This equation permits one to visualize the effect of agitation on the rate of dissolution. As the stirring rate is increased, the diffusion layer becomes thinner and therefore, the value of h is lowered and the dissolution rate is increased. It may also be observed that for a diffusion controlled reaction the rate of dissolution is dependent on the magnitude of the diffusion coefficient. In addition, the rate of dissolution of a diffusion controlled reaction increases linearly with an increase in concentration of reagent (7). The reaction rate for chemically dependent reactions increases in a nonlinear fashion with increasing reagent concentration and reaches a maximum.

Inhibition.—The reported observations of the effect of anions on the neutralization of aluminum hydroxide are apparently contradictory. If a given salt forms a water-soluble complex with aluminum hydroxide, as has been shown by Zolotukhin (8) for tartrate and citrate, more aluminum hydroxide will be dissolved at equilibrium. This will tend to increase the C_s term in both Eq. 2 and in the equation presented by Zdanovskii (9) for the dissolution rate of a chemically controlled process. Zdanovskii's equation may be written as:

$$\frac{dW}{dt} = \alpha A(C_s - C_y) \qquad \text{(Eq. 3)}$$

where α is the rate constant for the chemical reaction occurring at the interface, C_s is the concentration at saturation, C_y is the concentration in the bulk solution, and the other terms have the same definitions as previously presented. Since the rate of dissolution is proportional to the equilibrium solubility, it would be expected that the formation of a water-soluble complex would increase the dissolution velocity. On the other hand, if aluminum hydroxide is also flocculated by these salts, one would expect a decrease in surface area resulting in a decrease in rate. It would appear that flocculation and complexation are exerting opposite effects on the dissolution velocity.

It has been established that anions decrease neutralization rate (2, 10). This indicates either that the flocculating effect of the anions overshadows its ability to complex aluminum hydroxide, or that dissolution rate cannot be equated with neutralization rate in this system. The latter situation would exist if the hydrated aluminum ion complexed the anion with the subsequent release of protons. This phenomenon would thereby produce a buffer effect. If this were the case, the neutralization rate would not be related to dissolution rate. This type of complexation has been shown by Das and co-workers (11) for the reaction of ferric ions with citric, tartaric, and malic acids.

EXPERIMENTAL

Materials

The various experiments were conducted with aluminum hydroxide dried gel U.S.P., F-1000, lot 194, supplied by Reheis Co., Inc., Berkeley Heights,

N. J. This sample was divided into several portions, and each portion was stored at room temperature in tightly sealed glass jars. All other chemicals were reagent grade and were obtained from Fisher Scientific Co., New York, N. Y.

Methods

Neutralization Studies.—A 1.0-Gm. sample of AHDG was added to a 250-ml. beaker containing 100 ml. of 0.1 *N* HCl well stirred. The test mixture was maintained at 37 ± 1° by immersing the beaker in a constant-temperature water bath. Stirring was effected by an overhead stirrer. Unless otherwise stated, the rate of agitation was 150 r.p.m. The pH changes were recorded with respect to time, utilizing a Beckman Zeromatic pH meter. When inhibitors were used, they were first dissolved in the 0.1 *N* HCl.

Mathematical Treatment of Neutralization Data.—The results obtained from the neutralization experiments were plotted as pH *versus* time. The curves were then evaluated to yield the time of maximum rate of reaction. The maximum reaction velocity occurs at the inflection point of the pH-time curve. The evaluation procedure to obtain the time at which the inflection point occurs consisted of obtaining the second derivative of the pH-time plot and plotting this derivative [Δ (Δ pH)] *versus* time. The time at which the curve crosses the *x*-axis is the inflection time.

Flocculation Studies.—Flocculation studies designed to evaluate the changes in sedimentation rate in the presence of inhibitors were conducted as follows: a 1.0-Gm. sample of AHDG was added to 300 ml. of 0.1 *N* HCl contained in an Andreasen pipet (12). With the breather hole closed, the pipet was shaken vigorously 10 times and placed securely in a constant-temperature water bath at 37 ± 1°. A 10-ml. sample was collected every 2 min. for 10 min. A steady suction was applied until the pipet was filled. The stopcock was then closed and the sample transferred to a previously tared 150-ml. beaker. Pressure was applied to blow out the sample at high velocity. The pressure was repeated several seconds later to insure full collection. The sampling procedure must proceed rapidly. If the stopcock is not closed immediately upon collection of the 10-ml. sample the heavier particles will begin to fall out of the chamber. After withdrawal of the sample the stopcock must be opened slowly so that the liquid remaining in the sample tube can flow into the settling chamber without stirring the suspension.

The suspension in the beakers was evaporated to dryness, allowed to cool, and weighed on a Mettler balance, model H 15. Particular care was taken to prevent overdrying and charring of the organic acid. In the flocculation studies involving the use of sodium citrate and sodium tartrate, 50 ml. of a 2 *N* solution of either salt was added to a sufficient quantity of HCl to yield 300 ml. A correction factor to account for the weight of the salt was applied in each case.

Potentiometric—Complexation Studies.—Solutions containing 0.2 *N* aluminum chloride, sodium citrate, or sodium tartrate, as well as combinations of aluminum chloride and sodium citrate, and aluminum chloride and sodium tartrate were prepared in deionized water by adding 10 ml. of a given 0.2 *N* salt solution to 20 ml. of 0.2 *N* HCl. The resulting solution was titrated with 0.2 *N* NaOH. The pH was monitored with a Beckman Zeromatic pH meter. The solution was stirred during the test by a Teflon-coated magnetic stirrer. Sufficient time was allotted between each addition of alkali to ensure that equilibrium was reached.

Table I.—The Effect of Stirring Rate on the AHDG–HCl Neutralization

Stirring Rate, r.p.m.	Inflection Time, sec.
85	370
120	375
240	360

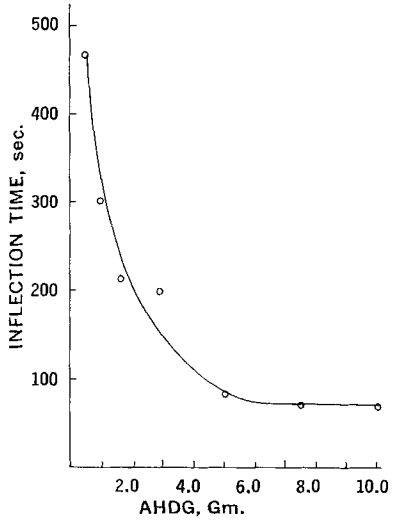

Fig. 1.—The effect of the amount of AHDG on the inflection time of the AHDG–HCl neutralization.

Table II.—The Effect of Various Anions on the Inflection Time (sec.) of the AHDG–HCl Neutralization[a]

Sodium Salt	Concn., eq./L. 10^{-3}	10^{-2}	10^{-1}
Citrate	630	1235	...
Tartrate	695	1020	>1860
Sulfate	...	460	270
Chloride	...	330	...
Acetate	...	320	...

[a] The inflection time for the control was 310 sec.

RESULTS AND DISCUSSION

Mechanism of Neutralization.—In an effort to establish whether the AHDG–HCl interaction was diffusion or chemically controlled, the effect of different agitation rates on the reaction velocity was studied. Experiments were conducted at stirring speeds ranging from 85 to 240 r.p.m. The results of this investigation are presented in Table I. Inspection of Table I indicates that the change in stirring rate had little effect on the neutralization

velocity. Therefore, it may be concluded that the value of the exponent *b* in Eq. 1 is approximately zero.

To further resolve the mechanism of the neutralization reaction, the reactant concentration was varied. AHDG was added to the reaction beaker in quantities ranging from 0.50–10.0 Gm. The results of these experiments are depicted in Fig. 1. It may be observed that neutralization rate is highly dependent upon the amount of antacid employed up to a maximum. Beyond this point (about 5 Gm.), it would appear that the neutralization rate is independent of the amount of AHDG present. It has been established (7) that in chemically controlled reactions, the reaction rate increases in a nonlinear fashion with increasing reagent concentration and reaches a maximum.

In view of the experimental results indicating that the value of exponent *b* in Eq. 1 approximates zero, and that the rate of reaction increases to a maximum with increasing AHDG concentration, it is reasonable to conclude that the reaction is predominantly chemically controlled.

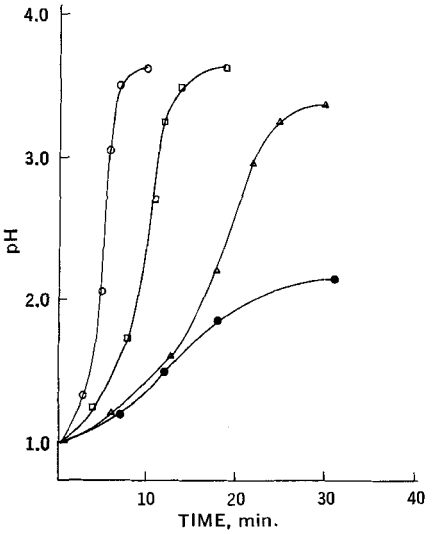

Fig. 2.—The effect of tartrate concentration on the AHDG–HCl neutralization. Key: O, 0.0001 *N*; □, 0.001 *N*; △, 0.01 *N*; ●, 0.1 *N*.

Mechanism of Inhibition.—Several salts were selected to be tested as inhibitors of AHDG, and were tested at several concentrations. The results of these investigations are listed in Table II.

Examination of the data contained in Table II reveals that the order of degree of inhibition exerted by the salts follows the Schulze-Hardy rule. Their descending order of effectiveness as coagulating agents follow their inhibitory activity, *viz.*, citrate = tartrate > sulfate > acetate = chloride. The fact that the experimental results follow this rule supports the mechanism of flocculation as a contributing factor in the inhibition process.

Figure 2 demonstrates the difference in neutralization velocities produced by different concentrations of sodium tartrate. It is typical of the effects observed with sodium citrate.

To confirm the flocculation mechanism for the inhibition of AHDG, sedimentation studies were conducted using the Andreasen pipet. Figure 3 shows that a significantly larger amount of AHDG was collected in the presence of citrate and tartrate as compared to the control. If flocculation did occur, one would expect the citrate- and tartrate-treated AHDG to settle faster due to a greater particle size. When a sample was withdrawn at a preset level there was more AHDG present at that level in the salt solutions. The results obtained from this study correlate to some degree with those obtained from the neutralization studies. It would appear that the salts act to reduce the surface area of the antacid, and thereby produce a concomitant reduction in neutralization velocity.

To investigate the possibility of anion–AHDG complexation with concurrent proton release, during the reaction of AHDG with sodium citrate and sodium tartrate, potentiometric titrations were performed. The results of these studies are shown in Figs. 4 and 5.

The inflection points of the sodium citrate and aluminum chloride curves in Fig. 4 are identical,

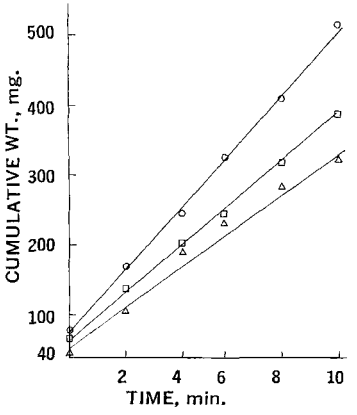

Fig. 3.—Cumulative weight of AHDG collected *versus* time. Key: △, AHDG; O, AHDG plus citrate; □, AHDG plus tartrate.

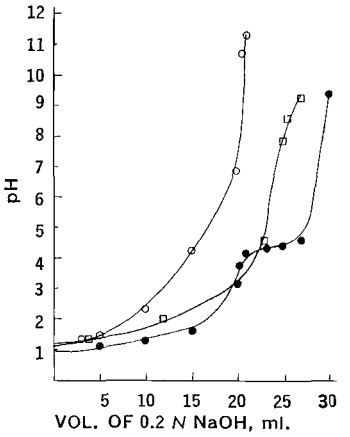

Fig. 4.—Potentiometric titrations of aluminum chloride (●), sodium citrate (O), and aluminum chloride plus sodium citrate (□).

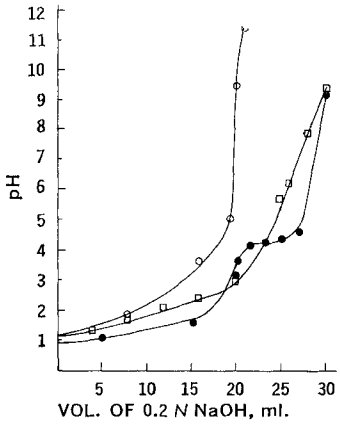

Fig. 5.—Potentiometric titrations of aluminum chloride (●), sodium tartrate (○), and aluminum chloride plus sodium tartrate (□).

both occurring at 20 ml. of alkali. However, the combination of these salts required more base for neutralization; the inflection point was displaced to the right, to about 24 ml. It is interesting to note that although the titration of aluminum chloride alone produced a precipitate of the hydroxide, no precipitate was observed in the presence of citrate or tartrate. This latter phenomenon suggests the presence of a water-soluble complex. In addition, since the system containing the aluminum complex required more alkali to produce neutralization than did either of the salts when tested separately, evidence is provided to indicate that the formation of this complex produces the release of excess protons and thereby decelerates the change of pH with respect to time. Similar results were found for the sodium tartrate–aluminum chloride potentiometric titration (Fig. 5).

In view of these findings we may conclude that a dual mechanism is operative to produce inhibition. AHDG reacts with sodium tartrate or citrate to produce a water-soluble complex with the concomitant liberation of protons. This effect coupled with flocculation causes a retardation in the rate of change of pH with time.

REFERENCES

(1) Desai, S., Gibaldi, M., and Kanig, J. L., *J. Pharm. Sci.*, **52**, 872(1963).
(2) Gibaldi, M., Kanig, J. L., and Amsel, L., *ibid.*, **53**, 1375(1964).
(3) Tartar, H. V., Bryan, C. C., and Shinn, H., *J. Am. Chem. Soc.*, **55**, 2266(1933).
(4) Van Name, E. A., and Hill, D. V., *Am. J. Sci.*, **36**, 543(1913).
(5) Wurster, D. E., and Taylor, R. W., *J. Pharm. Sci.*, **54**, 169(1965).
(6) Davion, M., "Etude sur la Vitesse de Dissolution des sels Cristallises," Thesis 370, University of Paris, Paris, France, 1953; through Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961).
(7) Burrows, W. H., Lewis, C. T., Jr., Saire, D. E., and Brooks, R. E., *Ind. Eng. Chem. Process Design Develop.*, **3**, 149(1964).
(8) Zolotukhin, V. K., *Russian J. Inorg. Chem.*, **5**, 915 (1960).
(9) Zdanovskii, A. B., *Zhur. Fiz. Khim.*, **25**, 170(1951); through *Chem. Abstr.*, **48**, 4291(1954).
(10) Nogami, H., and Nagai, T., *Chem. Pharm. Bull. (Tokyo)*, **10**, 741(1962).
(11) Das, H. K., Hohapatra, G., Mohapatra, S., Pattnaik, R. K., and Pani, S., *Proceedings of the Symposium on the Chemistry of Coordination Compounds*, part III, 28th Annual Session of the Indian Academy of Science, University of Agra, India, February 7 and 8, 1959, p. 102.
(12) Martin, A. N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, pp. 579–581.

Cytotoxicity of North Dakota Plants I

In Vitro Studies

By I. A. MUNI, W. H. BHATTI, L. J. SCHERMEISTER, and M. C. VINCENT

In this study, a method is presented for the direct determination of cytotoxicity and antitumor activity of plant materials *in vitro* using HeLa cells. Fifty poisonous plants representing 96 plant parts were tested by this method, out of which 25 plants showed limited cytotoxicity and six plants were found to be highly cytotoxic. This procedure can be used on a large scale prior to *in vivo* evaluation.

THE IMPORTANCE of plant screening for various chemical constituents of medical importance is well established. Plants have provided innumerable drugs which are being used for the prevention or cure of various diseases. This work was directed to the search for new drugs which may find application in the prevention or cure of cancer.

In vitro screening for cytotoxicity based on morphological evaluation was first reported by Biesele and associates (1) in 1952 using nonsynthetic media. Fjelde *et al.* (2) introduced human tumor cell lines for cancer chemotherapy.

Received May 9, 1966, from the School of Pharmacy, North Dakota State University, Fargo 58103.
Accepted for publication August 22, 1966.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
Abstracted in part from a thesis submitted by I. A. Muni to the North Dakota State University, Fargo, in partial fulfillment of Master of Science degree requirements.
The authors thank Dr. J. Dixon, Southern Research Institute, and Dr. K. Kajiwara, McArdle Laboratory, for the supply of HeLa cells.
This investigation was supported by institutional funds No. GU-NSF 922 from the National Science Foundation.

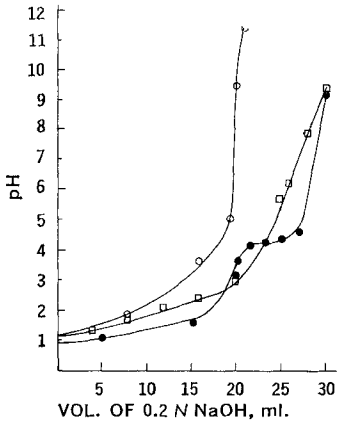

Fig. 5.—Potentiometric titrations of aluminum chloride (●), sodium tartrate (○), and aluminum chloride plus sodium tartrate (□).

both occurring at 20 ml. of alkali. However, the combination of these salts required more base for neutralization; the inflection point was displaced to the right, to about 24 ml. It is interesting to note that although the titration of aluminum chloride alone produced a precipitate of the hydroxide, no precipitate was observed in the presence of citrate or tartrate. This latter phenomenon suggests the presence of a water-soluble complex. In addition, since the system containing the aluminum complex required more alkali to produce neutralization than did either of the salts when tested separately, evidence is provided to indicate that the formation of this complex produces the release of excess protons and thereby decelerates the change of pH with respect to time. Similar results were found for the sodium tartrate–aluminum chloride potentiometric titration (Fig. 5).

In view of these findings we may conclude that a dual mechanism is operative to produce inhibition. AHDG reacts with sodium tartrate or citrate to produce a water-soluble complex with the concomitant liberation of protons. This effect coupled with flocculation causes a retardation in the rate of change of pH with time.

REFERENCES

(1) Desai, S., Gibaldi, M., and Kanig, J. L., *J. Pharm. Sci.*, **52**, 872(1963).
(2) Gibaldi, M., Kanig, J. L., and Amsel, L., *ibid.*, **53**, 1375(1964).
(3) Tartar, H. V., Bryan, C. C., and Shinn, H., *J. Am. Chem. Soc.*, **55**, 2266(1933).
(4) Van Name, E. A., and Hill, D. V., *Am. J. Sci.*, **36**, 543(1913).
(5) Wurster, D. E., and Taylor, R. W., *J. Pharm. Sci.*, **54**, 169(1965).
(6) Davion, M., "Etude sur la Vitesse de Dissolution des sels Cristallises," Thesis 370, University of Paris, Paris, France, 1953; through Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961).
(7) Burrows, W. H., Lewis, C. T., Jr., Saire, D. E., and Brooks, R. E., *Ind. Eng. Chem. Process Design Develop.*, **3**, 149(1964).
(8) Zolotukhin, V. K., *Russian J. Inorg. Chem.*, **5**, 915 (1960).
(9) Zdanovskii, A. B., *Zhur. Fiz. Khim.*, **25**, 170(1951); through *Chem. Abstr.*, **48**, 4291(1954).
(10) Nogami, H., and Nagai, T., *Chem. Pharm. Bull. (Tokyo)*, **10**, 741(1962).
(11) Das, H. K., Hohapatra, G., Mohapatra, S., Pattnaik, R. K., and Pani, S., *Proceedings of the Symposium on the Chemistry of Coordination Compounds*, part III, 28th Annual Session of the Indian Academy of Science, University of Agra, India, February 7 and 8, 1959, p. 102.
(12) Martin, A. N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, pp. 579–581.

Cytotoxicity of North Dakota Plants I

In Vitro Studies

By I. A. MUNI, W. H. BHATTI, L. J. SCHERMEISTER, and M. C. VINCENT

In this study, a method is presented for the direct determination of cytotoxicity and antitumor activity of plant materials *in vitro* using HeLa cells. Fifty poisonous plants representing 96 plant parts were tested by this method, out of which 25 plants showed limited cytotoxicity and six plants were found to be highly cytotoxic. This procedure can be used on a large scale prior to *in vivo* evaluation.

THE IMPORTANCE of plant screening for various chemical constituents of medical importance is well established. Plants have provided innumerable drugs which are being used for the prevention or cure of various diseases. This work was directed to the search for new drugs which may find application in the prevention or cure of cancer.

In vitro screening for cytotoxicity based on morphological evaluation was first reported by Biesele and associates (1) in 1952 using nonsynthetic media. Fjelde *et al.* (2) introduced human tumor cell lines for cancer chemotherapy.

Received May 9, 1966, from the School of Pharmacy, North Dakota State University, Fargo 58103.
Accepted for publication August 22, 1966.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
Abstracted in part from a thesis submitted by I. A. Muni to the North Dakota State University, Fargo, in partial fulfillment of Master of Science degree requirements.
The authors thank Dr. J. Dixon, Southern Research Institute, and Dr. K. Kajiwara, McArdle Laboratory, for the supply of HeLa cells.
This investigation was supported by institutional funds No. GU-NSF 922 from the National Science Foundation.

Eagle and Foley (3) described a technique for determination of cytotoxicity of chemical compounds for mammalian cells *in vitro* using semisynthetic media. Later they (4) reported cytotoxic activity of 180 compounds against the KB strain of human epidermoid carcinoma. Smith, Lummis, and Grady (5) determined the cytotoxic activity of various nucleic acids and vitamin antagonists, heavy metals, respiratory poisons, detergents, miscellaneous chemical solvents, and fermentation beers. Nitta (6,7) and other workers (8–11) have tested the different antibiotics for antitumor activity. A recent survey (12) indicates that about 400 plant species possess antitumor activity.

EXPERIMENTAL

Collection of Materials.—The investigational material was collected from wild growing plants throughout the State of North Dakota. Each plant under investigation was taxonomically classified and a sample of each specimen was retained in the herbarium for future reference.

The fresh plant material was air-dried at room temperature, separated into the respective plant parts, ground to a No. 40 powder, and stored in air-tight containers. When the presence of moisture interfered with grinding, the plant material was oven dried at 60°.

Preparation of Extracts.—Two extracts, aqueous and alcoholic, of all plant parts were prepared. The aqueous extract was prepared as follows. A 2-Gm. sample of dried material was macerated with 25 ml. of distilled water for 30 to 40 min. and filtered through Whatman No. 1 paper. The extracts were freeze dried and kept frozen until used. Prior to screening, each frozen extract, representing 2 Gm. of dried plant material, was dissolved in 2 ml. of isotonic saline. The pH of the solution was adjusted to 7.0 to 7.5 with 0.25 *N* HCl or 0.25 *N* NaOH. Then it was mixed with 0.5 ml. of 5X culture medium containing antibiotics and sterilized through UF-filtration. UF-filtration was accomplished by centrifuging in the closed system.

The same general procedure was used in preparing the alcoholic extracts, using 95% alcohol instead of water. However, to increase the solubility of the plant material, 2 drops of alcohol was added to the isotonic saline.

Preparation of Medium.—The culture medium recommended by Joseph (13) for the growth of HeLa cells was prepared as follows:

Medium 199 (10X)	8 ml.
Calf serum	20 ml.
Hank's BSS	72 ml.
Sodium bicarbonate solution, 7.5%	0.5 ml.
Potassium penicillin G	100 units/ml.
Streptomycin sulfate	100 mcg./ml.

The pH was found to be 7.0 to 7.5.

Fresh medium was prepared before running each set of samples and checked for sterility with alternative thioglycolate medium U.S.P. (14).

Cell Lines.—Stock cultures of HeLa cells were obtained from Southern Research Institute, Birmingham, Ala., and Microbiological Associates, Bethesda, Md. These cultures were maintained in Blake bottles containing culture medium 199 at 37°. Stock cultures were refed with fresh medium every 50 to 60 hr. A suspension of isolated cells was obtained by trypsinizing 75 to 80 hr. old cell cultures.

The number of cells present in the culture medium was determined in a hemocytometer. The required dilution was made with culture medium to obtain 40,000 to 50,000 cells/ml.

Screening Procedure.—Two-milliliter vinyl plastic cup trays were sterilized by immersing in 95% ethanol for 1 hr., and then dried for 4 to 6 hr. under ultraviolet light.

Cell suspensions were placed in a 125-ml. Pyrex flask, which was equipped with glass and rubber tubing connecting a 1-ml. Cornwall syringe. A three-way adapter was attached to the syringe to allow recirculation of medium that remained in the tubing between samples. The suspension was stirred either by circulation or by shaking the flask gently after every sample extraction.

Plant extracts were added to the cups in definite quantities, representing 4, 20, 40, 100, and 200 mg./ml. of dry plant material, using a syringe microburet. One-half milliliter of cell suspension was added to each cup and the volume was made up to 1 ml. with the addition of culture medium. A 30-gauge blunt-tip needle was used.

A series of controls were determined for each test series, such as sodium nitrofurantoin[1], methyl green, and Hank's basal salt solution for comparison. The plastic trays were covered with sterile plastic and sealed air-tight with strips of Scotch cellophane tape. All trays were incubated at 37°.

Evaluation of Data.—Toplin's evaluation method (15), accepted by CCNSC was used. The trays were examined after 75 to 80 hr. incubation for evidence of cytotoxicity. Cytotoxicity was evaluated by direct microscopic examination of cultures usually at 100 × magnification and, if necessary, at 430 × magnification. The cytotoxic end point (CE) and lethal end point (LE) were determined.

RESULTS AND DISCUSSION

Fifty poisonous plants, representing 96 plant parts, were tested for cytotoxicity using Gey's HeLa S-3 cells. The cytotoxic and lethal end point for all the plants tested is given for both alcoholic and aqueous extracts in Table I.

Toplin's direct microscopic method for cytotoxicity evaluation was used because it was simple, rapid, and reproducible.

The plant materials screened for cytotoxicity were used in concentrations representing 4 to 200 mg. of plant material per milliliter of culture medium. In Table II the comparison of *in vivo* results, as reported by Leiter *et al.* (16) and Hardinge *et al.* (17), with the *in vitro* cytotoxicity of the same plants at 200 mg. concentration indicated that the positive results were due to a general cellular, and not a specific, toxicity. For this reason, the highest concentration of plant material used was 200 mg./ml.

Both CE and LE values were necessary to determine the effectiveness of cytotoxic substances in the plant extracts. The effectiveness of these

[1] Marketed as Furadantin Sodium by Eaton Laboratories, Inc., Norwich, N. Y.

TABLE I.—CYTOTOXICITY SCREENING DATA[a]

Serial No.	Code No.	Scientific Name	Plant Part	Extract	Cytotoxicity Limits[b] CE	LE	Extract	Cytotoxicity Limits[b] CE	LE
1	64–146	*Achillea sibirica* Ledeb. (*Asteraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
2	63–68	*Actaea rubra Ait.* (*Ranunculaceae*)	L	Alc.	100–200	>200	Aq.	100–200	>200
3	63–62	*Apocynum androsaemifolium* L. (*Apocynaceae*)	L	Alc.	40–100	200	Aq.	100	200
4	64–108	*Apocynum sibiricum* Jacq. (*Apocynaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	100	200	Aq.	>200	>200
			R	Alc.	>200	>200	Aq.	>200	>200
5	63–32	*Ascelipas ovalifolia* Ded. (*Asclepiadaceae*)	L	Alc.	40	100–200	Aq.	200	>200
6	64–107	*Asclepias speciosa* Torr.	F	Alc.	200	>200	Aq.	>200	>200
			L	Alc.	20–40	200	Aq.	40	200
		(*Asclepiadaceae*)	R	Alc.	200	>200	Aq.	200	>200
7	64–106	*Asclepias syriaca* L.	B	Alc.	100–200	>200	Aq.	100–200	>200
		(*Asclepiadaceae*)	L	Alc.	40–100	>200	Aq.	40–200	>200
			S	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	100–200	>200
8	64–104	*Asparagus officinalis* L. (*Liliaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
9	63–31	*Astragalus caryocarpus* Ker. (*Febaceae*)	L	Alc.	20	100–200	Aq.	>200	>200
10	63–53	*Astragalus missouriensis* Nutt. (*Fabaceae*)	L-S	Alc.	100–200	>200	Aq.	>200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
11	63–34	*Astragalus tenellus* Pursh. (*Fabaceae*)	Fl-1	Alc.	20	100–200	Aq.	200	>200
			S	Alc.	40–100	200	Aq.	200	>200
12	64–116	*Celastrus scandens* L. (*Celastraceae*)	L	Alc.	100–200	>200	Aq.	>200	>200
			S	Alc.	100–200	>200	Aq.	>200	>200
13	63–29	*Cicuta maculata* L. (*Umbelliferae*)	L	Alc.	200	>200	Aq.	200	>200
			T	Alc.	>200	>200	Aq.	200	>200
14	64–126	*Eupatorium maculatum* L. (*Asteraceae*)	L	Alc.	40–100	200	Aq.	100–200	>200
			S	Alc.	100–200	>200	Aq.	200	>200
15	63–65	*Eupatorium perfoliatum* L. (*Asteraceae*)	L	Alc.	200	>200	Aq.	200	>200
16	64–124	*Euphorbia esula* L. (*Euphorbiaceae*)	L	Alc.	200	>200	Aq.	100–200	>200
			S	Alc.	40	200	Aq.	100–200	>200
17	63–80	*Fumaria officinalis* L. (*Fumariaceae*)	W	Alc.	40–200	>200	Aq.	100–200	>200
18	64–128	*Gaura coccinea* Pursh. (*Onagraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
19	62–20	*Glycyrrhiza lepidota* Nutt. (*Fabaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
20	64–121	*Gypsophila paniculata* L. (*Careophyllaceae*)	Fr-1	Alc.	40–200	>200	Aq.	40–200	>200
			R	Alc.	200	>200	Aq.	40–200	>200
			S	Alc.	40–200	>200	Aq.	100–200	>200
21	63–90	*Helenium autumnale* L. (*Asteraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	200	>200
22	64–119	*Lactuca pulchella* Pursh. (*Asteraceae*)	L	Alc.	100–200	>200	Aq.	200	>200
			S	Alc.	40–200	>200	Aq.	100–200	>200
23	63–64	*Lathyrus ochroleucus* Hook. (*Fabaceae*)	L	Alc.	100–200	>200	Aq.	40–200	>200
24	63–72	*Lobelia spicata* Lam (*Lobeliaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
25	64–143	*Lupinus argenteus* Pursh. (*Fabaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
26	63–59	*Mamillaria vivipara* Nutt. (*Cactaceae*)	L-S	Alc.	>200	>200	Aq.	>200	>200
27	63–75	*Menispermum canadensis* L. (*Menispermaceae*)	L	Alc.	40	200	Aq.	100–200	>200
			S	Alc.	200	>200	Aq.	>200	>200
28	64–100	*Nepeta cataria* L. (*Lamiaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
29	63–60	*Opuntia polycantha* Haw. (*Cactaceae*)	L-S	Alc.	100–200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
30	64–139	*Oxytropis lambertii* Pursh. (*Fabaceae*)	L	Alc.	>200	>200	Aq.	200	>200
			S	Alc.	>200	>200	Aq.	200	>200
31	63–67	*Penstemon grandiflorus* Nutt. (*Scrophulariaceae*)	L	Alc.	4	100–200	Aq.	20	200

Continued on next page.

TABLE I.—(*Continued.*)

Serial No.	Code No.	Scientific Name	Plant Part	Extract	Cytotoxicity Limits[b] CE	LE	Extract	Cytotoxicity Limits[b] CE	LE
32	63–50	*Penstemon nitidus* Dougl. (*Scrophulariaceae*)	L	Alc.	20	100–200	Aq.	>200	>200
33	63–33	*Polygonatum commutatum* Schutt. (*Liliaceae*)	L	Alc.	100–200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
34	62–8	*Psoralea argophylla* Pursh. (*Fabaceae*)	L	Alc.	40–100	200	Aq.	200	>200
35	64–103	*Rhus radicans* L. (*Anacardiaceae*)	L	Alc.	200	>200	Aq.	200	>200
			Rh	Alc.	100–200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	100–200	>200
36	64–144	*Rudbeckia hirta* L. (*Asteraceae*)	L	Alc.	40–100	200	Aq.	40	200
			S	Alc.	20–40	200	Aq.	>200	>200
37	64–105	*Rumex* sp. (*Polygonaceae*)	L	Alc.	40–200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	>200	>200
38	64–109	*Rumex venosus* Pursh. (*Polygonaceae*)	L	Alc.	4–40	100–200	Aq.	40	200
			S	Alc.	20–40	100–200	Aq.	20	40–200
39	63–66	*Sanguinaria canadensis* L. (*Papaveraceae*)	L	Alc.	200	>200	Aq.	200	>200
			Rh	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
40	64–115	*Silene cseri* Baum (*Caryophyllaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			R	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
41	64–102	*Silene noctiflora* L. (*Caryophyllaceae*)	Fl	Alc.	100–200	>200	Aq.	200	>200
			L	Alc.	100–200	>200	Aq.	100–200	>200
			R	Alc.	100–200	>200	Aq.	100–200	>200
			S	Alc.	200	>200	Aq.	200	>200
42	63–78	*Solanum carolinense* L. (*Solanaceae*)	W	Alc.	200	>200	Aq.	100–200	>200
43	64–140	*Stachys palustris* L. (*Laminaceae*)	Fl	Alc.	200	>200	Aq.	200	>200
			L	Alc.	200	>200	Aq.	200	>200
44	64–141	*Stipa comata* Trin. and Rupr. (*Graminaceae*)	W	Alc.	>200	>200	Aq.	>200	>200
45	64–142	*Thermopsis rhombifolia* Nutt. (*Fabaceae*)	L	Alc.	200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
46	64–101	*Triglochin maritima* L. (*Juncaginaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
47	63–83	*Veronica americana* Schwein. (*Scrophulariaceae*)	W	Alc.	>200	>200	Aq.	200	>200
48	64–147	*Vicia americana* Muhl. (*Fabaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
49	63–76	*Xanthium italicum* Mor. (*Asteraceae*)	L-S	Alc.	100–200	>200	Aq.	200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
50	63–37	*Zygadenus elegans* Pursh. (*Liliaceae*)	Fl	Alc.	100–200	>200	Aq.	200	>200
			L	Alc.	>200	>200	Aq.	>200	>200
			R	Alc.,	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200

[a] Alc, alcoholic extract; Aq, aqueous extract; B, buds; CE, cytotoxicity end point; F, fruit; Fl, flower; Fr-1, fruit and leaves; Fl-1, flowers and leaves; L, leaves; LE, lethal end point; L-S, leaves and stems; R, roots; Rh, rhizome; S, stems; T, tuber; W, whole plant. [b] Cytotoxicity limits expressed in mg. of dry plant material/ml. of culture.

TABLE II.—COMPARISON OF *In Vivo*[a] AND *In Vitro* RESULTS

Serial No.	Scientific Name	*In Vitro* CE	*In Vitro* LE
1	*Asparagus officinalis* L.	200	>200
2	*Eupatorium perfoliatum* L.	200	>200
3	*Glycyrrhiza lepidota* Nutt.	200	>200
4	*Lupinus argentius* L.	200	>200
5	*Nepeta cataria* L.	200	>200
6	*Sanguinaria canadensis* L.	200	>200
7	*Stachys palustris* L.	200	>200

[a] Reported in literature and in each case was tumor noninhibitory.

extracts in inhibiting HeLa cells was increased as the CE and LE values approached one another.

As shown in Table I, a CE value of 100 or lower was produced by 66 out of 192 extracts. Eight of these extracts had close CE and LE values and were considered highly inhibitory to HeLa cells. The most effective alcoholic extracts were obtained from the leaves of *Ascelpias ovalifolia*, *Astragalus caryocarpus*, *Penstemon grandiflorus*, *Penstemon nitidus*, and *Rumex venosus*, the stems of *Rumex venosus*, and the flowers and leaves of *Astragalus tenellus*. In the case of aqueous extracts, only the stem of *Rumex venosus* was included.

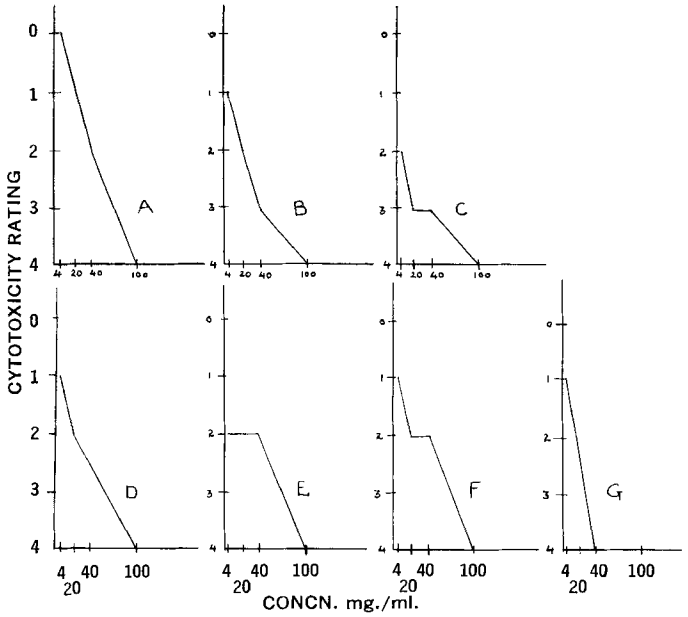

Fig. 1.—Graphical representation of cytotoxicity. Key: A, *Asclepias ovalifolia* leaves (alc.), B, *Astragalus tennellus* flowers and leaves (alc.) and *Astragalus caryocarpus* leaves (alc.); C, *Penstemon grandiflorus* leaves (alc.); D, *Penstemon nitidus* leaves (alc.); E, *R. venosus* leaves (alc.); F, *R. venosus* stem (alc.); G, *R. venosus* stem (aq.).

In order to determine which of the above plant extracts were more effective as cytotoxic agents, Toplin's plotting method was employed. These plots are presented in Fig. 1. From this figure it can be observed that plants which have greater cytotoxicity have a sharper slope. The sharpest slope was obtained with the aqueous extract of stems of *R. venosus*. All other effective extracts were alcoholic, and the decreasing order of cytotoxicity was as follows: leaves of *Ascelepias ovalifolia* and *Astragalus caryocarpus*, flowers and leaves of *Astragalus tenellus;* leaves of *Penstemon grandiflorus* and *Penstemon nitidus;* stems of *R. venosus* and the leaves of *R. venosus*.

The data in Table I indicate that the naturally occurring tumor inhibitory substances in these plants were more generally alcohol soluble. These alcohol soluble substances were more frequently found in leaves than in stems, while none were present in roots of those plants tested. Only the stems of *R. venosus* contained inhibitory substances which were soluble in both alcohol and water.

Of the 23 families of native plants tested, only four families contained species having cytotoxic constituents. They were *Polygonaceae, Asclepiadaceae, Fabaceae,* and *Scrophulariaceae.*

A slight inhibitory effect on HeLa cells was shown by 58 plant extracts. If the moderately cytotoxic plant extracts were further concentrated, other effective cytotoxic activities might be indicated.

The limitations of all *in vitro* studies are numerous and the correlation between antitumor activity in experimental animal tumors *in vivo* and cytotoxicity to mammalian cells *in vitro* can only be established by experiments.

SUMMARY

1. Gey's HeLa cells were used to determine *in vitro* cytotoxicity and antitumor activity of plant extracts.

2. Evaluation of cytotoxicity was based on microscopic observation of the cells after incubation with plant samples for 75 to 80 hr.

3. Fifty-eight out of 192 extracts tested were moderately cytotoxic in the range of 4 to 100 mg. of dry plant material per milliliter of culture media.

4. Eight plant extracts exhibited marked human cancer cell inhibitory activity.

5. More alcoholic extracts were found to be cytotoxic than the aqueous extracts for the plants tested.

REFERENCES

(1) Biesele, J. J., Berger, R. E., Clarke, M., and Weiss, L., *Exptl. Cell. Res., Suppl. No. 2,* **1952,** 279.
(2) Fjelde, A., Sorkin, E., and Rhodes, J. M., *Exptl. Cell. Res.,* **10,** 88(1956).
(3) Eagle, H., and Foley, G. E., *Am. J. Med.,* **21,** 739 (1956).
(4) Eagle, H., and Foley, G. E., *Cancer Res.,* **18,** 1017 (1958).
(5) Smith, C. G., Lummis, W. L., and Grady, J. E., *ibid.,* **19,** 847(1959).
(6) Nitta, K., *Japan J. Med. Sci. Biol.,* **10,** 277(1957).
(7) *Ibid.,* **10,** 287(1957).
(8) Smith, C. G., Lummis, W. L., and Grady, J. E., *Cancer Res.,* **19,** 843(1959).
(9) Owen, S. P., Dietz, A., and Camiener, G. W., *Antimicrobial Agents Chemotherapy,* **May 1962,** 772.
(10) Bhuyan, B. K., Dietz, A., and Smith, C. G., *ibid.,* **May 1962,** 184.
(11) DeBoer, C., Dietz, A., Lummis, N. E., and Savage, G. M. *Antimicrobial Agents Ann.,* **1960,** 17.
(12) Farnsworth, N. R., *J. Pharm. Sci.,* **55,** 225(1966).
(13) Joseph, J. M., in "Gradwohol's Clinical Laboratory Methods and Diagnosis," Frankel, S., and Reitman, S., eds., vol. 1, 6th ed., C. V. Mosby Co., St. Louis, Mo., 1963, pp. 857–864.
(14) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 856.
(15) Toplin, I., *Cancer Res.,* **19,** 959(1959).
(16) Leiter, J., Abbott, B. J., Rourke, A. R., Fitzgerald, D. B., and Schepartz, S. A., *ibid.,* **21,** 93(1962).
(17) Hardinge, M. G., Courville, D. A., Hardinge, M., Fujikawa, B., and Harvey, R., *ibid.,* **24,** 1(1964).

Effect of Hypothermia on the Chlorpromazine-Induced Bradycardia and Hypotension in the Rat

By K. F. FINGER, R. L. CAVANAGH, and E. E. MOOG

The administration of chlorpromazine (0.5 mg./Kg.) caused a rapid fall in blood pressure and a decreased heart rate in rats. A significant potentiation in both the extent and the duration of the heart rate effects was observed in hypothermic animals as compared to normothermic animals. No significant differences were observed in the blood pressure effects during the period of observation. It was also established that fatty acid mobilization, reflexly induced by chlorpromazine's hypotensive action, was inhibited in hypothermic animals but not in normothermic animals. These data suggest that chlorpromazine more effectively inhibits adrenergic mechanisms in the hypothermic animal and this mechanism may explain the potentiation of heart rate effects observed in this study.

CHLORPROMAZINE exerts a variety of pharmacological actions in both man and laboratory animals. Included among these are antiadrenergic, antihistaminic, and anticholinergic effects and its characteristic central nervous system depression (1). These actions manifest themselves as a decrease in spontaneous motor activity, altered behavioral patterns (primarily in response to external environmental factors), hypothermia, hypotension, and alterations in heart rate (1–3). Interest centered around the effects of chlorpromazine on blood pressure and heart rate with particular reference to the quantitative aspects of its antiadrenergic actions. During the course of preliminary investigations, it was noted that in regard to its effects on heart rate, chlorpromazine appeared to exert a prolonged action in hypothermic animals as compared to those which were maintained normothermic. The investigations into the possible reasons for these differences is the subject of this report.

EXPERIMENTAL

Methods.—Male Sprague-Dawley rats, weighing between 350 and 400 Gm., were employed in this study. They were anesthetized with urethan (approximately 1.0 Gm./Kg. i.p.), their femoral and carotid vessels cannulated for purposes of drug administration and blood pressure and heart rate recording, respectively, and either maintained normothermic by the use of a warming element or allowed to become hypothermic by omitting the warming procedure. Rectal temperatures were monitored for all animals utilizing the Yellow Springs Instrument Co. Telethermometer. Blood pressures and heart rates were determined from tracings obtained with the E and M Physiograph equipped with a Statham P23 transducer.

Control periods of sufficient length to assure a stabilized blood pressure and heart rate were continuously recorded prior to drug administration. Chlorpromazine, 0.5 mg./Kg., was administered i.v. in a volume of 0.1 ml./Kg. and control animals received 0.1 ml./Kg. of saline. Blood pressures and heart rates were monitored continuously for 20 min. immediately following drug administration and thereafter for several minutes at 20-min. intervals for approximately 3 hr. At least six animals were employed in each group and the data to be presented here are expressed as the mean value obtained ± the standard error of the mean.

For the determination of nonesterified fatty acids in plasma, the animals were treated in the manner described above for the cardiovascular experiments except that blood samples were withdrawn *via* the carotid cannula and the plasma assayed for fatty acids by a modification of the method of Dole (4).

RESULTS AND DISCUSSION

Body Temperature Changes.—The changes in body temperature produced by drug treatment and the experimental procedures are shown in Fig. 1. These results show that the procedures employed produced a highly significant difference in rectal temperature between those animals maintained with a warming element and those which were not provided with an external heat source. It is apparent from this figure that the use of the warming element maintained body temperature at a reasonably constant value, while those animals which were not heated became hypothermic. Chlorpromazine, at the low dosage employed in this study, produced an observable but relatively small reduction in rectal temperature.

Effect of Chlorpromazine on Blood Pressure.—The intravenous administration of 0.5 mg./Kg. of chlorpromazine produced a rapid fall in both systolic and diastolic pressure. In Fig. 2, the per cent of initial mean blood pressure has been plotted and the rapid decrease in blood pressure is evident. This hypotensive phase of chlorpromazine's actions was

Received April 25, 1966, from the Pharmacology Department, School of Pharmacy, University of Wisconsin, Madison.

Accepted for publication August 24, 1966.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This work was supported in part by grants from the National Aeronautics and Space Administration and the Wisconsin Alumni Research Foundation.

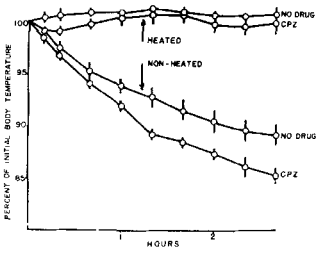

Fig. 1.—Rectal temperatures of rats. Arrows indicate temperature data curves for groups of rats, the upper two curves representing groups maintained with a warming element and the lower two curves those which were not heated.

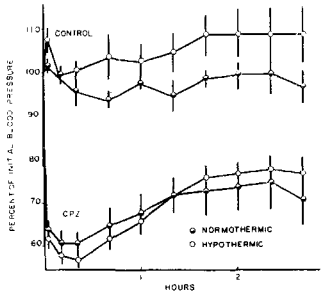

Fig. 2.—Effect of chlorpromazine on blood pressure of hypothermic and normothermic rats.

maintained throughout the period of experimental observation, although a moderate rise was observed to occur between 40 and 80 min. It is also apparent from Fig. 2 that the procedures themselves produced only minor alterations in blood pressure as can be seen in the control curves. It is interesting to note that the hypothermic control group showed slightly higher pressures than did the normothermic group, a result to be anticipated as a result of the induced hypothermia.

Effect of Chlorpromazine on Heart Rate.—Following the administration of chlorpromazine, a lag time occurred before the effects of chlorpromazine became evident on the heart rate. The lag time varied between 2 and 5 min. Following this brief lag period, the heart rate was slowed to an appreciable extent in both normothermic and hypothermic animals. These data are shown in Fig. 3. Twenty minutes after drug administration, the heart rates of the normothermic animals began to return toward normal values, while those of the hypothermic group decreased to even lower values. Indeed, the minimal value of heart rate in the hypothermic animals was still present 40 min. after drug administration, while the heart rate in the normothermic group had returned to approximately 85% of normal. The slope of the line describing the return toward normal values of the heart rate in the hypothermic group is also slightly less than that observed for the normothermic group. In addition, the mean heart rate of the hypothermic group did not return to control values during the period of observation but appeared to plateau out at approximately 85% of the initial value.

It is also interesting to note that there exists a difference in the time at which potentiation of chlorpromazine's heart rate effects are observed and the time of maximum decrease in body temperature. These data are interpreted to indicate that it is not the body temperature *per se* that is the important factor but rather the physiological events, yet unknown, which result in both a decrease in body temperature and the observed potentiation. It is not unusual for body temperature changes to lag behind other events inasmuch as the decrease in body temperature could be due to at least two factors: (*a*) decreased heat production and (*b*) increased heat loss, both of which would be relatively slow and continuous phenomena.

It is also apparent in Fig. 3 that the heart rate of the normothermic control (no drug) group remained quite stable, while a slight tachycardia was observed in the hypothermic control group.

Effect of Hypothermia and Chlorpromazine Treatment on Mobilization of Fatty Acids.—Two different experimental approaches were followed in an attempt to explain the effect of hypothermia on the chlorpromazine-induced changes in heart rate. They were (*a*) to attempt to explain the observed differences in terms of an altered rate of drug metabolism and (*b*) to attempt to obtain evidence indicating alterations in the ability of the chlorpromazine treated, hypothermic animals to respond to reflex homeostatic mechanisms.

The available analytical procedures were not sufficiently sensitive to quantitate the metabolic transformations of chlorpromazine at the dosage employed in this study (0.5 mg./Kg.). Utilizing higher doses, 10 and 30 mg./Kg., the authors were able to identify and quantitate parent drug, chlorpromazine sulfoxide, desmethyl-chlorpromazine, and its corresponding sulfoxide. Preliminary results indicated that while some differences were apparent in the concentrations of certain metabolites, a significant portion of the metabolized chlorpromazine was not accounted for by the moieties assayed. Furthermore, the rather high dosages employed in this phase of the study may have flooded enzymic systems, thus masking relatively small but significant alterations in metabolic rates. While this work is continuing, definitive statements on the rate of chlorpromazine metabolism in this system cannot be made.

The second experimental approach was based on the experimental finding reported by Costa *et al.* (5) and those of Pletscher *et al.* (6). These workers have shown that the release and/or uptake of biogenic amines is inhibited by chlorpromazine in hypothermic animals, but this effect is negated when the animals are maintained normothermic or heated. Costa and co-workers (5) were able to

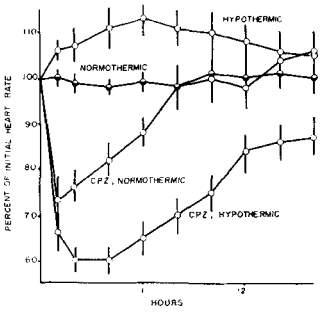

Fig. 3.—Effect of chlorpromazine on heart rate of hypothermic and normothermic rats.

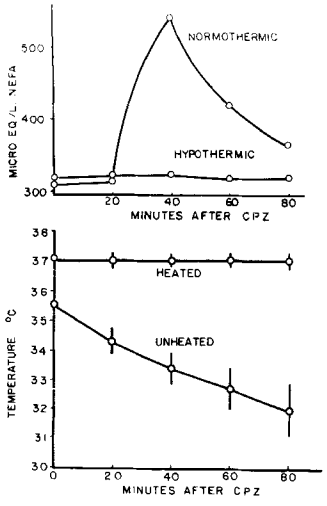

Fig. 4.—Top, effect of hypothermia on fatty acid mobilization in chlorpromazine treated rats. Chlorpromazine dose = 0.5 mg./Kg. i.v. Bottom, rectal temperatures obtained in both heated and unheated chlorpromazine treated rats.

show that chlorpromazine blocked the release of catecholamines by reserpine when the animals were hypothermic but that this action of reserpine was unaffected by chlorpromazine in normothermic animals. Similarly, Pletscher *et al.* (6), studying the uptake of tryptamine as influenced by drugs, found that body temperature had an influence on chlorpromazine's blockade of tryptamine uptake. To ascertain whether a similar effect was operative in this system, an experimental procedure was sought which was (*a*) under the primary control of the sympathetic nervous system and (*b*) sensitive enough to respond to homeostatic, sympathetic mechanisms in the anesthetized animals employed in the present study. Previous work (7–10) had shown that the mobilization of fatty acids from adipose tissue *in vivo* satisfied these criteria. Thus, it was decided to investigate the effect of hypothermia and chlorpromazine treatment on the mobilization of fatty acids *in vivo*.

The results of this phase of the study are shown in Fig. 4. The lower portion of the graph illustrates the changes in body temperature observed in the two groups of animals. Chlorpromazine was administered to both groups of animals at a dose of 0.5 mg./Kg. It is apparent in Fig. 4 that hypothermia effectively antagonized the elevation of plasma fatty acids. These results also show that the dose of chlorpromazine employed was not sufficient to block fatty acid mobilization *per se* inasmuch as significant elevations in plasma fatty acids were obtained in the chlorpromazine treated, normothermic animals. It would appear that an increased sympathetic outflow occurred as a result of the blood pressure lowering effects of the chlorpromazine treated group of animals, probably mediated by the release of catecholamines both locally in adipose tissue and systemically *via* adrenal medullary release. In the hypothermic, chlorpromazine treated animals, however, this manifestation of increased sympathetic tone was antagonized presumably by either inhibiting the release of catecholamines or by a more effective chlorpromazine antagonism of fatty acid mobilization.

It is well known that cold-stressed animals respond by mobilizing fatty acids (9, 10) and the hypothermia *per se* observed in these experiments therefore would have been expected to produce an elevation of nonesterified fatty acids. In contrast to the expected results with hypothermia, chlorpromazine, in sufficient dosage, would have been expected to antagonize fatty acid mobilization (11, 12), yet, in the dose employed, failed to do so in normothermic animals. The combination of chlorpromazine (at a low dose) and hypothermia, however, effectively antagonized fatty acid mobilization.

CONCLUSIONS

It is concluded that the chlorpromazine-induced bradycardia is potentiated by hypothermia with respect to both the extent and the duration of the response. The potentiation may be explained by a reduction in the ability of the hypothermic, chlorpromazine treated animal to respond to reflex homeostatic mechanisms. The antagonism of adrenergic mechanisms implicit in such an explanation may be due to either an effect on the release of adrenergic neurohumoral agents or by a more effective inhibition of adrenergic responses by chlorpromazine in the hypothermic animals.

REFERENCES

(1) Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., and Koetschet, P., *Arch. Intern. Pharmacodyn.*, **92**, 305 (1953).
(2) Kopera, J., and Armitage, A. K., *Brit. J. Pharmacol.*, **9**, 392(1954).
(3) Finkelstein, M., Spencer, W. A., Hammen, C. S., and Albert, S. N., *Federation Proc.*, **13**, 354(1954).
(4) Finger, K. F., Pereira, J. N., and Schneider, J. A., *Rev. Invest.*, **2**, 41(1962); Finger, K. F., Pereira, J. N., and Schneider, J. A., *Federation Proc.*, **21**, 175(1962).
(5) Costa, E., Gessa, A., and Brodie, B. B., *Life Sciences*, (No. 7) 315(1962).
(6) Pletscher, A., King, E., Staehler, H., and Gey, K. F., *Biochem. Pharmacol.*, **12**, 1065(1963).
(7) Werthiemer, E., and Shapiro, B., *Physiol. Rev.*, **28**, 451 (1958).
(8) Havel, R. J., and Goldfein, A., *J. Lipid Res.*, **1**, 102 (1959).
(9) Salvador, K. A., Colville, K. I., and Burns, J. J., *Ann. N. Y. Acad. Sci.*, **131**, 113(1964).
(10) Gilgen, A., Maickel, R. P., Nikodijevic, O., and Brodie, B. B., *Life Sciences*, (No. 12) 709(1962).
(11) Finger, K. F., Page, J. G., and Feller, D. R., *Biochem. Pharmacol.*, **15**, 1023(1966).
(12) Hulsmann, W. C., Fabius, A. J. M., and de Ruiter H., *Nature*, **202**, 1336(1964).

Absorption, Metabolism, and Excretion of Riboflavin-5′-phosphate in Man

By WILLIAM J. JUSKO and GERHARD LEVY

Evidence is presented which indicates that riboflavin-5′-phosphate (FMN) is absorbed from the gastrointestinal tract of man by specialized transport rather than by passive diffusion. Oral administration of the vitamin after a meal results in more extensive absorption which appears to be primarily due to a decrease in intestinal transit rate resulting in longer retention of FMN at absorption sites in the small intestine. Both FMN and riboflavin are excreted in the urine primarily, if not solely, as free riboflavin. The urinary recovery of riboflavin is the same when equimolar amounts of either form of the vitamin are administered orally in solution. The time course of urinary excretion of riboflavin after administration of high doses of FMN, together with data available in the literature, suggest that the vitamin is subject to enterohepatic cycling.

THE AUTHORS have recently found that the urinary recovery of riboflavin after oral administration to fasted normal humans decreases with higher doses and that absorption is limited mainly to the upper region of the gastrointestinal tract (1). The saturability and site-specificity of riboflavin absorption, together with other evidence summarized previously (1), led to the conclusion that the vitamin is absorbed mainly or solely by specialized transport rather than by passive diffusion. Unlike the results obtained when riboflavin was administered on an empty stomach, administration of the vitamin immediately after breakfast resulted in a constant per cent urinary recovery independent of dose. These results, which were obtained in the dose range of 5 to 30 mg., were interpreted as being due to decreased intestinal transit rate in the presence of food, resulting in prolonged retention of the vitamin at specialized absorption sites in the small intestine. If this interpretation is correct, it should be possible to observe saturation effects in the absorption of riboflavin even when the vitamin is administered on a full stomach, provided that sufficiently large doses are administered. The limited solubility of riboflavin itself made such a study unfeasible, since administration of the vitamin in suspension, rather than in solution, could lead to misleading results (1). This solubility problem is not encountered with riboflavin-5′-phosphate (FMN) and, provided that this form of the vitamin is subject to the same transport mechanism as is riboflavin itself, it should be possible to observe saturation effects when FMN is administered in sufficiently high doses after a meal. The purpose of the study to be described here was, therefore, to determine the mechanism of gastrointestinal absorption of FMN, and, if saturation effects become evident after oral administration of FMN on an empty stomach, to determine if similar saturation effects occur with sufficiently high doses when the vitamin is administered on a full stomach. In the course of the study, information has been obtained also concerning the metabolic fate of FMN and evidence will be presented which is indicative of the enterohepatic cycling of riboflavin.

Received September 6, 1966, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo 14214.

Accepted for publication October 7, 1966.

This investigation was supported in part by grant R01-AM08753-03 PET and a fellowship to William J. Jusko from the Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Absorption Study.—Four healthy male volunteers, 22 to 37 years of age, served as test subjects. The same subjects participated in the previous study (1). Each subject received doses of sodium FMN[1] equivalent to 5, 10, and 30 mg. of riboflavin dissolved in 100 ml. of 0.02 *N* acetic acid. The container was rinsed with a small amount of water which was also ingested. The vitamin solution was administered either on an empty stomach (after an overnight fast) or immediately after a standard breakfast. Three of the subjects received sodium FMN also in doses equivalent to 150 and 300 mg. of riboflavin. The breakfast, urine collections, and other details of the protocol were essentially the same as in the study of riboflavin absorption (1). The subjects were permitted to eat their usual lunches at the normal time, except when it was desired to determine the effect of withholding lunch on the excretion of riboflavin.

In another phase of the study, the effect of volume of gastrointestinal contents on the absorption of riboflavin was determined. Three of the subjects ingested 600 ml. of water on an empty stomach, followed by 30 mg. of riboflavin in 100 ml. of 0.02 *N* acetic acid (plus about 40 ml. of water to rinse the container) to approximate the volume of food and drink ingested when the vitamin was administered after the standard breakfast (1).

At least two 24-hr. blank urine collections, with

[1] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.

and without breakfast, were carried out in each subject.

Analytical Methods.—Riboflavin and FMN in the urine were determined fluorimetrically by the method of Burch *et al.* (2) and by a modified U.S.P. XVI assay procedure (3) using the Turner Fluorometer, model 111, with primary filter 47-B and secondary filter 2A-12. The modifications of the two assays have been described in a previous publication (1).

All data were corrected for blank values which averaged 0.6 mg./24 hr. by the Burch procedure and 1.0 mg./24 hr. by the modified U.S.P. procedure.

RESULTS

The urinary recoveries of riboflavin as a function of the administered dose of FMN, given either on an empty stomach or after a standard breakfast, are shown in Fig. 1. The curves shown in the figure are those obtained with the same subjects after ingestion of equivalent doses of riboflavin, as described in a previous publication (1). It is evident that the relation between dose and urinary recovery is essentially the same for FMN and riboflavin. There is a linear relationship between the dose of FMN and the amount of riboflavin excreted when doses of FMN from 5 to 30 mg. were ingested after a standard breakfast. The average recovery of riboflavin after oral administration of FMN in doses ranging from 5 to 30 mg. was 63%. This is essentially identical to the 62% recovery found previously when riboflavin itself was ingested after breakfast. When FMN was administered on an empty stomach (after an overnight fast), the per cent recovery of riboflavin in the urine decreased with increasing dose. This apparent saturation effect is also very similar to that found previously with riboflavin itself.

Since the similarity in the gastrointestinal absorption characteristics indicates that both forms of the vitamin are absorbed by the same mechanism,

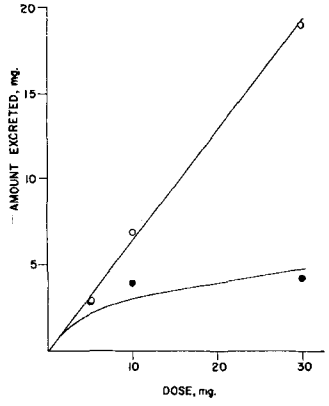

Fig. 1.—Urinary recovery of riboflavin after oral administration of FMN as a function of dose when given on an empty stomach (●) and after a standard breakfast (○). The curves are taken from Fig. 1 of *Reference 1* and represent data obtained after administration of riboflavin as such. Mean of four subjects. The two data points for the 5-mg. dose are superimposed.

TABLE I.—URINARY RECOVERY OF RIBOFLAVIN AFTER ORAL ADMINISTRATION OF FMN AFTER A STANDARD BREAKFAST

Subject	Urinary Recovery of Riboflavin, mg. Dose[a]: 5	10	30	150	300
J	2.08	5.21	20.1	22.8 16.2	22.5 (48.0)[b]
L	2.43	7.37	16.5	...	...
A	2.30	6.70	17.0	23.7	27.0 22.5[c]
M	4.77	8.09	22.4	22.5 23.5[c]	27.6
Mean	2.90	6.84	19.0	21.7	24.9
Dose, %	58.0	68.4	63.3	14.5	8.3

[a] Milligram riboflavin equivalent. [b] This value was not used in calculation of the average value. [c] Instances when subjects did not eat lunch.

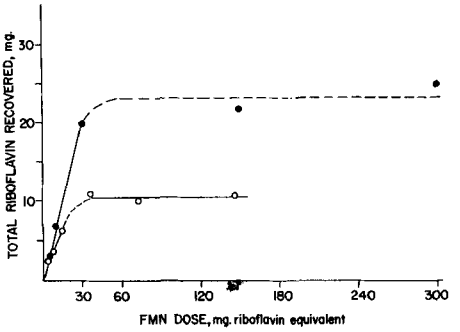

Fig. 2.—Urinary recovery of riboflavin after oral administration of FMN as a function of dose when given after a standard breakfast, showing the limited absorption capacity evident in the high dose range (●). Lower curve (○) taken from Fig. 2 of *Reference 4* (see text).

it was possible to take advantage of the greater solubility of FMN to determine if apparent saturation effects can be observed even if the vitamin is administered on a full stomach. Therefore, three subjects were given 150- and 300-mg. doses of riboflavin as sodium FMN after the standard breakfast. The relative urinary recovery decreased with increasing dose of FMN, demonstrating a definite upper limit in the gastrointestinal absorption of the vitamin (Table I, Fig. 2). This upper limit was somewhat above 30 mg. under the experimental conditions of the study. There was one unusually high recovery (48.0 mg. in subject *J* after a 300-mg. dose), but repetition of the experiment in the same subject resulted in a urinary recovery of 22.5 mg. The 48.0-mg. value was not included in the average data of Fig. 2 because of the large discrepancy relative to the remainder of the data. Figure 2 also includes data reported by Stripp (4) which appeared while the present study was in progress. Stripp's data also show a definite upper limit in the absorption of FMN. The absolute difference in the plateau values may be due to differences in the dietary regimen used in the respective studies.

The results of an experiment in which three subjects ingested 30 mg. of riboflavin (*a*) on an empty stomach, (*b*) after a standard meal, and (*c*) after

drinking a volume of water approximately equal to the volume of the standard breakfast are shown in Table II. The ingestion of 600 ml. of water before taking riboflavin increased the urinary riboflavin recovery from 15.0 to 21.6%, but the limited number of subjects does not permit determination of the significance of this difference. However, ingestion of riboflavin after breakfast resulted in an even greater recovery, namely 57.5%.

Analysis of the urines by both the Burch and modified U.S.P. methods permitted estimation of the respective amounts of riboflavin and FMN excreted after ingestion of either form of the vitamin. Riboflavin and FMN exhibit equal fluorescence intensities at equimolar concentrations in the modified U.S.P. assay, while the fluorescence intensity of riboflavin in the Burch assay is 3.4 times that of an equimolar concentration of FMN. The presence of significant amounts of riboflavin as FMN in the urine would result in appreciable differences between the results of the Burch and U.S.P. assays. There was no statistically significant difference in the urinary recovery of riboflavin as determined by the two assay methods. However, since the assay method is not sufficiently sensitive to permit determination of small amounts of FMN in the presence of considerably larger amounts of riboflavin, it can be concluded only that FMN is excreted largely, if not entirely, as free riboflavin.

All data presented in this report are based on the results obtained with the modified U.S.P. assay.

Figure 3 shows the time course of urinary excretion of riboflavin by subject *J* after oral administration of 30 and 300 mg. of riboflavin as FMN after breakfast. Maximum excretion of riboflavin after the 30-mg. dose occurred in the period 1 to 1.5 hr. after ingestion of the vitamin and was followed by an exponential decrease in the excretion rate with an apparent half-life of 1.1 hr. This was followed by a slower excretion phase after the 4th hr. The excretion curve obtained after ingestion of a 300-mg. dose of riboflavin as FMN after breakfast appeared to represent a plateau indicative of an upper limit in the absorption rate of FMN and the curve was drawn on the basis of that assumption. However, additional experiments in subject *J* and in other subjects have shown that the time course of urinary excretion rate after administration of large doses of FMN exhibits more than one maximum, and that the data for the upper curve in Fig. 3 represent two definite, although not very distinct, excretion rate maxima. More representative of the majority of results are the data shown in Fig. 4, where the excretion rate of riboflavin after administration of 150 and 300 mg. of FMN exhibited two distinct maxima.

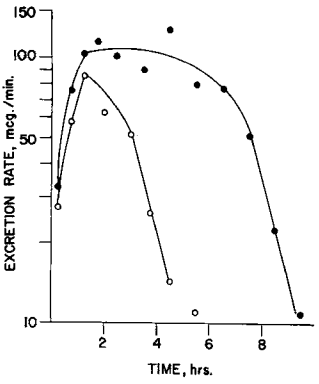

Fig. 3.—Urinary excretion rate of riboflavin as a function of time after oral administration of 30 mg. (○) and 300 mg. (●) of riboflavin as molar equivalents of FMN after a standard breakfast. Subject *J*.

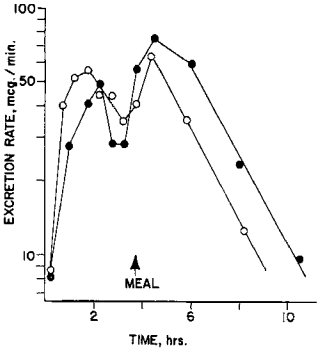

Fig. 4.—Urinary excretion rate of riboflavin as a function of time after oral administration of 150 mg. (○) and 300 mg. (●) riboflavin as molar equivalents of FMN after a standard breakfast. Subject *A*.

The second excretion maximum had a definite relation to lunch time in all of the experiments. When the experiments were repeated in two subjects with lunch being omitted, the second excretion rate maximum decreased appreciably in magnitude, but did not disappear entirely. This is shown in Fig. 5 for subject *A*. The amounts of riboflavin recovered in the urine after administration of large doses of FMN after breakfast were similar whether the subject ate lunch or omitted it (Table I).

TABLE II.—EFFECT OF TYPE AND VOLUME OF PREINGESTED MATERIAL ON URINARY RECOVERY OF RIBOFLAVIN AFTER ORAL ADMINISTRATION OF 30 mg. RIBOFLAVIN

	Conditions		
Subject	No Food	No Food, but 600 ml. Water	Std. Breakfast, Vol. Approx. 600 ml.
J	11.8	19.1	55.1
L	14.2	16.8	56.5
A	19.0	29.1	60.9
Mean	15.0	21.6	57.5

DISCUSSION

The results of the present study show that FMN has the same absorption and excretion characteristics as does riboflavin. The data suggest strongly that riboflavin and FMN are absorbed by the same specialized transport process which is saturable and located mainly or solely in the proximal region of the gastrointestinal tract. Just as with riboflavin, administration of FMN after a meal resulted in enhanced absorption of the vitamin,[2] and the post-

[2] The reader is reminded that this is a relatively specific effect and that food decreases the absorption of certain other drugs.

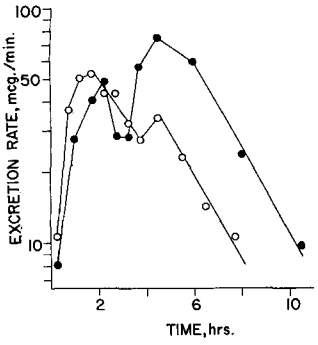

Fig. 5.—Urinary excretion rate of riboflavin as a function of time after oral administration of 300 mg. riboflavin as a molar equivalent of FMN after a standard breakfast with (●) and without (○) lunch. Subject *A*.

absorption excretion rate declined exponentially with a half-life of about 1.1 hr., followed by a slower component after about 4 hr. The latter may reflect the elimination of riboflavin from a "deep" compartment or it may be due to decreased renal clearance at low riboflavin concentrations, similar to the excretion characteristics of another B vitamin, pantothenic acid (5). It has now been possible to demonstrate by the use of large doses of FMN that saturation of the absorption process responsible for the gastrointestinal absorption of vitamin B_2 can be achieved even when the vitamin is administered after a meal. These results are consistent with the assumption that the more extensive absorption of riboflavin and FMN, when given on a full stomach, is due to prolonged retention of the vitamin at sites of absorption. The effect of food on the urinary recovery of riboflavin is not primarily a volume effect since the ingestion of an equivalent volume of water rather than food prior to riboflavin administration does not result in the marked increase in urinary recovery of riboflavin obtained when the vitamin is taken after a standard breakfast (Table II). However, the ingestion of a large volume of water may have a significant effect on riboflavin absorption, possibly by increasing the contact area of riboflavin with intestinal absorption sites.

The maximum amount that was recovered from the subjects under the experimental conditions was around 25 mg. as shown in Table I and Fig. 2, except for the one unusual value of 48.0 mg. The data reported recently by Stripp (4), which are also plotted in Fig. 2, show a lower maximum value. This difference in the maximum amount recovered is probably mainly due to the fact that a standard breakfast was ingested before administration of the vitamin in the present study, while the subjects in the Stripp study were "on an unrestricted diet."

The authors' previous data (1) and those of Stripp have shown that the upper limit in the urinary recovery of riboflavin is not due to a renal transport maximum, although there is evidence that riboflavin is excreted not only by glomerular filtration, but also by renal tubular secretion (6). Renal excretion rates over 450 mcg./min. (based on a 45-min. collection period) were observed by Stripp when 84 mg. of riboflavin was administered by intravenous infusion (4).

A review of the literature provides considerable information to explain the similarity in the absorption and excretion characteristics of FMN and riboflavin. There is extensive evidence showing that FMN is rapidly and almost completely dephosphorylated to free riboflavin in the small intestine. Okuda has found that FMN is rapidly decomposed enzymatically to free riboflavin in pancreatic juice, in homogenates of the small intestinal mucosa, and when injected into the lumen of the small intestine of rats (7, 8). Similar results were obtained by Turner and Hughes, who used isolated rat intestinal preparations (9). Okuda has found pronounced phosphomonoesterase activity in the jejunum and somewhat less in the duodenum and ileum of the rat (10). In this connection, it is of interest that betamethasone phosphate was found to undergo rapid dephosphorylation during gastrointestinal absorption and that only the free drug was found in the jugular blood of dogs after oral administration of this steroid ester (11).

Other studies reveal that riboflavin is eventually converted or reconverted to the phosphate ester (FMN) before it leaves the intestinal mucosa. Yagi and Okuda (12), Chen and Yamauchi (13), and Englard (14) have reported that riboflavin is phosphorylated enzymatically in the mucosa during absorption. Interestingly, Tedeschi and Canali (15, 16) find predominantly FMN in the *mesenteric* blood of rabbits after introduction of FMN or riboflavin into the intestine, while Stripp finds only the free vitamin in the *venous* blood of humans after oral administration of FMN (4). Thus, it appears that FMN is dephosphorylated in the intestinal lumen, rephosphorylated in the intestinal mucosa, transported in the blood to the liver as the phosphate ester, rapidly dephosphorylated in the liver, circulated as free riboflavin, and excreted mainly as such. This scheme represents the quantitatively important pathways in the metabolic disposition of large doses of the vitamin, and neglects the nutritionally more important but quantitatively minor biotransformation and retention of riboflavin in the form of FAD and various flavoproteins. The similarity in the absorption and excretion characteristics of riboflavin and FMN, respectively, can therefore be readily explained by the frequent and rapid interconversions of the two forms of the vitamin in the body. Such a pattern is by no means unique; Lawrence *et al.* have suggested that repeated hydrolysis and re-esterification in the intestinal mucosa occur during the absorption and storage of vitamin A (17).

Metabolism of Riboflavin and FMN.—Orally administered FMN was excreted in the urine mainly or solely as riboflavin. The same was true for riboflavin since no appreciable differences were found between the result of the Burch and modified U.S.P. assays of urines after oral administration of riboflavin (1). These findings are consistent with those of other investigators who could detect only riboflavin itself in the urine after oral or intravenous administration of riboflavin and FMN (4, 18, 19). However, Utsumi found that about 20% of an intravenous dose of FMN or riboflavin was excreted as FMN by ureter-cannulated patients with kidney disease (20). Since it is known that FMN is

hydrolyzed enzymatically in bladder urine (21), the results obtained by Utsumi may be due to by-passing of the bladder. It is of interest that Utsumi found FMN in the urine after intravenous administration of FMN as well as riboflavin (20). This may be due to some phosphorylation of riboflavin in the kidneys (22).

Apparent Enterohepatic Cycling of Riboflavin.—The second maximum in the excretion rate of riboflavin as a function of time (Figs. 3–5) appears to be due to the enterohepatic cycling of the vitamin. There is considerable evidence in the literature to support this hypothesis. In 1929, Makimura noted the presence of vitamins A, B, ascorbic acid, and vitamin D in the bile and proposed that enterohepatic cycling is responsible for the conservation of these vitamins (23). DePreux found riboflavin, mainly as FMN, in bile obtained by duodenal cannulation of healthy men (24). Tedeschi found mainly free riboflavin in the bile after introduction of FMN into the mesenteric circulation of rabbits (25). Yagi observed an accumulation of riboflavin in the small intestine of rats after subcutaneous injection of riboflavin and suggested that enterohepatic cycling was responsible (26). He provided additional data in support of this hypothesis in a recent study using ^{14}C-labeled riboflavin (27). Mizuhara (28) and Ono (29) perfused rabbit livers with blood containing riboflavin and dehydrocholic acid (the latter was used apparently to stimulate bile flow) and found that about 50% of the vitamin appeared in the bile.

Since Najjar *et al.* (30) found no increase in fecal riboflavin after intravenous injection of 5 to 30 mg. of riboflavin in man, and Stripp recovered 97% of an 84-mg. intravenous dose of riboflavin in the urine (4), it may be concluded that, in man at least, any riboflavin secreted into the bile is almost fully reabsorbed. It appears, therefore, that when large doses of riboflavin or FMN are administered, part of the absorbed vitamin is eliminated in the bile. When bile flow is stimulated by food, the vitamin enters the intestine and is reabsorbed, yielding the second peak in the excretion rate *versus* time plots obtained after riboflavin administration.

The possibility that the second peak in the excretion of riboflavin is due to delayed gastric emptying of a portion of the dose is ruled out by the similarity in the urinary recoveries of riboflavin in the experiments with and without lunch. Delayed gastric emptying of riboflavin would result in more extensive absorption of the vitamin in view of the saturability of its absorption mechanism.

In the two instances when the subjects repeated the experiments but did not eat lunch, the second excretion rate peak was considerably reduced in magnitude, but did not disappear entirely. Apparently, the thought of food at lunch time and the sight of others enjoying their lunch was sufficient to stimulate some flow of bile in the subjects. It is known that, while the emptying of the gallbladder normally follows the ingestion of certain foods, psychic influences will play a role. The sight and smell of food alone can produce a reflex stimulation of the gallbladder in both man and animals (31).

The second peak in the excretion rate of riboflavin was seldom seen with low doses (30 mg. or less) of riboflavin or FMN. While the reason for this is not readily apparent, it may be due to a concentration dependent relationship in the handling of riboflavin by the liver.

REFERENCES

(1) Levy, G., and Jusko, W. J., *J. Pharm. Sci.*, **55**, 285 (1966).
(2) Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457(1948).
(3) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 907.
(4) Stripp, B., *Acta Pharmacol. Toxicol.*, **22**, 353(1965).
(5) Roholt, K., and Schmidt, V., *Scand. J. Clin. Lab. Invest.*, **3**, 108(1951).
(6) Levy, G., and Jusko, W. J., *J. Pharm. Sci.*, **55**, 1322 (1966).
(7) Okuda, J., *Chem. Pharm. Bull. (Tokyo)*, **6**, 662(1958).
(8) *Ibid.*, **6**, 665(1958).
(9) Turner, J. B., and Hughes, D. E., *Quart. J. Exptl. Physiol.*, **47**, 124(1962).
(10) Okuda, J., *Chem. Pharm. Bull. (Tokyo)*, **7**, 295(1959); through *Chem. Abstr.*, **54**, 25133(1960).
(11) Symchowicz, S., Zeman, W. V., Williams, R. A., and Tabachnick, I. I. A., *Arch. Intern. Pharmacodyn.*, **158**, 360 (1965).
(12) Yagi, K., and Okuda, J., *Koso Kagaku Shin.*, **13**, 111 (1958); through *Chem. Abstr.*, **53**, 13226(1959).
(13) Chen, C., and Yamauchi, K., *J. Vitaminol. Japan*, **6**, 247(1960).
(14) Englard, S., *Federation Proc.*, **11**, 208(1952).
(15) Tedeschi, G. G., and Canali, V., *Ricerca Sci.*, **23**, 517 (1953); through *Chem. Abstr.*, **49**, 4104(1955).
(16) Tedeschi, G. G., and Canali, V., *Ricerca Sci.*, **23**, 851 (1953); through *Chem. Abstr.*, **47**, 10084(1953).
(17) Lawrence, C. W., Crain, F. D., Lotspeich, F. J., and Krause, R. F., *J. Lipid Res.*, **7**, 226(1966).
(18) DeRitter, E., Scheiner, J., Jahns, F. W., Drekter, K., and Rubin, S. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 1 (1955).
(19) Lane, M., Fahey, J. L., Sullivan, R. D., and Zubrod, C. G., *J. Pharmacol. Exptl. Therap.*, **122**, 315(1958).
(20) Utsumi, K., *Japan. J. Dermatol.*, **72**, 116(1962).
(21) Travia, L., *Quaderni Nutriz.*, **13**, 36(1953); through *Chem. Abstr.*, **50**, 8034(1956).
(22) Katagiri, M., *Vitamin*, **20**, 70(1960); through *Chem. Abstr.*, **61**, 16556(1964).
(23) Makimura, H., *Acta Med. Keijo*, **12**, 147(1929); through *Chem. Abstr.*, **24**, 4808(1930).
(24) DePreux, R., *Z. Ges. Exptl. Med.*, **110**, 12(1942).
(25) Tedeschi, G. G., *Boll. Soc. Ital. Biol. Sper.*, **30**, 663 (1954); through *Chem. Abstr.*, **49**, 1919(1955).
(26) Yagi, K., *J. Biochem.*, **41**, 757(1954).
(27) Yagi, K., Nagatsu, T., Nagatsu-Ishibashi, I., and Ohashi, A., *ibid.*, **59**, 313(1966).
(28) Mizuhara, S., *J. Japan Biochem. Soc.*, **22**, 11(1950); through *Chem. Abstr.*, **45**, 1661(1951).
(29) Ono, K., *Folia Endocrin. Jap.*, **26**, 322(1951); through *Biol. Abstr.*, **26**, 17581(1952).
(30) Najjar, V. A., Johns, G. A., Medairy, G. C., Fleishmann, G., and Holt, L. E., *J. Am. Med. Assoc.*, **126**, 357 (1944).
(31) Bockus, H. L., "Gastroenterology," vol. III, 2nd ed., W. B. Saunders Co., Philadelphia, Pa., 1965, p. 581.

Stability of Certified Dyes in Tablets III

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A colorimetric method is given for studying the stability of pharmaceutical tablet formulations. The data obtained were employed to calculate color difference units by using the Adams-Nickerson and the MacAdams equations. The fastness of the colorants in the particular formulation studied was rated relative to one another, and these ratings were compared to other pharmaceutical stability references. The methodology of studying color stability is applicable to many types of pharmaceutical preparations.

METHODS OF determining the stability of pharmaceutical colorants have appeared in several reports (1–5). Spectrophotometric measurements have provided a physical evaluation of color; but, in order to obtain a quantitative color measurement which relates to human color perception, colorimetry must be used. With the use of colorimetric instruments, the techniques of measurements and means of analyzing data have been continually expanding. The variation in physical form and composition of pharmaceutical dosage forms present additional problems in both instrumental methods and data analysis.

The pharmaceutical formulator has a need for predicting the fastness or stability of pharmaceutical colorants. Reference tabulations (6, 7) have served as his guide for color selection for many years. Modern instrumentation applied to simple, colored pharmaceuticals aged under standardized conditions provides useful data for the formulator. Similar studies have been carried out in the textile and paint industries (8–10) and furnish a guide for color stability testing of pharmaceuticals.

The purpose of this study is to describe a method of measuring color stability of pharmaceutical tablets. Color difference units are used to describe the chromaticity and lightness changes of the colors before and after aging. Particular colorants will be rated for color stability in the specific pharmaceutical composition studied. The fastness of the colorants will be rated relative to each other, and these ratings will be compared to those listed in other pharmaceutical color stability references (1, 6, 7). Differences in ratings of color stability in several references will be explained and reasons given why the color ranking proposed in this report is regarded as superior. The methodology involved is applicable to color stability testing of many pharmaceutical dosage forms.

EXPERIMENTAL

Colorants.—D&C and FD&C colorants were employed in several concentrations and are listed in Table I.

Formulations.—A basic formula was chosen and used throughout so that the relative inherent color stabilities could be determined. Mannitol was employed as the diluent because of its inertness and its ability to be uniformly colored. Magnesium stearate, 1%, was employed as the lubricant because of its wide usage. Since most formulations contained pigments, a dry blending and milling procedure was used for their preparation. In some instances, soluble colorants were used and these were dissolved in an appropriate solvent, and the resulting solutions were used to dye the mannitol. Tablets weighing 20 Gm. and measuring $2^1/_4$ in. in diameter were compressed on the Carver press at 20,000 lb./sq. in.

Exposure.—After initial measurement, tablets were exposed to cool, white fluorescent light measuring 1000 ft.-candles as determined by a model 614 Weston light meter. The fluorescent light was furnished by a metal cabinet which enclosed a bank of twelve 30-w. and four 20-w. General Electric bulbs (F30T12·CW·RS and F20T12·CW). Heat buildup was prevented by a constantly operating fan.

Measurement.—Colorimetric measurements were made at various time intervals that were appropriate depending on rapidity of color fading. The Color-Eye[1] model D-1 colorimeter was used with the nonspecular insert and white vitriolite standards. The following sets of equations were used to calculate the tristimulus values X_{CIE}, Y_{CIE}, and Z_{CIE}.

$$X_{CIE} = .69664\ X_{CE} + .17416\ Z_{CE} \quad \text{(Eq. 1)}$$

$$Y_{CIE} = .8937\ Y_{CE} \quad \text{(Eq. 2)}$$

$$Z_{CIE} = 1.0574\ Z_{CE} \quad \text{(Eq. 3)}$$

The subscript *CIE* denotes the true tristimulus

Received April 28, 1966, from Warner-Lambert Research Institute, Morris Plains, N. J. 07950.
Accepted for publication September 21, 1966.
Presented to the Industrial Pharmacy Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
The authors are grateful for the computer program and computations performed by Mr. R. A. Lamm of the Statistical Services Department.
Previous paper: Goodhart, F., Everhard, M., and Dickcius, D., *J. Pharm. Sci.*, **53**, 338(1964).
* Whitehall Laboratories, Division of American Home Products Co., Hammonton, N. J.

[1] Product of Instrument Development Laboratories, Division of Kollmorgen Corp., Attleboro, Mass.

TABLE I.—LISTING OF RELATIVE LIGHT STABILITY OF CERTIFIED COAL TAR COLORANTS

Colorant	Colorant Concn., %[a]	Exptl. Relative Stability[b]	Δe[c]	Stability Listed by Peacock[d]	Stability Listed by Lachman[e]	Chemical Type, *CI* No.[f]
D&C Red No. 30 Lake	.025–.075	*G*	1.1–2.6	6		Indigoid 73360
FD&C Blue No. 2 Lake	.013–.039	*G*	3.6–2.8	1	10	Indigoid Lake 73015
FD&C Yellow No. 5 Lake	.028–.084	*G*	5.0–5.9	5	9	PyrazoloneLake19140
D&C Yellow No. 10	.30	*G*	1.8	3	3	Quinoline 47005
D&C Yellow No. 11	.30	*G*	3.2	2		Quinoline 47000
D&C Orange No. 17	.162	*G*	3.4	5		Monoazo 12075
D&C Green No. 6	.097	*G*	3.4	4		Anthraquinone 61565
FD&C Red No. 2 Lake	.027–.081	*M*	6.5–14.6	4		Monoazo 16185
D&C Red No. 7 Lake	.031–.093	*M*	12.0–13.5	6		Monoazo 15850
D&C Red No. 36	.09–.27	*M*	8.5–9.8	6		Monoazo 12085
D&C Blue No. 6	.097–.291	*M*	13.4–16.1	6		Indigoid 73000
D&C Orange No. 5	.162	*M*	15.0	2		Fluoran
D&C Green No. 5	.100	*M*	9.8	5	5	Anthraquinone 61570
FD&C Red No. 3 Lake	.019–.057	*P*	30.0	3	1	Xanthene 45430
D&C Red No. 9 Lake	.270	*P*	16.9	6		Monoazo 15585
D&C Red No. 11 Lake	.180	*P*	20.0	5		Monoazo 15630
D&C Red No. 19 Lake	.270	*P*	30.0	3		Xanthene 45170
D&C Red No. 21 Lake	.080	*P*	30.0	2		Fluoran 45380A
D&C Red No. 28	.100	*P*	30.0	3		Xanthene 45410
FD&C Blue No. 1 Lake	.01–.03	*P*	30.0	3	6	Triphenylmethane 42090
FD&C Yellow No. 6 Lake	.06–.10	*P*	24.5–28.0	3		Monoazo 15985
FD&C Violet No. 1 Lake	.042	*P*	30.0	3	2	Triphenylmethane 10316
FD&C Green No. 3	.100	*P*	30.0	3	7	Triphenylmethane 42053

[a] Concentration of pure dye. [b] Based on 10 days exposure to cool white fluorescent light at 1000 ft.-candles. Less than 6 MacAdam units, good (*G*), less than 16 MacAdam units, moderate (*M*), and greater than 16 MacAdam units, poor (*P*). Tablet excipient, mannitol, lubricant magnesium stearate 1%. [c] Δe, MacAdam color difference at 240,000 ft.-candle hr. or 10 days at 1000 ft.-candle hr. exposure. Range of Δe's given when two colorants concentrations were used. [d] From *Reference 6*. [e] From high intensity fluorescent light (1). Best colorant given rating of 10 and next best successive whole number ratings less than 10. [f] From *Reference 7*.

values while the subscript CE denotes Color-Eye readings. The constants in the above equations are furnished for each piece of vitriolite standard.

Computation.—The Univac SS II computer was used for calculation of Adams-Nickerson color difference, ΔE. The Codic (11) was used to calculate MacAdam color differences ΔC and Δe.

DISCUSSION

Colorimetric Measurement.—Colorimetric measurement includes three factors: (*a*) objective measurement of the reflection or transmission characteristics of a material, (*b*) suitable weightings of the measurements dependent on the illuminant properties, and (*c*) inclusion of the sensitivity of the human eye to color. Since illuminant and the eye are included in determining the final data, colorimetry is *subjective*. This is not true of spectrophotometry which involves a purely physical measurement. The Color-Eye has been found to be a very satisfactory instrument for colorimetric measurements of pharmaceutical dosage forms.

Color Differences.—Past work on color stability in pharmaceutical systems has not come to grips with the concept of color difference. Fading has been described as a function of absorbance (1–3) and as a logarithmic function of the Kubelka-Munk equation (4, 5). Since these treatments have not been related to color in the subjective sense, it was suggested that basic colorimetry be applied to the determination of color fading (5). This technique is well developed and documented, and it is reasonable to study color in pharmaceuticals in this way. A number of color difference formulas have been developed (12), but at the present time only two are used extensively and will be described below.

Adams-Nickerson.—Several formulas for the degree of color difference were tested by Nickerson and Stultz (13). One of these was based on the combination of the Adams chromatic-value diagram combined with the Munsell value scale. Color difference, ΔE, is defined as:

$$\Delta E = 40\,\{(0.23\Delta V_y)^2 + [\Delta(V_x - V_y)]^2 + [0.4\,\Delta(V_z - V_y)]^2\}^{1/2} \quad \text{(Eq. 4)}$$

V_x, V_y, and V_z are Munsell value functions given in the following three equations:

$$\frac{X}{98.04} = 1.2219V_x - 0.23111V_x^2 + 0.23951V_x^3 - 0.021009V_x^4 + 0.0008404V_x^5 \quad \text{(Eq. 5)}$$

$$\frac{Y}{100} = 1.2219V_y - 0.23111V^2{}_y + 0.23951V_y^3 - 0.021009V_y^4 + 0.0008404V_y^5 \quad \text{(Eq. 6)}$$

$$\frac{Z}{118.10} = 1.2219V_z - 0.23111V_z^2 + 0.23951V_z^3 - 0.021009_z{}^4 + 0.0008404V_z^5 \quad \text{(Eq. 7)}$$

The terms X, Y, and Z are the tristimulus values obtained by colorimetric measurement. A tabulation of V_x, V_y, and V_z appears in Judd (12) as a function of the tristimulus values. However, the calculations are cumbersome and time consuming;

therefore, the use of a computer for solving the above equations is quite useful.

MacAdam.—The former color difference equations just described assume that color space may be satisfactorily transformed uniformly throughout the *CIE* diagram using one equation. The MacAdam formula (14, 15), however, establishes sets of transformation equations which define a uniform color space within a restricted portion of *CIE* space. The general formulas giving ΔC, the chromaticity difference, and Δe, the over-all color difference in MacAdam units are given below:

$$\Delta C = K(g_{11}\overline{\Delta x^2} + 2g_{12}\Delta x\Delta y + g_{22}\overline{\Delta y^2})^{1/2} \quad \text{(Eq. 8)}$$

$$\Delta e = (\Delta C^2 + g_{33}\Delta Y^2)^{1/2} \quad \text{(Eq. 9)}$$

Substituting Eq. 5 in Eq. 6 gives:

$$\Delta e = K^2(g_x\overline{\Delta x^2} + 2g_{12}\Delta x\Delta y + g_{22}\overline{\Delta y^2}) + g_{33}\overline{\Delta Y^2} \quad \text{(Eq. 10)}$$

In Eqs. 8–10, x and y are chromaticity coordinates, and Y is lightness. The Δ's refer to differences between sample and standard. The constants in the equations K, g_{11}, g_{22}, g_{33} have been determined experimentally and vary depending on the part of color space that includes the color under study.

Since Eq. 10 requires a great deal of time for calculation, simplified means have been devised for its solution. The use of charts for rapid calculation first was described by Davidson and Hanlon (15) and later was formalized by Simon and Goodwin (16). However, when many color differences are to be calculated, the use of a suitable computer is mandatory. A color difference computer, the Codic (11), is marketed by Davidson and Hemmendinger, Easton, Pa. This desk-top-size computer allows one to calculate ΔC (Eq. 5) and ΔE (Eq. 7) in several minutes. The tristimulus values of the sample and standard along with the constants are placed into the computer by means of potentiometer dials, and the chromaticities and color differences are calculated by nulling a meter. The Codic has precision surpassing visual sensitivity; therefore, it is an excellent tool for color matching work as well as fading studies.

Studies of the merits of various color difference formulas have been conducted. Ingle *et al.* (17) compared the loci of unit color differences for the MacAdam, Adams–Nickerson, and NBS color-difference equations. His comparison points out that the three formulas vary in their sensitivities and position throughout the chromaticity diagram. Berger and Brockes (18), in a study of eight color difference formulas, concluded that only the values obtained with the MacAdam equation agreed with the results of visual color comparison. The authors' data, therefore, are interpreted using the MacAdam color difference equation.

RESULTS

Since the Adams-Nickerson color difference also was calculated, a comparison of the numerical values of ΔE and Δe from the MacAdam equation was made. Often it was found that Δe was 2 or 3 times greater than ΔE, however, exceptions were frequent depending on the magnitude of the color differences. Figure 1 gives the various color differences for B6[2] at a concentration of 0.097%. The ratio of Δe to ΔE varies from about 2.28 at the lowest exposure time to about 2.65 at the highest exposure time. This variability illustrates the nonalignment of the two color difference formulas, and this illustration is typical of the results obtained from the other colorants studied. The chromaticity difference, ΔC, gives the color difference without regard to lightness change. It was found in this study that the largest part of the color change was that of chromaticity, and the magnitude of differences in Fig. 1 between ΔC and Δe were the exception rather than the rule. If the Adams-Nickerson equation had been used as a basis of comparison instead of the MacAdam, the over-all ranking of colorant stability that would have resulted would have been nearly the same since gross deviations were not obtained.

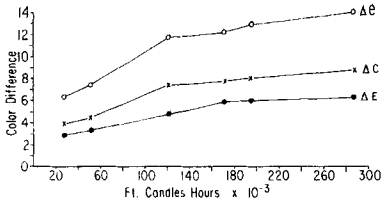

Fig. 1.—Plot of various color differences for D&C Blue No. 6, 0.097%, as a function of ft.-candle hr. exposure at 1000 ft.-candle .

In Fig. 2, MacAdam color differences are illustrated for the blue, green, and violet colorants. A wide range in stabilities can be seen for this group of colors varying from very small changes for B2 and G6 to very high and rapid color differences for B1 and V1.

A nearly consistent dependency of degree of fading on concentration can be seen in Fig. 2. Not all colorants were tested in two concentrations; but of those studied, it is seen that the color of the tablet containing the higher concentration fades to a greater extent over the same time period. The one exception is the colorant B2. This phenomenon readily is apparent visually by comparison of tablets of two colorant concentrations to their respective nonfaded standards. The lighter colored tablet after fading is closer to white, but the color difference is less than that found for the darker tablet. Godlove (19, 20) has written on the perceptibility and acceptability aspects of color acceptance with particular reference to "on tone" and "off tone" fading. He found, for instance, that the consumer is more disturbed by a change in hue than by equally perceptible changes in strength and brightness. Hue change is the exception rather than the rule in this work, but a similar question of acceptance is apparent when the same colorants in two concentrations are compared. While the tablet with the lighter color fades to a smaller degree, it may be less acceptable because of the tendency of its color to change toward white and hence diminution of color.

[2] Abbreviations for colorants will be used; the first letter of the colorant name is used for its designation and D&C and FD&C are omitted.

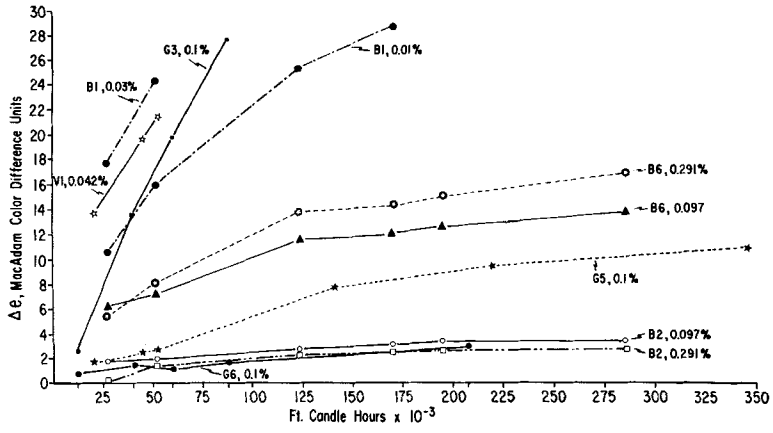

Fig. 2.—Plot of MacAdam color differences for blue, green, and violet colorants as function of ft.-candle hr. exposure at 1000 ft.-candles.

An arbitrary ranking of colorant stability in tablets is given in Table I. Also listed are the concentration ranges employed, the chemical type and CI number, along with stabilities listed by two other authors. The ranking is arbitrary and was deduced from the following considerations. A color showing fading of 6 MacAdam units after 240,000 ft.-candle hr. was given a good ranking (*G*), a color fading 16 MacAdam units after 240,000 ft.-candle hr. was given a moderate ranking (*M*), and color fading of more than 16 MacAdam units over the same period was listed as poor (*P*).

A maximum fading of 6 MacAdam units for a good rating is stringent since 2 to 3 MacAdam units are needed to give a visual color difference. Therefore those colorants in Table I having a good rating only show slightly perceptible color differences after 10 days (240,000 ft.-candle hr.). Testing for longer periods in many cases showed a leveling off of fading, and apparently the colors listed as good would not undergo further change under continued exposure. Some of the colorants given a moderate rating are satisfactory for product usage depending upon the use to which the colorant is put and its concentration. This would be especially true for R2 and R36. That group of colorants having poor stability are probably not satisfactory under any conditions of usage where the product is exposed to any light whatsoever. Among these that are highly unstable are R3, R19, R21, R28, B1, V1, and G3.

At the present time, guides to colorant stability have limited usefulness; and probably the best references are personal ones based on experience. Among the stability references are those found in Peacock's "The Application Properties of the Certified 'Coal Tar' Colorants" (6). The booklet lists the relative fastness of dyes to light as well as to oxidizing agents, reducing agents, and other chemicals. The substrate used in arriving at the rating is unknown but no doubt it had a definite effect, and this could explain the discrepancies. The same relative stabilities are listed in the *Encyclopedia of Chemical Technology* (7). Another listing of relative colorant stabilities has been given by Lachman *et al.* (1). This relative listing is based on reduction of absorbance and not on colorimetric data.

For comparison, Table I also lists colorant stabilities given by Peacock (6) and Lachman (1). It readily is seen that no correlation exists between the authors' ranking and those given by the above authors. The fact that this laboratory's data are based on colorimetry, not spectrophotometry, would explain the lack of correlation. The use of colorimetry affords a means of calculating subsequent color differences and relates directly to the actual psychophysical effect of color on the average human observer. Another point that must be kept in mind is that the substrate and additives may contribute a significant effect toward color stability. It is thought that the authors' colorimetric data should, however, be useful in predicting the stability of colorants in tablets.

Table II.—Color Differences for Duplicate Tablets Containing FD&C Red No. 3 Lake

hr.	ΔE		ΔC		Δe	
	1	*2*	*1*	*2*	*1*	*2*
			Concn., 0.019%[a]			
7	5.80	5.70	13.1	10.9	13.9	11.9
25	10.1	9.34	22.0	18.6	23.4	20.1
48	12.7	...	27.4	...	29.2	...
73	14.6	13.4	30	27.4	30	29.8
144	16.7	15.9		30		30
			Concn., 0.057%[b]			
7	8.7	9.04	12.9	17.6	13.8	18.8
25	14.0	14.9	28.3	30	30	30
48	17.8	18.1	30.0			

[a] Y = 73.2 for tablet *1* and 74.4 for tablet *2*. [b] Y = 61.7 for tablet *1* and 60.7 for tablet *2*.

No general relationship between the chemistry of colorants and stability has been found. However, the indigoid, pyrazolone, and quinoline types rank high in stability. Xanthene, fluoran, and triphenylmethane dyes rank low. Monoazo and anthraquinone colorants generally are intermediate. Functional groups and their positions no doubt are important factors in colorant stability.

Tablets of R3 were run in duplicate in order to obtain an estimate of reproduciblity. The data for the various color differences and two concentrations of the colorant are summarized in Table II. Color differences for each concentration are of different magnitudes as was noted previously. However, an inverse correlation between color difference and lightness, Y, is seen. For the lower concentration, tablet *1* has a lightness of 73.2 *versus* 74.4 for tablet *2*. Tablet *2* shows a smaller degree of fading in all instances, an effect related to the change in concentration. Even though the tablets contained the same amount of colorant, the compression of the powder gave tablets of variable lightness, a factor that alters stability to some extent. The same correlation can be seen for the higher concentration where tablet *1* is lighter than tablet *2*.

SUMMARY AND CONCLUSIONS

A method of testing color stability using a colorimetric procedure has been described. The same methodology is satisfactory for testing a variety of pharmaceutical products. A ranking of colorant stabilities in tablets is given which is intended to provide a guide for selecting a stable color for related formulations.

REFERENCES

(1) Lachman, L., Swartz, C. J., Urbanyi, T., and Cooper, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 165(1960).
(2) Lachman, L., Weinstein, S., Swartz, C. J., Urbanyi, T., and Cooper, J., *J. Pharm. Sci.*, **50**, 141(1961).
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(4) Everhard, M., and Goodhart, F., *ibid.*, **52**, 281(1963).
(5) Goodhart, F., Everhard, M., and Dickcius, D., *ibid.*, **53**, 338(1964).
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(7) *Encyclopedia of Chemical Technology*, **5**, 866(1964).
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(9) Schwen, G., and Schmidt, J., *Soc. Dyers Colourists*, **75**, 101(1959).
(10) Morton, T., *ibid.*, **65**, 597(1949).
(11) Operation Manual for Color Difference Computer, Davidson & Hemmendinger, Inc., Easton, Pa.
(12) Judd, D. B., and Wyszechi, G., "Color in Business, Science, and Industry," John Wiley & Sons, Inc., New York, N. Y., 1963.
(13) Nickerson, N., and Stultz, K., *J. Opt. Soc. Am.*, **34**, 550(1944).
(14) MacAdam, D., *ibid.*, **33**, 18(1943).
(15) Davidson, H., and Hanlon, J., *ibid.*, **45**, 617(1955).
(16) Simon, F. T., and Goodwin, W. J., Rapid Graphical Computation of Small Color Differences, Union Carbide Co., New York, N. Y.
(17) Ingle, G. W., Stockton, F. D., and Hemmendinger, H., *J. Opt. Soc. Am.*, **52**, 1075(1962).
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By R. S. MEDORA*, D. P. N. TSAO, and L. S. ALBERT

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THE FIRST work on tissue culture of the genus *Digitalis* was reported by Gautheret (1). Staba and co-workers (2–5) also described the growth and metabolism of static and suspension cultures of *Digitalis purpurea* L., *Digitalis lanata* Ehrh., and *D. purpurea* var. *gloxinaeflora* Hort., including a biotransformation study of digitoxigenin using cultures of *Digitalis mertonensis* Buxton and Darlington.

Steroids have been known to stimulate or inhibit growth in microorganisms and plant tissues (6). Tsao (7) found that 0.25% of sodium glycocholate stimulated and 5% concentrations inhibited glycoside production in *D. purpurea*. On the other hand, Chan and Tsao (8) found that sodium cholate inhibited glycoside

Received July 11, 1966, from the Department of Pharmacognosy, University of Rhode Island, Kingston.
Accepted for publication October 5, 1966.
Abstracted in part from a thesis submitted by R. S. Medora to the University of Rhode Island, Kingston, in partial fulfillment of Doctor of Philosophy degree requirements.
This research was supported in part by research grant MH-06511-02 from the National Institute of Mental Health, U. S. Public Health Service, Bethesda, Md.
The authors are grateful for gifts of Amerchol L-101 and Solulan C-24 from American Cholesterol Products, Inc., and for Carbowax 1000 from the Union Carbide Chemical Co., New York, N. Y.
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REFERENCES

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production in *D. purpurea* in concentrations of 0.5 and 2.5%. Much (9) has shown that 2% of cholesterol stimulated growth of digitalis while Tsao and Youngken (10) showed that 0.5 and 2% of cholesterol increased the glycosides in the leaves of *D. purpurea*. Buvat (11) and Levine (12) found that the degradation products of cholesterol produced more callus and organ growth in other species of plants.

These and other findings prompted the authors to investigate the effects of certain solubilized sterol mixtures and cholesterol on glycoside production when added to a medium for the callus growth of *D. mertonensis*.

EXPERIMENTAL

Sterol *A*,[1] sterol *B*,[2] and pure cholesterol[3] were selected as steroidal "precursors" to study their effects on the growth and glycoside production in the callus tissue of *D. mertonensis*. Sterol *A* (13,14) is an oil-soluble, hydrophilic, multisterol, nonionic surfactant, emolient, emulsifier, and comprises mainly the sterols from lanolin solubilized in a vehicle. Sterol *B* (13, 14) is a stable, active, water-soluble, ethoxylated derivative of cholesterol. The sterol *B* molecule is comprised of 25% pure cholesterol and 75% of the 24-molecule ethylene oxide side chain.

Preparation of the Media for Static Cultures.—Except where specified, all inorganic compounds used were of analytical reagent grade and the organic compounds[4] employed were of better available grades. A modification of White's (15) basic media was used for all experiments carried out with a semisolid media. In addition to vitamins of Eagle's solution (16), *myo*-inositol, biotin, and nicotinic acid in quantities of 200 mg., 1 mg., and 0.4 mg./L., respectively, were also included in the list of vitamins. Among others were sodium ferric ethylenediaminetetraacetate (NaFeEDTA), glycine, coconut water, sucrose, and the supplement suggested by Staba (2) for growth of digitalis callus tissue. Agar (0.7%) was added to give a semisolid consistency to the media, and 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-benzothiazoleoxyacetic acid (BTOA) in quantities of 1 mg. and 2 mg./L., respectively, were used to induce callus formation. The ingredients in formula 1 (Table I) were dissolved in glass distilled water and if necessary adjusted between pH 5.4–5.7 with potassium hydroxide. The media was then distributed into 125-ml. wide mouth conical flasks plugged with nonabsorbent cotten, covered with paper cups, and autoclaved. The ingredients of the supplement [formula 2 (Table I)] also were dissolved in glass distilled water, adjusted to the same pH as above, passed through a Millipore[5] HA filter and aseptically added to the luke warm autoclaved flasks. Totally, 20 ml. of semisolid sterile media (formulas 1 and 2) was then used in each flask to grow callus tissue.

TABLE I.—MEDIA FOR STATIC CULTURE

	mg./L.
Formula 1	
White's Modified Basal Media	
$MgSO_4 \cdot 7H_2O$	625
$Ca(NO_3)_2 \cdot 4H_2O$	200
Na_2SO_4 (anhydrous)	200
KNO_3	80
KCl	65
$NaH_2PO_4 \cdot H_2O$	19
$MnSO_4 \cdot H_2O$	4.5
$ZnSO_4 \cdot 7H_2O$	2.6
H_3BO_3	1.3
KI	0.75
$Na_2MoO_4 \cdot 2H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.003
Other Ingredients Used	
Sucrose	20,000
Agar[a]	7,000
Sodium ferric ethylenediaminetetraacetate[b]	50
Glycine	2
2,4-Dichlorophenoxyacetic acid	1
2-Benzothiazoleoxyacetic acid[c]	2
Coconut water	150 ml./L.
Eagle's Modified Formula for Vitamins (16)	
Formula 2	
Staba's Supplement (2)	

[a] Difco Laboratories, Detroit, Mich. [b] Geigy Industrial Chemicals, Ardsley, N. Y. [c] American Cyanamid Co., Princeton, N. J.

Conditions of Growth and Incubation.—MacLachlan (17) has noted that soybean seedling produced more sterols in the dark than in the light. Blackley and Stewart (18) reported that suspension cultures of *Hoplopappus* grown in the dark are more friable. It was the experience of the authors that static cultures grown in the dark were lighter in color and more friable. Friable cultures have been considered better for initiating suspension cultures (19). As the authors were interested in cultures which produced steroidal glycosides, all experiments were carried out in complete darkness.

All plant tissues were incubated in a Hotpack constant temperature room[6] model 1288-14 (operating temperatures 0 to 40°) adjusted to 27 ± 1°. The humidity of the incubator was maintained 50 to 80% by a De Vilbiss vaporizer[7] model 144.

Aseptic manipulations were carried out between Bunsen burners or in a sterile transfer chamber[8] which maintained positive air pressure and which was equipped with an ultraviolet lamp. The seeds of *D. mertonensis* were obtained from the Drug Plant Gardens, University of Washington, Seattle. They were sterilized by the method described by Staba (2). The sterile seeds were then distributed in Petri dishes, 9 cm. in diameter, which contained disks of Whatman No. 1 filter paper soaked in 10

[1] Marketed as Amerchol L-101 by American Cholesterol Products, Inc., Edison, N. J.
[2] Marketed as Solulan C-24 by American Cholesterol Products, Inc., Edison, N. J.
[3] Merck & Co., Inc., Rahway, N. J.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio, and Calbiochem, Los Angeles, Calif.
[5] Millipore Filter Corp., Bedford, Mass.
[6] Hotpack Corp., Philadelphia, Pa.
[7] The De Vilbiss Co., Somerset, Pa.
[8] Forma Scientific, Inc., Marietta, Ohio.

to 12 ml. of distilled water. The seeds were then incubated for 14 days. On the 14th day two germinated seedlings were aseptically transferred to each conical flask containing sterile media for static tissue culture. The seedlings were allowed to grow and develop into callus tissue. On the 37th day in all experiments the callus tissue from the first generation of growth was harvested, weighed (fresh weight), dried at 50° for 72 hr., and weighed again (dry weight).

Extraction of Callus from Static Culture.—The dried callus was weighed and coarsely ground. A tincture was prepared according to the U.S.P. method for digitalis tincture (20). The callus tincture was then stored in a refrigerator until used.

Extraction of the Culture Media.—On the basis of the moisture present in the media, enough alcohol was added to the media to give the same alcohol concentration as that of the menstruum used to extract the callus. Later, more menstruum was added, so that the total volume of menstruum corresponded to 2 ml. for each milliliter of the media at the start of incubation. The flasks with the media and menstruum were then shaken for 24 hr. The extractive from each lot was pooled separately, filtered through cheese cloth, and filtered twice through Whatman No. 1 filter paper. The clear extractive was evaporated to a syrupy consistency in a flash evaporator at temperatures below 50° and then lyophilized. The lyophilized extract was weighed and stored in a desiccator for future use. Two grams of the lyophilized extract was shaken for 8 hr. in 10 ml. of the menstruum. The solution was then filtered and the tincture stored for future use.

Colorimetric Assay of Baljet Positive Constituents[9] in the Callus and Media.—The assay method of the standard preparation of digitoxin in the U.S.P. XVII (21) was closely followed and a standard curve was plotted with micrograms of digitoxin *versus* absorbance. The total amounts of constituents in the callus and media tinctures were calculated as digitoxin and were determined by the modified method of Knudson and Dresbach (22) after preliminary decolorization of the tincture. To a suitable aliquot of decolorized tincture an equal volume of alkaline picrate was added. The absorbance of this solution was read on a Beckman DU spectrophotometer[10] at 495 mμ 16 min. after the addition of the alkaline picrate, against a blank using 0.5-cm. cells. The blank was prepared exactly the same way as the tincture, using aliquots of menstruum instead of the tincture.

Experiment A.—In the preliminary experiments, the concentration of 0.5% of sterol *A* was found to be optimum for the growth of the callus tissue on the basis of its dry weight. Therefore, 0.5% of sterol *A* was added to the media of some lots in this experiment. Equal quantities of the media for the growth of callus tissue were distributed in flasks with the Brewer automatic pipeting machine,[11] model 07-502, The flasks were divided into 6 lots. To the media of lots 1 and 4, 0.5% of sterol *A* was added while an equivalent amount of vehicle[12] was incorporated in lots 2 and 5. Lots 3 and 6 were used as controls. The media was prepared exactly as described earlier, except that sterol *A* was incorporated in formula 1 for lots 1 and 4 while the vehicle of sterol *A* was added to formula 1 for lots 2 and 5. Therefore, both sterol *A* and its vehicle were subjected to autoclaving with the rest of the media before ingredients of formula 2 were aseptically added. In all experiments, the callus tissue was grown in flasks of lots 1, 2, and 3, while lots 4, 5, and 6, respectively, were kept as controls for the above lots. For all experiments, the callus from each lot was harvested on the 37th day, dried, and the total constituents determined colorimetrically (Table II). The media tincture was also analyzed for the presence of total constituents (Table III).

Experiment B.—The lanolin fraction containing sterols in sterol *A* is about 1.2% (23). Sterol *B* is a molecule containing 25% by weight of pure cholesterol and 75% by weight of 24 moles of ethylene oxide side chain (14). On the basis of these data, enough sterol *B* was added to the nutrient media, such that the weight of cholesterol in it was approximately equivalent in weight to the total sterols present in sterol *A* previously added.

The side chain of sterol *B* is made up of 24 molecules of ethylene oxide and this is approximately equivalent to a molecule of polyethylene glycol 1000.[13] Therefore, polyethylene glycol 1000 was used in the media of one lot along with sterol *B* in another. Polyethylene glycol 1000 was added to the media of lots 2 and 5 in quantities equivalent to 75% of the sterol *B* added above. These lots were especially prepared to determine whether the 24 molecule ethylene oxide side chain of sterol *B* had an independent effect of its own on the callus tissue.

Totally, 6 lots were prepared for this experiment. Lots 1 and 4 contained 0.024% of sterol *B* in the media, lots 2 and 5 contained 0.018% polyethylene glycol 1000 and lots 3 and 6 were used as controls. The media was prepared the same way as described earlier. Sterol *B* and polyethylene glycol 1000 were added as extra ingredients to formula 1 of the media. The callus from each lot was harvested separately and the constituents were determined in the callus and media (Tables II and III).

Experiment C.—This experiment was carried out to determine whether pure cholesterol had any effect on the callus tissue. In the preliminary experiments, different methods were tried to incorporate cholesterol in the nutrient media in a fine suspension and the method which gave the finest suspension was employed. The ingredients of formula 1 were mixed together as described earlier and then cholesterol dissolved in chloroform was poured in the warm media agitating its container continuously. This gave the finest suspension of cholesterol. The flasks were then sterilized and the ingredients of formula 2 were added after filtration through a Millipore filter.

For this experiment 6 lots were prepared. To the flasks in lots 1 and 4 totally 0.1-ml. quantities

[9] Baljet positive constituents will be referred to as merely "constituents." Throughout the paper, constituents will be defined as total extractives of callus tissue and culture media which give a positive reaction to alkaline picric acid.

[10] Beckman Instruments, Inc., Fullerton, Calif.

[11] Baltimore Biological Laboratory Division, Becton, Dickinson and Co., Baltimore, Md.

[12] The vehicle was supplied by the American Cholesterol Co. and was devoid of the lanolin fraction.

[13] Marketed as Carbowax 1000 by Union Carbide Corp., New York, N. Y.

TABLE II.—ANALYSIS OF THE BALJET POSITIVE CONSTITUENTS[a] OF *D. mertonensis* CALLUS

Sample[b]	Treatment	Seedlings,[c] No.	Fresh Wt., mg.	Dry Wt. mg.	Dry Wt. % Difference	Baljet Positive Constituents mcg.	Baljet Positive Constituents Per mg. Dry Wt. mcg.	Baljet Positive Constituents Per mg. Dry Wt. % Difference
A1c	Sterol *A*	60	20110	1560	+13.87 *vs.* A3c +9.86 *vs.* A2c	2142	1.373	+31.76 *vs.* A3c +13.84 *vs.* A2c
A2c	Vehicle	60	18420	1420	+3.65 *vs.* A3c	1713	1.206	+15.73 *vs.* A3c
A3c	Nil	60	16560	1370		1428	1.042	
B1c	Sterol *B*	68	16750	1290	−20.86 *vs.* B3c	1713	1.327	+1.06 *vs.* B3c −5.75 *vs.* B2c
B2c	Polyethylene glycol 1000	68	24360	1520	−6.75 *vs.* B3c	2142	1.409	+7.23 *vs.* B3c
B3c	Nil	68	25710	1630		2142	1.314	
C1c	Cholesterol	38	7140	790	+21.54 *vs.* C3c +6.75 *vs.* C2c	999.6	1.265	+4.7 *vs.* C3c −6.4 *vs.* C2c
C2c	Chloroform	38	6830	740	+13.85 *vs.* C3c	999.6	1.351	+11 *vs.* C3c
C3c	Nil	38	4160	650		785.4	1.208	

[a] Calculated as digitoxin on the basis of the digitoxin standard curve. [b] The capital letters A, B, and C signify the experiment. The arabic numbers indicate treatment of the media, and "c" stands for callus extracts. Number 3 in all experiments is the control and indicates no treatment. [c] All seedlings were incubated for 37 days in the sterile nutrient media after preliminary germination of the seeds in Petri dishes for a period of 2 weeks.

TABLE III.—ANALYSIS OF THE BALJET POSITIVE CONSTITUENTS[a] OF THE MEDIA IN WHICH *D. mertonensis* CALLUS IS GROWN

Sample[b]	Treatment	Total Dry Extract from Media, mg.	Baljet Positive Constituents in 2 Gm. Extract mcg.	Baljet Positive Constituents in 2 Gm. Extract Corrected for Controls, mcg.	Baljet Positive Constituents in Total Extract, mcg.	Baljet Positive Constituents/mg. Extract mcg.	Baljet Positive Constituents/mg. Extract % Difference
A1m	Sterol *A*	13270	2523	1952	12951	0.975	−13.0 *vs.* A3m +13.9 *vs.* A2m
A2m	Vehicle	12710	2332	1716	10905	0.857	−23.5 *vs.* A3m
A3m	Nil	12560	2853	2237	14048	1.118	
A4m	Sterol *A*	6260	571				
A5m	Vehicle	6570	616				
A6m	Nil	5990	616				
B1m	Sterol *B*	12550	2616	2046	12838	1.022	+19.23 *vs.* B3m +16.25 *vs.* B2m
B2m	Polyethylene glycol 1000	12050	2283	1760	10604	0.88	+2.56 *vs.* B3m
B3m	Nil	12610	2330	1714	10806	0.856	
B4m	Sterol *B*	4350	570				
B5m	Polyethylene glycol 1000	4030	523				
B6m	Nil	3870	616				
C1m	Cholesterol	6590	1666	1190	3921	0.594	+47 *vs.* C3m +4 *vs.* C2m
C2m	Chloroform	7250	1618.4	1142.4	4141.2	0.571	+41 *vs.* C3m
C3m	Nil	7110	1285.2	809.2	2867.7	0.404	
C4m	Cholesterol	7000	476				
C5m	Chloroform	6860	476				
C6m	Nil	7740	476				

[a] Calculated as digitoxin on the basis of the digitoxin standard curve. [b] The capital alphabets A, B, and C signify the experiments. The arabic numbers indicate treatment of the media, and "m" stands for the media extracts. In lots No. 3 and 6 in each experiment the media was not treated. In all experiments, No. 1, 2, and 3 indicate callus grown lots and No. 4, 5, and 6, respectively, are controls of the above lots with no callus grown in them.

of 20% cholesterol solution in chloroform were added with a microburet. To flasks in lots 2 and 5, which were controls, 0.1 ml. of chloroform was added, and lots 3 and 6 served as controls without any adjuncts. The callus from each lot was harvested and dried. The tinctures of callus and media were assayed for their constituents (Tables II and III).

RESULTS AND DISCUSSION

Some of the seeds grown in distilled water sprouted on the 3rd day of incubation while more than 50% of the total viable seeds sprouted by the end of the first week. The seedlings were allowed to grow in the dark for 2 weeks; at the end of this period the radicle sizes varied from 1–2 in. The seedlings grown in the untreated media had the following pattern of growth. On the 6th day after the seedlings had been transferred to the nutrient media, a thickening was observed at the proximal end of the radicle, while the radicle tips of some seedlings showed slight browning. After the 10th day of in-

cubation, the thickening began to appear all over the radicles and they turned light brown. By the time the seedlings had been incubated for 12 days the thickening was very profuse and turned into a homogeneous mass of cells (callus). Some masses of callus also started to give off branches with fluffy protuberances. From this point on, the callus tissue grew larger in size, in the form of interrupted nodules, and started to turn darker in color. When this callus tissue from the first generation of growth was harvested on the 37th day and examined, it was found to be a friable, undifferentiated mass of homogeneous cells.

The callus grown in the media treated with sterol *A* grew in the form of clumps, was more friable, had more protuberances, and was lighter in color. Callus in the vehicle, cholesterol, chloroform, and polyethylene glycol 1000 treated lots had a more or less similar pattern of growth as the controls. In the case of sterol *B*-treated media, the seedlings turned yellow and then light brown even before callus growth was observed. Growth was poorer than the controls and callus tissue did not appear until the beginning of the 3rd week. The development of callus in the cholesterol-treated media was also delayed by about 2 days as compared with the controls.

In experiment *A*, sterol *A* used in 0.5% quantities in the media, showed an increase in the weights of fresh and dry callus as compared with its control (Table II). The callus from the lot treated with the vehicle showed an increase of 3.65% of the dry weight as compared with its control. However, the fact that the callus from the sterol *A*-treated media was 9.86% higher in weight than the one treated with the vehicle, showed that something else in sterol *A* besides the vehicle stimulated the growth of the callus.

In the colorimetric assay, the tincture prepared from the callus grown in the sterol *B*-treated media (Alc), showed an increase of 13.84% of the constituents per milligram dry weight of the callus compared to tincture of the callus grown in media treated with the vehicle (A2c). Compared to the tincture of the untreated callus (A3c), the increase in constituents in the tincture Alc is almost twice as much as that in A2c. Therefore, it could be surmised that, although the vehicle portion of sterol *A* has a stimulating effect on the production of constituents in the callus, the lanolin portion in sterol *A* also has a stimulating effect of its own.

The relatively high amounts of constituents in the media tinctures (Table III) are difficult to explain, particularly because the separated constituents of the media extract on a thin-layer plate are negative to Kedde's reagent. It seems, therefore, that while trying to extract the steroidal glycosides from the media, some portion of the media ingredients, along with other callus metabolites released in the media, were also extracted. Rowson (24) has pointed out that discrepancies do arise if impurities are present in the tinctures assayed for cardiac glycosides. The tincture of the untreated media (A3m), the tincture of the sterol *A*-treated media (Alm), and that of the vehicle-treated media (A2m) in which callus was grown gave a much higher reading for constituents than the tinctures of their respective controls. This indicates that during its growth the callus may have released certain constituents in the media. These constituents do not seem to resemble the digitalis glycosides as they were negative to Kedde's reagent.

From the evidence available, it is difficult to pinpoint which compounds in addition to digitalis glycosides produced a color with alkaline picrate. However, compounds such as glucose, ascorbic acid, creatinine, and lactams of α-guanidine acids, commonly found as plant and animal metabolites, are known to react with alkaline picrate (25, 26). Ascorbic acid has been known to occur in the genus digitalis (27) and Staba (3) has shown that *D. purpurea* suspension cultures hydrolyze sucrose to glucose and fructose. In spite of this, it is unlikely that the sugars are responsible for the color reaction if the absorbance is noted within 20 min. after the addition of the Baljet reagent (25).

The metabolites which react with alkaline picrate seem to be in higher quantities in tincture of untreated media than the sterol *A*-treated one. The reverse is true in the callus tincture, *i.e.*, there are greater amounts of the constituents in the callus from the treated media than from the untreated one. The A2m tincture seems to have smaller amounts of constituents compared with A3m. It seems possible, therefore, that in the absence of the vehicle or the vehicle with the lanolin fraction, more constituents are leached out of the callus cells into the media.

In experiment *B*, the presence of both sterol *B* and polyethylene glycol 1000 in the media seemed to inhibit growth of the callus tissue (Table II). However, the presence of polyethylene glycol 1000 in the media increased the constituents per milligram dry weight of the callus as compared to its control. On the other hand the presence of sterol *B* in the media did not seem to bring about a significant change in the constituents per miligram dry weight of the callus.

There was a significant increase in constituents in the media containing sterol *B*, although only a slight increase in constituents in the media containing polyethylene glycol 1000. The data obtained (Tables II and III) of the growth, constituents of callus, and release of constituents in the media for sterol *B* and polyethylene glycol 1000 treatment vary considerably. However, it is probable the 24-molecule ethylene oxide side chain on sterol *B* is not so inhibitory to growth of the callus as is the cholesterol moiety itself.

In experiment *C*, due to the breakdown in the refrigeration mechanism of the growth chamber, the temperatures in the chamber rose above 27° on various occasions. This retarded the growth of the tissue and the final yield of callus at harvest was less in weight compared to experiments *A* and *B*. Because of the fluctuation in temperature, the authors are skeptical about the results of experiment *C*.

The dry weight of the callus harvested from the cholesterol-treated media was 21.54% higher than the callus harvested from the untreated media; however, only 6.75% higher than the chloroform-treated media. The chloroform-treated lot showed an increase in 13.85% compared to its control. After autoclaving the media, it seems doubtful that enough chloroform stayed behind to produce an effect on the callus growth. However, if some did stay behind, the stimulating effect of less than

0.5% chloroform on the callus is not surprising in light of the findings that solvents such as alcohol in concentration of 0.2 to 0.3% enhanced Avena coleoptile elongation (28).

There is a decrease in the constituents in the callus harvested from the media treated with cholesterol as compared to the callus obtained from its chloroform control. However, in case of the media tincture, the condition is reversed. Because of fluctuation in temperature during experiment *C*, it is not possible to draw any definite conclusions.

From the over-all data of experiment *A*, it seems reasonable to recommend 0.5% sterol *A* as an extra adjunct to the media for the cultivation of static callus cultures of *D. mertonensis*.

SUMMARY

A method is described for the growth of callus tissue from the seedlings of *D. mertonensis* in tissue culture. Certain sterols were also studied for their effects on callus growth and digitalis-like glycoside production.

Increases in callus growth and constituents positive to the Baljet test were observed when a solubilized lanolin fraction sterol *A* was added to the nutrient media in amounts of 0.5%.

Cholesterol, as an adjunct to the culture media in quantities of 0.1%, stimulated callus growth. However, 0.024% of an ethoxylated derivative of cholesterol sterol *B* with a 24-molecule ethylene oxide side chain and a polymer of ethylene oxide (polyethylene glycol 1000) in quantities of 0.018% inhibited callus growth.

Constituents in the callus, positive to the Baljet test increased slightly in the presence of cholesterol and polyethylene glycol 1000. There was no significant change in the callus constituents when sterol *B* was added to the media.

REFERENCES

(1) Gautheret, R. J., "La Culture des Tissues Vegetaux. Techniques et Realisations," Mason et Cie, Paris, France, 1959, p. 42.
(2) Staba, E. J., *J. Pharm. Sci.*, **51**, 249(1962).
(3) Staba, E. J., and Lamba, S. S., *Lloydia*, **26**, 29(1963).
(4) Buchner, S. A., and Staba, E. J., *J. Pharm. Pharmacol.*, **16**, 733(1964).
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(6) Heftman, E., *Ann. Rev. Plant Physiol.*, **14**, 225(1963).
(7) Tsao, D. P. N., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 232(1957).
(8) Chan, F. T., and Tsao, D. P. N., *ibid.*, **46**, 228(1957).
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(14) Maso, H. F., personal communication.
(15) White, P. R., "The Cultivation of Animal and Plant Cells," 2nd ed., The Ronald Press Co., New York, N. Y., 1963, p. 59.
(16) Eagle, H., *Science*, **130**, 432(1959).
(17) MacLachlan, H. J., *J. Biol. Chem.*, **113**, 197(1936).
(18) Blackley, L. M., and Stewart, F. C., *Am. J. Botany*, **48**, 351(1961).
(19) Street, H. E., Henshaw, G. G., and Buiatti, M. C., *Chem. Ind.*, January **2**, 27(1965).
(20) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 189.
(21) *Ibid.*, p. 191.
(22) Knudson, A., and Dresbach, M., *J. Pharmacol. Exptl. Therap.*, **20**, 205(1923).
(23) Medora, R. S., Ph.D. Thesis, University of Rhode Island, Kingston, R. I., 1965, p. 76.
(24) Rowson, J. M., *Pharm. J.*, **119**, 71, 88(1954).
(25) Bell, F. K., and Krantz, J. C., Jr., *J. Pharmacol. Exptl. Therap.*, **83**, 213(1945).
(26) Waldi, D., in "Thin-Layer Chromatography. A Laboratory Handbook," Stahl, E., ed., Academic Press Inc., New York, N. Y., 1965, p. 499.
(27) Franjo, M. F., *Farm. Glasnik*, **6**, 1(1950).
(28) Mer, C. L., *Nature*, **183**, 1812(1958).

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Heats of transition and fusion have been determined for methylprednisolone and sulfathiazole by differential thermal analysis calorimetry. Methylprednisolone undergoes an endothermic transition at 209°, with heat of transition, 1380 ± 50 cal. mole^{-1}. Fusion occurs at 240°, with heat of fusion, 5350 ± 200 cal. mole^{-1}. Sulfathiazole undergoes an endothermic transition at 161°, with heat of transition, 1420 ± 40 cal. mole^{-1}. Fusion occurs at 200°, with heat of fusion, 5960 ±210 cal. mole^{-1}.

As part of a program investigating the phenomenon of polymorphism in pharmaceuticals it was desirable to obtain reliable data on the heats of transition and fusion for a large number of compounds. Adiabatic calorimetry (1), solubility measurements (2–4), and differential thermal analysis were among the techniques considered for use in these determinations. Calorimetry was rejected as being too time consuming for routine analyses. There are a num-

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REFERENCES

(1) Gautheret, R. J., "La Culture des Tissues Vegetaux. Techniques et Realisations," Mason et Cie, Paris, France, 1959, p. 42.
(2) Staba, E. J., *J. Pharm. Sci.*, **51**, 249(1962).
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ber of factors which tend to reduce the utility of the solubility method. Practically speaking, its use is restricted to systems exhibiting only two polymorphic forms. It is further limited to those systems in which transitions occur within a temperature range for which liquids having appropriate solvent properties are available if one is to obtain data that can be extrapolated with confidence.

Differential thermal analysis (DTA) is a technique in which the heat loss or gain resulting from physical or chemical changes occurring in a sample is recorded as a function of temperature or of time as the substance is heated at a uniform rate. Heat or enthalpic changes, either endothermic or exothermic, are caused by phase transitions—such as fusion, crystalline structure inversions, boiling, sublimation, and vaporization. Solid–solid transitions, fusion, and dehydration generally produce endothermic effects, whereas crystallization and oxidation produce exothermic effects (5).

In DTA a sample substance and a thermally inert reference substance are heated at a fixed rate by an external furnace. The junctions of a differential thermocouple are embedded in or near the sample and inert reference. As the temperature increases the electromotive forces developed by the junctions are amplified and compared by displaying on a recorder. Exothermic or endothermic changes are consequently recorded as deviations from a base line, and appear as peaks on one side of the base line or the other (6).

Although primarily a tool for qualitative analysis, DTA has been employed for estimating heats of reaction, fusion, and transition. These thermodynamic functions can be evaluated by integrating the endothermic or exothermic peak produced as the sample reacts, melts, or undergoes a solid-state transition. The area of the peak is proportional to the heat absorbed or evolved in the sample transformation. The heat involved in the process, ΔH, is given by

$$\Delta H = \psi \int_{t_1}^{t_2} \theta \, dt$$

where ψ is the proportionality constant, usually evaluated experimentally, or (rarely) calculated from instrument parameters; t_2 and t_1 are the times at the end and beginning of the peak; θ is the differential temperature; and t is the time.

Kung and Goddard (7) used this technique to obtain an estimate of the heat effects associated with the formation of a complex between sodium lauryl sulfate or myristyl sulfate and lauryl alcohol or myristyl alcohol. They pointed out that although the area of the DTA peak is primarily controlled by the enthalpy change involved, it is, at constant heating rate and sample size, also a function of the heat capacity and thermal conductivity of the sample. The latter property for solid substances is affected by the state of packing of the sample.

Varma (8) determined the heats of fusion for a group of organic compounds, including catechol and naphthalene. Care was taken to maintain the quantity of material employed in each determination exactly the same. Effects of thermal diffusivity of the different materials used in the analyses were ignored, however.

Sakurai and Yabe (9) have calculated the heat absorbed in the solid phase transition and fusion of *n*-hexadecanamide. Other quantitative applications of DTA have been reviewed periodically by Murphy (10).

There are a number of shortcomings associated with the use of conventional DTA equipment for the quantitative evaluation of thermodynamic functions, and several of these shortcomings have been pointed out by Speros and Woodhouse (6). In an attempt to overcome these objections, various design modifications have been proposed from time to time to improve the precision and accuracy of DTA instruments. The equipment used in this investigation incorporates modifications which were first proposed by Boersma (11). Holders for sample and reference are located in separate cavities in a nickel heating block. A resistance heating element is wound around this block, and chromel–alumel thermocouples contact the bottoms of the holders. This construction affords high resistance to heat transfer. When a sample undergoes transition or reaction, the heat developed is retained by the sample and its holder. As a result, the heat values obtained are independent of sample size, specific heat, and packing density.

The primary purpose of this investigation was to determine whether DTA calorimetry could be employed routinely, conveniently, and with confidence in the determination of thermodynamic parameters for organic crystalline materials undergoing polymorphic transformations. Two substances whose heats of transition had previously been determined by an independent method, methylprednisolone (3) and sulfathiazole (4), were selected for examination by DTA calorimetry.

EXPERIMENTAL

Materials.—Gallium, indium, tin, and zinc were

Fisher certified reagents. Ethanol U.S.P. was employed as recrystallization solvent for sulfathiazole, and Merck and Co. reagent grade acetone was employed as recrystallization solvent for methylprednisolone. Sulfathiazole N.F. XI was obtained from Merck and Co. Methylprednisolone[1] N.F. was supplied by Upjohn Laboratories.

Calibration of the Calorimeter Cell.—The instrument employed in these determinations was the Dupont 900 differential thermal analyzer equipped with a Dupont modular calorimeter cell. The cell was calibrated with gallium, indium, tin, and zinc. Six samples of each of these substances were weighed into aluminum sample liner holders fashioned with the aid of a dowel pin. The aluminum foil used for this purpose was 0.001 in. thick. No liner was employed in the reference holder.

The samples were weighed on a Cahn Electrobalance to ±0.002 mg. Sample sizes ranged from approximately 4 mg. for gallium to as much as 13 mg. for indium, the sample size being selected to produce a fusion peak whose area could be measured with an error of not more than 2%.

Samples of indium, tin, and zinc were each heated in the calorimeter cell from room temperature to 20–50° above the melting point. Samples of gallium were cooled to −40° with the aid of a dry ice–acetone mixture, then heated to 20° above the melting point. The heating rate used was 10° min.$^{-1}$.

In each case a graph of differential temperature as a function of reference temperature gave an essentially straight base line, with a single endothermic peak corresponding to fusion. The areas of these endothermic peaks were determined by use of a Keuffel and Esser Compensating Polar Planimeter. The area to be measured was found by drawing a line from the point where the thermogram first departed from the base line to the point where the base line was resumed following fusion.

The fusion temperature for each material was taken as the extrapolated onset temperature of the endothermic peak. The low temperature side of the fusion peak was extrapolated to the prefusion base line. Since the temperature response of the chromel-alumel thermocouples employed in the calorimeter cell is nonlinear, a small temperature correction (never more than 4°) had to be applied to obtain the corrected fusion temperature.

Calibration coefficients were calculated from the following equation:

$$E = \frac{\Delta H\ M\ a}{A\ T_s \Delta T_s}$$

where

E = calibration coefficient, mcal. °C.$^{-1}$ min.$^{-1}$,
ΔH = heat of fusion, mcal. mg.$^{-1}$,
M = sample mass, mg.,
a = heating rate, °C. min.$^{-1}$, 10° min.$^{-1}$,
A = peak area, in.^{2},
T_s = x-axis sensitivity, °C. in.$^{-1}$, 20° in.$^{-1}$, except for zinc, 50° in.$^{-1}$,
ΔT_s = y-axis sensitivity, °C. in.$^{-1}$, 0.5° in.$^{-1}$.

Heats of fusion employed in the above equation were as follows: gallium, 19.9 cal. Gm.$^{-1}$; indium, 6.79 cal. Gm.$^{-1}$; tin, 14.2 cal. Gm.$^{-1}$; and zinc, 27.0 cal. Gm.$^{-1}$ (12). Calibration coefficients and fusion temperatures obtained were as follows: gallium, 7.40 mcal. °C.$^{-1}$ in.$^{-1}$ and 29.8°; indium, 9.37 mcal. °C.$^{-1}$ in.$^{-1}$ and 156°; tin, 11.0 mcal. °C.$^{-1}$ in.$^{-1}$ and 231°; and zinc, 16.0 mcal. °C.$^{-1}$ in.$^{-1}$ and 418°. These data are plotted in Fig. 1.

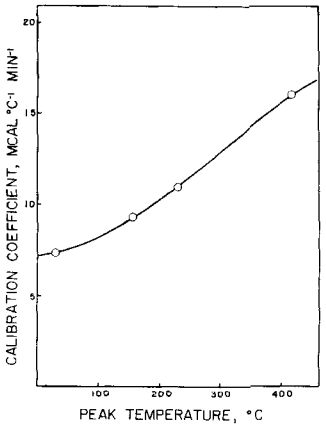


Fig. 1.—Calibration curve for the DTA calorimeter cell.

Measurement of Heats of Fusion and Transition.—Two crystal forms of sulfathiazole were obtained by the methods described by Milosovich (4). Form I, obtained by slow crystallization from warm alcohol consisted of hexagonal plates. Observed on a Kofler hotstage, these crystals underwent a transition to prisms at 155–162°, the exact temperature of the transition being dependent upon the heating rate employed. If a rapid heating rate was employed the crystals melted at 175° without undergoing a polymorphic transition. The melting point of the prismatic crystals was 201°, with decomposition. Form II was obtained by heating form I to 180° in an oven.

Form I of methylprednisolone was prepared by recrystallization from cold acetone. Form II was obtained by heating form I to 210° in an oven. Observed on a Kofler hotstage, form I was transformed to form II over the range 209–213°, with final melting occurring at 238–242°, with decomposition.

Infrared spectra of the polymorphs were taken as Nujol mulls and as potassium bromide pellets on a Beckman IR-10 spectrophotometer. Spectra of the two forms of methylprednisolone were identical with those reported by Higuchi *et al.* (3). The spectrum of sulfathiazole reproduced in the "Sadtler Standard Spectra" catalog is apparently that of form I (13).

Six samples of each polymorphic form were weighed into aluminum sample holder liners, and thermograms, graphs of differential temperature as a function of reference temperature, were obtained. The sulfathiazole samples employed weighed approximately 4.9 mg., and the methylprednisolone samples, approximately 6 mg. Typical thermograms for the four polymorphic forms investigated are shown in Figs. 2 and 3. In these thermograms, however, the y-axis sensitivity setting employed was 0.5° in.$^{-1}$ in the case of sulfathiazole and 0.2° in.$^{-1}$ in the case of methylprednisolone, whereas in the case of the thermograms from which area measurements were taken, only the fusion peaks

[1] The author is indebted to Dr. A. J. Taraszka for a gift of the methylprednisolone used in this study.

were determined at these settings. The transition peaks were determined at a higher sensitivity setting (0.2° in.$^{-1}$ for sulfathiazole and 0.1° in.$^{-1}$ for methylprednisolone) to facilitate measurement of the areas.

The temperatures of transition and fusion were determined by extrapolating the fusion endotherms to the prefusion base line and correcting this temperature for the nonlinear temperature response of the chromel–alumel thermocouples. The values of the calibration coefficients corresponding to each fusion and transition temperature were determined from Fig. 1.

Since, in the case of both sulfathiazole and methylprednisolone, transition from form I to form II occurs prior to fusion, the heat of fusion which is measured is the heat of fusion of form II. A separate determination of the heat of fusion of form II was made in each case, however, to determine whether there might be a kinetic effect which could influence the magnitude of the heat of fusion of a compound determined immediately following transition to a metastable form. Values of heats of fusion determined immediately following transition from form I to form II are listed under "Heat of Fusion, DTA, cal. mole^{-1}," while heats of fusion determined by heating form II from room temperature to fusion are listed separately. The values of the heats of transition and fusion are based on molecular weights 255.32 and 374.46 Gm. mole^{-1}, respectively, for sulfathiazole and methylprednisolone. (Table I.)

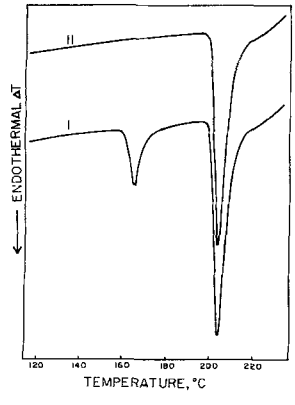


Fig. 2.—Thermograms for forms I and II of sulfathiazole.

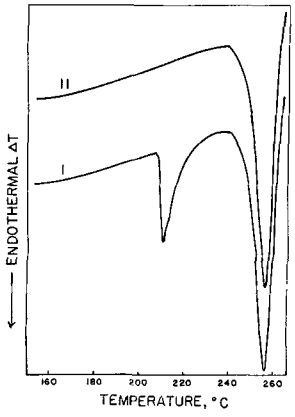


Fig. 3.—Thermograms for forms I and II of methylprednisolone.

TABLE I.—COMPARISON OF DTA CALORIMETRY AND SOLUBILITY ANALYSIS RESULTS

	Sulfathiazole	Methylprednisolone
Calibration coefficient, transition, mcal. °C.$^{-1}$ min.$^{-1}$	9.51	10.5
Calibration coefficient, fusion, mcal. °C.$^{-1}$ min.$^{-1}$	10.2	11.0
Heat of transition, DTA, cal. mole^{-1}	1420 ± 40	1380 ± 50
Temp. of transition, DTA, °C.	161	209
Heat of fusion, DTA, cal. mole^{-1}	5960 ± 210	5350 ± 200
Heat of fusion, DTA, form II, heated from room temp., cal. mole^{-1}	5970 ± 230	6020 ± 240
Fusion temp., °C.	200	240
Heat of transition, lit., cal. mole^{-1}	1744[a]	1600[b]
Temp. of transition, lit., °C.	94.5 ± 2.7[a]	118[b]

[a] *Reference 4.* [b] *Reference 3.*

RESULTS AND DISCUSSION

Clearly there is a large discrepancy in the transition temperatures obtained by the DTA calorimetry method and those obtained by the solubility method. If a heating rate of 10° min.$^{-1}$ is employed and crystals of sulfathiazole and methylprednisolone are observed on a Kofler hotstage, transition temperatures identical with those obtained by the DTA method are observed. The transition temperature of sulfathiazole is particularly sensitive to changes in the rate of heating, as has been noted by Miyazaki (14). Inoue and Saito determined the transition temperature of sulfathiazole by a qualitative DTA method. At a heating rate of 1° min.$^{-1}$ they obtained a transition temperature of 140° (15). Milosovich (4) demonstrated that form I is converted to II at 100° in glycerin suspension, and form II is converted to I at 90°. It appears that crystals of a polymorph uncontaminated with a second form, and heated in the solid state, can be subjected to considerable overheating or underheating before phase transition occurs. In spite of these large differences in transition temperatures, the values of the heats of transition for the two compounds are not far removed from those determined by the solubility method.

One of the advantages of the DTA calorimetry method is that it also affords a means of obtaining data on the heats of fusion of the compounds investigated. The two values of the heat of fusion for sulfathiazole are clearly within experimental error. There is, however, a considerable difference in the values obtained for methylprednisolone. The heat of fusion is significantly larger for form II heated from room temperature to fusion than for the same polymorph when the heat of fusion measurement is taken immediately following transition from form I to form II. This discrepancy may be due, in part, to the fact that methylprednisolone undergoes a highly exothermal decomposition reaction

immediately following fusion. This makes the precise determination of the area of the fusion peak difficult. It is possible, however, that the difference in the measured heats of fusion may indicate that some time is required following transition from form I to form II for orientation of the molecules of the crystal to occur. Since intermolecular attractive forces are not yet completely established when the fusion temperature is reached, less energy may be required to destroy the crystal lattice.

In their discussion of the mechanism of the phase transition occurring in methylprednisolone, Higuchi *et al.* speculate that the entropy difference between the two forms is a result of greater localization of the functional groups in the side chain in form I, resulting either from intermolecular or intramolecular interactions. A comparison of the infrared spectra of the two crystal forms as potassium bromide pellets reveals that there are large differences in the spectra in the regions designated as methylene stretching and bending regions (16). It seems likely, then, that there may be significant differences in the degree of participation of the methylene groups in the ring structure in intramolecular van der Waals' interactions.

In the case of sulfathiazole, the infrared spectra of forms I and II are strikingly different in the NH— stretching and bending regions. There are strong peaks in the spectrum of II corresponding to the NH— stretching vibration of the amino group (3360, 3480 $cm.^{-1}$) (17). These peaks are shifted to lower frequencies of vibration in form I. Such a shift is indicative of an increase in intermolecular hydrogen bonding (18). In addition, the intense NH_2— bending vibration at 1625 $cm.^{-1}$ in form II is reduced to a shoulder in form I. The SO_2— asymmetric stretching band is quite pronounced in form I (1320 $cm.^{-1}$), but is shifted to a lower frequency and is less intense in form II. The symmetric stretching band at 1135 $cm.^{-1}$ is little affected by the polymorphic transition.

These differences in infrared spectra provide a basis for explaining the greater solubility of form II. There is apparently a greater tendency for intermolecular hydrogen bonding in form I than in form II. Hence, there is less tendency for the molecules of sulfathiazole in form I crystals to form hydrogen bonds with the solvent. Similar interpretations have been suggested by other workers for the differences in solubility for two forms of chloroacetamide (19), and for the spectral shifts observed to occur in the various forms of estradiol (20).

SUMMARY

DTA calorimetry is a convenient method for obtaining heats of transition and fusion of pharmaceuticals. It should be particularly useful for obtaining these data when compounds which undergo several phase transitions prior to fusion must be examined. The method can also be recommended for those compounds that do not form ideal solutions with solvents appropriate for use in the solubility method. The transition temperatures observed in this method may, however, differ appreciably from those observed in the solubility method.

REFERENCES

(1) Westrum, E. F., Jr., and McCullough, E. F., in "Physics and Chemistry of the Organic Solid State," vol. 1, Interscience Publishers, Inc., New York, N. Y., 1963, p. 31.

(2) Lewis, G. N., and Randall, M., "Thermodynamics," 2nd rev., Pitzer, K. S., and Brewer, L., eds., McGraw-Hill Book Co., Inc., New York, N. Y., 1961, p. 415.

(3) Higuchi, W. I., Lau, P. K., Higuchi, T., and Shell, J. W., *J. Pharm. Sci.*, **52**, 150(1963).

(4) Milosovich, G., *ibid.*, **53**, 484(1964).

(5) Wendlandt, W. W., "Thermal Methods of Analysis," Interscience Publishers, Inc., New York, N. Y., 1964, p. 132.

(6) Speros, D. M., and Woodhouse, R. L., *J. Phys. Chem.*, **67**, 2164(1963).

(7) Kung, H. C., and Goddard, E. D., *ibid.*, **67**, 1965 (1963).

(8) Verma, M. C. P., *J. Appl. Chem.*, **8**, 117(1958).

(9) Sakurai, T., and Yabe, M., *J. Phys. Soc. Japan*, **13**, 5(1958).

(10) Murphy, C. B., *Anal. Chem.*, **36**, 347R(1964).

(11) Boersma, S. L., *J. Am. Ceram. Soc.*, **38**, 281(1955).

(12) Wagmann, D. D., Evans, W. H., Halow, I., and Dudley, R. M., in "American Institute of Physics Handbook," 2nd ed., McGraw-Hill Book Co., New York, N. Y., 1963, p. 4–170.

(13) "Sadtler Standard Spectra," vol. 1A, No. 311, the Sadtler Research Laboratories, Philadelphia, Pa., 1965.

(14) Miyazaki, H., *Japan J. Pharm. Chem.*, **19**, 133(1947).

(15) Inoue, M., and Saito, T., *J. Pharm. Soc. Japan*, **81**, 615(1961).

(16) Bellamy, L. J., "The Infra-red Spectra of Complex Molecules," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958, p. 13.

(17) Baxter, J. N., Cymerman-Craig, J., and Willis, J. B., *J. Chem. Soc.*, **1955**, 669.

(18) Colthup, N. B., Daly, L. H., and Wiberley, S. E., "Introduction to Infrared and Raman Spectroscopy," Academic Press Inc., New York, N. Y., 1964, p. 363.

(19) Katayama, M., *Acta Cryst.*, **10**, 468(1957).

(20) Smakula, E., Gori, A., and Wortiz, H. H., *Spectrochim. Acta*, **9**, 346(1957).

Detection and Separation of Penicillins by Thin-Layer Chromatography

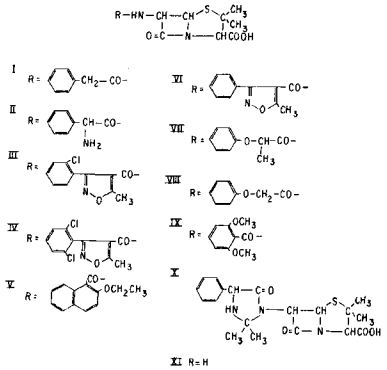
By IAIN J. MCGILVERAY and ROBERT D. STRICKLAND

Thin-layer chromatographic systems and spray reagents are described for the rapid differentiation of 10 penicillins. Comparative behavior of 18 other antibiotics under these conditions is presented together with other supporting data.

THE INTRODUCTION of new semisynthetic penicillins, two of which are official in U.S.P. XVII (1) (sodium methicillin and sodium oxacillin), has accentuated the need for convenient methods for microdetection and differentiation of members of the penicillin group. The current U.S.P. (1) and Canadian regulatory tests (2) depend upon solubility characteristics and time-consuming microbiological methods.

Paper and thin-layer chromatography have both been cited in the literature as methods for the separation of "natural" penicillins and other antibiotics (3–8). Detection techniques quoted include treatment with iodine (6, 7), iodine–sodium azide (3) and permanganate (9), and bioautography (4, 5, 8). The penicillins examined in these investigations included only those obtained from direct microbiological fermentation, although paper chromatography has been used in metabolic studies of semisynthetic analogs (10, 11). However, as far as the authors are aware, the differentiation of all the penicillins in current use—many of them manufactured by semisynthetic methods from 6-aminopenicillanic acid (6APA) (XI)—has not yet been achieved.

This paper describes some thin-layer chromatographic procedures which permit the identification of the following 10 penicillins: sodium benzylpenicillin (penicillin G) (I), sodium



ampicillin (II), sodium cloxacillin (III), sodium dicloxacillin (IV), sodium nafcillin (V), sodium oxacillin (VI), potassium phenethicillin (VII), potassium phenoxymethylpenicillin (penicillin V) (VIII), sodium methicillin (IX), and potassium hetacillin (X). Certain of these are well established clinically, while others show promise (12, 13). For purposes of comparison, the same procedures were applied to other readily available antibiotics (Table I).

EXPERIMENTAL

Preparation of Plates.—The chromatoplates (20 × 20 cm.) were coated to a thickness of 250 μ using the standard Desaga spreader. Silica Gel G (Merck) was applied according to Stahl's method (14). The plates were dried at room temperature and activated at 110° for 30 min. before use on the same day. The cellulose plates were coated with a smooth slurry prepared by stirring cellulose MN 300 without binder (Macherey and Nagel) (30 Gm.) mechanically at high speed for 15 min. with distilled water (200 ml.). They were then dried overnight at room temperature and used without preactivation.

The following systems were used. (*A*) Layer: cellulose MN 300, solvent: 0.1 *M* sodium chloride solution; (*B*) layer: cellulose MN 300, solvent: 0.3 *M* citric acid solution saturated with *n*-butanol [0.3 *M* citric acid (100 ml.) and *n*-butanol (20 ml.) were shaken and left to separate]; (*C*) layer: Silica Gel G, solvent: organic phase of isoamyl acetate–methanol–formic acid–water (65:20:5:10) (8); (*D*) layer: Silica Gel G, solvent: acetone–acetic acid (95:5) (9).

Spray Reagents.—(*a*) Ten per cent aqueous ferric chloride (20 ml.) and 5% aqueous potassium ferricyanide (10 ml.) were mixed with 20% sulfuric acid (70 ml.) and used on the day of preparation.

Received July 27, 1966, from the Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.

Accepted for publication September 19, 1966.

The authors thank Dr. Leo Levi for his interest in this work, Miss K. Fitzpatrick, Biologics Control Laboratory, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, for samples of sodium ampicillin, sodium methicillin, and potassium phenethicillin (Bristol Laboratories of Canada Ltd.), sodium benzylpenicillin and griseofulvin [Glaxo-Allenbury's (Canada) Ltd.], sodium novobiocin (Upjohn Co. of Canada), and ristocetin (Abbott Laboratories). They are indebted to the following manufacturers for generous gifts of additional samples used in this study: Ayerst Laboratories Ltd. for sodium ampicillin, sodium cloxacillin, and sodium dicloxacillin; Bristol Laboratories of Canada Ltd. for potassium hetacillin and sodium oxacillin; Burroughs Wellcome and Co. (Canada) Ltd. for polymyxin B sulfate; Cyanamid of Canada Ltd. for demethylchlortetracycline hydrochloride, chlortetracycline hydrochloride, and tetracycline hydrochloride; Glaxo-Allenbury's (Canada) Ltd. for cephaloridine; Hoechst Pharmaceuticals for pyrrolidinomethyltetracycline; Eli Lilly and Co. (Canada) Ltd. for vancomycin hydrochloride; Pfizer Company Ltd. for oxytetracycline hydrochloride; E. R. Squibb and Sons of Canada Ltd. for amphotericin B; Upjohn Co. of Canada for lincomycin hydrochloride; and John Wyeth and Brother (Canada) Ltd. for sodium nafcillin and sodium phenoxymethylpenicillin.

(*b*) Ninhydrin (0.3 Gm.) in acetone (100 ml.). (*c*) Fifty per cent sulfuric acid.

Preparation of Spotting Solutions.—Samples of each of the 10 penicillin powders (10 mg.) were dissolved in methanol (10 ml.). Similarly, samples (10 mg.) of the 18 antibiotics listed in Table I were shaken with methanol (10 ml.) and the supernatants spotted. Tenfold dilutions of the penicillin solutions with methanol were made when required for determining the sensitivity of the detection method.

Chromatographic Procedure.—Samples of the methanolic solution of each penicillin salt representing 1 mcg. (1 μl.) were applied to a plate by means of a Hamilton micro-syringe. The plates were inserted in a previously equilibrated filter paper-lined tank and the appropriate solvent allowed to rise to a height of 15 cm. (approximate times taken: system, *A*, 70 min.; *B*, 120 min.; *C*, 50 min.; and *D*, 20 min.). The plates were dried thoroughly in a stream of warm air except when system *C* was used, in which case drying was accomplished by heating at 120° for 20 min. and cooling before spraying. The spots were observed as blue areas on a yellow-green background after spraying lightly with reagent (*a*). Full color development took up to 5 min., the speed depending on the nature of the penicillins.

The sensitivity of this spray reagent was determined by spotting dilutions of the 10 penicillin solutions in amounts down to 0.04 mcg. with a Burroughs Wellcome Agla syringe on plates treated in the usual way in the four systems.

A plate loaded with the 10 penicillins was chromatographed as described above with system *A*, sprayed with reagent (*b*) after drying, and heated at 100° for 5 min. Only one violet spot corresponding to sodium ampicillin (II) was observed. Similarly, a plate sprayed with reagent (*c*) revealed only two yellow spots corresponding to sodium nafcillin (V) and sodium methicillin (IX).

The behavior of the 18 antibiotics listed in Table I in systems *A* to *D* was investigated. The methanol supernatants of these substances were applied to plates and chromatographed in the usual way. The plates were sprayed with reagent (*a*) after drying.

RESULTS AND DISCUSSION

Previous chromatographic investigations of the penicillins were not concerned with distinguishing between the different acyl derivatives of 6-aminopenicillanic acid (XI), although various methods have been described for paper chromatography of penicillins G and V (3, 7, 8). Some of the solvents cited in this early work were used in trials with different thin-layer chromatographic adsorbents during the present screening program. Thus, in this study, butyl acetate–*n*-butanol–acetic acid–water (80:15:40:24) used in paper chromatography by

TABLE I.—APPROXIMATE R_f VALUES[a] AND BEHAVIOR OF OTHER ANTIBIOTICS IN SYSTEMS *A* TO *D*

Antibiotic	System *A*	*B*	*C*	*D*
Tetracycline HCl	0.60[b]	0.76	0	0
Chlortetracycline HCl	0.54[b]	0.69	0	0
Oxytetracycline HCl	0.66[b]	0.75	0	0
Demethylchlortetracycline HCl	0.52[b]	0.67	0	0
Pyrrolidinomethyltetracycline	0.59[b]	0.74	0	0
Polymyxin B sulfate	N.S.[c]	N.S.	N.S.	N.S.
Vancomycin HCl	0.91[b]	0.95	0	0
Neomycin sulfate[e]	N.S.	N.S.	N.S.	N.S.
Tyrocidine HCl[f]	0	0	0.01(0.06)	0
Tyrothricin[f]	0	0	0.23	0
Gramicidin [f]	0	0	0.41	0.74[b]
Bacitracin[f]	N.S.	N.S.	N.S.	N.S.
Novobiocin	0	0	0.59	0.89
Amphotericin B	0	0.20	0	0
Cephaloridine	0.97[d]	0.90[b]	. . .[d]	0.79[b]
Griseofulvin	0.23	0.60	0.59	0.83
Ristocetin	0.74	0.97	0	0
Lincomycin HCl	0.96	0.95	0	0.13(0.03)

[a] R_f Values of secondary spots are shown in parentheses. [b] Streak. [c] No visible spot. [d] Long streak. [e] Purchased from K and K Laboratories, Plainview, N. Y. [f] Purchased from General Biochemicals Ltd., Chagrin Falls, Ohio.

TABLE II.—R_f VALUES[a] OF 10 PENICILLINS IN SYSTEMS *A* TO *D*

Substance	System *A*	*B*	*C*	*D*[b]
I, Benzylpenicillin Na	0.90	0.90	0.61(0.28)	0.58
II, Ampicillin Na	0.97	0.98(0.91)	0.12	0.15
III, Cloxacillin Na	0.65	0.38	0.64	0.77
IV, Dicloxacillin Na	0.47	0.22	0.65	0.77
V, Nafcillin Na	0.47(0.37)	0.22[c]	0.64	0.77
VI, Oxacillin Na	0.74	0.49	0.65(0.37)	0.63
VII, Phenethicillin K	0.84	0.73(0.55)	0.66	0.77
VIII, Phenoxymethylpenicillin K	0.82	0.76(0.56)	0.66	0.75
IX, Methicillin Na	0.93	0.93	0.52(0.18)	0.59
X, Hetacillin K	0.96	0.98	0.30(0.12)	0.64(0.15)

[a] Average of 10 plates. R_f values of secondary spots are shown in parentheses. [b] Results in this system are very susceptible to temperature variations. [c] With streaking.

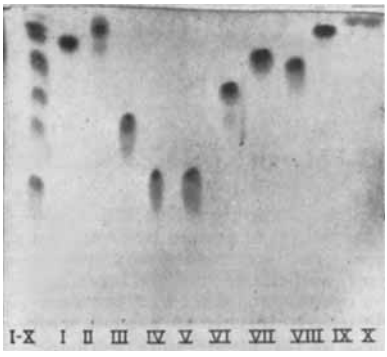


Fig. 1.—Chromatoplate of penicillins (I–X) in system *A*.

Fischer and Lautner (3), and butyl acetate–*n*-butanol–acetic acid–phosphate buffer pH 5.8–methanol (80:15:40:24:5) used by Nussbaumer in quantitative thin-layer chromatography of penicillins G and V (6) failed to separate the 10 analogs completely. Betina (8) in an extensive survey of antibiotic chromatography utilized various solvent systems for penicillins G and V and quoted 3% ammonium chloride and isoamyl acetate–methanol–formic acid–water (system *C*) as giving the best paper chromatographic separations. The authors found that 0.1 *M* sodium chloride was superior to the ammonium chloride system and system *C* was of limited value for separating the different derivatives by thin-layer chromatography.

The R_f values of the 10 penicillin salts with the four systems used are recorded in Table II. The limit of detection with spray (*a*) was found to be in the range of 0.1 to less than 0.04 mcg. System *A* was the system of choice for the separation of the analogs but failed to separate benzylpenicillin (I) from methicillin (IX); ampicillin (II) from hetacillin (X); dicloxacillin (IV) from nafcillin (V), and phenethicillin (VII) from phenoxymethylpenicillin (VIII) (Fig. 1). System *C* was found useful for the separation of I, II, IX, and X, but of all the solvent/adsorbent modifications tried, none separated penicillin IV from V or VII from VIII. However, nafcillin (V) was identified by spraying the chromatographed plate with spray reagent (*c*) when it appeared as an intense yellow spot. Methicillin was the only other penicillin which gave this color but at a very different R_f value. The use of (*a*) after this treatment, revealed the other analogs as previously described. As expected, ampicillin (II) with a primary amino group was the only penicillin to give the ninhydrin reaction which may, therefore, be applied as a confirmatory test for this substance. System *B* was a useful alternative solvent, although some of the spots exhibited variable R_f values due to streaking effects.

Ampicillin (II) and its derivative hetacillin (X) gave similar R_f values in the above systems. Hardcastle *et al.* (9) reported R_f values of 0.15 and 0.65, respectively, for these compounds as free acids in solvent system *D* and noted that dimethylformamide (DMF) should be used as the spotting solvent since hetacillin decomposes in methanol. The authors compared DMF and methanol as spotting solvents for all 10 penicillin salts in the four systems and found that the R_f values for all compounds except hetacillin were not appreciably altered by the different solvents when both freshly prepared and solutions "aged" for 1 week were used. For potassium hetacillin, there was no change in R_f when systems *A* and *B* were used. However, with systems *C* and *D* two spots were evident for the salt with both freshly prepared methanol and DMF spotting solutions. The major, faster running, spot (R_f values 0.30 and 0.64 in systems *C* and *D*, respectively) was found to be diminished in intensity while the slower running spot (R_f values 0.12 and 0.15) was found to be increased in "aged" solutions of both methanol and DMF. Thus, it would appear that hetacillin or its salts decomposed to yield ampicillin in both methanol and DMF (in which the salt is only slowly soluble) on storage and that differentiation of hetacillin from ampicillin is best accomplished using fresh solutions in system *D*. The R_f values in this system, as noted in Table II, vary markedly with changes in temperature, hence, system *D* is quoted only for the differentiation of ampicillin (II) and hetacillin (X).

A number of other solvent systems and adsorbents were tried during the course of this work. Acidic and alkaline solvent mixtures were generally found to give double spot formation and tailing (as with system *B*) probably attributable to hydrolysis. The use of ion-exchange layers such as diethylaminoethylcellulose and buffered layers proved disappointing.

The approximate R_f values of 18 antibiotics in these systems quoted in Table I indicate that these drugs do not interfere with this method of penicillin differentiation. Many of the antibiotics were only slightly soluble in methanol and with such substances the supernatant was used for spotting. The spray reagent (*a*) was much less sensitive in all cases.

These rapid diagnostic procedures will ensure that the more common penicillins are not readily substituted for the more expensive and recently introduced analogs and should also prove useful when applied to the detection of cross-contamination of pharmaceutical formulations by penicillins (15). The application of these procedures to the latter problem is currently under study in these laboratories.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) "Office Consolidation of the Food and Drugs Act and Regulations," Queen's Printer and Controller of Stationery, Ottawa, Ontario, Canada, 1965, p. 86.
(3) Fischer, R., and Lautner, H., *Arch. Pharm.*, **294**, 1(1961).
(4) Nicolaus, B. J. R., Coronelli, C., and Binaghi, A., *Farmaco (Pavia), Ed. Prat.*, **16**, 349(1961).
(5) Nicolaus, B. J. R., Coronelli, C., and Binaghi, A., *Experientia*, **17**, 473(1961).
(6) Nussbaumer, P. A., *Pharm. Acta Helv.*, **38**, 758(1963).
(7) Khokhlov, A. S., and Blinova, L. N., *Antibiotiki*, **8**, 35(1963); through *Chem. Abstr.*, **61**, 2908*a*(1964).
(8) Betina, V., *J. Chromatog.*, **15**, 379(1964).
(9) Hardcastle, G. A., Johnson, D. A., Panetta, C. A., Scott, A. I., and Sutherland, S. A., *J. Org. Chem.*, **31**, 897 (1966).
(10) Acred, P., and Brown, D. M., *Brit. J. Pharmacol.*, **21**, 339(1963).
(11) Stewart, G. T., "The Penicillin Group of Drugs," Elsevier Publishing Co., New York, N. Y., 1965, p. 124.
(12) *Ibid.*, p. 17, *et seq.*
(13) "New Drugs," American Medical Association, Chicago, Ill., 1965, p. 9.
(14) Stahl, E., "Thin-Layer Chromatography," Academic Press Inc., New York, N. Y., 1965, p. 31.
(15) Cox, W. H., and Greenwell, B. E., *J. Pharm. Sci.*, **54**, 1076(1965).

Synthesis of Some Disubstituted Cyanoacetamides as Potential Anticonvulsants

By HERBERT F. SCHWARTZ, II, LEE F. WORRELL, and JAIME N. DELGADO

A series of 17 cyanoacetamides was synthesized for pharmacologic evaluation. Several synthetic methods were studied: *C*-alkylation, acylation of amines by ester, and acylation of amines by amides. The *C*-alkylation of cyanoacetamides was investigated in several solvent systems using various bases, and the results are reported. This method was adopted for the alkylation of the cyanoacetamides in a pressure-reaction vessel and utilized formamide and/or dimethylsulfoxide as solvent with potassium hydroxide as the basic reagent. Several of the compounds were obtained by the pyrolytic acylation of amine by amide.

IN AN EARLIER paper Schwartz and Doerge (1) reported the preparation and investigation of some disubstituted malondiamides. Preliminary pharmacological studies showed several of these to possess anticonvulsant activity and simultaneously to be devoid of sedative-hypnotic properties. Furthermore, the disubstituted cyanoacetamide intermediates used in the preparation of the malondiamides were observed to possess higher anticonvulsant activity and, like the malondiamides, to be devoid of sedative-hypnotic activity. Table I summarizes these pharmacological data (2).

The significant activity of 2-ethyl-2-propyl- and 2,2-diethylcyanoacetamide against both electroshock and subcutaneous pentylenetetrazol tests suggests that further molecular modification might lead to more active compounds. Such modification might serve as the basis for the study of structure-activity relationships among cyanoacetamides. Accordingly, a series of *N*-alkylated and similar *N*-cyclic derivatives from the aforementioned ethylpropyl-, and diethylcyanoacetamides was prepared by several synthetic methods which involved *C*-alkylation, aminolysis, and the acylation of amines by amides.

Since preliminary and previous laboratory experience and literature surveys indicated classical methods of *C*-alkylation and *N*-alkylation to be undesirable and impractical in the preparation of these disubstituted cyanoacetamides, alternate synthetic methods were studied.

DISCUSSION

C-Alkylation.—The *C*-alkylation of cyanoacetamides was investigated in several solvents using various bases by preparing previously known mono- and disubstituted cyanoacetamides and several related esters with the hope of finding a more suitable method.

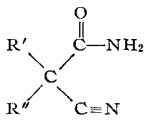
Under the conditions described under *Experimental*, cyanoacetamide has been found to act in a manner qualitatively similar to other active methylene compounds and on an acidity and reactivity basis, theoretically, may be categorized with malonic ester. The initial introduction of low molecular weight primary substituents (R-X less than 4 carbon atoms) into these types of compounds produced a mixture of monoalkylated and dialkylated product with starting material. Such mixtures are known to be difficult to purify by ordinary fractional distillation. The corresponding cyanoacetamides, although solids, are relatively water soluble and also produce a mixture which is difficult to separate by fractional crystallization.

Table II summarizes the results of this work. Since primary alkyl halides were used, dialkylation was the main side reaction detected. *N*-Alkylation was not observed.

Of the 17 compounds listed in Tables III and IV, only compound *2* (*N*-methyl-2,2-diethylcyanoacetamide) has previously been reported in the literature. Fourteen of the 16 compounds were prepared by application of the method of *C*-alkylation described above, utilizing potassium hydroxide in formamide or dimethylsulfoxide or in admixtures of these. Most of the desired compounds are derivatives of either diethyl- or ethylpropylcyanoacetamide and may be derived from their common intermediate ethylcyanoacetamide. Initially all reactions were carried out in formamide because of its lesser tendency toward dialkylation. Furthermore, when ethyl bromide was the alkylating agent, no diethylcyanoacetamide was produced and purification was easily accomplished. In earlier work, therefore, conversion to dialkylated compound was accomplished in a separate step, starting with purified ethylcyanoacetamide in formamide solution. In several instances it was possible to add the second set of reagents and to introduce the second alkyl substituent after having allowed the first stage to become essentially neutral, without separating and isolating the intermediate. Since dialkylation is of no concern when diethylcyanoacetamide is the desired product, and since dimethylsulfoxide supported a more vigorous reaction and gave excellent yields, this solvent was ultimately chosen for dialkylation in those situations when the alkyl groups were identical. When they

Received April 25, 1966, from the Pharmaceutical Chemistry Laboratories, College of Pharmacy, University of Texas, Austin 78712.
Accepted for publication September 20, 1966.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

TABLE I.—PHARMACOLOGICAL DATA ON CYANOACETAMIDES STUDIED[a,e,f]



Compd.	R′	R″	Max. Tolerated Oral Dose, mg./Kg.	SED[b]	SMES[c]	RSCM[d]
2658 L	C_2H_5	C_2H_5	200	0	3+	4+
2655 L	C_2H_5	n-C_3H_7	75	0	2+	3+
2663 L	C_2H_5	i-C_3H_7	80	0	4+	+
2659 L	C_2H_5	n-C_4H_9	70	0	+	0
2652 L	C_2H_5	i-C_4H_9	125	0	3+	2+
2660 L	C_2H_5	n-C_5H_{11}	125	0	4+	+
2654 L	C_2H_5	i-C_5H_{11}	175	0	2+	0

[a] The male albino rat, dosed orally, was used in all the tests. [b] SED, sedative: the ability of the compound to control the stimulating action of 30 mg./Kg. ephedrine sulfate injected subcutaneously is recorded on a kymographic record. [c] SMES, supramaximal electroshock test: the ability of the compound to abolish the hind limb extensor reflex produced by a current of 150 ma. delivered for 0.3 sec. to the brain *via* the optic pathway is recorded. [d] RSCM, rat subcutaneous pentylenetetrazol test: the ability of the compound to block completely the convulsions produced by pentylenetetrazol injected subcutaneously at a level of 70 mg./Kg. is noted. [e] Rating system: in all the tests, 0 to + indicates essentially no detectable activity of the compound to produce the desired effect. A rating of 2+ to 3+ indicates fair to good activity. A 4+ rating indicates complete control of desired effect and from an activity standpoint is the maximum attainable rating. [f] These pharmacological data abstracted from a personal communication to R. F. Doerge from R. W. Cunningham, then with Lederle Laboratories, Division of American Cyanamid Co.

were dissimilar, the usual order of alkylation for this series became ethyl- then propyl-, stepwise in the same reaction vessel, in formamide, without isolation of the intermediate.

Both *N*-methyl-2-ethylcyanoacetamide and *N*-dimethyl-2-ethylcyanoacetamide failed to react with the addition of the second portion of base and propyl bromide. Reversal of the order of introduction of the alkyl groups provided *N*-dimethyl-2-ethyl-2-propylcyanoacetamide in a slightly depressed yield but the corresponding *N*-methyl-compound was obtained in low yield and poor quality. Conversion of *N*-methylcyanoacetamide by a single molar quantity of propyl bromide gave a mixture of propyl- and dipropyl-substituted product. The desired *N*-methyl-2-ethyl-2-propylcyanoacetamide was ultimately obtained in fair to good yield by ethylating *N*-methyl-2-propylcyanoacetamide.

Generally, enolate salts appeared to be considerably less soluble in dimethylsulfoxide than in formamide, and their solubility was noted to decrease with increase in the weight of the *N* substituent. In those instances when precipitation of the enolate became troublesome, it was found advantageous to add the more polar formamide as an aid in solvation. Homogeneous solutions or satisfactory results were obtained in most instances with a 1:3 ratio of formamide to dimethylsulfoxide.

The propylation of 1-(2-cyanobutyryl)-piperidine in formamide failed until a mixture of formamide and dimethylsulfoxide was used.

Acylation of Amine by Ester.—Several of the desired members of the series arose by applying the aminolysis reaction to unsubstituted ester to obtain *N*-alkylated unsubstituted amide which was later *C*-alkylated. All of the piperidine, pyrrolidine, and morpholine derivatives were prepared in this manner. Of these unalkylated amide intermediates only 1-(2-cyanoacetyl)-pyrrolidine has not been found in the literature.[1]

[1] Compound *10* is not shown in tables.

Acylation of Amine by Amide.—The Galat-Elion acylation method (10) applied to disubstituted cyanoacetamides produced three substances not previously reported in the literature (Table IV): *N* - methyl - 2,2 - diethylcyanoacetamide,[2,3] *N*-methyl - 2 - ethyl - 2 - propylcyanoacetamide,[2] *N* - propyl - 2,2 - diethylcyanoacetamide, and *N*-propyl - 2 - ethyl - 2 - propylcyanoacetamide. Additionally, attempts were made to prepare *N* - dimethyl - 2,2 - diethylcyanoacetamide[2] and 1 - (2- cyano - 2 - ethylbutyryl) - piperidine.[2] The heating period varied from 4 to 6 hr. but occasionally, guided by the lack of precipitated ammonium chloride, the reaction time was extended overnight. The external (mantle) temperature was maintained so that the liquid appeared to be boiling gently, usually between 190° and 230°. Each reagent was melted separately and maintained in a molten condition for a period of time to insure minimal water content. Since disubstituted cyanoacetamides sublime readily, an attempt was made to counteract this loss by starting with 10% excess. Attempts to *N*-dialkylate using secondary amine salts, in a similar manner, repeatedly gave depressed yields of *N*-monoalkylated product. Considerable dealkylation apparently had taken place under these conditions (11, 12). The carbon and hydrogen analysis of *N*-methyl-2-ethyl-2-propylcyanoacetamide prepared by this method was no better than when prepared by *C*-alkylation as in Table III.

EXPERIMENTAL[4]

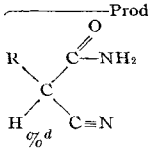
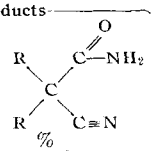
Unsubstituted amide intermediates were prepared from ester by a method similar to that to

[2] Preparation of this compound is possible *via* the unsubstituted ester to *N*-alkylated amide, then *C*-alkylation *via* the novel method.

[3] Known compound (9).

[4] Melting point determinations were made using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Elemental analyses were conducted by Dr. K. W. Zimmerman, University of Melbourne, Melbourne, Australia, and by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

TABLE II.—*C*-ALKYLATION IN SEVERAL SOLVENTS USING VARIOUS BASES

Expt.	Solvent	Vol. Solvent,[a] ml.	Alkyl Halide, Gm.	Basic Reagent[b]	Substrate	Ratio[c]	Size of Run, mole	Products:  % [d]	Products:  %
1	Formamide	30	Benzyl chloride	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.1	43–46[e]	26–28[h]
2	Formamide	30	Benzyl chloride	Sodium methoxide	Cyanoacetamide	1:1:1.1	0.1	17–34[e]	34–36[h]
3	Formamide	60	Benzyl chloride	Sodium carbonate	Cyanoacetamide	1:1:1.1	0.1	17–32[e]	1–7[h]
4	Formamide	30	Benzyl chloride	Potassium phenoxide	Cyanoacetamide	1:1:1.1	0.1	35[e]	9[h]
5	Formamide	40	Ethyl bromide	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.4	62–74[f]	0
6	Formamide	40	Ethyl bromide	Potassium hydroxide	Cyanoacetamide	1:2:2.2	0.2	29–49[f]	0–8[i]
7	Formamide	40	Ethyl bromide	Sodium hydroxide	Cyanoacetamide	1:1:1.1	0.2	58–71[f]	0
8	Formamide	20	Ethyl bromide	Sodium methoxide	Cyanoacetamide	1:1:1.1	0.2	38–45[f]	0
9	Formamide	30	Ethyl bromide	Potassium hydroxide	2-Ethylcyano-acetamide	1:1:1.1	0.2	...	69–75
10	Formamide	90	Propyl bromide	Potassium hydroxide	2-Ethylcyano-acetamide	1:1:1.1	0.3	...	77
11	Dimethyl-sulfoxide	60	Benzyl chloride	Sodium methoxide	Cyanoacetamide	1:1:1.1	0.1	17–31[e]	30–32[h]
12	Dimethyl-sulfoxide	20	Benzyl chloride	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.1	23–29[e]	27–30[h]
13	Dimethyl-sulfoxide	40	Benzyl chloride	Potassium hydroxide	Cyanoacetamide	1:2:2.2	0.1	3[e]	94[h]
14	Dimethyl-sulfoxide	60	Ethyl bromide	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.2	53–58[f]	0–4[j]
15	Dimethyl-sulfoxide	60	Ethyl bromide	Potassium hydroxide	Cyanoacetamide	1:2:2.2	0.1	0	64–78[j]
16	*N*,*N*-Dimethyl-formamide	30	Benzyl chloride	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.1	23–25[e]	32–33[h]
17	Ethylene-diamine	30	Benzyl chloride	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.1	16–18[e]	15–21[h]
18	Ethanol 99.5%	80	Ethyl bromide	Potassium hydroxide	Cyanoacetamide	1:1:1.1	0.4	59[f]	23[j]
19	Formamide	100	*n*-Butyl bromide	Potassium hydroxide	Ethyl acetoacetate	1:1:1.1	0.3	Ethyl butyl-acetoacetate 39–49[g]	Ethyl dibutyl-acetoacetate 0

[a] The volume of solvent stated is for the size of run indicated and in the ratio shown. Since quantities larger than those shown have not been attempted, caution is urged when exceeding these conditions by the pressure-bottle method. [b] Potassium and sodium hydroxide were in pellet form. Sodium methoxide was commercial powder. [c] Amide or ester, basic reagent, alkyl halide, respectively. [d] Identity confirmations were by melting point and mixed melting point (Mel-Temp apparatus) of known samples based on carbon and hydrogen determinations and infrared analyses. Pressure-bottle reaction at room temperature unless otherwise specified. [e] M.p. 127–128°. Shimo and Asami report 128–130°, 38–43% yield (3). [f] M.p. 112–113°. Shimo and Asami report 105–108°, 42–65% yield (3). Shimo, Wakamatsu, and Inoue report 112.5–113.5°, 24% yield (4). Schwartz and Doerge report 113–114°, 36–46% yield (1). [g] Heated to about 95°, b.p. 113–115°/15 mm., n_D^{23} 1.4297. Zaugg *et al.* report 95–100°/10 mm., 59–72% yield (5). Marvel and Hager report 112–117°/16 mm., 69–72% yield (6). [h] M.p. 165–165.5°. Shimo and Asami report 162–164°, 59–74% yield based on dialkylation as the main reaction (7). [i] Stepwise addition of base and alkyl halide without isolation of monosubstituted product gives only diethylcyanoacetamide (69%). [j] M.p. 120–121°. Schwartz and Doerge report 121.5–122°, 62% yield (1). Doerge and Wilson report 120–121°, 63% yield (8).

be given for 1-(cyanoacetyl)-pyrrolidine. Unspecified, known, *C*-alkylated intermediates were prepared by the *C*-alkylation methods below.

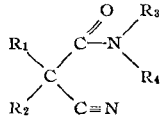
1 - (2 - Cyanobutyryl) - piperidine.—Potassium hydroxide (26 Gm. of 85%; 0.4 mole) and 1-(cyanoacetyl)-piperidine (61 Gm.; 0.4 mole) were added to 120 ml. of formamide contained in a 500-ml. Pyrex pressure-bottle.[5] The container was stoppered and placed on a Parr apparatus, and shaken for 1 hr. at which time ethyl bromide (48 Gm.; 0.44 mole) was added and shaking resumed. The container and cage soon became hot and potassium bromide began to precipitate. After 4 hr. of shaking, the reaction was essentially completed and had cooled. The bromide was removed and the filtrate diluted with a volume of water equal to about 1/4 of its original volume. Refrigeration (−10° to −17°) for 12 to 24 hr. gave 60 Gm. (83%) of crystals which when recrystallized from a mixture of ether and petroleum ether or from isopropanol–water had a melting point of 59–60°.

Anal.—Calcd. for $C_{10}H_{16}N_2O$: C, 66.63; H, 8.95. Found: C, 67.17; H, 8.97.

N - Dimethyl - 2 - ethylcyanoacetamide.—*N* - Dimethylcyanoacetamide (22.4 Gm.; 0.2 mole) and potassium hydroxide (13.0 Gm. of 85%; 0.2 mole) were added to 40 ml. of formamide contained in a 500-ml. Pyrex pressure-bottle. The container and its contents were shaken on a Parr apparatus for 1 hr., after which time ethyl bromide (24.0 Gm.; 0.22 mole) was added and the shaking resumed. The exothermic reaction began in a few minutes. The shaking was continued for 4 hr. during which time potassium bromide precipitated and the reaction mixture cooled. After the shaking period, the precipitate was separated, the reaction mixture and the filtrate neutralized with diluted hydrochloric acid, then transferred to a Rinco evaporator operating under vacuum and steam. After the low boiling substances had been removed, the

[5] Tested to 125 lb.

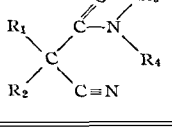
TABLE III.—2,2-DIALKYLCYANOACETAMIDES BY *C*-ALKYLATION[a]



No.	R_1	R_2	R_3	R_4	Formula	Calcd.	Found	Amide Substrate	Recrystallizing Solvent	Refract. Index, M.p./B.p. °C.	Yield, %
1	H	C_3H_7	CH_3	H	$C_7H_{12}ON_2$	C, 59.97 H, 8.63	60.07 8.55	*N*-Methyl-cyanoacetamide	Water or aqueous ethanol	65.5–66.5	41
2[b]	C_2H_5	C_2H_5	CH_3	H	$C_8H_{14}ON_2$	C, 62.30 H, 9.15	62.70 9.21	*N*-Methyl-cyanoacetamide[c]	Water	97–98	84
3	C_3H_7	C_2H_5	CH_3	H	$C_9H_{16}ON_2$	C, 64.25 H, 9.59	64.52 10.38	*N*-Methyl-2-propyl-cyanoacetamide	Water or isopropyl ether	86.5–87.5	68–82
4	C_3H_7	C_3H_7	CH_3	H	$C_{10}H_{18}ON_2$	C, 65.89 H, 9.98	66.10 9.80	*N*-Methyl-cyanoacetamide	Water or aqueous ethanol	111–112	16
7	H	C_2H_5	CH_3	CH_3	$C_7H_{12}ON_2$	C, 59.97 H, 8.63	60.02 8.65	*N*-Dimethyl-cyanoacetamide		120–121 at 4 mm. n_D^{23} 1.4616	43–48
8	C_2H_5	C_2H_5	CH_3	CH_3	$C_9H_{16}ON_2$	C, 64.25 H, 9.59	64.31 9.77	*N*-Dimethyl-cyanoacetamide[d]		92–93 at 7mm. n_D^{23} 1.4606	48
9	C_2H_5	C_3H_7	CH_3	CH_3	$C_{10}H_{18}ON_2$	C, 65.89 H, 9.96	66.31 10.24	*N*-Dimethyl-cyanoacetamide[d,e]		111–112 at 4 mm. n_D^{23} 1.4605	44[g]
11	H	C_2H_5	N⌒		$C_9H_{14}ON_2$	C, 65.03 H, 8.49	65.18 8.20	1-(Cyanoacetyl)-pyrrolidine		160–161 at 8 mm. n_D^{24} 1.4868	70–89
12	C_2H_5	C_2H_5	N⌒		$C_{11}H_{18}ON_2$	C, 68.00 H, 9.34	67.84 9.25	1-(2-Cyanobutyryl)-pyrrolidine[d]		127–128 at 3 mm. n_D^{24} 1.4816	31–47
13	C_2H_5	C_3H_7	N⌒		$C_{12}H_{20}ON_2$	C, 69.19 H, 9.68	69.43 9.73	1-(2-Cyanobutyryl)-pyrrolidine[d,f]		133–134 at 3 mm. n_D^{24} 1.4810	70
14	H	C_2H_5	N⌒		$C_{10}H_{16}ON_2$	C, 66.63 H, 8.95	67.17 8.97	1-(Cyanoacetyl)-piperidine	Isopropanol or ether–pet. ether	59–60	83–100
15	C_2H_5	C_2H_5	N⌒		$C_{12}H_{20}ON_2$	C, 69.19 H, 9.68	69.00 9.56	1-(Cyanoacetyl)-piperidine[c]		122–123 at 6 mm. n_D^{21} 1.4846	82–95[g]
16	C_3H_7	C_2H_5	N⌒		$C_{13}H_{22}ON_2$	C, 70.23 H, 9.97	69.77 9.93	1-(2-Cyanobutyryl)-piperidine[d]		137–138 at 3 mm. n_D^{23} 1.4829	78–100
17	H	C_2H_5	N⌒O		$C_9H_{14}O_2N_2$	C, 59.32 H, 7.74	59.47 7.91	4-(Cyanoacetyl)-morpholine		164–165 at 4 mm. n_D^{24} 1.4915	69
18	C_2H_5	C_2H_5	N⌒O		$C_{11}H_{18}O_2N_2$	C, 62.83 H, 8.63	62.93 8.82	4-(Cyanoacetyl)-morpholine		138–139 at 5 mm. n_D^{23} 1.4858 m.p. 35–36°	43[g,h]

[a] Solvent formamide unless otherwise specified. [b] Conrad and Zart report m.p. 102° (9). [c] Solvent dimethylsulfoxide. [d] Solvent a mixture of formamide and dimethylsulfoxide. [e] Propyl bromide, then ethyl bromide. [f] With formamide–dimethylsulfoxide mixture obtained 79% yield. [g] Two consecutive steps without isolation of monoalkyl intermediate, yield based on dialkylated product. [h] With formamide–dimethylsulfoxide mixture obtained 55% yield.

TABLE IV.—2,2-DIALKYLCYANOACETAMIDES—ACYLATION OF AMINES BY AMIDES



No.	R_1	R_2	R_3	R_4	Formula	Calcd.	Found	Amide Substrate	Recrystallizing Solvent	Refract. Index, M.p./B.p.	Yield, %
2[a,b]	C_2H_5	C_2H_5	CH_3	H	$C_8H_{14}ON_2$	C, 62.30 H, 9.15	62.70 9.21	2,2-Diethyl-cyanoacetamide	Water	97–95°	38–61
3[b]	C_3H_7	C_2H_5	CH_3	H	$C_9H_{16}ON_2$	C, 64.25 H, 9.59	64.52 10.38	2-Ethyl-2-propylcyano-acetamide	Isopropanol or ether–pet. ether	86.5–87.5°	60–62
5	C_2H_5	C_2H_5	C_3H_7	H	$C_{10}H_{18}ON_2$	C, 65.89 H, 9.96	65.46 9.85	2,2-Diethyl-cyanoacetamide	Aqueous isopropanol	71–71.5°	69–78
6	C_3H_7	C_2H_5	C_3H_7	H	$C_{11}H_{20}ON_2$	C, 67.30 H, 10.27	67.61 10.47	2-Ethyl-2-propylcyano-acetamide	Aqueous ethanol or isopropanol	65.5–66.5°	70–90

[a] Conrad and Zart report m.p. 102° (9). [b] Also prepared by *C*-alkylation method.

material was fractionated under reduced pressure, using a 14-cm. heated column with multiple receiver. Pure *N*-dimethyl-2-ethylcyanoacetamide distilled between 120–121° at 4 mm. pressure (n_D^{23} = 1.4616, 13.5 Gm.; 48.3%).

Anal.—Calcd. for $C_7H_{12}N_2O$: C, 59.97; H, 8.63. Found: C, 60.02; H, 8.65.

4-(2-Cyanobutyryl)-morpholine.—This compound was prepared in 69% yield from 4-(cyanoacetyl)-morpholine in the same manner as *N*-dimethyl-2-ethylcyanoacetamide above, except that the formamide solvent was increased to 80 ml. for 0.2 mole (b.p. 164–165° at 4 mm., n_D^{24} = 1.4915).

Anal.—Calcd. for $C_9H_{14}N_2O_2$: C, 59.32; H, 7.74. Found: C, 59.47; H, 7.91.

1-(2-Cyanobutyryl)-pyrrolidine.—This substance was prepared in 70–89% yield from 1-(cyanoacetyl)-pyrrolidine in an identical manner using 80 ml. of formamide for 0.2 mole (b.p. 160–161° at 8 mm., n_D^{24} = 1.4868).

Anal.—Calcd. for $C_9H_{14}N_2O$: C, 65.03; H, 8.49. Found: C, 65.18; H, 8.20.

N - Methyl - 2 - ethyl - 2 - propylcyanoacetamide.[6]—Except for the change of starting amide *N*-methyl-2-propylcyanoacetamide (56.8 Gm.; 0.4 mole) the preparation was almost identical with that for 1-(2-cyanobutyryl)-piperidine. The filtered reaction product was neutralized with a minimum of diluted hydrochloric acid. Sufficient isopropyl alcohol was then added to obtain a homogeneous solution which was chilled to produce crystals (55 Gm.; 81.8%). The melting point after recrystallization was 86.5–87.5°.

Anal.—Calcd. for $C_9H_{16}N_2O$: C, 64.25; H, 9.59. Found: C, 64.52; H, 10.38.

N - Methyl - 2 - propylcyanoacetamide and N-Methyl - 2,2 - dipropylcyanoacetamide.—Potassium hydroxide (13.0 Gm. of 85%; 0.02 mole) and *N*-methylcyanoacetamide (19.6 Gm.; 0.2 mole) were added to 60 ml. of formamide contained in a 500-ml. Pyrex pressure-bottle. The container was stoppered, placed on a Parr apparatus, and shaken for 1 hr. The dissolution was exothermic and complete, and gave an orange-colored solution. Propyl bromide (27 Gm.; 0.22 mole) was added and the two-phase mixture returned to the shaker. Within a few minutes the container and its protective cage became warm, and potassium bromide began to precipitate. The shaking was continued for 4 hr., after which time the bromide was removed and the filtrate shaken with an equal volume of hot water. The resulting two layers were separated and when chilled (−10° to −17°) for 12 to 24 hr. yielded crystals.

Alternatively, the filtrate was diluted with a volume of water equal to about 1/4 of its original volume and refrigerated as above. Extraction of the resulting crystals (16 Gm.; 57%) with hot water gave 41% of *N*-methyl-2,2-dipropylcyanoacetamide and 16% of *N*-methyl-2-propylcyanoacetamide based on starting amide. The latter was recrystallized from aqueous ethanol or from diisopropyl ether, m.p. 65.5–66.5°.

Anal.—Calcd. for $C_7H_{12}N_2O$: C, 59.97; H, 8.63. Found: C, 60.07; H, 8.55.

N-methyl-2,2-dipropylcyanoacetamide was recrystallized from hot water or hot aqueous ethanol, m.p. 111–112°.

[6] Also prepared by acylation of the amine by the amide.

Anal.—Calcd. for $C_{10}H_{18}N_2O$: C, 65.89; H, 9.98. Found: C, 66.10; H, 9.80.

N - Dimethyl - 2,2 - diethylcyanoacetamide.—*N*-Dimethylcyanoacetamide (22.4 Gm.; 0.2 mole) and potassium hydroxide (13.0 Gm. of 85%; 0.2 mole) were added to 80 ml. of solvent (formamide–dimethylsulfoxide, 1:3). At the end of 2 hr. shaking on a Parr apparatus, a small amount of basic reagent remained undissolved, and some metal enolate had precipitated. To this mixture, contained in a Pyrex glass pressure-bottle, was added ethyl bromide (24.0 Gm.; 0.22 mole) and the shaking continued. As the ethyl bromide reacted and the temperature rose, all insoluble material dissolved, and the mixture became a biphasic liquid. As the reaction progressed, potassium bromide precipitated and the liquid became homogeneous. At the end of 4 hr. insoluble bromide was removed and the filtrate returned to the reaction flask. The pattern of adding reagents was repeated: potassium hydroxide (13.0 Gm. of 85%; 0.2 mole) was added and the container shaken for 1 or 2 hr. Ethyl bromide (24.0 Gm.; 0.22 mole) was added and the mixture shaken for an additional 4 hr., after which time the insoluble bromide was removed. The filtrate was diluted with a volume of water equal to about 1/4 of its volume, neutralized with diluted hydrochloric acid, and extracted with 3 or more portions of ether. The combined ether extract was washed with water and dried over anhydrous sodium sulfate. Ether solvent was removed *via* the Rinco evaporator under reduced pressure. The residual oil was fractionally distilled under reduced pressure using a Fabco microdistillation apparatus with a heated column of about 14 cm. and a multiple receiver. Alternatively, the filtrate, after the removal of bromide, was neutralized with diluted hydrochloric acid, treated at the Rinco evaporator to remove low boiling substances, then fractionally distilled. Solvents distilled prior to the product. The yield was 48% of pure *N*-dimethyl-2,2-diethylcyanoacetamide obtained between 92–93° at 7 mm. pressure (n_D^{23} = 1.4606).

Anal.—Calcd. for $C_9H_{16}N_2O$: C, 64.25; H, 9.59. Found: C, 64.31; H, 9.77.

N - Dimethyl - 2 - ethyl - 2 - propylcyanoacetamide.—This compound was prepared in the same manner as *N*-dimethyl-2,2-diethylcyanoacetamide. Propyl bromide (27.0 Gm.; 0.22 mole) was added in the first step and ethyl bromide (24.0 Gm.; 0.22 mole) in the second. The crude yield was 44%; the pure product had a b.p. range of 111–112° at 4 mm. pressure (n_D^{23} = 1.4605).

Anal.—Calcd. for $C_{10}H_{18}N_2O$: C, 65.89; H, 9.96. Found: C, 66.31; H, 10.24.

1 - (2 - Cyano - 2 - ethylvaleryl) - piperidine and 1 - (2 - Cyano - 2 - ethylvaleryl)-pyrrolidine.—These compounds were both prepared in a manner analogous to the preparation of *N*-dimethyl-2,2-diethylcyanoacetamide except that since the starting materials were 1-(2-cyanobutyryl)-piperidine and 1-(2-cyanobutyryl)-pyrrolidine, respectively, only one step with base and alkyl halide was necessary. 1 - (2 - Cyano - 2 - ethylvaleryl)-piperidine was obtained almost quantitatively; the pure material boiled at 137–138° at 3.5 mm. pressure (n_D^{23} = 1.4829).

Anal.—Calcd. for $C_{13}H_{22}N_2O$: C, 70.23; H, 9.97. Found: C, 69.77; H, 9.93.

1 - (2 - Cyano - 2 - ethylvaleryl) - pyrrolidine (70–80% yield) distilled between 133–134° at 3 mm. pressure (n_D^{24} = 1.4810).

Anal.—Calcd. for $C_{12}H_{20}N_2O$: C, 69.19; H, 9.68. Found: C, 69.43; H, 9.73.

1 - (2 - Cyano - 2 - ethylbutyryl) - piperidine, 1-(2 - Cyano - 2 - ethylbutyryl)-pyrrolidine, and 4-(2 - Cyano - 2 - ethylbutyryl) - morpholine.—All three were prepared and isolated in essentially the same manner as *N*-dimethyl-2,2-diethylcyanoacetamide, with small differences in detail. 1-(2-Cyano-2-ethylbutyryl)-piperidine was prepared from 1-(cyanoacetyl)-piperidine by dialkylation, without isolation of the intermediate, in dimethylsulfoxide (60 ml./0.1 mole). The yield was 82–95%. The pure liquid distilled between 122–123° at 6 mm. (n_D^{23} = 1.4846).

Anal.—Calcd. for $C_{12}H_{20}N_2O$: C, 69.19; H, 9.68. Found: C, 69.00; H, 9.56.

1 - (2 - Cyano - 2 - ethylbutyryl) - pyrrolidine was prepared by monoalkylation of 1 - (2 - cyanobutyryl)-pyrrolidine in dimethylsulfoxide solvent (40 ml./0.1 mole). The vigorous reaction was moderated by chilling the enolate suspension to a solid prior to the addition of ethyl bromide. The pure substance boiled between 127–128° at 3 mm. (yield 31–47%, n_D^{24} = 1.4816).

Anal.—Calcd. for $C_{11}H_{18}N_2O$: C, 68.00; H, 9.34. Found: C, 67.84; H, 9.25.

4 - (2 - Cyano - 2 - ethylbutyryl) - morpholine was obtained by dialkylation without isolation of intermediate in formamide (43% yield, 40 ml. of solvent/0.1 mole) or in a somewhat improved yield (55%) in formamide–dimethylsulfoxide mixture (40:60; 100 ml./0.2 mole). The purified product distilled between 138–139° at 5 mm. (n_D^{23} = 1.4858) and solidified on standing, m.p. 35–36°.

Anal.—Calcd. for $C_{11}H_{18}N_2O_2$: C, 62.83; H, 8.63. Found: C, 62.93; H, 8.82.

Acylation of Amine by Ester.—*1-(Cyanoacetyl)-pyrrolidine.*—Chilled ethyl cyanoacetate (22.6 Gm.; 0.2 mole) was added to an excess of pyrrolidine (28.5 Gm.; 0.4 mole) as it was being swirled in a flask. The rate of reaction was controlled by means of an ice bath. As the reaction vigor abated, the flask was permitted to stand with an occasional shaking. Within an hour the reaction mixture solidified. The crystals were collected by filtration. Further amounts of less pure crystals were obtained by chilling the mother liquor. The crude material was recrystallized from isopropanol and after purification melted at 71.5–72.5°. The crude yield with a twofold excess of amine was 90.5%; decreasing the amine to 10% excess depressed the yield to 68%.

Anal.—Calcd. for $C_7H_{10}N_2O$: C, 60.85; H, 7.30. Found: C, 60.42; H, 7.18.

Acylation of Amine by Amide.—*N-Propyl-2-ethyl-2-propylcyanoacetamide.*—The reaction apparatus consisted of a round-bottom, 1-neck distilling flask fitted with a Standard-Taper adapter, the plain end of which passed through a large rubber stopper closing the larger end of an inverted percolator (25 cm., 250 ml.). The flask was heated using a Glas-col mantle controlled by a Powerstat; the approximate temperature of the mantle was monitored using a Fisher pyrometer.

Dry propylamine hydrochloride (28.7 Gm.; 0.3 mole) placed in the dry flask was heated to melt. Mild suction was applied to the top of the inverted percolator. 2 - Ethyl - 2 - propylcyanoacetamide (50.9 Gm.; 0.33 mole) contained in a beaker was melted separately. Both chemicals were maintained molten for a period of time to insure minimal moisture content in order to reduce the hydrolysis reaction.

After pouring the amide into the amine, the temperature was raised and maintained at about 200° for 2 to 4 hr. The partially cooled reaction mixture was diluted with isopropanol and the insoluble ammonium chloride removed by filtration. Sublimed amide and ammonium chloride and condensed amine trapped in the upper chamber of the apparatus were discarded. Evaporation of the isopropanol solution gave a 70–90% yield of crude product. Fractional crystallization employing water and isopropanol enabled the separation of unchanged amide. Pure *N*-propyl-2-ethyl-2-propylcyanoacetamide melted at 65.5–66.5°.

Anal.—Calcd. for $C_{11}H_{20}N_2O$: C, 67.30; H, 10.27. Found: C, 67.61; H, 10.47.

N - propyl - 2,2 - diethylcyanoacetamide.—This compound was prepared in approximately the same manner by heating with the *secondary* amine salt and 2,2-diethylcyanoacetamide (69–78% yield, m.p. 71–71.5°).

Anal.—Calcd. for $C_{10}H_{18}N_2O$: C, 65.89; H, 9.96. Found: C, 65.46; H, 9.85.

N - Methyl - 2 - ethyl - 2 - propylcyanoacetamide.[7]—This substance was prepared by the method above by heating the amide with the *secondary* amine salt. The *N*-methyl product was obtained in 60–62% yield. The analysis of the compound prepared by this method was unacceptable.

SUMMARY

In the course of this investigation 17 new compounds have been synthesized: *N*-methyl-2-propylcyanoacetamide;[8] *N* - methyl - 2 - ethyl - 2 - propylcyanoacetamide; *N* - methyl - 2,2 - dipropylcyanoacetamide; *N* - propyl - 2,2 - diethylcyanoacetamide; *N* - propyl - 2 - ethyl - 2 - propylcyanoacetamide; *N* - dimethyl - 2 - ethylcyanoacetamide;[8] *N* - dimethyl - 2,2 - diethylcyanoacetamide; *N*-dimethyl - 2 - ethyl - 2 - propylcyanoacetamide; 1 - (cyanoacetyl) - pyrrolidine;[8] 1 - (2 - cyanobutyryl) - pyrrolidine;[8] 1 - (2 - cyano - 2 - ethylbutyryl) - pyrrolidine; 1 - (2 - cyano - 2 - ethylvaleryl) - pyrrolidine; 1 - (2 - cyanobutyryl)-piperidine;[8] 1 - (2 - cyano - 2 - ethylbutyryl)-piperidine; 1 - (2 - cyano - 2 - ethylvaleryl) - piperidine; 4 - (2 - cyano - butyryl) - morpholine;[8] 4 - (2 - cyano - 2 - ethylbutyryl) - morpholine. The pharmacological data and structure-activity relationships of 12 of these are discussed in a separate paper to be published.

Several synthetic methods were developed and applied to the preparation of these *N*-substituted-2,2-dialkylcyanoacetamides and *N*-cyclic dialkylated cyanoacetamides. *C*-Alkylation of 2-cyanoacetamide was accomplished by a method which utilized potassium hydroxide and the appropriate alkyl halide in formamide or in dimethylsulfoxide or in mixtures of these. These exothermic reactions were

[7] Prepared also by *C*-alkylation.
[8] Not tested for anticonvulsant activity.

conducted without additional heating, in a stoppered and shielded Pyrex pressure-vessel, by shaking on a Parr apparatus. Both mono- and dialkylations were accomplished within 4 to 8 hr. The results of the influence of several solvent systems and bases on the alkylation reaction are tabulated.

Many 2,2-disubstituted cyanoacetamides prepared by the above method were converted to *N*-alkyl-2,2-dialkylcyanoacetamides by a method similar to that of Galat and Elion (10) for the acylation of amine by amide. While this method was effective for *N*-alkyl derivatives, it failed for *N*-dialkyl products when using secondary amine salts. Recourse to acylation of amine by ester, using appropriate aliphatic secondary amine (or in the instances of cyclic derivatives, pyrrolidine, piperidine, and morpholine), provided the desired amides which were subsequently alkylated at C-2.

REFERENCES

(1) Schwartz, H. F., and Doerge, R. F., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 80(1955).
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(3) Shimo, K., and Asami, R., *Sendai Japan Tohoku Univ., Sci. Rep.*, **9**, 328(1957).
(4) Shimo, K., Wakamatsu, S., and Inoue, T., *J. Am. Chem. Soc.*, **26**, 4868(1961).
(5) Zaugg, H. E., Dunnigan, D. A., Michaels, R. J., Swett, L. R., Wang, T. S., Sommers, A. H., and DeNet, R. W., *J. Org. Chem.*, **26**, 644(1961).
(6) Marvel, C. S., and Hager, F. D., *Org. Syn.*, **1**, 248 (1947).
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(9) Conrad, M., and Zart, A., *Ann. Chem.*, **340**, 329 (1905).
(10) Galat, A., and Elion, G., *J. Am. Chem. Soc.*, **65**, 1566 (1943).
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(12) Hickinbottom, W. J., "Reactions of Organic Compounds," Longmans Green and Co., New York, N. Y., 3rd ed., 1957, p. 416.

Lawsone Derivatives III

Cyclizations Involving Side Chains

By H. MACHATZKE, W. R. VAUGHAN*, CHARLOTTE L. WARREN, and G. R. WHITE

In continuing efforts to prepare a stable 3-ω-bis-β-chloroethyl aminoalkyl-2-hydroxy-1,4-naphthoquinone the preparation and reactions of several ω-substituted alkyllawsones have been examined. The ultimate objective of the research was not achieved as a consequence of unexpectedly facile cyclizations, either of the 3-side chain to the lawsone nucleus or by macrocyclic dimerization. The nature of these cyclizations is discussed, and the structures of the cyclic products are delineated.

THE OBJECTIVES of this continuing research have been discussed in a previous paper (1), and it should be noted in this respect that lawsone derivatives have for a considerable number of years been of interest as potential chemotherapeutic agents, particularly as antimalarials (2–4) and as antitumor compounds (1).

The purpose of the present paper is to report reactions which interfere with the preparation of 3-ω-bis-2′-hydroxyethylaminoalkyl-2-hydroxy-1,4-naphthoquinones. Two types of interfering cyclizations were encountered: (*a*) a previously recognized one in which a 3-ω-chloroalkyl-side chain closes to the 2- or 4-oxygen of 2-hydroxy-1,4-naphthoquinone (lawsone), and (*b*) an unexpected dimerization of a 3-ω-carboxyalkyllawsone to a macrolactide.

Received July 21, 1966, from the Department of Chemistry, University of Michigan, Ann Arbor.
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This investigation was supported by research grant CA 02961 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
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DISCUSSION

Four 3-ω-chloroalkyllawsones were investigated (I) as alkylating agents for bis-β-hydroxyethylamine (diethanolamine). And in each case the reaction desired was simple alkylation of diethanolamine, but in none was this achieved. In the case of I*a* simple cyclization to the 2-hydroxyl, as reported by Moser (4), was observed. But a mixture of II and the isomeric III (8, 9-benzo-7, 10-dioxo-2-oxabicyclo-[4.4.0]deca-1[6], 8[9]-diene and 9,10-benzo-7, 8-dioxo-2-oxabicyclo[4.4.0]-deca-1[6], 9[10]-diene, respectively) was shown to be present. The structure of III is assigned on the basis of its infrared spectrum, microanalysis, and acid isomerization[1] (5) to II. The formation of III in about 10% yield was demonstrated by comparision of the infrared spectrum of the product with spectra of pure II and pure III. And when I*b* was used in place of I*a*, the yield of III increased to about 40% at the expense of II. But when I*c* was used in place of I*a* with diethanolamine a considerably more complex reaction was encountered, with production of III accounting for but 14% of the initial I*c*, and the only other readily characterizable product (IV) being the result of 1,4-addition of diethanolamine to lawsone with reduction. Scheme I represents a rationale for the

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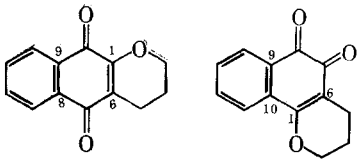
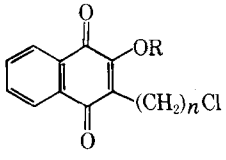
[1] *Cf.* conversion of β-lapachone to α-lapachone.

formation of the two isolated products, III and IV. The structure assigned to IV is inferred from its infrared spectrum, which shows no quinone absorption but which has a broad hydroxyl band, which in turn is confirmed as an alcohol function by preparation of a 1-napthylurethan.

It is noteworthy that a cyclization of I*c* can occur, apparently without the intervention of a base other than a hydroxylic solvent. For when an attempt was made to alkylate bis-β-hydroxyethylsulfide with I*c* by heating at reflux temperature for 10 days in 80% ethanol, a mixture was obtained which was identified as consisting of 78% III (isolation, infrared spectrum, and melting point) and about 20% II. The authors suggest the reactions outlined in Scheme II account for this phenomenon.



II III

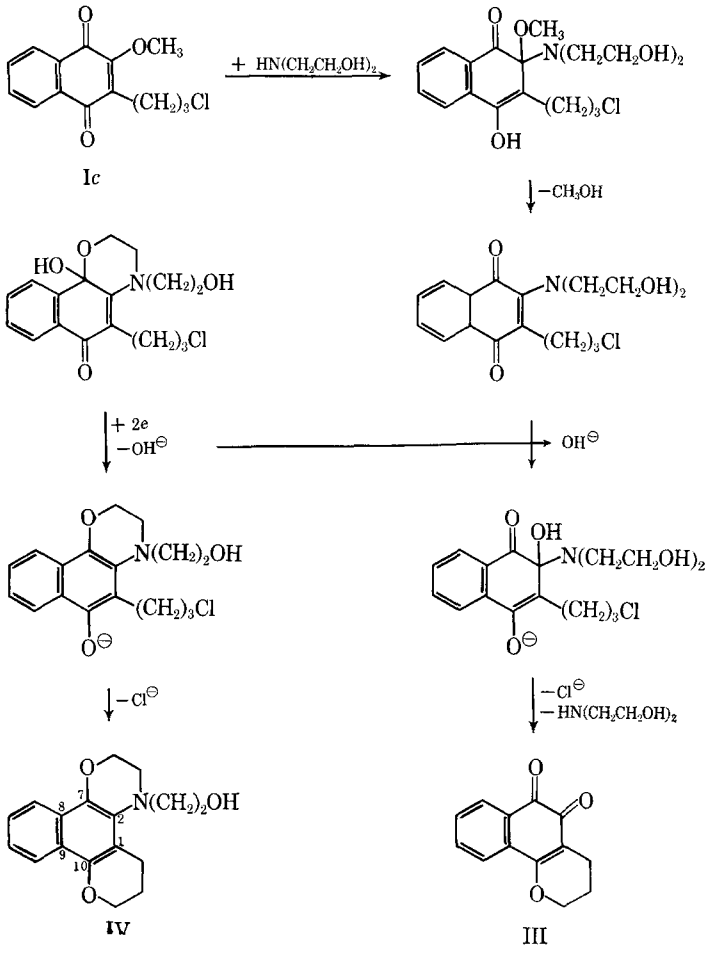


a, $n = 3$, R = H
b, $n = 3$, R = $COCH_3$
c, $n = 3$, R = OCH_3
d, $n = 4$, R = H

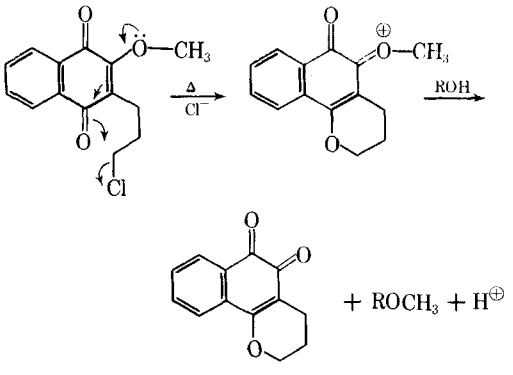
I

The conversion of III to II in the presence of an acid catalyst is an established type of reaction (*cf.* identification of III under *Experimental*). It should be noted that the production of III in the reaction of I*c* with diethanolamine, as suggested in Scheme I may in fact be the result of minor competition of the reactions of Scheme II with the conjugate addition-reduction leading to IV.

Although Fieser (6) has reported that 3-(10-bromodecyl)-2-hydroxy-1,4-naphthoquinone may be converted quantitatively to a tertiary amine by treatment with diethylamine, no characterizable product could be isolated from the reaction between I*d* and diethanolamine. Presumably reactions analogous



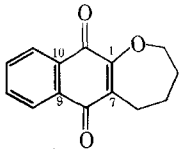
Scheme I



III

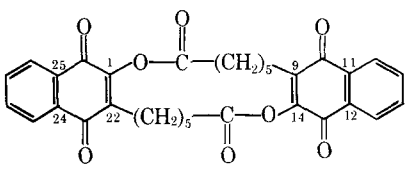
Scheme II

to those proposed in Scheme I occurred, complicated by the fact that competition between cyclizations and dimerizations is to be expected, since intramolecular cyclization would lead to seven-membered rings. That the suggested intramolecular cyclization to a seven-membered ring is not impossible is to be seen in the results of an attempt to convert *Id* to the corresponding iodide by treatment of its sodium salt with sodium iodide in aqueous solution. The only characterizable product, V (9,10-benzo-8,11-dioxo-2-oxabicyclo[5.4.0]undeca-$1^{7},9^{10}$-diene), exhibits an infrared spectrum similar to that of II.



V

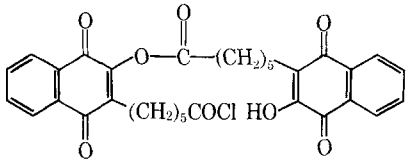
While precedent for the foregoing cyclizations was at hand, the behavior of 6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanoic acid (VI)(1) with thionyl chloride is entirely unprecedented. In initial experiments involving these two reagents there separated from the mixture a compound, VII, which analyzed for $C_{32}H_{28}O_8$. And acetylated VI (VIII) (1) also reacted with thionyl chloride to produce VII. The analysis and molecular weight of VII leave no doubt that it is the consequence of a dimeric condensation, and the infrared spectrum of VII exhibits absorptions characteristic of the 1,4-napthoquinone system as well as of a vinyl ester. Consequently, it seems most reasonable to describe VII as 11,12,24,25-dibenzo-2,15-dioxa-3,10,13,16,23,26-hexaoxotricyclo-[20,4,$0^{9,14}$]hexaeicosa-$1^{22},9^{14},11^{12},24^{26}$-tetraene (the lactide of VI).



VII

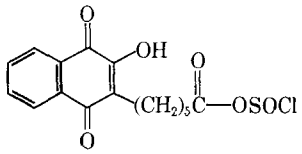
Subsequent attempts to duplicate the original experiments with both VI and VIII led to inconsistent results, and consequently a careful investigation of reaction conditions was undertaken. Of paramount importance in arriving at reproducible results was the observation that VI may be obtained in different forms which depend upon the solvent used for recrystallization: thus, one of these melts at 113° when recrystallized from benzene, another melts at 94° when recrystallized once from aqueous methanol and at 112° upon further recrystallization from the same solvent. Furthermore, the lactide (VII) apparently exists also in different crystal forms: m.p. 240° from benzene–petroleum ether (b.p. 40–60°), and m.p. 210° from benzene (which affords a nearly white product possessing the same infrared spectrum as the higher melting form, but nevertheless giving a mixed melting point with the latter of 224°). The details of this study appear in Table I.

The isolation of two compounds, the acid chloride (X) of the dimeric half ester, and what appears to be



X

the chlorosulfite of VI (XI),



XI

is to be noted (Table I and *Experimental*), but neither substance could be obtained sufficiently pure for microanalysis.

Attempts to convert VII, IX, X, and XI to the bis-β-hydroxyethylamide by reaction with diethanolamine were for the most part unsuccessful and will be reported in a subsequent paper. But by contrast, the simple amide of VI (XII) was readily obtained by refluxing VI with thionyl chloride and pouring the crude product (IX) into excess concentrated aqueous ammonia. The resultant mixture is deep blue and becomes red upon acidification. The anilide (XIII) of VI was also prepared from IX.

The preparation of VIII is readily achieved by treating VII with acetic anhydride–boron trifluoride etherate (1) and VIII may be converted to its acid chloride (albeit not in sufficient purity for analysis) by refluxing VIII with thionyl chloride using high dilution in chloroform. The identity of the product (XIV) as the acid chloride is inferred by examination of its infrared spectrum.

From the data presented here the sequence of reactions leading to production of VII may be inferred to be as follows:

$$\text{VI} + \text{SOCl}_2 \xrightarrow{(a)} \text{XI} \xrightarrow{(b)} \text{IX} \xrightarrow{(c)} \text{X} \xrightarrow{(d)} \text{VII}$$

TABLE I.—REACTIONS OF VI WITH THIONYL CHLORIDE[a]

Acid			Thionyl Chloride		Product				
mmole	M.p., °C.	Recryst. Solvn.	ml.	mmole	Compd.	Yield, %	Color	Solvn.	M.p., °C.
1	113	C_6H_6	1.44	20	VII	45	Yellow	A[b]	233–240
1	113	C_6H_6[c]	1.44	20[d]	VII	61	Yellow	A[b]	242–249
3.47	94	aq. MeOH	5.0	79.4[d]	IX	86	Yellow	...	Oil[e,f]
2.6	112	aq. MeOH	6.0	95.3[d]	X				Oil[g]
3.0	112	aq. MeOH	6.0	95.3[d]	X	92			Oil[h]
1.7	104	CH_3NO_2	5.0	79.4	VII	87	White	C_6H_6[i]	200[j]
1.0	113[k]	C_6H_6	1.44	20[l]	?[m]				

[a] General conditions: reagents mixed and refluxed 40 min.; evaporated *in vacuo;* benzene added and distilled off. [b] A, benzene–petroleum ether (b.p. 40–60°). [c] If analytically pure, refluxing with thionyl chloride for 40 min. affords about 60% VII, m.p. 240°. [d] Freshly distilled thionyl chloride. Careful purification of the reagent in the usual manner did not change results from those obtained using redistilled commercial product. [e] Anilide (XIV) prepared. (See under *Experimental.*) [f] Chief contaminant is X (infrared spectrum and chemical behavior). [g] Infrared spectrum similar to that of IX but contains additional bands at 1770 cm.$^{-1}$ (v.s.) and 3080 cm.$^{-1}$ (W). [h] Infrared spectrum same as for X but appears to lack band at 686 cm.$^{-1}$. [i] Washed with petroleum ether. [j] Infrared spectrum identical to that of VII but mixed m.p. 224°. [k] Under "high dilution" conditions in refluxing chloroform the product appears to be XI (infrared spectrum and chemical behavior). [l] VI dissolved in 100 ml. benzene and mixture refluxed 90 min. [m] Infrared spectrum of product suggests no reaction occurred, but product "tacky."

Two of the steps in this reaction sequence (*c*) and (*d*) involve acylation of the hydroxyl group of lawsone by an acyl chloride produced *in situ* by the action of thionyl chloride upon an acid. This, of course, is not the usual manner of acylating either an alcohol or enol, and consequently it seemed desirable to obtain evidence that such a process is indeed a reasonable one, and to this end the reactions of lawsone with acetic acid and thionyl chloride were investigated.

When equivalent amounts of acetic acid and lawsone are mixed and refluxed with thionyl chloride, the product consists principally of 2-chloro-1,4-naphthoquinone. But if excess acetic acid is used and the reaction with thionyl chloride is maintained at ice-bath temperature for 3 hr. and then refluxed for 1 hr., a 67% yield of 2-acetoxy-1,4-naphthoquinone is readily obtained. Thus, it is possible to bring about the kind of reaction implied by the dimerization (*c*) and the cyclization (*d*). However, the reason why it appears to be possible to halt the reaction series at IX when the lower melting form of VI is used remains obscure. Likewise the reproducible formation of the two different crystal forms of VII from samples of VI differing in purity also remains obscure.

The bimolecular dehydration of lawsone to di-1,4-naphthoquinon-2-yl ether by dicyclohexylcarbodiimide is an interesting extension of the use of this latter reagent.

EXPERIMENTAL[2]

2-Acetoxy-3-(3′-chloropropyl)-1,4-naphthoquinone (Ib).—To a suspension of 1.0 Gm. of 2-hydroxy-3-(3′-chloropropyl)-1,4-naphthoquinone (*Ia*) (4) in 5 ml. of acetic anhydride was added 1 ml. of boron trifluoride-etherate, and after 20 min. at room temperature, the mixture was poured into water, whereupon yellow crystals separated. The product was recrystallized from aqueous methanol: 1.13 Gm. (95%), m.p. 98–99°.

[2] Melting points uncorrected. Kofler block method indicated by superscript *k* where used. Microanalyses by Spang Microanalytical Laboratory, Ann Arbor, Mich. Infrared spectra of Nujol mulls obtained with Perkin-Elmer model 21 double beam spectrophotometer. The authors are indebted to Mr. B. E. Wenzel and Mr. Gunter Schütze for preparation of certain of these spectra.

Anal.—Calcd. for $C_{15}H_{13}ClO_4$: C, 63.5; H, 4.95; Cl, 12.1. Found: C, 63.72; H, 4.91; Cl, 12.21.

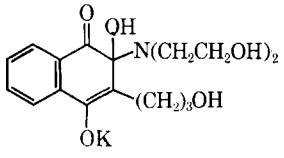
Reaction of 3-(3′-Chloropropyl)-2-hydroxy-1,4-naphthoquinone (Ia) (4) with Diethanolamine.—A solution of 1.0 Gm. of *Ia* (0.004 mole) and 0.42 Gm. of diethanolamine (0.004 mole) in 100 ml. of absolute ethanol was refluxed for 3 hr., during which time the color of the solution changed from red to yellow. Upon cooling and filtering, there was deposited 0.750 Gm. of yellow crystals, m.p. 215–216°, which were identified by mixed melting point as 8,9-benzo-7,10-dioxo-2-oxabicyclo[4.4.0]deca-1^6,8^9-diene (II). [Reported m.p. 220–221°(4).]

Evaporation of the red filtrate to about 5 ml., followed by cooling, afforded orange crystals (0.15 Gm., m.p. range > 15°). This material subsequently was shown by identity of infrared spectra to be identical with 9,10-benzo-7,8-dioxo-2-oxabicyclo[4.4.0]deca-1^6, 9^{10}diene (III) produced in the following experiment.

Reaction of 3-(3′-Chloropropyl)-2-methoxy-1,4-naphthoquinone (Ic) (1) with Diethanolamine.—A solution of 2.65 Gm. of *Ic* (10 mmoles) and 1.05 Gm. of diethanolamine (10 mmoles) in 100 ml. of absolute ethanol containing 1.40 Gm. (10 mmoles) of potassium carbonate was refluxed for 20 hr. The solution was then filtered and concentrated *in vacuo,* the residual oil being dissolved in 5 ml. of absolute ethanol. Addition of dry ether precipitated 1.5 Gm. of a brown crystalline product, m.p. 150° dec.

Anal.—Calcd. for $C_{17}H_{22}KNO_6$: C, 54.4; H, 5.90; K, 10.4; N, 3.73. Found: C, 55.8; H, 5.24; K, 12.1; N, 2.98.

This corresponds approximately to the following possible product (*cf.* Scheme I):



The filtrate from the preceding dilution was evaporated to an oil which was diluted with hydrochloric acid and extracted with ether, which upon concentration afforded orange-red crystals which were recrystallized from absolute ethanol: 0.3 Gm.

(14 %), m.p. 181–182°. The infrared spectrum is the same as that for III isolated above.

Anal.—Calcd. for $C_{13}H_{10}O_3$: C, 72.89; H, 4.71. Found: C, 72.72; H, 4.91.

Upon heating with concentrated hydrochloric acid for 0.5 hr. (5)[1] this substance was converted into II, as shown by mixed melting point (215–216°) and comparison of infrared spectra.

The acidic aqueous filtrate from the preceding isolation of III was neutralized with sodium hydroxide and the mixture then was extracted with ether from which separated a dark oil. This subsequently solidified and was then recrystallized from absolute ethanol: 0.3 Gm. (14 %), m.p. 128–129°, of 3-aza-8,9-benzo-6,11-dioxatricyclo-[8.4.$0^{2,7}$] tetradeca-1^{10}, 2^7, 8^9-triene (IV).

Anal.—Calcd. for $C_{17}H_{19}NO_3$: C, 71.60; H, 6.70; N, 4.91. Found: C, 71.40; H, 6.61; N, 4.82.

Infrared spectrum shows no carbonyl absorption but a broad band in the 3000 cm.$^{-1}$ region. The product upon reaction with 1-napthylisocyanate affords a urethan, m.p. 140–141°.

Anal.—Calcd. for $C_{28}H_{26}N_2O_4$: C, 74.00; H. 5.78; N, 6.18. Found: C, 74.04; H. 5.82; N, 6.45.

Reaction of 3-(4′-Chlorobutyl)-2-hydroxy-1,4-naphthoquinone (Id) (4) with Sodium Iodide–Sodium Carbonate.—A solution of 3.0 Gm. of Id (11.5 mmoles) and 2.0 Gm. of sodium iodide (13 mmoles) in 100 ml. of acetone containing 2.0 Gm. (20 mmoles) of sodium carbonate was refluxed for 20 hr. and then filtered. The filtrate was evaporated *in vacuo* and the residue dissolved in 10 ml. of methanol. Dilution of the methanol solution with 100 ml. of water and acidification with hydrochloric acid caused separation of an oil which crystallized on cooling in the refrigerator. Recrystallization from aqueous methanol afforded 2.5 Gm. of impure crystals, m.p. 64–70°, which could not be further purified by recrystallization. Trituration of this material with dilute sodium hydroxide, until the basic washings were colorless, left a yellow material which was then thrice recrystallized from aqueous methanol: 0. 5. Gm. (19%), m.p. 100–101°, mol. wt. (Rast) 212, 222 (calcd. 228), 9,10-benzo-8,11-dioxo-2-oxabicyclo[5.4.0]undeca-1^7, 9^{10}-diene (V).

Anal.—Calcd. for $C_{14}H_{12}O_3$: C, 73.70; H, 5.31. Found: C, 73.78; H, 5.41.

The infrared spectrum is characteristic of a 1,4-naphthoquinone.

6-(2-Hydroxy-1,4-naphthoquinon-3-yl)hexanoic Acid (VI).—This compound was prepared by a previously published procedure (1). It was usually recrystallized from benzene (m.p. 113°), but recrystallization from aqueous methanol (m.p. 112°) afforded a product which behaved differently with thionyl chloride, although no difference between the two was detectable upon infrared analysis. Two (presumably less pure) products (m.p. 94°) from aqueous methanol or from nitromethane (m.p. 104°) also appeared to behave in still another different manner with thionyl chloride. Consequently, it is important to note the source of VI in the succeeding experiments.

11,12,24,25-Dibenzo-2,15-dioxa-3,10,13,16,-23,26-hexaoxotricyclo[20.4.$0^{9,14}$]hexaeicosa-1^{22},9^{14},-11^{12},24^{25}-tetraene (VII) (Lactide of VI).—The following procedure is typical, and results of runs with VI of various origins are shown in Table I.

A mixture of VI (m.p. 113° from benzene) (288 mg., 1.0 mmole) and freshly distilled thionyl chloride (1.5 ml., 20 mmoles) was refluxed for 40 min., then evaporated under reduced pressure to dryness, the last traces of thionyl chloride being removed by addition and distillation of benzene. The solid residue (252 mg., 93.0%) was recrystallized from benzene–petroleum ether (b.p. 40–60°) to give 179 mg. (66%) of VII as yellow crystals, m.p. 244°. Mol. wt. calcd.: 540.5. Found (Rast): 515.

Anal.—Calcd. for $C_{32}H_{28}O_8$: C, 71.15; H, 5.23. Found: C, 71.41; H, 5.43.

Using essentially the same procedure for VIII, the acetyl ester of VI (1), VII was produced in 75% yield, m.p. 238–240°, with infrared spectrum identical to that of VII prepared from VI.

6-(2-Hydroxy-1,4-naphthoquinon-3-yl)hexanoyl Chloride (IX).—Most attempts to prepare IX from VI by treatment of the acid with thionyl chloride without resorting to high-dilution conditions afforded only VII. However, reactions which lead to IX from VI are recorded in Table I, the product never being obtained pure, but its existence being inferrable from infrared analysis and subsequent reaction. It should be noted that the α-methyl derivative of IX has been obtained from the α-methyl derivative of VI without the difficulties encountered in the present work (7).

6-{2-[6′-(2′-Hydroxy-1′,4′-naphthoquinon-3′-yl)-hexanoyl]-1,4-naphthoquinon-3-yl}hexanoyl Chloride (X).—This compound was not isolated in sufficiently pure condition for microanalysis. Its existence is inferred from its infrared spectrum which contains a very strong peak at 1770 cm.$^{-1}$ (vinyl ester carbonyl) and a band at 3080 cm.$^{-1}$ (hydroxyl). Otherwise the spectrum resembles the spectrum of IX. (See Table I.)

6-(2-Hydroxy-1,4-naphthoquinon-3-yl)hexanoyl Chlorosulfite (XI).—The preparation of XI employed conditions identical to those reported below for XIV, starting from VI, from 2.88 Gm. of which in 100 ml. of chloroform and 200 Gm. of thionyl chloride in 1 L. of chloroform, mixing requiring 72 hr. and refluxing being continued for 48 hr., there was obtained 4.04 Gm. of a brown intractable oil, whose infrared spectrum suggests XI. It afforded positive test for sulfur, supporting the spectrographic inference. The infrared spectrum shows bands at 3400 cm.$^{-1}$, 1745 cm.$^{-1}$, 1400 cm.$^{-1}$, and 1200 cm.$^{-1}$, the last two bands being characteristic of the —SO_2Cl group (8). There is no absorption characteristic of an acid chloride.

Reaction of crude XI (4.04 Gm.). with diethanolamine (10.5 Gm., tenfold excess) at 150° for 2 hr. afforded a dark blue product which was acidified with 200 ml. of concentrated hydrochloric acid, the resulting solution being continuously extracted with ether for 6 days. The ethereal extract was filtered, dried, and evaporated to give a dark red oil, paper chromatography of which (butanol–ethanol–water, 4:3:3) showed but one red band. The infrared spectrum is similar to that of the expected diethanolamide, to be reported in a subsequent paper.

6-(2-Hydroxy-1,4-naphthoquinon-3-yl)hexanamide (XII).—A mixture of 4.00 Gm., (1.38 mmoles) of VI and freshly distilled thionyl chloride (70 ml.) (*cf.* Table I, for preparation leading to IX) was refluxed for 40 min. and then concentrated under reduced pressure to an oil (IX) which was added

dropwise to an ice-cold ammonia solution (130 ml., sp. gr. 0.880) and then stirred overnight. Next 100 ml. of 5% hydrochloric acid was added, followed by addition of 1:1 hydrochloric acid until the pH was 7 (30 ml.). The yellow precipitate (3.51 Gm.) was filtered off and washed with 50 ml. of water, the filtrate being extracted with chloroform (six 25-ml. portions). Evaporation of the chloroform extracts and combination of the residue with the residual solid from filtration followed by recrystallization from toluene afforded 2.76 Gm. (69%) of XII, m.p. 154°.

Anal.—Calcd. for $C_{16}H_{17}NO_4$: C, 66.88; H, 6.11. Found: C, 66.95; H, 5.96.

6-(2-Hydroxy-1,4-naphthoquinon-3-yl)hexananilide (XIII).—A sample of crude IX (0.88 Gm., 0.29 mmole) was dissolved in 12 ml. of benzene to which was added 0.27 Gm. of aniline, the mixture being refluxed for 2 hr. After cooling, the solution was washed with water (three 4-ml. portions), with filtration after each wash, from a copious brown precipitate, which was recrystallized from ethanol–petroleum ether (b.p. 40–60°) to give 0.45 Gm. (44%) of XIII as yellow rhombs, m.p. 159–160°.

Anal.—Calcd. for $C_{22}H_{21}NO_4$: C, 72.71; H, 5.83; N, 3.86. Found: C, 72.45; H, 6.03; N, 3.89; ash, 0.29.

6-(2-Acetoxy-1,4-naphthoquinon-3-yl)hexanoyl Chloride (XIV).—A solution of 3.3 Gm. of VIII (0.01 mole) in 100 ml. of chloroform was dropped into a vigorously stirred refluxing solution of 100 Gm. of thionyl chloride in 1 L. of chloroform over an 8-hr. period. The solvent and excess thionyl chloride were then removed *in vacuo* and benzene added and distilled off. After filtration of the oily residue through sintered glass (residue 100 mg. of dark red crystals) it was dissolved in a solution of ammonia in ethanol and allowed to stand overnight. Upon removal of the solvent there remained a crystalline ester, m.p. 71–73° (reported 72–74°), whose infrared spectrum was superimposable upon that of the ethyl ester of VI (1).

The infrared spectrum of XII has bands at 1880–1800 $cm.^{-1}$, (acid chloride), 1790 $cm.^{-1}$ (vinyl acetate carbonyl), 1685–1695 $cm.^{-1}$ (quinone), 1185 $cm.^{-1}$ (phenolic acetate). No trace of the macrocyclic lactide (VII) was detectable.

Reaction of Lawsone with Acetic Acid in Thionyl Chloride.—A solution of 1.75 Gm. (0.01 mole) of lawsone, 10 ml. of thionyl chloride, and 3 ml. (0.05 mole) of acetic acid was stirred in an ice bath for 3 hr. and then reflxed for 1 hr. Volatile material was removed *in vacuo*, and residual thionyl chloride was removed by addition and distillation of benzene, and then the residue was triturated with cold methanol from which there was obtained on evaporation 0.45 Gm. of 2-chloronaphthoquinone (9) (infrared). The methanol-insoluble pale yellow residue was again treated with methanol, leaving 1.45 Gm., (67%) of 2-acetoxy-1,4-naphthoquinone, m.p. 127–129° K.). [Reported (10) 130°.] With less acetic acid only 2-chloro-1,4-naphthoquinone could be isolated.

Di-1,4-naphthoquinon-2-yl Ether.—To a solution of 1.74 Gm. (0.01 mole) of lawsone in 50 ml. of ethyl acetate and 10 ml. of acetone was added a solution of 0.95 Gm. of dicyclohexylcarbodiimide (0.005 mole) in 20 ml. of acetone. After stirring at room temperature for 2 hr., the solution was filtered, and the filtrate evaporated to dryness. The brown-orange residue was treated with dimethylformamide and filtered, leaving a greenish yellow material which was washed with water, methanol, and ether, and finally was dissolved in chloroform. Addition of ether precipitated 0.45 Gm. of green-yellow crystals, m.p. ~340° dec.

Anal.—Calcd. for $C_{20}H_{10}O_5$: C, 72.75; H, 3.06. Found: C, 72.79; H, 2.84.

REFERENCES

(1) Machatzke, H., and Vaughan, W. R., *J. Pharm. Sci.*, **53**, 730(1964).
(2) Fieser, L. F., Heymann, H., and Seligman, A. M., *J. Pharmacol. Exptl. Therap.*, **94**, 112(1948).
(3) Paulshock, M., and Moser, C. M., *J. Am. Chem. Soc.*, **72**, 5073(1950).
(4) Moser, C. M., and Paulshock, M., *ibid.*, **72**, 5419 (1950).
(5) Hooker, S. C., *ibid.*, **58**, 1168(1936).
(6) Fieser, L. F., *et al.*, *ibid.*, **70**, 3211(1948).
(7) *Ibid.*, **70**, 3208(1948).
(8) Bellamy, L. J., "The Infrared Spectra of Complex Molecules," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1962, pp. 360–363.
(9) Fieser, L. F., *J. Am. Chem. Soc.*, **70**, 3170(1948).
(10) Thiele, J., and Winter, E., *Ann.*, **311**, 347(1900).

Structure–Activity Analysis of Tetrahydrofolate Analogs Using Substituent Constants and Regression Analysis

By ELINOR MILLER and CORWIN HANSCH

The binding to dihydrofolate reductase of a series of substituted pyrimidines and triazines is shown to be dependent on the electron-contributing and lipophilic character of the substituents, as expressed by the parameters π and σ. The configuration of binding is variable, and is determined by the necessity of placing the most lipophilic substituent in a hydrophobic region of the enzyme. When two or more lipophilic substituents are present, competition between them for the hydrophobic site is dominated by the more lipophilic group, while the less lipophilic group contributes to hydrophobic bonding to a slight extent. Suggestions are made for the design of a new tetrahydrofolate analog with possible application as a nonclassical antimetabolite.

RECENT WORK has shown that biological response to congeneric drugs may be quantitatively correlated with molecular structure by means of substituent constants (1, 2). Two of the more useful parameters are Hammett's σ constant for electronic effects of substituents, and π, which is related (3) to the lipophilic character of a substituent ($\pi = \log P_X - \log P_H$ where P_X is the octanol–water partition coefficient of a derivative and P_H is that of the parent molecule). The combination of σ and π, as well as the steric parameter (4) of Taft (E_s), has been applied to a variety of compounds and systems, including the action of penicillins on bacteria (5), the relative sweetness of aromatic nitroamines (6), the binding of organic compounds by proteins (7, 8), the inhibition of photosynthesis by amides (9), the inhibitory activity of phosphate esters on cholinesterase (10), the microsomal demethylation of tertiary amines (11), and the interaction of enzymes and substrates (12).

In the present paper substituent constants and regression analysis are applied to series of substituted pyrimidines (13, 14) (Tables II and III) and triazines (15) (Table IV). These results come from the very interesting and extensive work of Baker and his co-workers on analogs of tetrahydrofolic acid designed to act as nonclassical antimetabolites (16) in the inhibition of dihydrofolate reductase. Baker's early studies were concerned primarily with steric and functional group requirements for enzyme binding (17, 18), but it has recently become apparent that hydrophobic bonding regions on the enzyme may also aid in binding the substrate (15). The purpose of this report is to show that many of Baker's deductions can be treated in a quantitative fashion, and in this way the relative importance of different substituent effects can be more precisely delineated.

Received August 19, 1966, from the Department of Chemistry, Pomona College, Claremont, Calif. 91713.

Accepted for publication September 27, 1966.

The work was supported by research grant GM-07492 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors are indebted to Susan Anderson, Smith Kline & French Research Associate, for determining the partition coefficient of diphenhydramine.

METHOD

If ΔF°_{BR} represents the free-energy change in some biological response which can be attributed to a single physical or chemical reaction, then as a first approximation this free-energy change can be factored as follows:

$$\Delta F^\circ_{BR} = \Delta F^\circ_{L/H} + \Delta F^\circ_{\text{elect.}} + \Delta F^\circ_{\text{steric}} \sim \ln k_{BR} \quad \text{(Eq. 1)}$$

In Eq. 1, k_{BR} may represent a rate or equilibrium constant, while $\Delta F^\circ_{L/H}$ represents that portion of the free-energy change which can be attributed to hydrophobic bonding (19), $\Delta F^\circ_{\text{elect.}}$ represents the electronic contribution, and $\Delta F^\circ_{\text{steric}}$ represents the spatial demands of reactants and products on the free-energy change. The effect of substituents on the change in free energy of a reference molecule can then be represented as in Eq. 2:

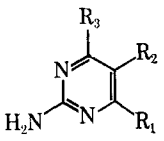
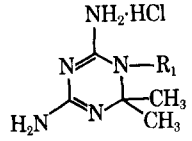
$$\delta_x \Delta F^\circ_{BR} = \delta_x \Delta F^\circ_{L/H} + \delta_x \Delta F^\circ_{\text{elect.}} + \delta_x \Delta F^\circ_{\text{steric}} \sim \delta_x \log k_{BR} \quad \text{(Eq. 2)}$$

Now substituent constants from model systems can be utilized in an extrathermodynamic approach (4) to evaluate substituent effects on k_{BR} as shown in Eq. 3.

$$\log BR \equiv \log \frac{1}{C_x} = k\pi + \rho\sigma + k'S + k'' \sim \delta_x \log k_{BR} \quad \text{(Eq. 3)}$$

In Eq. 3, C_x is the molar concentration of derivative x causing an equivalent biological response under standard conditions. π, σ, and S are extrathermodynamic constants for the respective substituent effects described in Eq. 2. The constants π and σ represent two parameters which are useful for hydrophobic and electronic effects; others can be used (3). This report will not attempt to evaluate steric effects included in S.

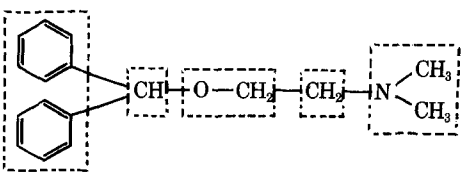
TABLE I.—STRUCTURE-ACTIVITY CORRELATIONS FOR TETRAHYDROFOLATE ANALOGS[a]

Pyrimidines	Compd.	Ref.	Eq.	n[b]	r[c]	S.D.[d]
	*1–16*	(13)	$\log \frac{1}{C}$[e] $= -5.002 - 5.162\sigma$ (Eq. 6)	16	0.760	1.076
	1–16	(13)	$\log \frac{1}{C} = -1.970 + 0.302\pi$ (Eq. 7)	16	0.328	1.565
	1–16	(13)	$\log \frac{1}{C} = -6.951 + 0.457\pi - 5.820\sigma$ (Eq. 8)	16	0.903	0.741
$R_3 = NH_2$	*5–16*	(13)	$\log \frac{1}{C} = -7.326 + 0.465\pi - 6.301\sigma$ (Eq. 9)	12	0.885	0.723
$R_3 = NH_2$, $\pi(R_1) = 0$	*5–16*	(13)	$\log \frac{1}{C} = -2.191 + 0.717\pi - 1.022\sigma$ (Eq. 10)	12	0.897	0.688
$R_3 = NH_2$, $\pi(R_2) = 0$	*5–16*	(13)	$\log \frac{1}{C} = -10.806 + 0.598\pi - 11.498\sigma$ (Eq. 11)	12	0.760	1.011
	5–16	(13)	$\log \frac{1}{C} = -8.144 + 0.800\pi_L$[f] $- 7.339\sigma$ (Eq. 12)	12	0.936	0.546
	5–16	(13)	$\log \frac{1}{C} = -6.686 + 0.512\pi_s$[g] $- 7.260\sigma$ (Eq. 12*a*)	12	0.735	1.054
	5–16	(13)	$\log \frac{1}{C} = -7.928 + 0.251\pi_s + 0.737\pi_L - 7.206\sigma$ (Eq. 13)	12	0.953	0.502
$R_1 = OH$, $R_3 = NH_2$	*17–26*	(14)	$\log \frac{1}{C} = -12.350 + 0.424\pi - 8.703\sigma$ (Eq. 14)	10	0.764	0.917
Omit R_2 = ethyl, *i*-amyl	*17–24*	(14)	$\log \frac{1}{C} = -17.864 + 0.103\pi - 13.724\sigma$ (Eq. 15)	8	0.899	0.523
Triazines						
						
pigeon liver	*28–39*	(15)	$\log \frac{1}{C} = -2.488 + 0.962\pi - 2.326\sigma$ (Eq. 16)	12	0.881	0.715
Omit R_1 = ethyl, *i*-amyl	*28–37*	(15)	$\log \frac{1}{C} = -2.215 + 0.861\pi - 1.724\sigma$ (Eq. 17)	10	0.930	0.499
E. coli	*27–36, 38, 39*	(15)	$\log \frac{1}{C} = -2.223 + 0.715\pi - 2.941\sigma$ (Eq. 18)	12	0.880	0.507

[a] All compounds are assayed in a pigeon liver enzyme system unless otherwise noted. [b] n is the number of points used in the regression. [c] r is the multiple correlation coefficient. [d] S.D., standard deviation. [e] C is the micromolar concentration causing 50% inhibition. [f] π_L is the π value of the most lipophilic substituent. [g] π_s is the π value of the second most lipophilic substituent.

The authors have taken σ from the compilation of Jaffé (20), making the assumption that although values for σ will change for substituents on a heterocyclic nucleus, they will do so in a parallel fashion. Using σ^+ or σ_m instead of σ_p did not give improved correlations.

While considerable evidence has accumulated to show that σ is an additive constant, an example illustrating the additive character (3) of log P and π is instructive. The experimental value of log P for a well-known antihistamine[1] is 3.07. Log P for diphenhydramine[1] can be calculated by taking advantage of the additivity of log P and π shown in I.



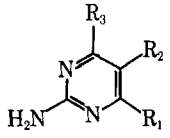
3.40 obs.
+ 4.26 + 0.30 −0.98 + 0.50 −0.95 = 3.13 calcd.

I

[1] Marketed as Benadryl by Parke-Davis Co., Detroit, Mich.

In I, log *P* for benzene (21) is taken as 2.13. Hydrogen is 0. The trisubstituted CH is given the value of 0.30 which is obtained by subtracting 0.50 for each methyl group in the isopropyl (π = 1.30) moiety. The value of CH_3 or CH_2 is 0.50. The value of −0.98 is that for an aliphatic methoxy

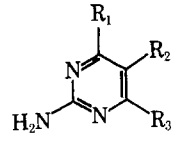
TABLE II.—DATA USED IN DERIVATION OF EQS. 6–11, 14–15 (TABLE I)



Compd.	R_1	R_2	R_3	$\Sigma\pi$	$\Sigma\sigma$	$\Sigma\pi$ $\pi(R_1)=0$	$\Sigma\pi$ $\pi(R_2)=0$	Obs.[a] log $\frac{1}{C}$	Calcd. log $\frac{1}{C}$
1	CH_3	$C_6H_5NH(CH_2)_3$	OH	2.29	−0.70	...	...	−2.90[b]	−1.83[c]
2	CH_3	$C_6H_5NH(CH_2)_3$	H	2.90	−0.34	...	...	−2.68	−3.65
3	CH_3	$C_6H_5(CH_2)_4$	OH	4.02	−0.70	...	...	−1.47	−1.04
4	C_6H_5	$C_6H_5(CH_2)_3$	H	5.76	−0.17	...	...	−3.58	−3.33
5	CH_3	$C_6H_5(CH_2)_4$	NH_2	3.48	−1.00	2.98	−0.65	1.57	0.46
6	CH_3	$C_6H_5NH(CH_2)_3$	NH_2	1.75	−1.00	1.25	−0.65	−0.34	−0.33
7	C_6H_5	$C_6H_5(CH_2)_4$	NH_2	5.11	−0.83	2.98	0.98	−0.04	0.22
8	C_6H_5	$C_6H_5NH(CH_2)_3$	NH_2	3.38	−0.83	1.25	0.98	0.06	−0.58
9	CH_3	H	NH_2	−0.65	−0.83	−1.15	−0.65	−3.04	−2.42
10	C_6H_5	H	NH_2	0.98	−0.66	−1.15	0.98	−2.20	−2.66
11	CH_3	*n*-C_4H_9	NH_2	1.35	−0.99	0.85	−0.65	−0.30	−0.57
12	C_6H_5	*n*-C_4H_9	NH_2	2.98	−0.82	0.85	0.98	−1.46	−0.82
13	*p*-$C_6H_5C_6H_4$	H	NH_2	3.11	−0.66	−1.15	3.11	−1.46	−1.67
14	*n*-C_3H_7	$C_6H_5(CH_2)_4$	NH_2	4.48	−0.96	2.98	0.35	1.68	0.69
15	$C_6H_5CH_2$	$C_6H_5NH(CH_2)_3$	NH_2	3.88	−0.83	1.25	1.48	−0.58	0.24
16	$C_6H_5CH_2$	$C_6H_5(CH_2)_4$	NH_2	5.61	−0.83	2.98	1.48	0.47	1.03
17	OH	$C_6H_5(CH_2)_4$	NH_2	2.37	−1.19	...	...	−0.92[d]	−1.28[e]
18	OH	$C_6H_5(CH_2)_3$	NH_2	1.87	−1.19	...	...	−1.78	−1.34
19	OH	H	NH_2	−1.76	−1.02	...	...	−4.11	−4.05
20	OH	*n*-C_8H_{17}	NH_2	−0.26	−1.15	...	...	−2.65	−2.11
21	OH	*n*-C_4H_9	NH_2	+0.24	−1.18	...	...	−1.60	−1.64
22	OH	*n*-C_6H_{13}	NH_2	1.24	−1.18	...	...	−1.48	−1.54
23	OH	*n*-C_8H_{17}	NH_2	2.24	−1.18	...	...	−1.68	−1.44
24	OH	$(CH_3)_2C{=}CHCH_2$	NH_2	0.24	−1.15[f]	...	...	−1.23	−2.06
25	OH	C_2H_5	NH_2	−0.76	−1.15	...	...	−4.23	−2.67
26	OH	*i*-C_5H_{11}	NH_2	0.54	−1.18	...	...	−0.60	−1.95

[a] *C* is the micromolar concentration causing 50% inhibition. [b] Calculated from data in *Reference 13*. [c] Calculated from Eq. 8. [d] Calculated from data in *Reference 14*. [e] Calculated from Eq. 15. [f] Value estimated.

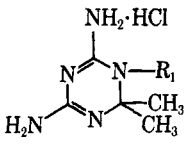
TABLE III.—DATA USED IN DERIVATION OF EQS. 12, 12*a*, AND 13 (TABLE I)



Compd.	R_1	R_2	R_3	Most Lipophilic R	π_L[a]	Obs. log $\frac{1}{C}$	Calcd. log $\frac{1}{C}$
5	CH_3	$C_6H_5(CH_2)_4$	NH_2	R_2	4.13	1.57	1.38
6	CH_3	$C_6H_5NH(CH_2)_3$	NH_2	R_2	2.40	−0.34	0.06
7	C_6H_5	$C_6H_5(CH_2)_4$	NH_2	R_2	4.13	−0.04	0.17
8	C_6H_5	$C_6H_5NH(CH_2)_3$	NH_2	R_2	2.40	0.06	−1.15
9	CH_3	H	NH_2	R_1	0.50	−3.04	−2.59
10	C_6H_5	H	NH_2	R_1	2.13	−2.20	−2.56
11	CH_3	*n*-C_4H_9	NH_2	R_2	2.0	−0.30	−0.32
12	C_6H_5	*n*-C_4H_9	NH_2	R_1	2.13	−1.46	−1.42
13	*p*-$C_6H_5C_6H_4$	H	NH_2	R_1	4.26	−1.46	−0.94
14	*n*-C_3H_7	$C_6H_5(CH_2)_4$	NH_2	R_2	4.13	1.68	1.09
15	$C_6H_5CH_2$	$C_6H_5NH(CH_2)_3$	NH_2	R_1	2.63	−0.58	−0.26
16	$C_6H_5CH_2$	$C_6H_5(CH_2)_4$	NH_2	R_2	4.13	0.47	0.88

[a] π_L = π value of most lipophilic substituent; *i.e.*, the largest π value. [b] *C* is the micromolar concentration causing 50% inhibition predicted by Eq. 12.

TABLE IV.—DATA USED IN DERIVATION OF EQS. 16–18 (TABLE I)



Compd.	R_1	log $\frac{1}{C}$[a] Pigeon Liver Obs.[b]	Pigeon Liver Calcd.[c]	*E. coli* Obs.[b]	*E. coli* Calcd.[d]	π	σ^*
27	C_6H_5	...	...	−0.48	−1.00	2.13	0.10[e]
28	$C_6H_5CH_2$	−0.52	−0.33	−1.53	−0.99	2.63	0.22
29	$C_6H_5(CH_2)_2$	0.15	0.34	−0.58	−0.22	3.13	0.08
30	$C_6H_5(CH_2)_3$	1.52	0.88	1.22	0.31	3.63	0.02
31	$C_6H_5(CH_2)_4$	1.40	1.31	0.68	0.67	4.13	0.02[e]
32	CH_3	−1.87	−1.78	−1.68	−1.87	0.50	0.00
33	n-C_3H_7	−1.04	−0.72	−0.67	−0.80	1.50	−0.12
34	n-C_4H_9	0.44	−0.27	−0.32	−0.41	2.00	−0.13
35	n-C_6H_{13}	0.50	0.59	0.04	0.30	3.00	−0.13[e]
36	n-C_8H_{17}	0.85	1.45	0.57	1.02	4.00	−0.13[e]
37	$C_6H_4(C_4H_9$-$n)$-p	1.22	1.17	...	...	4.13	0.10[e]
38	C_2H_5	−2.34	1.18	−1.79	−1.22	1.00	−0.10
39	i-C_5H_{11}	1.22	−0.01	0.16	−0.20	2.30	−0.13[e]

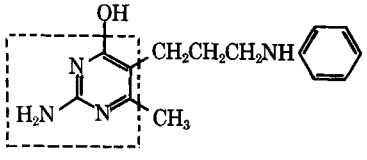
[a] C is the micromolar concentration causing 50% inhibition. [b] Calculated from data in *Reference 15*. [c] Calculated from Eq. 17. [d] Calculated from Eq. 18. [e] Value estimated.

group (3). The figure of −0.95 is that for an aliphatic dimethylamino fragment (3). The good agreement between observed and calculated log P exemplifies the additive character of π.

For the work in hand one does not have or need to use log P for the pyrimidine or triazine unit. Since π is an additive constant when strong group interactions are absent, partition coefficients may be estimated from previously determined values for functional groups.

$$\log P = \pi_1 + \pi_2 \ldots + \pi_i$$

where π_i represents logs of partition values for the components of a molecule. In series of compounds where a portion of the molecule is held constant, that portion may be disregarded and $\Sigma\pi$ for the substituents is then used for the correlation. For example, $\Sigma\pi$ for the molecule in II (number 1 in Table II) was calculated as shown in II and Eqs. 4 and 5.



constant

II

$$\Sigma\pi = \pi OH + \log P\ C_6H_5NH_2 + 3\pi CH_2 + \pi CH_3 \quad \text{(Eq. 4)}$$

$$\Sigma\pi = -0.61 + 0.90 + 3(0.50) + 0.50 = 2.29 \quad \text{(Eq. 5)}$$

$\Sigma\sigma$ was calculated for each molecule by adding the individual σ values. π for the H_2N group was taken as −1.15. For groups such as $C_6H_5CH_2CH_2CH_2$— and $C_6H_5NHCH_2CH_2CH_2$— it was assumed that the phenyl and phenylamino functions would be sufficiently insulated from the ring so that they played no electronic role. Taft's σ^* values (4) were used for the nonaromatic substituents in Table IV. The use of σ from the benzene system is an approximation which is particularly bad for the OH group since in the nitrogen heterocycles keto–enol tautomerism is so important. From the data in Tables II, III, and IV the equations in Table I have been derived by the method of least squares using an IBM 360/40 computer.

DISCUSSION

Rather good correlations were found to result using simple two-parameter equations employing only π and σ. In no instance did a significant improvement in correlation result when either a π^2 or σ^2 term or both π^2 and σ^2 were added. This is what one would normally expect (12) in an *in vitro* system using purified enzymes.

Baker and his colleagues have concluded (13) that the dihydrofolate reductase inhibitors are bound to the enzyme by the interaction of ring electrons with an electron-deficient site, and by hydrophobic interaction of one or more side chains. The equations in Table I strongly support these conclusions. All of the equations have positive coefficients for π, indicating that the more lipophilic the side chain, the more effective the compound is as an inhibitor. The negative coefficient associated with σ means that the more electron-releasing the substituent (the more it tends to increase the electron density on the ring), the more effective the derivative is as an inhibitor. The importance of the use of the two parameters is best seen by considering Eqs. 6–8 in Table I. The two single-parameter equations account for only 58% ($r^2 = 0.58$) and 11% of the variance in the data, while the two-parameter equation accounts for more than 81% of the vari-

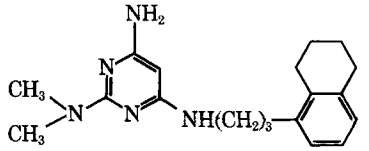
ance. In deriving these equations, only one compound [R_3=OH, R_2=$C_6H_5(CH_2)_4$, R_1=n-C_3H_7] was not used because it gave a poor fit in relation to the other data. The correlation obtained for the other 16 different derivatives in which changes were made in three different substituents is surprisingly good, especially when one considers no account has been taken of steric interactions of substrate and enzyme which classical enzyme theory has characterized as being so highly important (lock and key theory).

In order to evaluate more precisely the role of hydrophobic bonding, the 12 derivatives were taken in which changes were made only in R_1 and R_2. Equation 9 gives the result, treating the substituents R_1 and R_2 in simple additive fashion. First the hypothesis was tested that substituents in only one of the positions (R_1 or R_2) could fit into the hydrophobic area of the enzyme and thus contribute to inhibitory power of the derivative. This was done by setting R_1 equal to zero in Eq. 10, and R_2 equal to zero in Eq. 11. The higher correlation obtained with Eq. 10 would seem to confirm Baker's feeling (18) that the 6-position substituent is unable to contact the hydrophobic bonding area. However, the fact that one obtains essentially the same correlation with Eqs. 9 and 10 seems inconsistent. If, in fact, 6-position substituents contribute nothing to binding, an equation which includes their π values would include a varying and meaningless addition to π and should show a poor correlation. This inconsistency makes doubtful the theory that only one conformation is possible, but the relatively high correlations obtained suggested that hydrophobic binding may be *primarily* controlled by the larger lipophilic substituent. Inspection of the groups in positions 5 and 6, as shown in Table II, reveals that position 5 has more of the larger substituents. This could account for the fact that Eq. 10 gives a better correlation than Eq. 11. Equation 12 gives the best correlation. The hypothesis underlying Eq. 12 implies that substituted pyrimidines bind to dihydrofolate reductase in any of several conformers and that the choice of conformer is determined by the necessity of placing the most lipophilic group in contact with the hydrophobic bonding region of the enzyme. The most lipophilic group thus provides the dominating force in determining the binding configuration. Equation 12 confirms this hypothesis by rationalizing 87% of the variance in the data. Equation 12*a* shows that the contribution to the binding by the second most lipophilic substituent correlates about 50% of the variance in the data. However, when these two terms are included in a single equation (Eq. 13), the slight improvement in correlation is not statistically significant (F test).

Equations 14 and 15 correlate a series of trisubstituted pyrimidines which differ from the previous group chiefly in having an OH group in place of the lipophilic function at R_1. They appear to be intrinsically less active (the intercepts are more negative). Although this series appears very similar in structure to that considered in Eqs. 6–13, combining the two sets of data resulted in a very poor correlation (r = 0.776), indicating that the two series have different modes of binding. The coefficients of Eq. 15 indicate an increased dependence on σ and a decreased importance of π, which might result from a greater dependence on hydrogen bonding. In Eq. 15 the ethyl and isoamyl groups which were poorly correlated have been omitted. The ethyl group was about 30 times less active than predicted and the isoamyl was about 10 times more active than predicted. Why these groups are not adequately accommodated by the correlation is not clear. However, a reasonable suggestion by Baker (14) is that a conformational change may be caused in the enzyme in proceeding from ethyl to n-butyl. This brings out one of the great advantages of regression analysis, however, since the spotting of such exceptional structures indicates areas for further research. This same characteristic of the ethyl and isoamyl groups is apparent again in the substituted triazine series.

The coefficients in the best equations for pyrimidines (Eq. 12) and for the triazines (Eq. 17) are quite similar to each other. Hydrophobic bonding in the triazines seems to be somewhat more important, as indicated by the larger coefficient of π, and electronic bonding is somewhat less important as indicated by the smaller coefficient of σ. In general, the 90% confidence limits on the σ terms are much greater, no doubt because the differences in σ values are small compared to the experimental error in measuring σ. F tests also indicate lower significance for σ terms in the triazines. Of the more than 20 derivatives tested by Baker, the authors have not been able to treat those in which a phenyl group was attached directly to the ring nitrogen because of the uncertainty in electronic interaction. Delocalization of the lone pair electrons on nitrogen by the phenyl ring can greatly affect the lipophilic character (21) of the molecule, as well as the electron density on the ring which appears to react with an electron-deficient species. For the substituents which were considered, species differences in enzymes appear to be rather small (*cf.* Eqs. 16 and 18), although in some instances Baker has found quite marked differences. The species differences mentioned by Baker are, however, supported by the authors' finding that response for isoamyl and ethyl substituents are moderately well predicted for the *E. coli* enzyme (Eq. 18) but not for the enzyme from pigeon liver (Eqs. 14–17).

The equations in Table I should enable one to design more active inhibitors. Greater activity should result from compounds having larger positive $\Sigma\pi$ and more negative $\Sigma\sigma$. To increase the electron density on the ring, one could move the amino group Baker has incorporated into the side chain to a ring position in the 5- or 6-position and attach the largest possible lipophilic substituent to it. The possibility in III seems most logical.



III

In the variation of Baker's general type of inhibitor (III), the authors have added the quite lipophilic cyclohexyl moiety to the phenyl ring, hoping, of course, that the enzymic hydrophobic area is large enough to accommodate it. Another approach

would be to use a pentamethyl- or pentachlorophenyl group on the side chain. Placing methyl groups on one of the amino groups would lower affinity for the aqueous phase, even though hydrophobic bonding in the direct sense might not be possible at this position. We have indicated attachment of the side chain at the 6-position to make maximum use of the electron-releasing effect of the amino function. Since a better correlation was obtained using σ_p rather than σ_m, it is assumed that it is the high electron density on the 1,3,5-positions which is most important. This may not be the case, however. Since so few strong electron-releasing groups were tested, it is not possible to isolate this factor with confidence. In any case, it would be advantageous to separate the two functions so that steric interactions do not twist either of the nitrogen atoms out of the ring-plane and thus prevent effective overlapping of p-orbitals.

While the above design might be very effective in *in vitro* work, such a highly lipophilic compound would probably not make the best drug. For *in vivo* use, one would first have to determine π_0 (2) and then work close to this value, making maximum use of σ, in designing derivatives. The problem of penetration of the blood-brain barrier which appears to be a limiting factor in the clinical use of the tetrahydrofolate analogs, aminopterin and amethopterin (22), could be circumvented by proper lipophilic design of the drug (23).

The good correlations obtained for the structure-activity relationships of these varied series of compounds show that the additive nature of π makes it a useful and reliable parameter in drug design. The large reduction in variance obtained by using the linear combination of σ with π confirms the value of the use of σ constants in heterocyclic systems, even though the values were obtained from benzene. Although it is likely better correlations would be obtained if pKa values for the drugs in question could be used, the values of Jaffé's σ are probably good enough for practical purposes. If one wished to isolate as precisely as possible the steric factors, it would then be essential to determine pKa values. The rather good mathematical correlation the authors have obtained with the wide variety of bulky substituents used by Baker is further evidence for the importance of the role of flexibility in enzyme action (24) which in recent years has become so apparent. The authors' correlations provide a mathematical basis for the essential conclusions of Baker's studies and reinforce these conclusions by deriving them in an independent way.

REFERENCES

(1) Hansch, C., and Fujita, T., *J. Am. Chem. Soc.*, **86**, 1616(1964).
(2) Hansch, C., Steward, A. R., Iwasa, J., and Deutsch, E. W., *Mol. Pharmacol.*, **1**, 205(1965).
(3) Iwasa, J., Fujita, T., and Hansch, C., *J. Med. Chem.*, **8**, 150(1965).
(4) Leffler, J. E., and Grunwald, E., "Rates and Equilibria of Organic Reactions," John Wiley & Sons, Inc., New York, N. Y., 1963.
(5) Hansch, C., and Deutsch, E. W., *J. Med. Chem.*, **8**, 705(1965).
(6) Deutsch, E. W., and Hansch, C., *Nature*, **211**, 75(1966).
(7) Hansch, C., Kiehs, K., and Lawrence, G., *J. Am. Chem. Soc.*, **87**, 5770(1965).
(8) Kiehs, K., Hansch, C., and Moore, L., *Biochemistry*, **5**, 2602(1966).
(9) Hansch, C., and Deutsch, E. W., *Biochim. Biophys. Acta*, **112**, 381(1966).
(10) *Ibid.*, **126**, 117(1966).
(11) Hansch, C., Steward, A. R., and Iwasa, J., *J. Med. Chem.*, **8**, 868(1965).
(12) Hansch, C., Deutsch, E. W., and Smith, R. N., *J. Am. Chem. Soc.*, **87**, 2738(1965).
(13) Baker, B. R., and Shapiro, H., *J. Pharm. Sci.*, **55**, 308(1966).
(14) Baker, B. R., Ho, B.-T., and Santi, D. V., *ibid.*, **54**, 1415(1965).
(15) Baker, B. R., and Ho, B.-T., *ibid.*, **55**, 470(1966).
(16) Baker, B. R., *ibid.*, **53**, 347(1964).
(17) Baker, B. R., Santi, D. V., Almaula, P., and Werkheiser, W. C., *J. Med. Chem.*, **7**, 25(1964).
(18) Baker, B. R., and Shapiro, H., *ibid.*, **6**, 664(1963).
(19) Kauzmann, W., *Advan. Protein Chem.*, **14**, 37(1959).
(20) Jaffé, H. H., *Chem. Rev.*, **53**, 191(1953).
(21) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(22) Freireich, E., *Ann. Internal Med.*, **59**, 939(1963).
(23) Hansch, C., Steward, A. R., and Iwasa, J., *Mol. Pharmacol.*, **1**, 87(1965).
(24) Koshland, D. E., *Cold Spring Harbor Symp. Quant. Biol.*, **27**, 473(1963).

Analysis of Promethazine Hydrochloride in Syrups

By ALBERT R. SPERLING

An improved method for the determination of promethazine hydrochloride in official syrups is presented. Column chromatography has been used to separate the active ingredient from those of its degradation products that interfere with the present official analysis. Determination and identification are by ultraviolet and infrared spectrophotometry, respectively. The procedure is rapid and accurate. One of the major breakdown products of promethazine hydrochloride in aqueous solution is identified.

THE U.S.P. XVII (1) assay for promethazine hydrochloride syrup is not adaptable to all commercial products. The viscosity of some syrups prevents efficient extraction, and the results of analysis are poor. Another disadvantage of the assay is that certain degradation products of promethazine hydrochloride which may be present will interfere with the analysis. No provision has been made for the separation or removal of these materials. Steele (2) attempted an analysis of various phenothiazine derivatives by using a strongly acidic ion-exchange resin. Good results were obtained on pure materials but the application to dosage forms was less successful. Turi (3) used a diatomaceous earth[1] chromatographic column with 1% tartaric acid as the stationary phase to separate promethazine hydrochloride and other phenothiazines from mixtures containing other therapeutic agents. Only tablet and capsule dosage forms were studied.

The present paper describes a simple method for the chromatographic separation, on diatomaceous earth columns, of promethazine hydrochloride from official syrups, and the removal of interfering degradation products. The active principle is determined and identified by ultraviolet and infrared spectrophotometry, respectively. Also identified is the major degradation product from an aqueous solution of promethazine hydrochloride.

METHOD

Apparatus.—A Beckman DK-2 recording spectrophotometer and chromatographic columns, 20 × 200 mm., were used.

Received July 29, 1966, from the Spectrophotometric Research Branch, Division of Pharmaceutical Chemistry, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C. 20204.

Accepted for publication October 5, 1966.

[1] Marketed as Celite 545 by Johns-Manville Corp., New York, N. Y.

Reagents.—Chloroform, A.C.S., reagent grade; chloroform, A.C.S., reagent grade saturated with water, containing 1% v/v acetic acid; ether U.S.P., saturated with water; 1 *N* sulfuric acid solution; 1 *N* sodium hydroxide solution; diatomaceous earth.

Preparation of Chromatographic Column.—Place a pledget of glass wool at the bottom of a chromatographic column. To 4 Gm. of diatomaceous earth, add 1 ml. of 1 *N* sulfuric acid, and mix well. Transfer to the column and tamp down moderately.

Preparation of Standard Solution.—Dissolve 6 mg. of U.S.P. promethazine hydrochloride reference standard, accurately weighed, in 200 ml. of water-saturated chloroform containing 1% v/v acetic acid.

Preparation of Sample Solutions.—For syrups containing 1 to 2 mg./ml. of active ingredient, dilute the syrup with an equal volume of water in a volumetric flask. For syrups containing about 5 mg./ml. of active ingredient, dilute 1 part of syrup with 4 parts of water in a volumetric flask. To measure the viscous syrup more easily, pipet the appropriate volume of water into the flask and then bring to volume with syrup.

Procedure.—*Assay.*—Transfer a 10-ml. aliquot of sample solution to a 125-ml. separator and allow the pipet to drain well. If necessary flush the pipet with a small amount of water (less than 2 ml.). Extract the solution with three 45-ml. portions of chloroform and filter the extracts through a pledget of glass wool. Evaporate the filtrate to dryness on a steam bath with the aid of a stream of air. Remove immediately from the steam bath, add 2 ml. of water to the residue, and mix well. The entire residue will not dissolve. To this mixture add 3 Gm. of diatomaceous earth and mix thoroughly. Transfer this diatomaceous earth-sample mixture to the previously prepared chromatographic column, tamp moderately, and add a pledget of glass wool. Wash the column with 150 ml. of water-saturated ether and discard this eluate. Rinse the sample beaker and elute the column with water-saturated chloroform containing 1% v/v acetic acid; collect the eluate in a 200-ml. volumetric flask. Dilute to volume with chloroform. Determine the absorbances of the chloroform solutions of the sample and standard solutions in

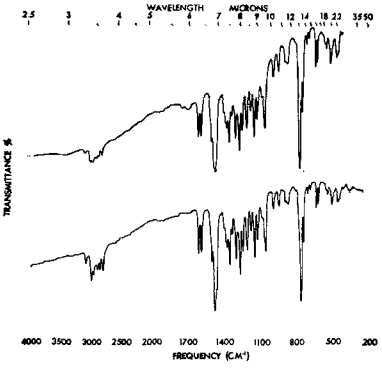


Fig. 1.—Infrared spectra of promethazine standard (bottom) and promethazine isolated from the sample (top).

1-cm. cells at the wavelength of maximum absorption at about 302 mμ and at the wavelength of minimum absorption at 360 mμ, using water-saturated chloroform containing 1% v/v acetic acid as a blank. Calculate the quantity of promethazine hydrochloride in milligrams by the formula

$$\frac{C(A_u^{302} - A_u^{360})}{V(A_s^{302} - A_s^{360})} = \text{mg./ml. of syrup}$$

where A_u and A_s are the absorbances of the unknown and standard solutions at the specified wavelengths, C is the concentration of the standard solution in mg./200 ml., and V represents the volume of syrup present in the 10-ml. aliquot of the sample solution.

Identification.—Transfer a volume from both standard and sample chloroform solutions, equivalent to about 1 to 2 mg. of promethazine, to 125-ml. separators. Wash each solution successively with 10 ml. of 1 *N* sodium hydroxide and 10 ml. of water. Evaporate each chloroform layer to dryness in an agate mortar and dry the residue for 30 min. in a vacuum desiccator over phosphorus pentoxide. Prepare a potassium bromide disk of the residue. Identify the sample by comparing the infrared absorption spectrum of the sample with that of the standard (Fig. 1).

RESULTS AND DISCUSSION

Table I gives the recoveries of U.S.P. reference standard promethazine hydrochloride which was added to a simulated and a commercial syrup. The recoveries agree within 1.4% of the amounts added. Table II shows the average results of analysis of various commercial samples. Samples *A*, *B*, *C*, *E*, and *F* are declared to contain 1.25 mg./ml. and sample *D* 5 mg./ml. Very low results were obtained when syrups *B* and *F* were analyzed by the U.S.P. method, but after dilution with an equal volume of water, results were satisfactory. All of the results agreed within 3.2% of the declared content except sample *A*, which assayed only 70% of the declared amount. Preliminary investigation showed that this low result was due to partial decomposition of the syrup. When an attempt was made to analyze this syrup by the present U.S.P. (1) method, degradation products

Table I.—Recovery of Reference Standard Added to Simulated and Commercial Syrups

Sample	Recoveries, %
A[a]	100.2
	99.7
B[a]	100.0
	100.0
E[a]	98.1
	98.6
F[b]	98.9
	99.5

[a] Commercial syrups. [b] Simulated syrup.

Table II.—Analysis of Promethazine Hydrochloride in Commercial Syrups

Sample	Found, mg./ml.	Labeled Amt., % Proposed Method	Labeled Amt., % U.S.P. Method	Labeled Amt., % Modified U.S.P. Method[a]
A	.882	70.6		
	.881	70.5		
B	1.259	100.7	49.5	96.3
	1.252	100.1	51.8	95.6
C	1.255	100.4		
	1.252	100.1		
D	5.057	101.1		
	5.097	101.9		
E	1.233	98.6		
	1.229	98.3		
F	1.210	96.8	69.5	96.9
	1.216	97.3	62.4	96.2

[a] The syrup was diluted with an equal volume of water, then analyzed by the official U.S.P. method

interfered with the analysis because no provision had been made for their removal or separation. This interference (Fig. 2, curve 2) can be seen by the change in the ultraviolet spectrum from that of the reference standard (Fig. 2, curve 1). An interfering peak occurs at about 335 mμ and the main peak is shifted to about 292 mμ with an increase in absorptivity. With the proposed procedure, the promethazine is trapped on the diatomaceous earth in the sulfuric acid layer while the ether wash removes the interfering degradation products. The eluate is then determined and identified.

According to Waaler (4) the thermal decomposition products of promethazine in aqueous solution are 10-methylphenothiazine, acetaldehyde, and dimethylamine. Yamamoto and Fujisawa (5) report the same decomposition when a 0.5% solution is

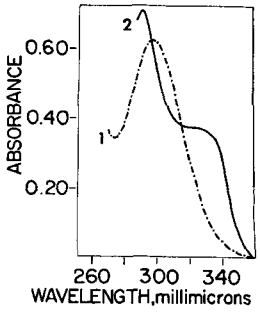


Fig. 2.—Ultraviolet spectra of promethazine in 0.1 *N* acid (curve 1) and in the presence of degradation products (curve 2).

exposed to 20-w. cool white fluorescent light in the presence of air, for a period of 2 or 3 days. In the course of this investigation it has been found that when an aqueous solution of promethazine hydrochloride of similar concentration is exposed to sunlight for longer periods of time, phenothiazine is one of the major degradation products. After extraction of the aqueous solution, this compound was identified by thin-layer chromatography and by its infrared spectrum.

SUMMARY

A procedure has been developed for the analysis of promethazine hydrochloride in syrups. Column chromatography is combined with extraction to separate the active ingredient from interfering degradation products and excipients. Phenothiazine has been identified as one of the degradation products of promethazine.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 529.
(2) Steele, J. W., *Can. Pharm. J.*, **February 1964**, 59.
(3) Turi, P., *J. Pharm. Sci.*, **53**, 369(1964).
(4) Waaler, T., *Pharm. Acta Helv.*, **35**, 168(1960).
(5) Yamamoto, R., and Fujisawa, S., Proceedings: 23rd Congress of Pharmaceutical Science, Münster, Germany, 1963.

Technical Articles

Evaluation of the Suitability of Butadiene–Acrylonitrile Rubbers as Closures for Parenteral Solutions

By J. SHANKER, M. GIBALDI*, J. L. KANIG, A. P. PARKER†, and L. LACHMAN

The sorption, leaching, tensile, and other properties of a series of butadiene–acrylonitrile rubbers from the medium-high acrylonitrile group were evaluated to determine the suitability of this copolymer system as elastomer closures for multiple-dose vial injectable preparations. These elastomers were found to exhibit considerable sorption of antibacterial preservatives from solution and leaching of extractives to the solutions when compared with a butyl rubber control. Partial identification of the leached materials was possible by chemical analysis. By reducing the amount of accelerator in one of the elastomer formulations by 25 per cent, a 35 per cent reduction in the amount of extractive leached from the elastomer resulted. This rubber formulation also exhibited a lower tensile strength and greater ultimate elongation. The butadiene-acrylonitrile elastomers possessed very low porosity and exhibited a low degree of water vapor transmission.

THERE HAS been considerable development in elastomer chemistry and technology during the last two decades, and a variety of synthetic elastomers are now in common use. A few of the more important uses of elastomers by the pharmaceutical industry are as packaging materials, surgical gloves, tubing for medical use, and vial closures.

These materials make excellent closures for multidose vials containing parenteral solutions because of their unique combination of properties—namely, pierceability and resealability (1). Unfortunately, the favorable qualities are also accompanied by various undesirable characteristics. It has long been known that rubber closures may yield substances to the preparation for which it is used as a stopper, a property known as leaching (2, 3). The closure can also sorb components from the preparation (3). In addition, it has been reported that materials can be lost by vapor transmission through the closure, chemical incompatibility between the product and closure could take place, physical instability of the elastomer could result, and coring or cutting away of rubber particles by the hypodermic needle is a frequent occurrence (1, 4, 5–7).

It is evident from the foregoing brief literature review that most of the studies appearing in the

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TABLE I.—COMPOSITION OF BUTADIENE–ACRYLONITRILE COPOLYMERS[a]

1002	1022	1032	1042
Butadiene	Butadiene	Butadiene	Butadiene
Acrylonitrile	Acrylonitrile	Acrylonitrile	Acrylonitrile
Mono and dioctylated diphenylamines mixture	DTBPC[b]	DTBPC[b]	Mono and dioctylated diphenylamines mixtures
Sodium salt of a fatty acid	Sodium salt of a fatty acid	Rosin acid soap	Alkyl aryl sulfonic acid salt

[a] In addition to the components listed above, sodium and potassium salts, mercaptans, sulfates, peroxides, fatty acids, amines, and disproportionated rosins are present in these copolymers in amounts of 1% or less. [b] 2,6-Ditertiary butyl *p*-cresol.

literature to date have dealt with factors affecting sorption, leaching, and various other properties of several natural and synthetic elastomeric products (1–12). Recently, some information has been presented concerning the influence of mylar, silicone, and epoxy linings on the sorption and leaching tendencies of closures (1, 13). However, except for these reports, there has been very little information presented in the literature regarding the development of rubber closures that would meet the broad and diversified requirements of the pharmaceutical industry.

The present study was undertaken to evaluate the sorption, leaching, and physical properties of a group of elastomers not commonly used as closures for parenteral multiple-dose containers. It was also the intent of this study to explore ways to improve the properties of these elastomers by formulation modification, if the results indicated certain shortcomings for use as closures for multiple-dose vials. The long-range objective of the investigational program is to develop a closure which will possess the major characteristics demanded by the pharmaceutical industry, namely, be nonadditive, nonsorptive, nonreactive, and yet possess the requisite physical properties needed for satisfactory use as a closure for multiple-dose parenteral preparations.

EXPERIMENTAL

Materials

Elastomers.—The elastomers selected belong to the nitrile rubber group and are butadiene–acrylonitrile copolymers.[1] Four of these formulations, coded 1008, 1009, 1010, and 1011, were employed. These were identical in composition, except for the antioxidants and emulsifying agents which are different in each case (Table I). A representative formula is as follows: butadiene–acrylonitrile copolymer, zinc oxide, sulfur, channel black, di-(2-ethyl hexyl)phthalate, diethylene glycol, tetraethylthiuram disulfide,[2] tetramethylthiuram disulfide,[3] stearic acid.

[1] Marketed as Hycar by the B. F. Goodrich Chemical Co., Cleveland, Ohio.
[2] Marketed as Ethyl Tuads by the R. T. Vanderbilt Co., New York, N. Y.
[3] Marketed as Methyl Tuads by the R. T. Vanderbilt Co., New York, N. Y.

The respective nitrile rubber present in each elastomer compound is as follows:

Elastomer Compd.	Nitrile Rubber No.
1008	1022
1009	1032
1010	1042
1011	1002
1008′[4]	1022

The nitrile rubber constitutes the bulk of the elastomer compound. The other ingredients which act as reinforcing agents, colorants, accelerators, plasticizers, and fillers are present in much smaller amounts.

Butadiene–Acrylonitrile Copolymers.—The nitrile rubbers are the basic crude polymers. They are spongy, yellowish-white solid materials. The composition of each polymer is recorded in Table I.

Solvents.—Alcohol U.S.P.; chloroform, certified reagent (Fisher Scientific Co.); petroleum ether (40–60) (Sargent and Co.), double distilled before use; polyethylene glycol 300 (Ruger Chemical Co.).

Components of Elastomer Compounds.—Butadiene–acrylonitrile rubber and di-(2-ethyl hexyl) phthalate (B. F. Goodrich Chemical Co.), a mono- and dioctylated diphenylamines mixture,[5] tetraethylthiuram disulfide, tetramethylthiuram disulfide; sulfur (Olin Mathieson Chemical Corp.); channel black (J. M. Huber, Inc.); diethylene glycol (Carbide and Carbon Co.); stearic acid and zinc oxide (National Bureau of Standards).

Miscellaneous Reagents and Chemicals.—Benzyl alcohol, certified reagent and anhydrous sodium sulfate, certified reagent (Fisher Scientific Co.); *p*-chloro-β-phenylethyl alcohol (Ciba Pharmaceutical Co.); zinc diethyldithiocarbamate[6] and zinc dimethyldithiocarbamate;[7] anhydrous calcium sulfate;[8] epoxy adhesive sealant.[9]

Standard Solutions.—Benzyl alcohol 1.0% (w/v solution) in standard citrate–phosphate buffer (0.275 *M*), pH 4; *p*-chloro-β-phenylethyl alcohol 0.3% (w/v solution) in standard citrate–phosphate buffer (0.275 *M*), pH 4; standard phosphate buffer solutions (0.2 *M*), pH 4–9.

Equipment.—Micro Kjeldahl steam distillation

[4] Elastomer 1008′ is a modified form of 1008. It contains 25% less of tetraethylthiuram disulfide and tetramethylthiuram disulfide, respectively.
[5] Marketed as Age Rite Stalite by the R. T. Vanderbilt Co., New York, N. Y.
[6] Marketed as Ethyl Zimate by the R. T. Vanderbilt Co., New York, N. Y.
[7] Marketed as Methyl Zimate by the R. T. Vanderbilt Co., New York, N. Y.
[8] Marketed as Drierite by the W. A. Hammond Drierite Co., Xenia, Ohio.
[9] Marketed as Hysol by the Hysol Corp., Olean, N. Y.

unit; Beckman ultraviolet spectrophotometer, model DB; Perkin Elmer infrared spectrophotometer, model 421; Beckman pH meter, model Zeromatic; Instron Universal Testing Equipment, model TM; Aminco Winslow Mercury Porosimeter; Hotpack constant humidity chambers; hydraulic cutting press for ASTM tensile studies.

Sorption of Preservatives

Preparation of Samples.—The vulcanizates were obtained in sheets of uniform thickness (0.4 cm.). These were cut into small pieces of uniform dimensions (0.4 × 0.5 × 1.0 cm.^{3}) using a paper cutter. The cut pieces were washed by immersion in a 1% solution of sodium lauryl sulfate using continuous agitation for 10 min. The detergent solution was decanted and the sample was washed repeatedly with fresh warm water. Fifteen to 20 washings were used. The sample was finally washed with 10 successive quantities of distilled water. The washed samples were dried in a hot air oven between 60–70° and stored at room temperature.

Sorption Properties.—Sorption of *p*-chloro-β-phenylethyl alcohol (0.3% solution in citrate–phosphate buffer) and benzyl alcohol (1.0% solution in citrate–phosphate buffer) was investigated. An amount of the prepared vulcanized samples (elastomer compounds 1008, 1009, 1010, 1011, and butyl rubber stoppers[10]) equal to 2.5 Gm. was transferred to a 25-ml. conical flask containing 10 ml. of the preservative solution. Triplicate samples of each elastomer compound were prepared for each preservative solution, and control solutions were run simultaneously. The samples were stored at room temperature and shaken once or twice daily to remove the air bubbles which settle on the elastomer samples. At the end of the experimental period (1–3 weeks) solutions from all the samples were withdrawn. The preservative content was ascertained by spectrophotometric absorbance measurement at 257 mμ and 267 mμ for benzyl alcohol and *p*-chloro-β-phenylethyl alcohol, respectively.

Direct absorbance measurement was not possible due to the presence of leached materials from the several samples. A reasonable degree of purification was obtained by steam distillation of the solutions using a micro Kjeldahl distillation unit. One or 2 ml. of solution was transferred to the distillation unit. The distillate was collected directly in a 25-ml. volumetric flask. About 20 ml. of the distillate was collected each time and the final volume was adjusted to 25 ml. with distilled water. The butyl rubber was found to possess some steam volatile materials, and a correction factor was applied according to the method suggested by Lachman *et al.* (14).

Leaching of Extractives

Leaching behavior of the samples was investigated by allowing the prepared elastomer samples to stand in contact with the selected solvents. Duplicate samples of each of the elastomer compounds and their basic nitrile rubber polymers were prepared by transferring 2.5 Gm. of the sample in a 25-ml. conical flask. To each flask, 10 ml. of distilled water, 10% ethyl alcohol, or 50% aqueous polyethylene glycol 300 were added. The flasks were sealed and stored at room temperature (23–28°) and shaken once or twice daily. At the end of the experimental period (1–6 weeks) solutions containing the leached materials were withdrawn and filtered through a sintered-glass funnel of medium porosity. Their absorbance was checked in the visible and ultraviolet range.

Effect of Surface Area.—Since elastomer compounds 1008, 1009, 1010, and 1011 were found to be less porous than the butyl rubber, it was considered of interest to investigate the effect of surface area on the leaching behavior. Sample 1008 was selected for the study. The investigation was conducted in two ways. In one set of experiments, the weight of the samples was kept constant at 2.0 Gm. and they were cut into large, small, and very small pieces to obtain a small (6.5 cm.^{2}), medium (8.9 cm.^{2}), and large (10.2 cm.^{2}) surface area, respectively. In the other set of experiments, samples weighing 1 Gm., 1.5 Gm., and 2 Gm., respectively, were cut in calculated sizes to obtain the same surface area. All the samples were tested in distilled water at room temperature in accordance with the procedure described earlier.

Effect of pH.—In order to evaluate the effect of pH on the leaching properties of the nitrile rubber vulcanizates, two of the samples which showed the lowest degree of leaching were selected. Butyl rubber was used for comparison. The procedure followed was identical to that in the determination of leaching in the pure solvents. The pure solvent was replaced with the same amount of standard phosphate buffer solution (0.2 *M*) at pH 4, 5, 6, 7, 8, and 9, respectively.

Identification of the Leached Materials

Spectrophotometric Analysis.—Ultraviolet absorption spectra of all the materials used in the synthesis of the basic nitrile rubber polymers and in the several elastomer formulations were obtained in distilled water, 10% ethyl alcohol, and 50% aqueous polyethylene glycol 300. Most of the ingredients are only sparingly soluble in these solvents. Consequently, saturated solutions were prepared by allowing 5 Gm. or 5 ml. of the compound to stand with 25 ml. of the solvent for 48 hr. with occasional shaking. The solutions were filtered through a medium porosity sintered-glass funnel and diluted wherever necessary. The ultraviolet absorption spectra of these solutions was compared with the absorption spectra of the leached material from the basic nitrile polymers and vulcanized elastomer formulations.

Chemical Analysis.—One-hundred grams of elastomer compound 1008 was cut in small pieces. It was washed and dried according to the procedure described previously. The dried sample was transferred to a glass refluxing assembly and refluxed for 2 hr. with 400 ml. of distilled water. The aqueous extract so obtained was transferred, while still hot, to a 1-L. beaker. The remaining elastomer compound was washed twice with 50-ml. portions of hot distilled water. The washings were combined with the first portion. The combined aqueous extract appeared milky with a white colloidal material dispersed in it. The extract was allowed to cool to room temperature. A portion of the extract settled

[10] Butyl Rubber Stoppers manufactured by West and Co., Inc., were used for comparative evaluation.

out as a thin white precipitate, but the rest of it remained suspended. The dispersed material was not retained on ordinary filter paper.

The aqueous extract was transferred to a 1-L. separator. It was extracted with successive 100-ml. portions of petroleum ether by shaking for 25 min. each time. The extraction was continued until 1 ml. of the petroleum ether portion did not leave any observable residue on evaporation to dryness. A total of five extractions was required. All the petroleum ether portions were combined and washed twice by shaking with 50-ml. portions of distilled water. The petroleum ether extract was then dried by allowing it to stand in contact with 50 Gm. of anhydrous sodium sulfate for 24 hr. with occasional shaking. The dried extract was filtered through filter paper, and the solvent was distilled off. The residual solvent was removed by keeping the extract in a vacuum desiccator for 24 hr. The final residue weighed 15 mg. and was a pale viscous liquid. An infrared absorption spectrum of the material was obtained.

The aqueous extract left after extraction with petroleum ether was heated at 80° to remove the residual petroleum ether. It was allowed to cool to room temperature and was extracted repeatedly with 100-ml. portions of chloroform until 1 ml. of the chloroform portion left no residue on evaporation to dryness. A total of four extractions was sufficient. The chloroform extract was washed, dried, and the solvent was removed as described above for the petroleum ether extraction.

The final residue weighed 17 mg. and occurred as a brownish amorphous mass. This was redissolved in a small amount of fresh chloroform. Most of the material dissolved, but a small portion of the insoluble brown residue separated out. Infrared absorption spectrum of the insoluble residue was taken in liquid petrolatum[11] and that of the soluble portion in chloroform.

The aqueous extract left after extraction with petroleum ether and chloroform was distilled under reduced pressure to approximately 50 ml. This was evaporated to dryness and heated strongly on a direct flame. The residue did not decompose or char, indicating that it was an inorganic material. This residue was further analyzed for inorganic components according to standard semimicro techniques.

Physical Testing of Rubber Samples

Water Vapor Transmission and Sorption.—Preparation of Samples.—The vulcanized elastomer compounds were cut into circular disks of uniform diameter (1.8 cm.) from sheets of uniform thickness (0.4 cm.) using an electric cork borer. These disks were washed by immersing them in a 1% solution of sodium lauryl sulfate and stirring for 10 min. The detergent solution was decanted and the sample washed repeatedly with fresh warm water. Final washing was performed with 10 successive quantities of deionized water. The washed samples were air dried (25–28°) and stored at room temperature in a desiccator.

Evaluation of Moisture Sorption.—The prepared disks were individually tied with a thin copper wire and with a portion of the wire, a hook was formed by which the disks were hung in a 90% ± 3% relative humidity cabinet set at 26°. The individual samples were removed from the relative humidity chamber after 1, 2, 3, and 4 hr. and 3, 5, 7, 11, and 71 days and weighed.

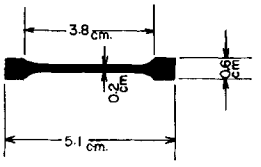

Fig. 1.—Dimensions of dumbbell samples used for the determination of tensile properties.

Evaluation of Water Vapor Transmission.—To 10-ml. glass vials, approximately 5 Gm. of anhydrous calcium sulfate was added. The disks of the elastomer formulations were sealed along the top rim of the vials with an epoxy adhesive sealant and allowed to set for 20 min. The vials were then crimped with three-piece aluminum vial caps. The outer and center portions of the aluminum seal were detached, exposing a uniform surface area of rubber beneath the cap. The sample vials were weighed (0 time sample) and placed into a 90% relative humidity chamber at 26°. Another set of 10-ml. glass vials, which did not contain calcium sulfate, was sealed in the same manner with the four different rubber disks and placed into the relative humidity chamber as controls. Samples with and without anhydrous calcium sulfate were removed from the relative humidity chambers at 1, 2, 3, and 4 hr. and 3, 5, 7, 11, and 71 days and weighed.

Tensile Properties.—The vulcanized sheets were cut into uniform "dumbbell" shaped samples having the dimensions shown in Fig. 1, by use of a hydraulic cutting press. These were tested for tensile strength, ultimate elongation, and tension set in accordance with modified ASTM procedures (15). The modification consisted of altering the minimum distance between grips (bench marks) at the start of the test from that recommended by the ASTM. For all samples with the exception of 1008′, a 2-cm. distance was used, while for 1008′ 0.5 cm. was employed. Using the recommended bench mark distances of the ASTM with the model TM Instron which has a maximum travel of 60 cm. for its tensil fixture, no break could be obtained with any of the samples. Consequently, it was necessary to reduce bench mark distances until a point of rupture within the travel distance of the equipment was obtained. The rate of jaw separation for each of these measurements was 50 cm./min.

The *tensile strength* of a material may be defined as the force required to rupture a dumbbell sample of unit area. It may be calculated as follows:

$$\text{tensile strength} = \frac{\text{force at rupture } (F)}{\text{cross sectional area of unstretched sample } (A)}$$

The *ultimate elongation* (E) of a sample can be defined as the maximum elongation immediately prior to sample rupture and is expressed as follows:

$$E(\%) = \frac{L - L_0}{L_0} \times 100$$

[11] Marketed as Nujol by Plough Laboratories, Memphis, Tenn.

where L = observed distance between stretched bench marks at point of rupture,
L_0 = original distance between unstretched bench marks.

The bench mark for the rubber dumbbell samples is the distance between the jaws clamping the sample.

The *tension set* is determined by stretching the sample at a uniform rate of speed (0.6 cm./sec.) for 15 sec. The sample is held in this stretched position for 10 min. At the end of the 10-min. period, the sample is released and allowed to rest for an additional 10 min. with no external force applied. At the end of the rest period, the distance between the bench marks is again measured. *Tension set* is defined as the extension remaining after a sample has been stretched and allowed to retract in a specified manner, expressed as a percentage of original length.

Porosity Studies.—The vulcanized samples of the butadiene–acrylonitrile copolymer compounds were cut into small cubical pieces (0.4 cm. × 0.4 cm. × 0.4 cm.) and the porosity determined by mercury diffusion using the Aminco-Winslow porosimeter (16).

Each sample is placed in the penetrometer which is in turn attached to the porosimeter. A vacuum pump evacuates the system and fills the penetrometer with mercury. Pore sizes of 17 μ or larger in diameter can be determined under these conditions.

TABLE II.—SORPTION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL

Sample	Preservative Absorbed, % 1 Wk.	2 Wk.	3 Wk.
Butyl	14.5	15.6	16.9
1008	78.0	78.3	78.3
1009	75.5	76.4	76.5
1010	76.5	78.2	78.7
1011	75.1	75.8	76.0
1008′	77.8	78.4	78.5

TABLE III.—SORPTION OF BENZYL ALCOHOL

Sample	Preservative Absorbed, % 1 Wk.	2 Wk.	3 Wk.
Butyl[a]	5.6	7.1	8.9
1008	32.1	34.8	35.2
1009	32.5	35.2	35.5
1010	33.0	35.8	36.1
1011	31.9	35.5	36.2
1008′	34.5	35.0	35.0

[a] The values were obtained after applying correction for the steam volatile impurities leached by the butyl rubber. Corrected absorbance, $A_c = A_{257} - (A_{253} - A_{263})$ (14).

The penetrometer is then transferred to the pressure chamber of the porosimeter where pressures up to 5000 p.s.i. can be applied to the mercury and where pore sizes down to 0.035 μ in diameter can be measured. As the pressure is increased, mercury is forced into smaller and smaller pores, the volumes of which are continuously indicated by the level of mercury in the graduated stem of the penetrometer.

RESULTS AND DISCUSSION

Sorption of Preservatives.—The results of the sorption study are presented in Tables II and III. All the butadiene–acrylonitrile copolymer compounds show considerable sorption of *p*-chloro-β-phenylethyl alcohol and benzyl alcohol from buffered aqueous solutions of the preservatives. There is very little difference in the loss of preservative after 2 or 3 weeks, indicating equilibrium has been attained at 2 weeks or sooner. The amount of *p*-chloro-β-phenylethyl alcohol sorbed is almost twice the amount of benzyl alcohol during the same period of time. Elastomer compound 1008′, the modified sample, showed a degree of sorption similar to that of the rest of the samples belonging to the acrylonitrile group. In comparison to the butyl rubber, the formulations under investigation caused considerably more sorption of the preservatives.

The sorption properties of elastomers is a direct consequence of their ability to "dissolve" other materials. The extent to which a preservative would be sorbed from an aqueous solution would thus depend on the partitioning tendencies of the preservative between water and the elastomer. The long chain, high molecular weight organic molecules of the elastomers preferentially sorb hydrophobic organic materials from aqueous systems. A nonpolar substance, with a limited water solubility, would usually be more soluble in the elastomer composition than in water. Thus, it is reasonable to expect greater sorption of *p*-chloro-β-phenylethyl alcohol than benzyl alcohol which is more polar than the former and has greater water solubility.

It has been proposed that sorption is a function of the basic polymer and is not influenced by the fillers to any considerable extent (17). All the samples evaluated in this investigation showed a remarkably similar degree of sorption as would be expected with acrylonitrile–butadiene ratios in the same relative range. This similar degree of sorption displayed by these formulations suggests that the difference in the molecular structure of the particular

TABLE IV.—ULTRAVIOLET ABSORPTION MAXIMA OF THE LEACHED MATERIALS FROM POLYMER SAMPLES

Sample	Wavelength, mμ Distilled Water	10% Ethyl Alcohol	50% Polyethylene Glycol
Nitrile rubber 1002	244	244	280; 208
Nitrile rubber 1022	224	224	277; 207
Nitrile rubber 1032	275; 266; 246	275; 266; 246	274; 266; 248; 212
Nitrile rubber 1042	224	224	288; 224–208
Elastomer compn. 1008	244	244	248; 208
Elastomer compn. 1009	244	244	248; 208
Elastomer compn. 1010	244	244	248; 208
Elastomer compn. 1011	244	244	248; 208
Elastomer compn. 1008′	244	244	248; 208

TABLE V.—ULTRAVIOLET ABSORPTION MAXIMA OF THE SOLUTION OF INGREDIENTS USED IN THE ELASTOMER FORMULATIONS

Ingredient[a]	Wavelength, mμ: Distilled Water	10% Ethyl Alcohol	50% Polyethylene Glycol 300
Acrylonitrile	260	260	292
	220	220	222
Mono- and dioctylated diphenylamines	280	280	284
	244	244	208
DTBPC	275	275	276
			207
Rosin acid soap	275	275	275
	266	266	266
	260	260	212
Alkyl aryl sulfonic acid salt	261	261	262
	255	255	256
	223	223	223
Diethylene glycol	262	262	264
Di-(2-ethyl hexyl) phthalate	276	276	284
			232
			210
Tetraethyl thiuram disulfide	268	269	275
Tetramethyl thiuram disulfide	250	250	246
	206	206	210

[a] Only those ingredients are listed whose solutions had well-defined maxima. Others (zinc oxide, sulfur, stearic acid, etc.) either did not absorb or had no maxima.

TABLE VI.—LEACHED COMPONENTS IDENTIFIED IN 50% POLYETHYLENE GLYCOL 300

Butadiene–Acrylonitrile Copolymer Sample	Leached Component
1002	Mono- and dioctylated diphenylamines mixture[a]
1022	2,6-Ditertiary butyl *p*-cresol
1032	Rosin acid soap[a]
1042	Mono- and dioctylated diphenylamines mixture

[a] These were also identified to be the components present n distilled water and 10% ethyl alcohol extractives.

basic nitrile rubber polymers is not large enough to alter significantly their affinity for the preservative molecules.

Leaching of Extractives.—The polymers show a considerable degree of leaching at room temperature in distilled water, 10% ethyl alcohol, and 50% aqueous polyethylene glycol 300. Leaching was observed both from the basic polymers (*i.e.*, the butadiene–acrylonitrile copolymers) and the finished elastomer formulations. The absorption patterns of the leached materials from the butadiene–acrylonitrile copolymers differed from one another as is evident from the ultraviolet absorption maxima presented in Table IV.

The leached materials from the various vulcanized elastomer compounds, unlike the material leached from the butadiene–acrylonitrile copolymers, had identical absorption patterns in all three solvents (Table IV). The degree of absorbance, however, was different with sample 1008 exhibiting the least leaching.

The leached materials from the butadiene–acrylonitrile copolymers could be identified by a comparison of their ultraviolet absorption spectra with that of the component ingredients. The leached materials from all four samples were found to be single ingredients and their identification was relatively simple. A comparison with the wavelengths of maximum absorbance of the component ingredients, presented in Table V, led to the identification of the leached materials. These are recorded in Table VI. The materials leached in distilled water and 10% ethyl alcohol were found to be the same as in 50% polyethylene glycol 300 in the case of basic nitrile rubber polymers 1002 and 1032. In the case of basic nitrile rubber polymers 1022 and 1042, the materials leached in 50% ethyl alcohol and in water could not be identified.

Although the absorption pattern of the leached materials from the various vulcanized elastomer compounds was identical in all three solvents, it was not similar to the absorption pattern of any of the pure component ingredients, and no identification was possible by their comparison. A bulk extract was, therefore, prepared and fractionated into different solubility groups by solvent-solvent extraction. Three different fractions were obtained, namely, a petroleum ether extract, a chloroform extract, and an inorganic portion. The residue from the petroleum ether extract was examined for its infrared absorption in its original form. This gave an indication of the presence of phenyl groups (at 702, 740, 1575, and 1595 $cm.^{-1}$) and ester groups (at 1710–1720 $cm.^{-1}$). The only component in the elastomer formulations containing these groups is di-(2-ethyl hexyl) phthalate. Its presence was confirmed by taking an infrared spectrum of the pure compound which was found to coincide with the petroleum ether extractive.

The residue from the chloroform extract occurred as a brownish mass and appeared to be impure as it did not go into solution completely upon the addition of chloroform. The insoluble residue did not show any identifiable group on the infrared spectrum besides a hydrocarbon chain and could not be identified. The chloroform soluble portion gave an indication of the presence of —C=S groups. One source of these could be the thiuram-type accelerators, *i.e.*, the tetraethyl thiuram disulfide and the tetramethyl thiuram disulfide. However, an infrared spectrum of the pure compound did not resemble that of the extract. It was, therefore,

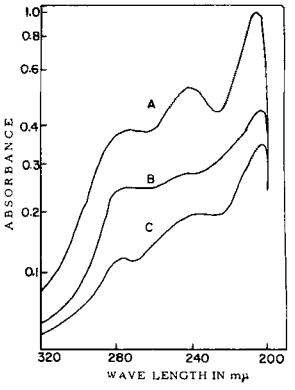

Fig. 2. — Ultraviolet absorption spectra of the aqueous extract of elastomer 1008 and zinc diethyldithiocarbamate. Key: A, aqueous extract diluted with 50% aqueous polyethylene glycol 300; B, saturated solution of zinc diethyldithiocarbamate in distilled water; C, aqueous extract diluted with distilled water.

suspected to be a reaction product of these materials and was identified as a mixture of zinc diethyl/methyl dithiocarbamate. These products are known to be formed during vulcanization process (18). These appear to be the primary leached components responsible for strong absorbance in the ultraviolet region. An ultraviolet absorption spectrum of the aqueous extract obtained after large dilution was in excellent agreement with the absorption spectrum of the dithiocarbamates (Fig. 2). The inorganic portion was identified to be zinc sulfide which is also formed during the vulcanization process.

Based on these findings, elastomer formulation 1008 was modified by reducing the concentration of tetraethyl and tetramethyl thiuram disulfide 25%. This sample, now called 1008′, demonstrated a 35% reduction in the degree of leaching. Figures 3, 4, and 5 represent the results of the leaching study over a period of 6 weeks in distilled water, 10% ethyl alcohol, and 50% aqueous polyethylene glycol 300, respectively. As is evident from the figures, the degree of leaching of the acrylonitrile rubbers is much higher than the butyl rubber samples. Furthermore, the degree of leaching in 50% aqueous polyethylene glycol 300 was much higher than in distilled water or 10% ethyl alcohol.

Leaching of extractives by the elastomers is much more complex than might appear initially. Attempts to prevent or reduce leaching by modification of the formulation most likely has received considerable attention by the rubber stopper manufacturers, but since this has not been reported in the literature, it is difficult to assess the progress made. The degree of reduction in leaching achieved by the modification of the formulation in the present investigation is by no means adequate; nevertheless, the results are quite encouraging, particularly in view of the fact that a reduction of the tetraethyl and tetramethyl thiuram disulfides alone brought about a 35% reduction in leaching. It may be possible to further reduce leaching by decreasing the di-(2-ethyl hexyl) phthalate and zinc oxide which have also been found to be leached from the rubber. There is, however, a finite limitation to the extent of reduction beyond which the physical

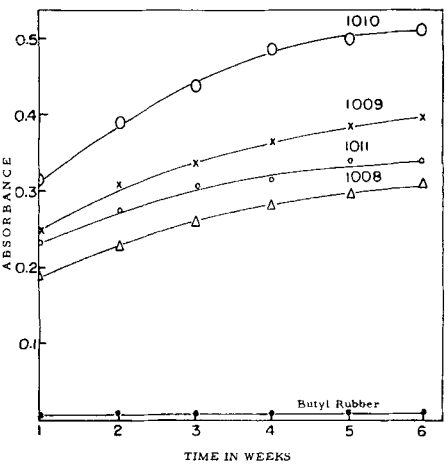

Fig. 3.—Relative leaching from elastomer samples in water at room temperature.

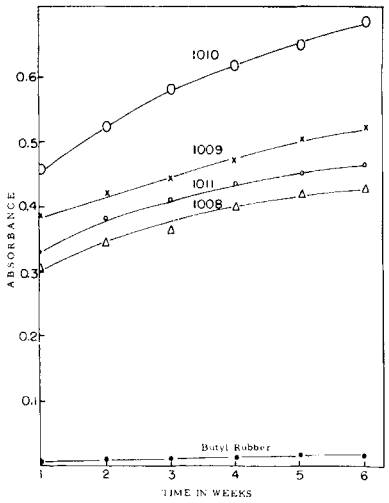

Fig. 4.—Relative leaching from elastomer samples in 10% aqueous ethyl alcohol at room temperature.

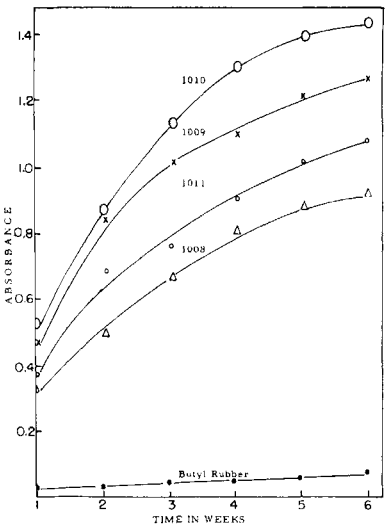

Fig. 5.—Relative leaching from elastomer samples in 50% aqueous polyethylene glycol 300 at room temperature.

properties of the elastomer may be adversely affected.

Effect of Surface Area and pH on Leaching.—The amount of leaching was found to be directly proportional to the surface area and completely independent of weight as illustrated by the graphs in Fig. 6. The leaching increased as the surface area of rubber exposed to the solvent was increased, even though the weight of the sample was maintained constant. On the other hand, samples with the same surface area, but of different weights, had the same degree of leaching. An increase in the surface area of the samples allows greater contact with the solvent, resulting in a faster rate of dissolution. The fact that leaching was not a function of the mass employed indicates that the leaching process in the butadiene–acrylonitrile copolymer compounds is essentially a surface phenomenon.

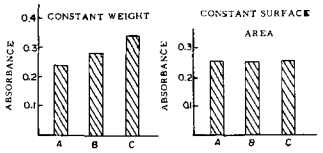

Fig. 6.—Effect of surface area on leaching. Weight: A, 1.0 Gm.; B,1.5 Gm.; C, 2.0 Gm. Surface area: A, 6.5 cm.²; B, 8.9 cm.²; C, 10.2 cm.².

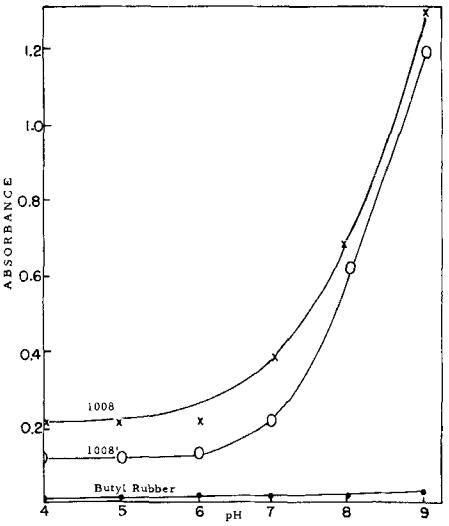

Fig. 7.—Effect of pH on leaching.

This may be a consequence of the low porosity of the samples.

The pH of the solvent had a profound effect on leaching. In the acidic range, the degree of leaching is relatively low. As the pH value increases, an increase in the degree of leaching is observed (Fig. 7).

Water Vapor Transmission.—The data summarized in Table VII indicate that for the storage period of the test, elastomer formulation 1011 shows the greatest tendency for water vapor permeation, while elastomer formulation 1008′ the least.

Water Sorption.—The results from the water sorption studies are summarized in Table VIII. It is evident from these data that the five elastomer formulations show sorption of about the same magnitude. However, it would appear that the relationship found for the water vapor transmission studies exists here too, but the magnitude of the differences are not so great.

Tensile Properties.—It is evident from the data in Table IX that a reduction in the tetraethyl and tetramethyl thiuram disulfide concentration by 25% in formulation 1008′ over formulation 1008 results in a considerably less tight cure (undercure) as evidenced by the lower tensile strength and greater ultimate elongation. From the curves in Fig. 8, illustrating the Hooke's law relationship of the five rubber formulations, this is further substantiated by the lower slope of the curve for formulation 1008′ as compared to formulation 1008. It would be expected that elastomer formulation 1008′ would exhibit higher coring, could be more difficult to insert into vials, and possibly exhibit greater seizure with the glass wall of the vial, and very objectionable compression set.

Porosity Studies.—Porosity measurements performed on the five rubber formulations using the mercury porosimeter at a maximum of 5000 p.s.i. showed essentially no porosity down to 0.035 μ in diameter. This can possibly explain why low water vapor transmission was found for these rubber samples in the water vapor transmission studies discussed earlier. In addition, this low porosity could also be the reason why leaching was found essentially to be a surface phenomenon and not influenced by the weight of the rubber sample.

TABLE VIII.—WATER VAPOR SORPTION OF ELASTOMER FORMULATIONS, mg.

Time	1008′	1008	1009	1010	1011
1 hr.	1.3	1.8	1.6	1.3	1.5
2 hr.	1.5	2.1	1.9	1.5	1.8
3 hr.	2.4	3.0	2.8	2.8	2.7
4 hr.	3.0	3.8	3.5	3.7	3.6
3 days	5.0	5.3	6.5	6.1	7.6
5 days	7.4	7.5	8.2	9.7	10.1
7 days	9.4	10.7	9.2	11.9	11.2
11 days	10.3	11.5	9.7	12.7	12.2
71 days	10.3	11.9	9.9	13.7	13.8

TABLE IX.—TENSILE CHARACTERISTICS OF ACRYLONITRILE RUBBER SAMPLES

Formulation	Tensile Strength, lb./sq. in.	Ultimate Elongation, %	Tension Set, %
1008′	875	5180	4.0
1008	1250	1570	12.5
1009	2090	1600	10.0
1010	1700	1289	7.5
1011	1640	1014	5.0

TABLE VII.—WATER VAPOR TRANSMISSION THROUGH ELASTOMER FORMULATIONS, mg.

	1008′		1008		1009		1010		1011	
Time	Sample[a]	Control[b]	Sample	Control	Sample	Control	Sample	Control	Sample	Control
1 hr.	0.6	0.3	0.5	0.7	0.9	0.6	0.7	0.5	0.9	0.5
2 hr.	0.7	0.4	0.6	0.8	1.1	0.7	0.8	0.5	1.1	0.6
3 hr.	0.9	0.7	1.2	1.1	1.5	1.2	1.5	1.1	1.8	1.0
4 hr.	1.4	1.2	2.6	1.6	2.0	1.6	2.1	1.6	2.3	1.6
3 days	4.7	3.8	4.2	4.2	5.1	4.5	5.6	5.0	6.1	5.0
5 days	5.0	4.6	5.3	4.9	6.5	5.5	6.1	6.1	7.6	6.3
7 days	7.4	8.7	7.5	7.0	8.2	7.7	9.7	8.4	10.1	8.5
11 days	9.4	10.5	9.4	9.0	10.0	9.5	12.0	10.5	11.8	10.4
71 days	28.1	16.8	32.2	15.2	32.4	14.4	36.0	17.7	39.7	16.9

[a] Sample = sample with anhydrous calcium sulfate. (See *Footnote 8.*) [b] Control = no anhydrous calcium sulfate. (See *Footnote 8.*)

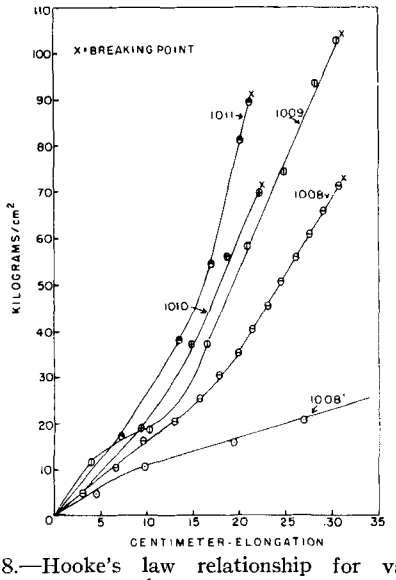

Fig. 8.—Hooke's law relationship for various elastomers.

SUMMARY AND CONCLUSIONS

The information gained from this study may be summarized as follows.

1. Considerable sorption of preservative was found to take place for each of the five elastomer formulations evaluated.

2. The magnitude of the loss of preservative was found approximately the same for the several formulations.

3. Significant extractive was leached from each of the formulations and were identified.

4. A reduction of 25% in the concentration of tetraethyl and tetramethyl thiuram disulfides in elastomer formulation 1008 resulted in a 35% reduction in extractives leached from the elastomer.

5. Water vapor transmission and sorption studies indicated that elastomer formulation 1008′ gave the best results and formulation 1011 the poorest.

6. A reduction of 25% in concentration of tetraethyl and tetramethyl thiuram disulfides in formulation 1008 gave a rubber formulation which exhibited two-thirds the tensile strength and about three and a half times the ultimate elongation. It would be anticipated that closures made from this formulation would not function satisfactorily because of probable undercure.

7. All the elastomer formulations in the study exhibited a very low degree of porosity and small pore size.

REFERENCES

(1) Hopkins, G. H., *J. Pharm. Sci.*, **54,** 138(1965).
(2) Berry, H., *J. Pharm. Pharmacol.*, **5,** 1008(1953).
(3) Lachman, L., Weinstein, G., Hopkins, G., Slack, S., Eisman, P., and Cooper, J., *J. Pharm. Sci.*, **51,** 224(1962).
(4) Royce, A., and Sykes, G., *ibid.*, **9,** 814(1957).
(5) Hopkins, G., West Co. Technical Report, No. 8, Appendices I, II, III, IV, 1955–1956–1958.
(6) *Ibid.*, No. 9, 1958.
(7) *Ibid.*, No. 10, 1963.
(8) Wing, T. W., *J. Pharm. Pharmacol.*, **7,** 648(1955).
(9) Weiner, S., *ibid.*, **7,** 118(1955).
(10) Wing, T. W., *ibid.*, **8,** 734(1956).
(11) Milosovich, G., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **45,** 758(1956).
(12) Kaestener, D., *Bull. Parenteral Drug Assoc.*, **10,** 22 (1956).
(13) Lachman, L., Sheth, P. B., and Urbanyi, T., *J. Pharm. Sci.*, **53,** 211(1964).
(14) Lachman, L., Urbanyi, T., and Weinstein, S., *ibid.*, **52,** 244(1963).
(15) ASTM Standards, Part 28, "Rubber; Carbon Black; Gaskets," April 1965, pp. 198–209.
(16) Frevel, L. K., and Kressley, L. J., *Anal. Chem.*, **35,** 1492(1963).
(17) Wing, T. W., *J. Pharm. Pharmacol.*, **8,** 738(1956).
(18) Dogadkin, B. A., and Shevshuev, V. A., *Rubber Chem. Technol.*, **35,** 1(1962).

Instrumented Rotary Tablet Machines I

Design, Construction, and Performance as Pharmaceutical Research and Development Tools

By E. L. KNOECHEL, C. C. SPERRY, H. E. ROSS, and C. J. LINTNER

Two conventional rotary tablet compressing machines, the Stokes models 540-35 and BB2-27, have been instrumented to measure both compressional and ejectional forces. This was accomplished by attaching strain gauges to key components of the presses and displaying the electrical outputs of the strain gauges on a dual-beam oscilloscope fitted with a camera. The compressional and ejectional forces developed during the tableting operation are thus obtained under dynamic conditions. The design, construction, and performance of these instrumented rotary tablet machines (IRTM) are described and illustrated.

ALTHOUGH COMPRESSED tablets have been in use for many years, fundamental research concerning tableting was initiated only little more than a decade ago. Since that time, attempts have been made to isolate and evaluate the effects of many of the variables involved in the complex process of producing a tablet. Some of the most significant information has been obtained with instrumented single punch tablet machines where the compressional and ejectional forces involved in the tableting operation could be accurately detected, recorded, measured and, if necessary, reproduced.

The instrumentation of single punch compressing machines was first described by Brake in 1951 (1) and later by Higuchi, Nelson, and Busse (2), Gagnon (3), Markowski (4), and most recently by Shotton and Ganderton (5).

By attaching strain gauges to the upper punch of a single stroke Colton 4B press,[1] Brake (1) was able to measure and record the compressional force exerted in the tableting process.

Higuchi and co-workers (2) modified a Stokes model A3 tablet machine[2] and attached strain gauges and load cells directly to the press. This instrumentation permitted the simultaneous recording of any two of the following variables as functions of compression time: (*a*) the absolute force exerted by the upper punch; (*b*) the absolute force transmitted to the lower punch; and (*c*) the absolute displacement of the upper punch. They were also able to determine the force of ejection.

Gagnon (3) attached strain gauges to the lower punch of the instrumented press used by Brake. This enabled Gagnon to detect and measure the lower punch compressional force. Markowski (4) further refined this system to measure the ejectional forces as well as upper and lower punch forces.

Shotton and Ganderton (5) attached strain gauges to the upper punch and lower punch holder of "a Lehman single punch eccentric tableting machine, driven at a constant speed through a Kopp Variator." They were able to measure the upper and lower punch compressional forces as well as the ejectional force.

The value of these instrumented single punch tablet machines in investigating fundamental aspects of tableting has been clearly established (1–21). However, single stroke machines are usually used only for research and pilot batches, for small volume production items, or for large size specialty tablets such as boluses. Today, rotary tablet machines are the conventional type of compressing equipment in the pharmaceutical industry. It was felt that methods were needed to measure and record the compressional and ejectional forces involved in the preparation of tablets on high speed rotary presses under production conditions.

This paper discusses the instrumentation of two widely used rotary tablet presses, a Stokes model 540-35 and a modified Stokes model BB2-27.[2] The instrumentation permits the simultaneous detection and recording of (*a*) the compressional forces during the formation of the tablets in their dies and (*b*) the ejectional forces during the removal of the tablets from their dies. This communication describes and illustrates the design, construction, and performance of these machines.

REQUIREMENTS OF DESIGN

In instrumenting rotary tablet machines, two distinct problems were encountered: (*a*) finding the means of detecting the compressional and ejectional forces and (*b*) finding an adequate method of moni-

Received May 16, 1963, from the operating units of Product Research and Development and Mechanical Engineering, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication August 5, 1966.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.
Instrumentation of rotary tablet machines is claimed in U.S. pat. 3,255,716 (June 14, 1966).
The authors acknowledge the technical assistance of Miss Jane Patterson, Mr. C. R. Hine, Mr. D. D. Noble, Mr. M. Flegal, and members of the Tablet Production Unit.

[1] Arthur Colton Co., Detroit, Mich.
[2] F. J. Stokes Corp., Philadelphia, Pa.

toring (*i.e.*, amplifying, displaying, recording, and measuring) the detected forces.

Force Detecting System.—To detect the forces, a system was required that: (*a*) could be readily attached to a conventional high speed rotary tablet press; (*b*) could operate over a wide range of thickness, die fill, overload, and speed settings; (*c*) would not interfere with the normal operations of the press; (*d*) would permit the detection of individual forces for each station; (*e*) would permit the use of any size or shape punches and dies; (*f*) could be readily transferred to other machines of the same type.

Commercially available strain gauges were selected as the force sensors. Because of the simplicity, sensitivity, reproducibility, reliability, versatility, durability, and short recovery time of strain gauges, the first three requirements were fulfilled.

It was obvious, however, that if the punches of each station were to be instrumented in a manner similar to that used to gauge the single punch machines, complex electronic arrangements would be required to "pick up" and transmit the impulses from each set of gauges. If some portion of the press remotely linked to the punches could be gauged to detect the desired forces as they occurred at each station, requirements (*d*) and (*e*) would be achieved. The last requirement would also be fulfilled if the members selected for gauging were conveniently transferable to another machine. Since cast iron and similar materials do not exhibit constant values of the modulus of elasticity[3] and Poisson's ratio,[4] the gauges would have to be attached to steel portions of the press.

Monitoring System.—To monitor the detected electrical impulses, a system was required that: (*a*) could follow at least two simultaneous or exceedingly rapid, consecutive events (*e.g.*, the compression and ejection events); (*b*) could respond to a tablet production rate of at least 2,000 tablets per minute (TPM); (*c*) would follow and record either individual or a consecutive series of compression and/or ejection events; (*d*) could respond to and measure compressional forces ranging from 200 to 10,000 lb.; (*e*) would be sensitive enough to amplify and measure the lower magnitude ejectional force impulses. As the ejectional forces are usually smaller than compressional forces, the electrical output of the gauges would be lower; (*f*) would allow some sort of permanent record to be readily obtained at any time if desired; (*g*) would be versatile, relatively easy to operate, and yet would not interfere with or limit the normal operation of the press; (*h*) would be stable, durable, portable, and self-contained (*i.e.*, not dependent upon a wide variety of associated equipment or procedures, such as photographic development, etc.).

The monitoring system meeting the above requirements consists of a high sensitivity dual-beam oscilloscope, such as a Tektronix model 502 fitted with a Tektronix model C-12 camera.[5] The camera, equipped with a *f*/1.9 lens having a 1 to .9 object-to-image ratio, is fitted with a Polaroid roll film back.

[3] Modulus of elasticity in compression is the constant which expresses the ratio of unit stress to unit deformation for all values of unit stress not exceeding the proportional limit of a material.

[4] Poisson's ratio, also known as the factor of lateral contraction, is the ratio of lateral unit deformation within the elastic limit.

[5] Tektronix, Inc., Beaverton, Oreg.

The electronic traces of two events can be displayed simultaneously on this oscilloscope. Each beam can be adjusted separately over a wide range of vertical sensitivity settings (20 v./cm. to 200 μv./cm.). Because the sweep range selection is large (5 sec./cm. to 1 μsec./cm.), either an individual or a series of impulses can be displayed. The persistency of the image on the screen is controlled by the intensity setting of the beam, and a green filter can be inserted to prolong the image. Impulse heights can be estimated directly from the graticule which is calibrated in 1-cm. divisions with 2-mm. markings at the baselines.

With the camera attached to the oscilloscope, a permanent record can be obtained at any time without interfering with the visual observation of the screen by taking a picture through the beam splitting mirror. The photographic prints, which are obtained in less than 30 sec., reproduce the graticule markings as well as the electronic traces to $^{9}/_{10}$ their original size. Fast speed film permits the capture of very rapidly occurring events or sequences with extremely good resolution. Film which produces high contrast transparencies can also be used.

Portability of the entire monitoring system is obtained by mounting the camera, oscilloscope, batteries, and Wheatstone bridge circuits on a wheeled cart (see Fig. 5).

INSTRUMENT DESIGN AND CONSTRUCTION

This phase of the investigation consisted of selecting the proper press components to be gauged, installing the gauges on the selected components, and confirming that the gauges were responsive to the stresses produced.

The Stokes model 540-35 was the first rotary press to be instrumented in our laboratory.

540-35 Press.—The essential features of the system to detect compressional forces for this press are shown in Fig. 1. The mechanical design of the press overload system is such that oil under preset pressure forces one end of a lever system against an adjustable stop screw. The opposite end of the lever supports the upper compression roll. As tablets are compressed, the force on the punches operates through

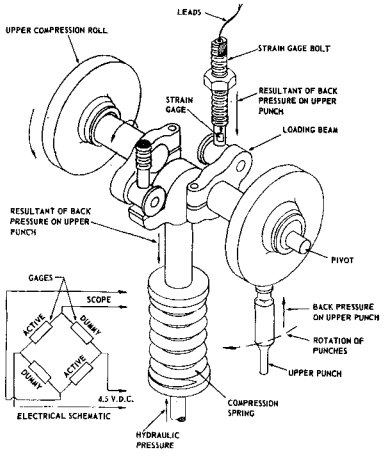

Fig. 1.—Instrumentation of 540-35 press. (Detection of compressional force.)

the upper compression roll and the lever to reduce the force applied to the adjusting screw. To detect compressional forces for this press, semiconductor type strain gauges were attached directly to the lower end of a specially fabricated adjusting screw of the overload system. The gauges, which were attached on opposite sides of the screw, reflect the tablet compressional force as a function of the reduction of force on the screw. The plane passing through the strain gauges must be perpendicular to the axis of the roller on the overload system.

Modifications to the screw included a uniform "necked-down" section for application of the gauges and a hole running axially through the center of the screw to convey the lead wires from the machine. These alterations also increased the sensitivity of the strain gauged area of the screw to stress. The gauges were attached to the screw by the Kulite-Bytrex Corp., Newton, Mass., whose calibration of the screw indicated that an output of 7.5 μv. was generated per input volt per pound of applied force. The geometry of the release mechanism causes a ninefold reduction in detected stress, *i.e.*, 9 lb. of force on the punch reduces the force on the gauged screw by 1 lb. As indicated in the electrical schematic drawing (Fig. 1) for the compressional force instrumentation of the 540-35 press, the input voltage of the Wheatstone bridge circuit is 4.5 v. Thus, with this arrangement an absolute force of 270 lb. applied to the punches will generate a gauge output of 1.0 mv.

When the gauges are connected to the upper beam of the oscilloscope, the force of compression in pounds can be determined for each of the 35 stations as tablet formation occurs. The force of compression is an absolute force at the point of measurement. However, the indicated compressional force values include the friction in the upper punch guide and overload system. This frictional value could not be discerned when the signal on a strain gauged slug in the die cavity and the indicated force on the instrumented machine screw were compared. In this experiment the generation of a known load of 5000 lb. in the die cavity produced an equivalent reading on the instrumented screw.

The instrumentation of the ejection system for this press is shown in Fig. 2. A steel bracket was made to hold the two specially fabricated bolts to which semiconductor strain gauges were attached by the Kulite-Bytrex Corp. This bracket was mounted directly below the ejectional track or cam so that this track was supported .002 in. above the track bed by the strain gauged bolts. The track, although held in position by its original mounting bolts, was free to move vertically. When the track was struck by a lower punch in pushing a tablet from its die, the strain gauged bolts supporting the track detected this event.

Although the gauges were calibrated in terms of millivolts of output per volt of input per pound of applied force (1 mv./4.5 v./25 lb.), the ejectional force determined is a relative measurement of the actual force involved. Because a lever system is involved, an absolute equivalency would be possible only if the punches always struck the ejectional track at the exact same point and accomplished complete ejection when they did. Unfortunately, these conditions cannot be fulfilled because of the following reasons. (*a*) The contact point is changed

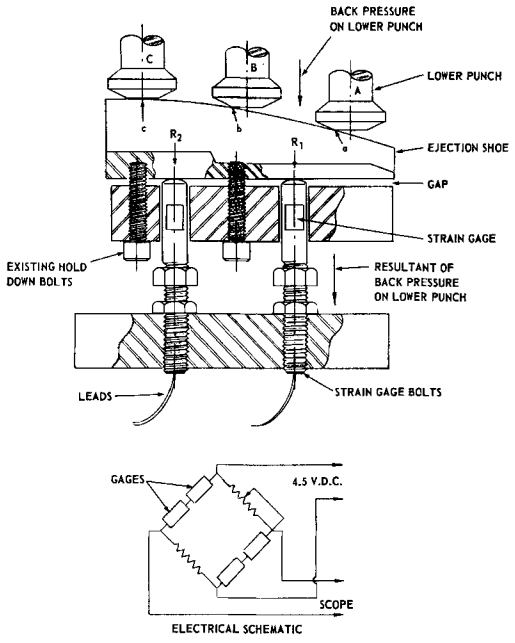

Fig. 2.—Instrumentation of 540-35 press. (Detection of ejectional force.)

when the machine settings, which vary with the formulation being compressed, are altered. (*b*) Random variations in the dimensions of the tools and press components also displace the contact point. (*c*) Complete ejection does not occur instantaneously at the initial point of contact.

When the gauges are connected by a Wheatstone bridge circuit to the lower beam of the oscilloscope (see Fig. 2), an ejectional force impulse will be detected as a single punch "A" strikes the track at point "a." This impulse represents the force of the contact which was applied to the entire track and created a reaction R_1 and R_2 in the two strain gauges. Disregarding the minimal horizontal shear force due to the angle of approach, the sum of R_1 and R_2 equals the applied force of ejection. Therefore, irrespective of the locus of this force on the track, the algebraic sum of the two reactions will always equal the force applied to the track by the punches.

Since two to three punch heads are in contact with the track at any time, the detected electrical impulse is a summation of the forces resulting at various points along the surface of the ejectional track. The literature pertaining to the instrumentation of single stroke presses (2, 4–7, 12, 13, 15) indicates that the force required to propel the tablet from its die is smaller than the force initially required to "break" the tablet loose from the die wall. If this is so, then the force applied by punch "A" as it first contacts the track will be much larger than the forces applied by punches "B" and "C" as they slide across the track. Our studies showed that this is the case. When ejection occurs, the oscilloscopic trace produced shows a sharp initial peak that slowly decreases until the next peak is generated (see Fig. 6).

Since the ejection event occurs somewhat later than the compression event for a given station, the

peak ejectional force impulse is not displayed on the oscilloscope's screen directly below the compressional force impulse for that tablet. Rather, the ejectional impulse is displaced from its compressional impulse by five impulses for the 540-35 press.

One "side" of this 540-35 press was instrumented and thus only the compressional and ejectional forces occurring during this half of the revolution can be monitored. Obviously, the components of the opposite side of the press could be instrumented in a similar manner if desired.

Model BB2-27.—One of the major advantages of the 540 press—its high tablet production rate—unfortunately becomes a distinct drawback in research and development work. Although the press can be slowed to produce as few as 700 TPM, a great deal of tablet stock is still required for a satisfactory run. For this reason a tablet press with a lower tablet output was sought.

A Stokes model 538 press coating tablet machine was converted to a single stage rotary tablet press. The design of this press was originally based on the standard BB2-27 press. By changing the lower cam track design, the converted press performs like one-half of a standard BB2-27 machine. Thus, tablets are made on this press (termed $^1/_2$ BB2-27) in the conventional manner during one-half of the cycle. During the remainder of the cycle following ejection, the punches remain elevated with the lower punches level with the bed surface and the upper punches held in their uppermost position until the rear pull-down tracks are reached. The die filling, compressing, ejecting, and take-off sequence was not altered. Although this press has a capacity of only 375–750 TPM, the operation of this press and a standard Stokes BB2 press are identical. So that speed selections could be standardized, a tachometer was also attached to the press. This machine, then, is a very useful tool for tablet research and development studies and was instrumented in a similar manner to that used for the 540-35 press.[6]

A diagrammatic sketch showing the instrumentation and the electrical schematic outline for the compressional force detecting and monitoring system is shown in Fig. 3. Force applied to the lower punch during tablet formation is transmitted through the lower compression roll and the lever arm to produce a strain on the eyebolt of the pressure regulatory mechanism. The first step to instrument this press was to detach the entire lower, threaded portion of this cast iron eyebolt and to replace it with a specially fabricated steel section. This new part was threaded at the lower end as usual to accept the adjusting wheel and reduced in cross-sectional area at the upper end to increase its sensitivity to stress. Metal foil type strain gauges attached to this "necked-down" area measure the compressional force as tablet formation occurs. Our calibration of the gauges and our calculation of the mechanical advantage indicated that a force of 3,000 lb. on the

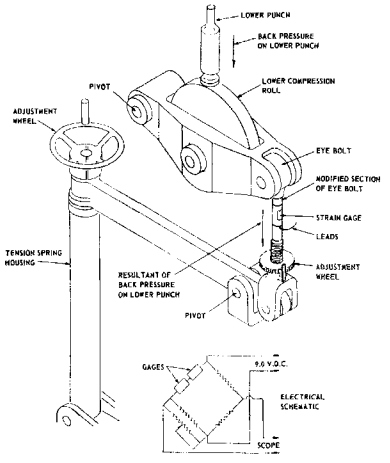

Fig. 3.—Instrumentation of BB2-27 press. (Detection of compressional force.)

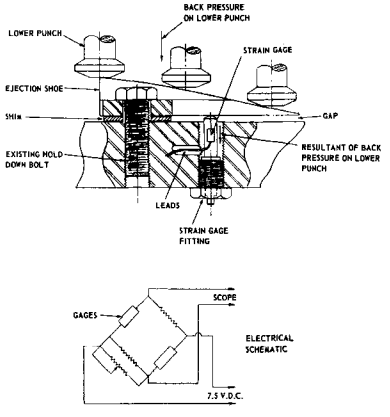

Fig. 4.—Instrumentation of BB2-27 press. (Detection of ejectional force.)

lower punch produced a 1 mv. output by the gauges with 9 v. applied to the Wheatstone bridge circuit.

The installation of detectors to measure the ejectional force for the $^1/_2$ BB2-27 press was similar to that of the 540-35 press. Figure 4 shows this instrumentation and the electrical schematic diagrams for the monitoring system. The standard ejection track was shimmed to .002 in. above the track bed and the existing hold-down screw was used to secure it at the rear end. A bolt to which metal foil strain gauges were attached was positioned to support the front half of the ejectional track. Thus, the force generated when a punch hits the track while ejecting a tablet is transmitted to the bolt. Our calibrations indicated that, at a point directly above the gauged bolt, a force of 250 lb. was required to generate a gauge output of 1 mv. when 7.5 v. were applied to the Wheatstone bridge circuit. At a point near the leading edge of the track, the same force generates twice this electrical output, or 2 mv. However, because two to three punches are always in contact with the track at any given time, the electrical display is a composite of the forces being produced by these punches. Thus, as with the

[6] Subsequent to our developments, the authors found that Brake (1) had briefly explored the partial instrumentation of a Stokes B2 rotary tablet press. Although Brake's instrumentation of the single stroke machine has been mentioned in the literature, to our knowledge no previous reference has been made to his work concerning the measurement of compressional forces on a rotary tablet press. Another investigator working with a B2 press (22) claimed that "during the tableting process the pressures exerted on the punches were measured and increased twice." Unfortunately, just how this was accomplished was not explained. The forces measured ranged from only about 1500 to 4000 lb.

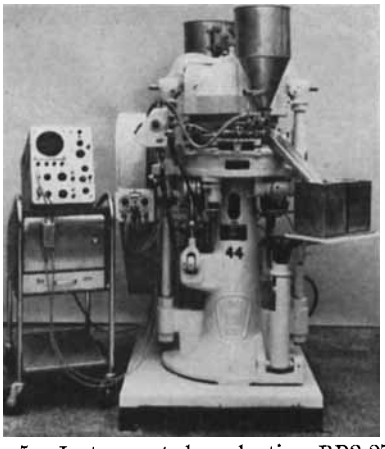

Fig. 5.—Instrumented production BB2-27 rotary tablet machine showing oscilloscope, control panel, and connections to strain gauges.

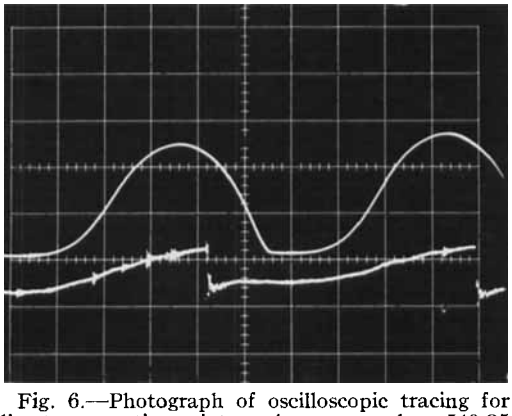

Fig. 6.—Photograph of oscilloscopic tracing for direct compaction mixture *A* compressed on 540-35 IRTM with Force-Flo-Feeder operating at 1000 TPM using 35-$^{7}/_{16}$ in. full oval punches. Calibration: upper trace (C.F.), one large division = 2700 lb.; lower trace (E.F.), one large division = 25 lb.; sweep, 20 msec./large division; left to right.

540-35 IRTM, only a relative measure of the ejectional force is obtained with this system.

Again, the dual-beam oscilloscope was used to amplify and display the generated impulses, and the camera was used to record the oscilloscopic displays for the studies. With this press the ejectional force impulse trailed the compressional force impulse by three impulses.

Both "sides" of two standard production BB2-27 presses have been instrumented in the described manner to measure compressional and ejectional forces. These IRTM's are in routine production use today (see Fig. 5). Furthermore, the eyebolts and ejectional tracks of a Manesty Bicota[7] have been instrumented in a manner similar to that shown in Figs. 3 and 4.

TYPE AND NATURE OF DATA OBTAINABLE

Figure 6 is a photograph of the compressional and ejectional force traces produced during tableting of a direct compaction mixture, *A*, containing three active ingredients with the 540-35 IRTM. The upper trace records the compressional force impulses and the lower trace records the ejectional force impulses. The latter is inverted, *i.e.*, its magnitude increases in a downward direction. For this photograph the horizontal sweep of the beams was increased so that only two consecutive compression and ejection events are shown. The compressional force for the first tablet (impulse to the left side of the photograph) is about 6480 lb. For the second tablet it is 7020 lb. For the first ejectional event displayed, the initial peak height represents a force of about 27 lb., while a force of about 32 lb. is registered for the ejection of the second tablet. As explained, the ejection events shown do not correspond to the compressional curves depicted.

By decreasing the horizontal sweep and making a time exposure, the photograph shown in Fig. 7 was obtained. In this run, however, the upper punches were removed from stations 31 and 33, accounting for the "blanks" in both tracings. It can be observed that an ejectional force impulse is displaced to the right by five impulses from its compressional force counterpart. The usual variation in both the compressional and ejectional force impulse heights was accentuated because the experimental direct compaction mixture *A* being compressed did not flow well. The uniform compressional pulse heights at the right side of the picture occurred because the press "unloaded" as the tablets were compressed, *i.e.*, the development of excessive pressures was automatically prevented by the hydraulic-mechanical overload system of the press. When this occurs, there is a plateau in the individual compressional force curve since additional force cannot be applied to the tablet or punches.

The oscilloscopic displays obtained from the $^{1}/_{2}$ BB2-27 IRTM are similar to those shown for the 540-35 IRTM. An example is shown in Fig. 8 where the horizontal sweep has been increased on the left half to show the characteristics of individual impulses and then decreased on the right half to

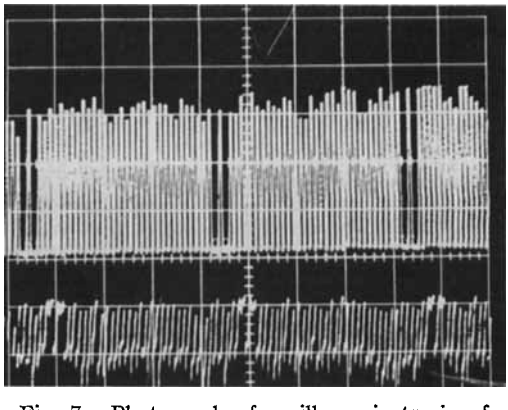

Fig. 7.—Photograph of oscilloscopic tracing for direct compaction mixture *A* compressed on 540-35 IRTM with Force-Flo-Feeder operating at about 1000 TPM using 35-$^{7}/_{16}$ in. full oval punches (upper punches removed from stations 31 and 33). Calibration: upper trace (C.F.), one large division = 2700 lb.; lower trace (E.F.), one large division = 25 lb.; sweep, 1 sec./large division; left to right.

[7] Manesty Machines Ltd., Liverpool, England.

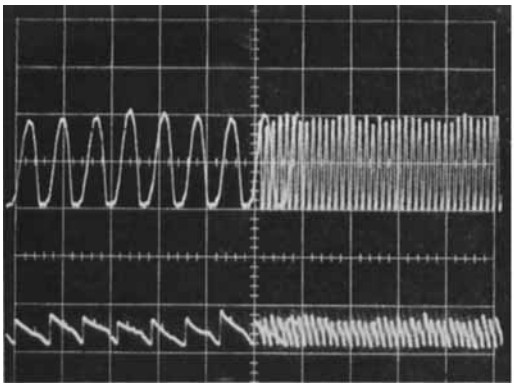

Fig. 8.—Photograph of oscilloscopic tracing for direct compaction mixture *B* compressed on BB2-27 IRTM with Force-Flo-Feeder operating at a speed of about 430 TPM using 27-$^{7}/_{16}$ in. full oval punches. Calibration: upper trace (C.F.), one large division = 3080 lb.; lower trace (E.F.), one large division = 125 lb.; left sweep, 0.2 sec./large division; right sweep, 1 sec./large division; left to right.

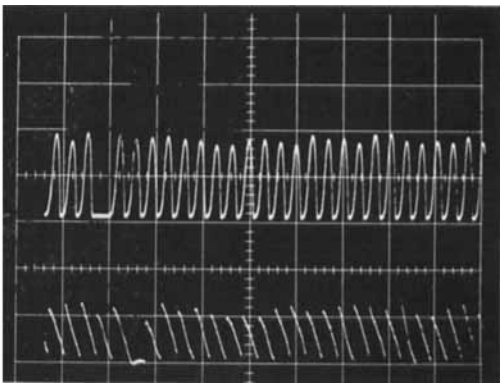

Fig. 10.—Photograph of oscilloscopic tracing of conventional granulation D compressed on BB2-27 IRTM operating at about 400 TPM using 26-$^{7}/_{16}$ in. full oval punches (upper punch removed from station 1). Calibration[8]: upper trace (C.F.), one large division = 2360 lb.; lower trace (E.F.), one large division = 83 lb.; sweep, 0.5 sec./large division; left to right.

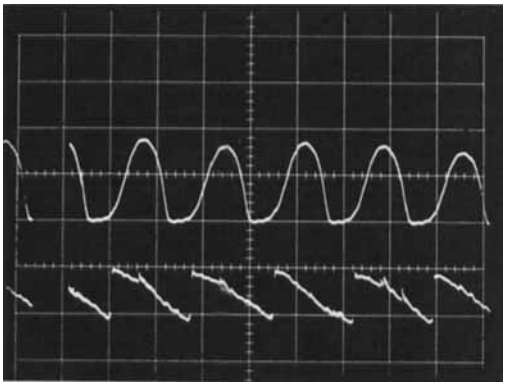

Fig. 9.—Photograph of oscilloscopic tracing for conventional granulation D compressed on a BB2-27 IRTM operating at about 400 TPM using 27-$^{7}/_{16}$ in. full oval punches. Calibration[8]: upper trace (C.F.), one large division = 2360 lb.; lower trace (E.F.), one large division = 83 lb.; sweep, 0.1 sec./large division; left to right.

"squeeze" the series of impulses together. These compressional force impulses are quite uniform. One explanation is that this particular direct compaction mixture *B*, containing the same three active ingredients as *A*, flowed extremely well. The ejectional force tracings are not inverted in Figs. 8, 9, and 10; hence, the magnitude of the force increases in an upward direction.

Figures 9 and 10 are photographs obtained while a conventional granulation D containing the same three active ingredients was being compressed on the $^{1}/_{2}$ BB2-27 IRTM.[8] Figure 9 shows the individual impulses, while Fig. 10, obtained by decreasing the sweep and removing the upper punch from one station, shows the displacement of the ejectional force impulse to the right of the corresponding compressional force impulse by three impulses. There was more compressional force uniformity for this granulation than there was for the poorly flowing direct compaction mixture *A* shown in Fig. 7. However, the good flowing direct compaction mixture *B* pictured in Fig. 8 showed the most nearly uniform compressional force impulse heights of the three.

Various methods can be used to measure the individual heights for a series of impulses. Photographic prints or transparencies can be projected onto a calibrated grid or measured directly by a specially scaled transparent ruler. The mean of a series of impulse heights can be determined and used to calculate the force involved in the preparation of that sample of tablets. For example, in Fig. 8 the mean pulse height for the left side of the picture was found to be 1.71 cm. Since 0.9 cm. is equivalent to 3080 lb., the average compressional force generated during the compression of these tablets is 1.71 × 3080 ÷ 0.9 or about 5850 lb. The average ejectional force calculated in a similar manner is 0.52 × 125 ÷ 0.9 or about 72 lb. Compressional and ejectional force data can be obtained in this manner to define or describe an individual tableting run. All force measurements can be converted into pressure values by simple calculation involving the area of the punch faces. It is important to remember that the compressional force measurements are in terms of absolute force at the point of measurements and include any slight friction components of the upper punch guide and overload systems. The ejectional force measurements are relative values with respect to the machine, the machine settings, and the tools. The ejectional force is related to the stock, the size and shape of the punches, the depth of compression, and the size or weight of the tablets being produced.

The material presented describes the type and nature of data that can be obtained with instrumented rotary tablet machines. Some of the ways that

[8] The calibrations differ between Figs. 9 and 10 and Fig. 8 because deliberate alterations to the circuitry and cross-sectional area were made to increase the sensitivity of the system. Presently, by adjusting the circuitry, the sensitivity has been further increased for the BB2-27 IRTM.

such data have been used to study a variety of tableting problems will be presented in part II of this series (26).

SUMMARY

The proposed design requirements to measure and monitor compressional and ejectional forces developed on rotary tablet machines have been fulfilled by the two instrumented presses described here. The forces are detected by strain gauges attached to strategic components of the press remotely located from the sites of action. The detected forces are monitored on a dual-beam oscilloscope connected through Wheatstone bridge circuits to the individual compressional and ejectional force sensing strain gauges. Diagrams are included to show the strain gauging and electrical circuits. Permanent records are obtained by taking pictures of the transmitted displays with a Polaroid-backed oscilloscope camera. Photographs are used to illustrate the type and nature of compressional and ejectional force data obtainable from the instrumented presses.

Thus, two instrumented rotary tablet machines, the Stokes models 540-35 and 1/2 BB2-27, are now available for research and development projects. Other tablet machines, similarly equipped, have been used to monitor routine tablet production runs.

ADDENDUM

Since the time this paper was prepared and presented at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION in May 1963, additional studies on the instrumentation of rotary tablet machines have been reported.

Shotton and co-workers (23) bonded strain gauges to the shanks of one upper and one lower tablet punch and used radio telemetry to transmit the compressional force to the recorder. The press was a Manesty D-3, 16 punch, rotary tablet machine in which all of the tablet punches had been removed except for a single punch and die set. The machine had to be hand turned.

Fuhrer (24) discussed the pressure distribution in tableting with a study on compression curves in rotary tableting presses.

Recently, Wray *et al.* (25) reported on an instrumented Stokes BB2 rotary tablet machine similar to that described in this paper. These workers were able to monitor simultaneously the upper and lower compressional forces from both a set of instrumented punches and instrumented upper and lower compression rolls and ejection forces from an instrumented lower punch and a redesigned ejection cam.

The rotary tablet machine instrumented by Shotton is a research tool incapable of operating at high speeds. The instrumented rotary tablet machines described in this paper and the one subsequently modified by Wray may be utilized to study typical tablet development and production problems under dynamic conditions.

REFERENCES

(1) Brake, E. F., "Development of Methods for Measuring Pressures During Tablet Manufacture," M.S. Thesis, Purdue University, West Lafayette, Ind., 1951.

(2) Higuchi, T., Nelson, E., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 344(1954).

(3) Gagnon, L. P., "A Correlation of Physical Aspects in Tablet Production," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1957.

(4) Markowski, S. L., "The Evaluation of Tablet Lubricants Using a Conventional Type Tablet Machine Instrumented with Strain Equipment," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1958.

(5) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **12**, Suppl. 87T(1960).

(6) Nelson, E., "Measurement of Force and Displacement Variable in the Tablet Compression Process," Ph.D. Dissertation, University of Wisconsin, Madison, Wis., 1954.

(7) Nelson, E., Naqvi, S. M., Busse, L. W., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 596(1954).

(8) Higuchi, T., Elowe, L. N., and Busse, L. W., *ibid.*, **43**, 685(1954).

(9) Elowe, L. N., Higuchi, T., and Busse, L. W., *ibid.*, **43**, 718(1954).

(10) Swintosky, J. V., Kennon, L., and Tingstad, J., *ibid.*, **44**, 112(1955).

(11) Nelson, E., Busse, L. W., and Higuchi, T., *ibid.*, **44**, 223(1955).

(12) Raff, A. M., Arambulo, A. S., Perkins, A. J., and Deardorff, D. L., *ibid.*, **44**, 290(1955).

(13) Nelson, E., *ibid.*, **44**, 494(1955).

(14) Strickland, W. A., Jr., "A Study of the Fundamental Aspects of Tablet Lubrication and the Quantitative Evaluation of Lubricants," Ph.D. Dissertation, University of Wisconsin, Madison, Wis., 1955.

(15) Strickland, W. A., Jr., Nelson, E., Busse, L. W., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 51(1956).

(16) Nelson, E., Arndt, J. R., and Busse, L. W., *ibid.*, **46**, 257(1957).

(17) Strickland, W. A., Jr., Higuchi, T., and Busse, L. W., *ibid.*, **49**, 35(1960).

(18) Daoust, R. G., "A Study of the Combined Effects of Binders, Lubricants, and Lubricant Conventions in Tablet Making," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1960.

(19) Munden, B. J., DeKay, H. G., and Banker, G. S., *Drug Std.*, **28**, 12(1960).

(20) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **12**, Suppl. 93T(1960).

(21) *Ibid.*, **13**, 145T(1961).

(22) Filleborn, V. M., *Am. J. Pharm.*, **120**, 233(1948).

(23) Shotton, E., Deer, J. J., and Ganderton, D., *J. Pharm. Pharmacol.*, **15**, Suppl. 106T(1963).

(24) Fuhrer, C., *Pharm. Ind.*, **25**, 674(1963).

(25) Wray, P. E., Vincent, J. G., Moller, J. W., and Jackson, G. J., "Rotary Tablet Machine Instrumentation," presented to the Industrial Pharmacy Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

(26) Knoechel, E. L., Sperry, C. C., and Lintner, C. J., *J. Pharm. Sci.*, **56**, 116(1967).

Instrumented Rotary Tablet Machines II

Evaluation and Typical Applications in Pharmaceutical Research, Development, and Production Studies

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The instrumented rotary tablet machines (IRTM's) previously described have been used to study many phases of the tableting process. The performance of rotary tablet machines with respect to compressional force generated at various settings of the machine controls and to the tablets produced was analyzed and graphically described. Additional studies established the specific relationships between the dimensions and physical properties of the tablets and the compressional forces used to prepare them for a number of different tableting mixtures. These findings are compared with similar relationships obtained by others who used instrumented single punch tablet presses, specialized "static" compression methods, or empirical methods of measuring compressional force. In most cases the results obtained for rotary machines were similar to those previously reported, but in a few instances contradictory behaviors were observed. It was concluded that the excessive amount of punch-to-punch and cycle-to-cycle variation in compressional and ejectional forces that was unexpectedly found to exist for rotary tablet machines is primarily due to unequal filling of the individual dies with stock. Some applications where IRTM's were used to investigate typical tablet development and production problems are described.

THE INSTRUMENTATION of conventional rotary tablet machines so that compressional and ejectional forces can be determined under dynamic, production-type conditions has been described in the first paper of this series (1). Along with the details of their design and construction, the type and the nature of the data which can be obtained from instrumented rotary tablet machines (IRTM's) were also presented.

In the present report a series of experiments evaluated the performance of rotary tablet machines with respect to the tablets and the compressional force levels produced over the entire range of the various machine settings.

Following these investigations, another series of experiments examined the relationships between compressional force and the physical properties of the tablets produced from a variety of different tableting mixtures. These findings were compared to those previously found by others working with instrumented single stroke tablet machines (2–18), static compressional devices (19–24), empirical methods of measuring "compression" (25–29), and rotary presses (2, 30, 31).

When the basic performance of the rotary presses had been established, the IRTM's were used to study some typical tablet development and production problems.

Data from specific tableting runs as well as general observations from the studies are presented to describe the various ways that the IRTM's were used.

EXPERIMENTAL

Materials.—Since the main purpose of this paper is to describe the value of the IRTM, the details of the formulations and their preparation are being omitted. However, a variety of experimental and standard production granulations as well as a number of experimental direct compaction mixtures were studied. All formulations contained therapeutic concentrations of physiologically active ingredients. As a result, data on the compressional characteristics of actual production-type materials were collected.

Methods of Measurement and Equipment.—(*a*) Hardness values are the average for 5 to 20 tablets determined on a Strong-Cobb hardness tester[1] operated from a compressed air line.

(*b*) Fracture resistance values are also the average for 5 to 20 tablets determined by using the modified anvil and plunger for the Strong-Cobb hardness tester previously described by Endicott *et al.* (32).

(*c*) Friability values are the weight loss in per cent determined after 20 tablets were rotated in the Roche Friabilator for 4 min. at 25 r.p.m. (33).

(*d*) Disintegration times, determined by U.S.P. procedure, apparatus, and official plastic disks, are reported either as the average time or the total time required for six tablets to disintegrate. In some cases the disks were not used. Generally, the fluid used was 750 ml. of deionized water at ~37°; when a different fluid was used, this is indicated.

(*e*) Dissolutive rates, expressed in terms of the

Received May 16, 1963, from the operating units of Product Research and Development and Mechanical Engineering, The Upjohn Co., Kalamazoo, Mich.

Accepted for publication September 8, 1966.

Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

Instrumentation of rotary tablet machines is claimed in U. S. pat. 3,255,716 (June 14, 1966).

The authors acknowledge the technical assistance of D. D. Noble, Jane Patterson, and C. Hine, and the members of the Tablet Production Unit and Statistical Section of the Scientific Administration Staff during the course of these studies.

Previous paper: Knoechel, E. L., Sperry, C. C., Ross, H. E., and Lintner, C. J., *J. Pharm. Sci.*, **56**, 109(1967).

[1] Strong-Cobb & Co., Inc., Cleveland, Ohio.

time required for a given percentage of the drug(s) to be dissolved (*e.g.*, $T_{50\%}$) from the tablet, were determined either by using the procedures and equipment described previously by Schroeter *et al.* (34) or by using the "automated dissolution rate apparatus" described by Schroeter and Wagner (35).

(*f*) Mean tablet weights were obtained by weighing 90 to 100 tablets on torsion balances and performing the appropriate calculations. Individual tablet weights were determined to the nearest 0.1 mg. on a Mettler Gramatic balance.[2]

(*g*) Mean tablet thicknesses were obtained either by measuring the total length (to the nearest 0.01 in.) of a column of 60 to 100 tablets stacked face to face in a V-shaped trough (one side of which was a 2 ft. steel ruler) and making appropriate calculations or by measuring individual tablets with dial micrometers[3,4] (estimated to the nearest 0.0005 or 0.0001 in., respectively) and averaging the values obtained.

(*h*) Compression ratios (CR), a measure of a tablet's apparent density, were calculated either by dividing the weight of the individual tablet by its own thickness or by dividing the mean weight by the mean thickness for a tablet sample and expressing the results in "mg./in." (27, 29). The term compression ratio is used here because of its historical significance and widespread usage. To convert to mg./mm., divide the compression ratio in mg./in. by 25.4.

(*i*) Compressional and ejectional force values were obtained from the Stokes 540-35 IRTM, the Stokes 1/2 BB2-27 IRTM, or the instrumented ejectional track of the Manesty Bicota machine and the associated electronic and photographic equipment described previously (1). Mean values were determined by averaging 50 to 130 individual impulse heights (estimated to the nearest millimeter) from the photograph obtained for the determination and making appropriate calculations as described previously (1).

(*j*) Settings for the press controls were designated as follows.

Speed Settings.—These were determined either by reading the press tachometers (calibrated in tablets/min. (TPM) for the 540-35 and 1/2 BB2-27 presses) or by measuring the r.p.m. of the head and converting this to TPM for the conventional BB2-27 press.

For a 540-35 press the rate ranges from 700 to ~2400 TPM (2 tablets/station/revolution). For the 1/2 BB2-27 press the rate ranges from ~350 to 700 TPM (1 tablet/station/revolution). Since the conventional BB2-27 press produces two tablets/station/revolution, it has twice the rate (700 to 1400 TPM) of the 1/2 BB2-27 press.

Weight Cam Settings.—These were expressed in terms of "notches of weight added." For both the 540-35 and the BB2-27 presses one complete rotation of this control equals 40 notches.

Thickness Settings.—These were expressed in terms of revolutions of the appropriate control for the 540-35 press on which pointers and specially calibrated dials were installed. For the BB2-27 presses the settings were in terms of "notches;" these were converted to degrees of rotation by the factor, 7 notches = 45°, or 56 notches = one complete revolution.

(*k*) Punches and dies were regular production tools. However, the length of each punch was measured with a platform dial micrometer[5] and the diameter of each punch and die was determined by using the Sheffield Precisionaire Gage.[6] Each individual tool was labeled and its dimensions recorded. The tools were then arranged so that upper and lower punches and dies with uniform clearances were obtained for each station. These were installed so that the total length of upper and lower punches first decreased from and then increased back to the total length of the punches in the first station.

Procedures.—*Evaluation of Rotary Tablet Machine Performance.*—For these studies the general procedure for conducting the runs was as follows.

All control settings were made, checked, and "locked-in." Hopper(s) were filled to a preselected level. The press was run at the desired settings for at least 2 min. before tablets were collected or photographs taken. Then at least 125 tablets were collected (only from the instrumented side of the 540-35 IRTM) while the time exposure was being made of the oscilloscopic tracings. These tablets were bottled and labeled and the photograph identified. Then the press control being evaluated was readjusted to the next setting—with no changes being made to the others—and the entire procedure was repeated. For the "speed" control study the upper and lower limits were those of the press itself. For the weight cam and thickness control studies the upper limit occurred when the press "unloaded" because it had reached its mechanical capacity, while the lower limit was reached when tablets became too fragile to handle.

After the collections were finished for all the runs, the various measurements on the tablets and photographs were performed.

Establishment of Relationships Between Physical Properties of Tablets and Compressional Force.—Essentially the same procedure as described above was used to carry out these studies. Only the thickness control setting was adjusted to produce the compressional force changes. Except as noted, all tablet measurements were made within a short time after their compression.

Applications for Tablet Development and Production Studies.—The various procedures used for these studies will not be described in detail in this section since they will be evident when the results of the individual studies are discussed.

RESULTS AND DISCUSSION

Evaluation of the Performance of Rotary Tablet Machines.—These studies, designed to quantify the general rules of thumb that tablet men have long used in working with rotary presses, established the relationship between specific settings of the speed, weight cam, and thickness controls of the presses and of the compressional force generated.

Figures 1 and 2 typify the photographs obtained

[2] Mettler Instrument Corp., Princeton, N. J.
[3] Federal model 22P-30, Federal Product Corp., Providence, R. I.
[4] Starrett model 1010-E, L. S. Starrett Co., Athol, Mass.
[5] Federal Platform Dial Micrometer graduated in 0.0001 in., Federal Products Corp., Providence, R. I.
[6] Sheffield Products, Dayton, Ohio.

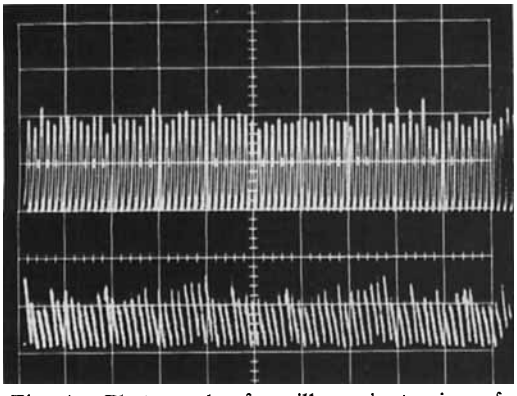

Fig. 1.—Photograph of oscilloscopic tracing of conventional granulation E compressed on 1/2 BB2-27 IRTM at 420 TPM using 27-7/16 in. full oval tablet punches. Calibration: upper trace (C.F.), one large division = 1,540 lb.; lower trace (E.F.), one large division = 125 lb.; sweep, 1 sec./large division, left to right.

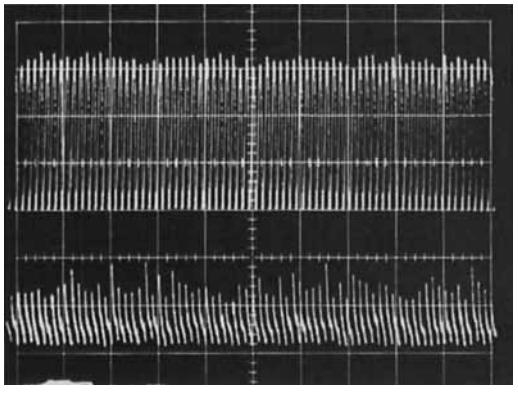

Fig. 2.—Photograph of oscilloscopic tracing of conventional granulation E compressed on 1/2 BB2-27 IRTM at 420 TPM using 27-7/16 in. full oval punches. Calibration: upper trace (C.F.), one large division = 3080 lb.; lower trace (E.F.), one large division = 125 lb.; sweep, 1 sec./large division, left to right.

for this series; here a conventional granulation E was compressed on the 1/2 BB2-27 IRTM at two thickness settings that differed by 1 rev. of the control. The compressional and ejectional force values and the dimensions of the tablets from the samples collected as the two time exposures were being made are listed below.

	Fig. 1[7]	Fig. 2[8]
Thickness setting, °	360	720
Thickness, mean, in.	0.2388	0.2338
Weight, mean, mg.	516.5	515.0
Compression ratio, mg./in.	2163	2203
Compressional force, lb.		
—mean	2910	9730
range	2465–3540	9240–10160
Ejectional force, lb.		
—mean	141	170
—range	100–200	125–238

[7] See run 1 in Table IV.
[8] See run 9 in Table IV.

In this manner the effects of various press adjustments were determined for this granulation and other tablet formulations. It was found that these effects are related mainly to the mechanical aspects of the presses and not necessarily to the composition of the formulations.

Speed Control.—The data listed in Table I were obtained when only the speed control setting of an IRTM was adjusted (by 200 TPM increments) during the compression of a previously slugged and granulated conventional granulation F. Figure 3 shows plots of the press speed *versus* (*a*) the mean tablet weight and (*b*) the mean compressional force developed. As the speed increased, the die fill decreased, the tablet weight diminished, and the compressional force being generated decreased. The apparent density and thickness of the tablets also decreased as the press speed increased. In fact, for this run the decrease in thickness was directly proportional to the decrease in weight. Furthermore, the relationship between tablet weight and compressional force approaches linearity.

In another study the weight of tablets produced and the compressional forces generated were determined for each setting when the press speed was increased by increments. Appropriate adjustments were then made to the weight cam control to obtain tablets of the initial weight at that particular speed. In all cases the compressional force being generated returned to the initial level when tablets with the proper weight were made. It should be pointed out, however, that although the compressional force levels remained constant, the dwell time (*i.e.*, the actual time that the tablet stock was subjected to the compressional force) became increasingly shorter as the press speed increased.

Weight Cam Control.—The data in Table II were obtained when the weight cam control of an IRTM was adjusted by "4 notch" increments to produce heavier tablets from conventional granulation D. In Fig. 4 it will be observed that as the fill weight cam setting was advanced, the actual weight of the tablet produced increased linearly with the setting. Figure 4 also shows that as the weight cam setting was adjusted the compressional force increased until the capacity of the press was reached (~4

TABLE I.—INFORMATION PERTAINING TO TABLETS PRODUCED WHEN SETTING OF SPEED CONTROL WAS ADJUSTED[a]

Run No., Order	Speed, TPM[b]	Wt., mg.	Thickness, in.	Compression Ratio, mg./in.	Compressional Force, lb.
1	700	595.4	.2536	2347	4555
2	900	589.7	.2531	2329	4350
3	1100	589.0	.2526	2331	4015
4	1300	577.5	.2510	2300	3570
5	1500	562.6	.2476	2272	2580
6	1700	557.0	.2464	2260	2555
7	1900	552.5	.2461	2245	2390
8	2100	535.2	.2434	2198	1770
9	2300	521.6	.2414	2160	1490
10	2400	521.2	.2420	2153	1555
11	700	597.4	2538	2353	4660

[a] Controls: constant settings for weight cam and thickness adjustments. Material: previously granulated and slugged conventional granulation F. Punches: 7/16 in. full oval. Machine: 540-35 IRTM. [b] TPM = tablets/min. On this press 70 tablets are made per revolution.

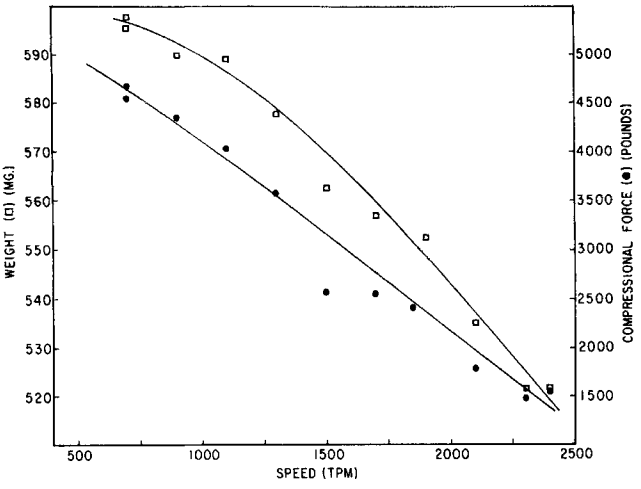

Fig. 3.—Speed *versus* weight (mean) (□) and compressional force (mean) (●).

TABLE II.—INFORMATION PERTAINING TO TABLETS PRODUCED WHEN SETTING OF WEIGHT CAM CONTROL WAS ADJUSTED[a]

Run No., Order	Wt. Cam, Total Notches[b]	Wt., mg.	Thickness, in.	Compression Ratio, mg./in.	Compressional Force, lb.
1	0	448.7	.2224	2018	825
2	4	462.2	.2248	2056	1040
3	8	473.7	.2260	2096	1225
4	12	484.8	.2272	2134	1530
5	16	496.2	.2290	2167	1900
6	20	507.7	.2306	2202	2410
7	24	519.1	.2324	2234	2895
8	28	530.3	.2349	2258	3385
9	32	541.2	.2373	2281	NA
10 (repeat)	32	540.6	.2373	2278	4270
11	36	552.1	.2399	2301	5000
12	40	563.7	.2427	2323	5780
13	44	576.8	.2464	2341	6840
14	48	589.4	.2502	2356	7540
15[c]	52	601.9	.2536	2373	8160
17[d]	54	608.1	.2555	2380	8235
16[d]	56	615.2	.2572	2392	8235

[a] Controls: constant settings for thickness and speed (~700 TPM) adjustments. Material: conventional granulation D. Punches: 7/16 in. full oval. Machine: 540-35 IRTM. [b] One revolution of weight control knob = 40 notches. [c] At this setting many stations "unloaded." [d] At these settings all stations "unloaded."

tons). Less than 1.5 rev. of the weight cam control produced the entire range of fill weights (448.7 to 615.2 mg.) and compressional forces (825 to 8,235 lb.). As the weight of the tablets increased, their thickness and apparent density also increased, even though the thickness control setting was not altered during the run.

Furthermore, it can be seen that a significant portion of the curve which would be obtained with a plot of weight *versus* compressional force would be linear. This points out that the compressional force generated is apparently dependent upon the weight of the tablets or the die fill. Brake (2) and Rehberg (31) anticipated this possibility for rotary presses, and similar relationships were reported by others (8, 10, 11, 15) in their work with instrumented single stroke presses.

Thickness Control.—Regulation of the thickness control to bring the upper and lower punches closer together or farther apart at the instant of compression is the usual way that a press is adjusted to control the properties of the tablets to be made once the desired fill weight is obtained. The press speed is usually optimized and is not ordinarily used to control the properties of the tablets. The data in Table III were collected when tablets were compressed at different thickness settings from an experimental granulation I on an IRTM. The two plots in Fig. 5 show the relationships between thickness control setting and (*a*) the thickness of the tablets produced and (*b*) the compressional force generated. Adjusting the thickness control to "apply more pressure"

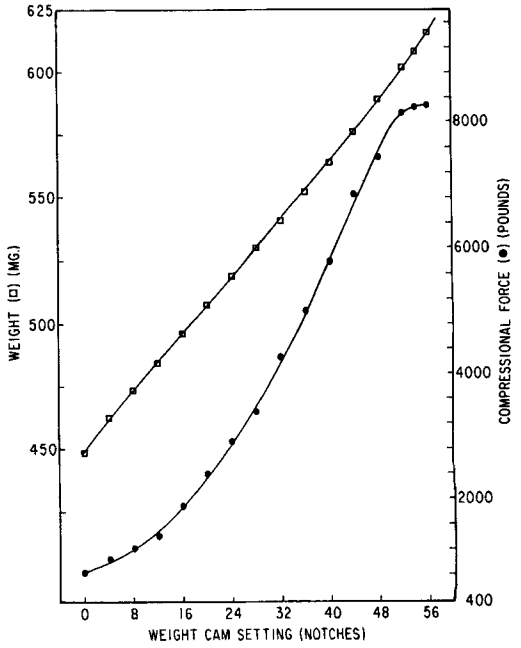

Fig. 4.—Weight cam setting *versus* weight (mean) (□) and compressional force (mean) (●).

resulted in thinner tablets being compressed at higher force levels.

The entire range of compressional forces was attained with less than 2 rev. of the thickness control. The compressional force did not change uniformly throughout the range of the thickness settings. The last 160° of rotation to the control produced as much a change in force as did the first 440°. Thus, adding a notch of "pressure" to the thickness control is not equivalent to adding a constant increment of force to the compression.

Another important observation is that the thickness decreased rapidly at first, and then leveled to a constant value, even though the pressure applied to the mass continued to increase. Since the weight of the tablets remained relatively constant during the run, the apparent density of the tablets thus increased to an asymptotic value as the thickness control was continually adjusted to produce thinner tablets. The significance of this phenomenon will be discussed later.

On the basis of these and other experiments, relationships for the press settings can be generally summarized as follows.

Increasing the press speed causes the die fill to decrease, a factor which causes the tablet weight and compressional force being generated to decrease. The tablets, therefore, become successively thinner and less dense and are ejected with a decreasing amount of force.

Adjusting the weight cam control setting to increase the die fill results in successively higher compressional and ejectional forces being generated while thicker and denser tablets are also produced. In support of these findings, Nelson *et al.* (5) showed that for a single stroke press the ejectional force

TABLE III.—INFORMATION PERTAINING TO TABLETS PRODUCED WHEN SETTING OF THICKNESS CONTROL WAS ADJUSTED[a]

Run No., Order	Thickness Setting, °[b]	Wt., mg.	Thickness, in.	Compression Ratio, mg./in.	Compressional Force, lb.
1	360	579.7	.1930	3004	3455
2	320	583.7	.1946	2999	3080
3	280	580.0	.1955	2967	2525
4	240	579.1	.1970	2940	1935
5	200	579.0	.1991	2908	1635
6	160	581.8	.2018	2883	1455
7	120	580.6	.2043	2842	1210
8	80	583.6	.2073	2815	1120
9	40	582.1	.2100	2772	1025
10	0	580.8	.2131	2725	780
11 (repeat)	360	580.9	.1935	3002	3605
12	400	582.7	.1935	3011	4225
13	440	582.8	.1932	3017	4835
14	480	580.0	.1924	3015	5590
15	500	581.1	.1922	3023	6050
16	520	578.8	.1917	3019	6655
17	540	580.0	.1924	3015	6860
18	560	582.3	.1926	3023	7265
19	580	584.5	.1929	3030	8075
20	590	584.2	.1930	3027	8210
21	600	584.5	.1929	3030	8465
22 (repeat)	360	576.5	.1930	2987	3185

[a] Controls: constant settings for weight cam and speed (~2000 TPM) adjustments. Material: experimental granulation I. Punches: 15/32 in. quarter oval. Machine: 540-35 IRTM. [b] At 0° punches were farthest apart at time of impact; at 600° they were nearest to each other.

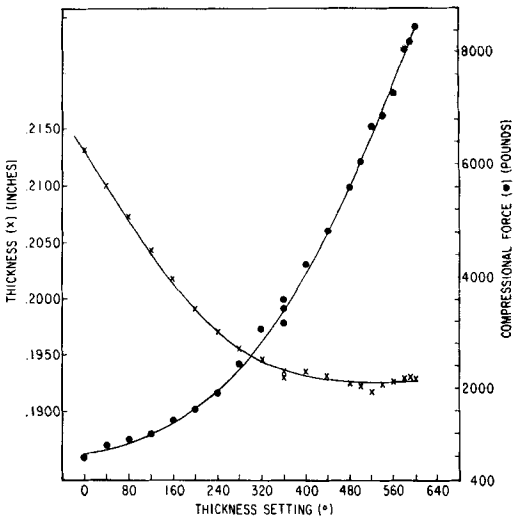

Fig. 5.—Thickness setting *versus* thickness (mean) (×) and compressional force (mean) (●).

increased linearly with an increase in tablet weight (and resulting thickness) when the compressional force was maintained constant.

Adjusting the thickness control setting to bring the punches closer together for the punching event causes successively thinner and denser tablets to be produced up to a point. The compressional and ejectional forces developed increase continuously throughout the entire range of press settings, even though the thickness decreases and the density increases, until each attains a nearly constant level.

Correlation of Compressional Force and Physical Properties of the Tablets.—For all the materials studied, nonlinear, but generally reproducible, relationships were found to exist between the relative setting of the thickness control and the generated compressional force. However, in attempting to correlate the physical properties of the tablets with the force used to compress them, two distinct types of behavior, predictable and nonpredictable, were found to exist for all the materials.

(*a*) The relationship between certain physical dimensions of the tablets, *e.g.*, thickness and apparent density, as well as the force required for ejection and the compressional force used in their preparation, are relatively independent of the actual material being compressed. Since all the materials behaved similarly, the compressional force profiles with respect to these properties are usually predictable.

(*b*) The relationship between certain other physical characteristics of the tablets, *e.g.*, hardness, friability, disintegration, and dissolutive rate values and the force used to compress them, are more dependent on the composition of the particular stock being tableted. Since compositions vary greatly between formulas, the effect that different levels of compressional force will have on the properties of the tablets is unpredictable. The compressional force profile must be determined for each material.

General or Predictable Relationships.—A specific case typifying predictable behavior is depicted by the data in Table IV and the plots shown in Fig. 6

TABLE IV.—INFORMATION PERTAINING TO TABLETS COMPRESSED AT DIFFERENT COMPRESSIONAL FORCE LEVELS[a]

Run No., Order	Thickness Setting,[b]	Wt., mg.	Thickness, in.	Compression Ratio, mg./in.	Compressional Force, lb.	Ejectional Force, lb.
1	360	516.5	.2388	2163	2910	141
2	405	516.5	.2368	2181	3740	148
3	450	516.0	.2354	2192	4080	136
4	495	516.0	.2342	2203	4975	159
5	540	515.5	.2335	2208	5700	160
6	585	515.5	.2335	2208	6870	163
7	630	516.0	.2337	2208	7700	161
8	675	516.0	.2339	2206	8930	175
9	720	515.0	.2338	2203	9730	170
10 (repeat)	360	515.5	.2380	2166	3080	146
11	315	516.5	.2399	2153	2150	143
12	270	517.5	.2408	2147	1810	119
13	225	517.5	.2431	2129	1450	104
14	180	515.2	.2462	2093	1150	85
15	135	517.3	.2498	2071	1020	87
16	90	516.6	.2533	2039	815	83
17	45	516.0	.2568	2009	685	70
18 (repeat)	360	514.6	.2377	2165	3035	114

[a] Controls: constant settings for weight cam and speed (360 TPM) adjustments. Material: conventional granulation E. Punches: 7/16 in. full oval. Machine: 1/2 BB2-27 IRTM. [b] Measured in terms of "notches" of control setting; 7 notches = 45° or 56 notches/rev. At 0° the punches were farthest apart at time of compression; at 720° the punches were nearest to each other.

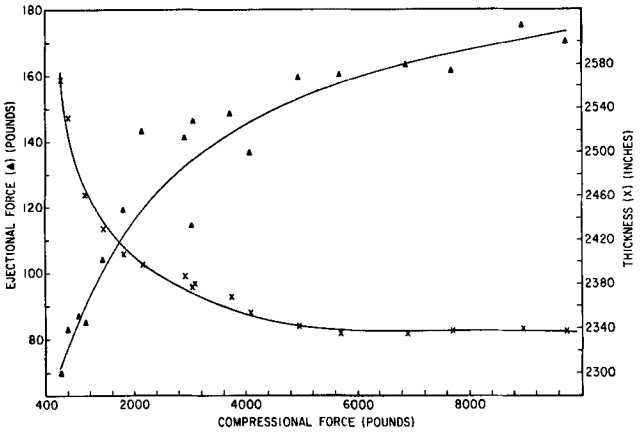

Fig. 6.—Compressional force (mean) *versus* ejectional force (▲) and thickness (×).

of compressional force *versus* (*a*) ejectional force and (*b*) mean tablet thickness. Here a conventional granulation E was compressed at various compressional force levels on an IRTM.

Higher compressional forces were generated as thinner (and consequently denser) tablets were produced. Note that the tablet thickness hardly changed as the compressional force increased from 5000 to almost 10,000 lb. Thus, although the compressional force almost doubled, the apparent density of the tablets (*i.e.*, CR α1/thickness) did not increase beyond a certain asymptotic value.

The results were in general qualitative agreement with those previously found by investigators who worked with "static" methods of compression (20–22) or instrumented single stroke machines (14, 16, 18). These workers empirically plotted the density (real or apparent) or porosity *versus* the logarithm of compressional force to obtain linear relationships at all but the higher force levels. Similar semilogarithmic plots using our data are also more linear, except where tailing occurs at higher compressional force levels.

The apparent density and thickness of the tablets tend to approach constant values as the compressional force is increased. The common practice used to determine the correct degree of compression for a given tablet is to measure a few tablets as they are made and compare their thickness to a previously set standard. This procedure should be questioned since tablets with the "correct" thickness could be compressed over a wide range of compressional forces if the "acceptable standard" was within the plateau region. Certain tablet properties may be more dependent on the actual force at which the tablets were compressed than they are on the thickness or apparent densities of the tablets. The value of being able to measure the actual force of compression with an IRTM during a tableting operation, rather than attempting to determine these values by indirect methods, is obvious.

As the compressional force increased, the force

TABLE V.—INFORMATION PERTAINING TO TABLETS COMPRESSED AT DIFFERENT COMPRESSIONAL FORCE LEVELS[a]

Run No., Order	Prev. Desig.[b]	Thick. Set	Mean Values Determined for the Tablets of Each Run: Wt., mg.	Thick., in.	CR, mg./in.	Comp. Force, lb.	DT,[c] min.	Hard., Kg.	F.R., Kg.	Fria.,[d] % Wt. Loss	For the 2 Rep. Tablets Tested[e] Av. of 2 Tablets w/o Disks, min. DT	$T_{50\%}$	$T_{90\%}$
1	A	700°	594.6	.2513	2366	7655	35.3	12.1	9.8	...	56	32	62.5
2	B	720°	596.1	.2516	2369	7975	33.8	12.6	9.9	.17	54	35	58
3	C	680°	592.9	.2511	2361	7225	32.5	12.3	9.5	.21	52	31.5	59
4	D	660°	591.2	.2507	2358	6735	33.2	11.6	9.7	.17	46.5	26	53
5	...	640°	590.2	.2509	2353	6350							
6	E	620°	592.1	.2513	2356	6080	27.2	11.4	9.0	.21	42.5	25	48
7	F	600°	590.1	.2510	2350	5500	27.8	12.2	8.9	.18	37.7	20	43
8	G	560°	592.3	.2517	2353	4715	24.8	11.2	8.4	.15	33	20	41
9	H	520°	590.0	.2531	2331	4275	23.8	11.7	8.5	.17	31	22.5	44
10	I	480°	592.7	.2542	2331	3650	20.7	11.1	8.5	...	27	14.5	31
11	...	440°	594.9	.2557	2326	3370							
12	J	400°	595.5	.2568	2318	2845	13.7	7.7	6.3	.13	19	12	25.5
13	K	360°	593.4	.2582	2298	2170	4.7	6.2	6.1	.17	15	10	19.5
14	L	320°	593.2	.2603	2278	1685	3.3	5.1	4.1	...	5	5	12
15	...	280°	588.2	.2621	2244	1500							
16	M	240°	595.0	.2650	2245	1345	2.5	2.5	2.9	.15	2	3	9
17 (repeat)	...	320°	590.8	.2600	2272	1790							
18	...	200°	591.1	.2675	2209	950	1.5						
19	N	160°	581.0	.2691	2159	730	1.0	.3	.5	Badly crumbled	1	2.5	8.75
20	...	120°	580.9	.2718	2137	605							
21	O	80°	580.0	.2740	2116	520	1	...	...	Badly crumbled	1	2	8.5
22	...	40°	584.0	.2771	2107	480							
23	P	0°	586.0	.2801	2092	415	1	...	...	...	1	2.5	9.5
24	Gran.	...	590.0	...	...	...	0	...	...	...	...	1.7	8.5

[a] Controls: constant settings for weight cam and speed (700 TPM) adjustments. Material: previously slugged granulation F containing phenacetin, aspirin, and caffeine. Punches: 7/16 in. full oval. Machine: 540-35 IRTM. [b] Tablets from these runs were designated by these letters in *Reference 34*. [c] Average time for 6 tablets to disintegrate with disks in pH 1.3 fluid (HCl–NaCl, $\mu = 0.1$). [d] These values were determined about 6 months later than others (after storage at room temperature). [e] Average times required for the 2 tablets tested individually. Fluid used was HCl–NaCl, pH 1.3 with $\mu = 0.1$.

required to eject the tablets also increased even though the tablets became thinner (Fig. 6). An apparent linear relationship was obtained when the ejectional force was plotted against the logarithm of the compressional force. Shotton and Ganderton (16), working with an instrumented single stroke press, found a similar relationship for unlubricated sodium chloride crystals.

If tablet thickness is plotted *versus* ejectional force, a curvilinear relationship with a negative slope is obtained; this is the converse of that reported by Nelson *et al.* (5) and discussed earlier. These anomalous findings are apparently explained because a higher force of compression increases the ejectional force more than a smaller tablet thickness reduces it (23). Furthermore, Nelson used an unlubricated granulation with a single stroke press and kept the compressional force constant while varying the tablet weight to change the thickness. In the present study where a lubricated granulation was

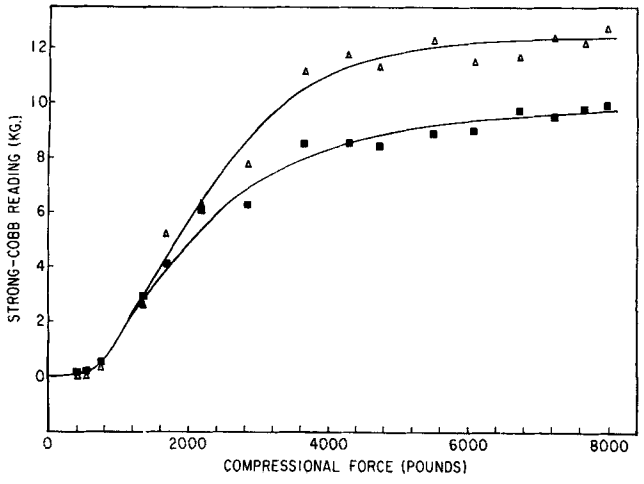

Fig. 7.—Compressional force *versus* hardness (△) and fracture resistance (■).

run on a rotary machine, the weight was held constant but the compressional force varied as the tablet thickness was changed.

For all the materials studied, the thickness and apparent density of the tablets level off even though the compressional and ejectional forces continue to increase. Because different materials reach their density plateaus at different compressional forces or with less absolute change in density, the rate of increase of tablet density with increasing compressional force possibly may be used as a measure of the bonding strength of the tablet. Elowe *et al.* (22) suggested that this in turn might be a criterion of the ease with which tablets can be compressed and reflects on the physical properties of the tablets after compression. No attempt has been made in these studies to test the validity of this proposal.

Specific or Unpredictable Relationships.—As indicated, general relationships between the compressional force and the physical properties of the tablets, such as hardness, friability, disintegration time, and dissolutive rate, were not found for the various materials studied. The specific compressional force profile for a material, or the exact way in which properties vary with compressional force, is often unpredictable and must be determined for each tableting mixture.

The following illustrates the establishment of such specific compressional profiles for one of the mixtures studied. The data in Table V were obtained when an experimental mixture containing aspirin, phenacetin, and caffeine, which had been previously wet granulated and slugged, was compressed on an IRTM. As usual, as the thickness setting was successively adjusted "to increase the pressure," thinner and denser tablets were produced—up to a point—but the compressional force generated increased continuously. Figure 7 shows that the values for tablet hardness and fracture resistance also increased to plateaus when they were plotted against compressional force. This finding confirms similar ones reported previously for "static" compression methods (20–22) and instrumented single stroke machines (15, 16, 18). Plots of the hardness or fracture resistance *versus* the logarithm of compressional force would also be linear except at the higher force levels where tailing occurs. Linear relationships would also be found for the tablets of this series if their apparent densities were plotted against either their hardness or fracture resistance values; similar findings have been reported for a specialized single stroke press (20) and a rotary press (31).

Although the thickness, apparent density, hardness, and fracture resistance values for the tablets of this run reach plateaus, the disintegration times (determined with and without plastic disks) and the $T_{50}\%$ and $T_{90}\%$ values for dissolutive rates continued to increase as the compressional force increased. These relationships are shown in Fig. 8. Tablets compressed with a force of about 5,800 lb. had a compression ratio of about 2,353 mg./in. and a thickness of .2517 in. while tablets compressed at about 7,600 lb. had similar values. The tablets compressed at the lower force required 40 min. for 90% of their ingredients to dissolve while those made at the higher force needed over 60 min. to release the same proportion of their ingredients. Furthermore, their disintegration times were somewhat different (28 *versus* 36 min.), yet there was very little difference in the hardness and fracture resistance values of the two samples.

For this series of tablets significant linear correlations were established between the various values for disintegration times and dissolutive rates (34). Obviously, linear correlations can also be obtained between any of these values and the compressional forces used to prepare the tablets.

Others (27, 29) have also shown that the disintegration time for certain tablets continues to increase as the compressional force is increased, even though the compression ratio of the tablets has reached a maximum. Similarly, in separate studies, Higuchi *et al.* found semilogarithmic relationships between the distintgration time and the compressional force for certain tablets made by static compression methods (20, 21) despite the fact that their porosities approached asymptotic values.

Unfortunately, the friability values for these tablets were determined quite some time after the tablets were prepared. Since compressed

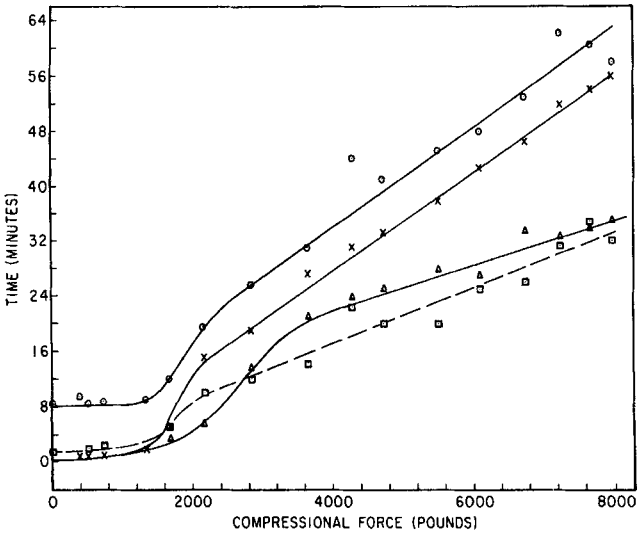

Fig. 8.—Compressional force (mean) *versus* $T_{90}\%$ (O) disintegration time w/o disks (×), disintegration time with disks (△), and $T_{50}\%$ (□).

tablets, especially those made from wet granulations, have a tendency to "set-up" upon storage (28, 29), the values listed do not reflect the true friabilities of the tablets at "time zero." Initially, the tablets made at compressional forces of less than 2,000 lb. were quite fragile.

As indicated, the above relationships were specific for the particular material in question. Different relationships with respect to the compressional force used to prepare the tablets were found for the other materials studied. For example, as the compressional force was increased over the range of 500 to 9,000 lb., the properties of the tablets were found to vary as follows.

The *hardness* values may increase continuously; increase and then level off; increase, level off, and then increase again, or increase to a maximum and then decrease. Similarly, the *fracture resistance* values may increase continuously; increase and then level off; increase to a maximum and then decrease; or not necessarily parallel the hardness values. The *friabilities* may decrease sharply from high values to plateau at lower ones; decrease more slowly from high values to plateau at lower ones; decrease to a minimal value and then increase again; or not necessarily be correlated with the hardness or fracture resistance values.

The *disintegration times* (with disks) may increase continuously; increase continuously with only a short plateau; increase only slightly and level off over a wide range; increase to a maximum and then decrease; decrease slightly, then increase continuously; or not necessarily be correlated with hardness or friability. The *dissolutive times* may increase from low values to level off at high values; increase from very short times to level off at only slightly longer times; vary randomly at intermediate values with no apparent relation to compressional force; increase from moderately long times to level off at very long times; increase to a maximal time and then decrease again; or not necessarily be correlated with disintegration times.

A unique finding of these studies was that for one of the conventional tablet granulations E, maxima were found for the hardness, fracture resistance, and disintegration and dissolutive times with respect to compressional forces. The friability reached a minimal value. As the compressional force increased, however, the hardness, fracture resistance values, and disintegration and dissolutive times decreased while the friability values increased.

Examples of many of these different types of behaviors have been reported previously by other investigators using a variety of methods to measure tablet properties and the degree or force of compression for single stroke machines (2–12, 15–18), specialized static compressional methods (19–23), empirical methods of varying compression (24–29), and in three cases, rotary machines (2, 30, 31).

Thus, for all materials, although tablets cease to become thinner or more dense when the compressional force is increased beyond a given point, predictions cannot always be made regarding the relationships between the compressional force and other physical properties of the tablets. Certain characteristics, *e.g.*, hardness, fracture resistance, disintegration time, dissolutive rate, and friability, may be affected more by the nature of the formulation or the medicaments than they are by the compressional force used to prepare them. This fact has not always been clearly established in the literature.

Interdependency of Tablet Uniformity and Force Variations.—For the foregoing studies the mean values from a large number of individual measurements and determinations were used to establish the various relationships. However, some general statements can be made regarding the individual compressional and ejectional force values observed during the runs.

Usually rotary tablet machines are thought to provide more uniform compression than do the single stroke machines. Contrary to expectations, however, large punch-to-punch and cycle-to-cycle variations in compressional and ejectional forces were found to exist. Brake also mentioned that the individual compressional forces varied more than expected during the exploratory work with his partially instrumented rotary tablet machine (2). Rehberg (31) also anticipated this possibility.

The degree of variation found in this study was not the same for all runs; for some the compressional force variation was observed to be as high as ±40% of the mean. In other runs much greater uniformity was displayed. Furthermore, the variation is usually larger at the lower compressional force levels and smaller at the higher compressional force levels because "unloading" of the press occurs at some stations; this tends to eliminate the high peaks that otherwise would be observed. For instance, in Fig. 1 the variation in compressional force is about ±18%, but in Fig. 2, at the higher compressional force level, the variation is only about ±5%.

The variation observed is also smaller when good flowing granulations or direct compaction mixtures are compressed with the aid of mechanical die filling equipment. Some of the most "uniform" compressional force tracings were observed when direct compaction mixtures made up of essentially lubricated spray-dried lactose were compressed.

It has been shown in Figs. 3 and 4 that the compressional force developed is directly related to the weight of the tablets being compressed. These data, the above observations, and previous findings by other workers who used single stroke (2, 8, 10, 11, 15) or rotary (2, 31) tablet presses indicate that the prime reason for the tablet-to-tablet and cycle-to-cycle variation in compressional force levels noted with a rotary tablet machine is apparently that the individual dies are being filled unequally. Rehberg (31) found that the weight variations of tablets made with rotary presses were normal distributions. Although compressional forces were not measured, Rehberg postulated that the compressional force was proportional to the weight of the tablet made. Thus, anything which causes more uniform filling of the dies should produce a more uniform compressional force impulse picture. Because the finer mesh particles of direct compaction mixtures fill the dies more uniformly than the coarser material of the conventional granulations, more uniform compressional force impulse heights are observed when the former materials are compressed (8, 36–38). (See Figs. 12 and 13 also.)

In addition to the fact that unequal die filling affects compressional force uniformity, evidence from these studies suggests that small differences

in the punch and die dimensions may also be a contributing factor by influencing not only the volume of fill (tablet weight) but the degree of compression (tablet thickness) as well. A die fitted with a short lower punch should receive a larger charge than a die fitted with a long one; thus, this station would deliver a heavier tablet. A long upper punch will always exert a higher compressional force than a short one when opposed by lower punches of the same length with dies filled with equal charges. Obviously, in sets of tools for presses with 16 to 51 stations a wide variety of combinations is possible. When the effect of the variations in die and punch diameters is introduced, the problem becomes even more complex. Others have pointed out that such variations exist within a set and from set to set as well (40–42).

The punch-to-punch and cycle-to-cycle variations in ejectional force occur for at least two reasons, because variations in compressional force occur and because variations in the lengths and the clearances of the tools exist for almost every set-up. In addition, tools wear unevenly (40, 42). It can be seen in Fig. 9 (where this was demonstrated for two different compressional force levels) that compression at uniform force levels (by adjusting the "overload" setting point to a lower value) does not produce tablets which are ejected with uniform forces. As expected, a higher ejectional force was required for the tablets produced at the higher compressional force level.

Results from some preliminary work as well as literature supplied by equipment manufacturers suggest that, if the overload system of the press were set so that the press continuously "unloaded" and all the tablets were compressed at the same force, tablets with uniform densities would be obtained. It has also been reasoned that tablets with uniform densities would have uniform physical characteristics, *i.e.*, disintegration times, dissolutive rates, hardness values, and friabilities. Less tablet-to-tablet variation in a lot would be observed. However, when this was attempted experimentally, there was no dramatic increase in uniformity of tablet densities or other properties because such a procedure does not improve the uniformity of flow or die filling. Actually, the presses could be damaged unless they were equipped with specialized "unloading" systems.

However, it is obvious that tablet-to-tablet variations as well as lot-to-lot variations with respect to the physical properties of the tablets may be due in some cases to the variation in compressional force used in their preparation. Since weight variations contribute mainly to the compressional force variations, the former may also be considered to influence greatly the physical properties of the individual tablets (8, 11, 15, 30, 31, 39). In the study mentioned previously, Rehberg (31) showed that for a series of three different tablet formulations compressed on a rotary press at constant settings, their thickness, density, hardness, and disintegration time all increased linearly as the tablet weight increased. He concluded that this occurred because heavier tablets were compressed at higher forces. Brake also suggested that hardness and disintegration might be correlated with the compressional force used to prepare the tablets (2). Thus, anything that can be done to insure compression of equal die fills at uniform and reproducible force levels will contribute greatly to the production of tablets which are uniform within a lot and from lot to lot (7, 8, 11, 31, 36, 37, 43, 44).

Applications of the IRTM for Tablet Development and Production Studies.—The foregoing material described the use of the IRTM for some types of basic or research studies. An extension of these studies led to the use of IRTM's to study both developmental and production tableting problems on the "floor" rather than at the bench. Some of these uses are described below. Where applicable, references to previously published investigations of a related nature are included in the discussions.

Tablet Development Studies.—(*a*) The IRTM has been used to determine the optimum lubrication system for a given stock by screening lubricants and

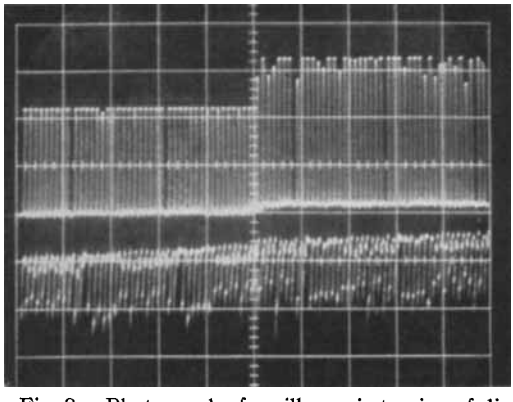

Fig. 9.—Photograph of oscilloscopic tracing of direct compaction mixture *A* compressed on 540-35 IRTM with Force-Flo-Feeder (FFF) at about 1000 TPM using 17-$^{7}/_{16}$ in. full oval punches. The upper punches were removed from every other station. Calibration: upper trace (C.F.), one large division = 2,700 lb.; lower trace (E.F.), one large division, = 25 lb.; sweep, 1 sec./large division, left to right. Overload setting on left side, 3 tons; on right side, 4 tons.

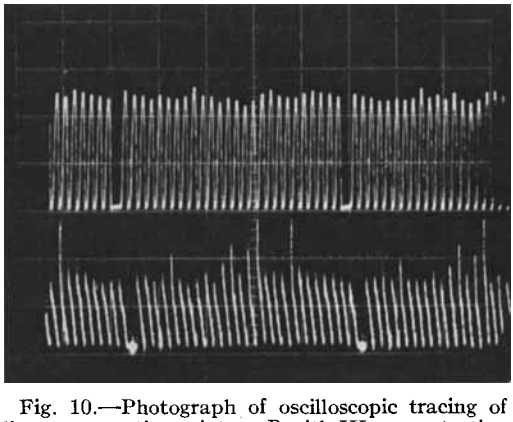

Fig. 10.—Photograph of oscilloscopic tracing of direct compaction mixture *B* with IX concentration of lubricant compressed on $^{1}/_{2}$ BB2-27 IRTM with FFF at about 660 TPM using 26-$^{7}/_{16}$ in. full oval punches (upper punch removed from one section). Calibration: upper trace (C.F.), one large division = 1540 lb.; lower trace (E.F.), one large division = 125 lb.; sweep, 0.5 sec./large division, left to right.

lubricant concentrations (3–5, 9–11, 13, 14, 17, 24). Figure 10 shows the compressional and ejectional forces generated for a given direct compaction mixture *B*. When the lubricant concentration was doubled with all the other variables being kept constant, the force required to eject the tablet was reduced to the level shown in Fig. 11. Note that with the increase in lubricant, the ejectional force impulses also became more uniform. When the lubricant concentration was tripled, only a slight additional decrease in ejectional force was observed. Thus, on the basis of ejectional force level, the optimum lubricant concentration for this formulation was considered to be between 2 to 3 times the initial concentration.

(*b*) The IRTM has been used to study and compare the relative flowability of various tableting mixtures. One way to evaluate how well a particular stock flows is to determine the weight variation of a collected sample of tablets. When the mixture flows well, the individual tablet weight variation is usually low; but if the flowability is poor, the individual weight variation will be high. As previously indicated, since the compressional force is directly proportional to the tablet weight or die fill, the degree of uniformity of the compressional force impulses should be a measure of the relative flowability of the mixture being tableted.

This is demonstrated by the following experiment in which 500-mg. tablets of a direct compaction mixture *C* and a conventional granulation E containing the same three active ingredients were each compressed on an IRTM. About 125 tablets were collected as the photographs of the compressional force impulses were being made during each run. One hundred tablets from each sample were weighed individually to the nearest 0.1 mg. The tablets compressed from the direct compaction mixture were found to have a lower weight variation than the tablets made from the standard granulation (coefficient of variation 0.52% *versus* 0.92%).

Figure 12 is the photograph obtained during the compression of the direct compaction mixture while the photograph in Fig. 13 was made when the conventional granulation was compressed. Data from these photographs show that the coefficient of variation for the compressional force impulses is less for the direct compaction mixture (4.49% *versus* 6.15%).

Both with respect to weight variation of individual tablets and compressional force impulse variation, the flowability of the direct compaction mixture was found to be superior to that of the conventional granulation.

Thus, the IRTM provided an instantaneous and continuous indication of the relative flowability of a tableting material in contrast to the more time-consuming method of weighing a representative number of individual tablets after a run has been finished (31, 36, 37, 39, 43, 44).

(*c*) The IRTM has been used to compress similar

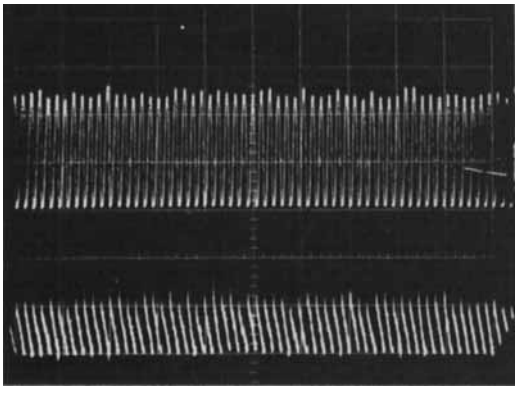

Fig. 11.—Photograph of oscilloscopic tracing of direct compaction mixture *B* with 2X concentration of lubricant compressed on $^1/_2$ BB2-27 IRTM with FFF at about 660 TPM using 26–$^7/_{16}$ in. full oval punches (upper punch removed from one section). Calibration: upper trace (C.F.), one large division = 1540 lb.; lower trace (E.F.), one large division = 125 lb.; sweep, 0.5 sec./large division, left to right.

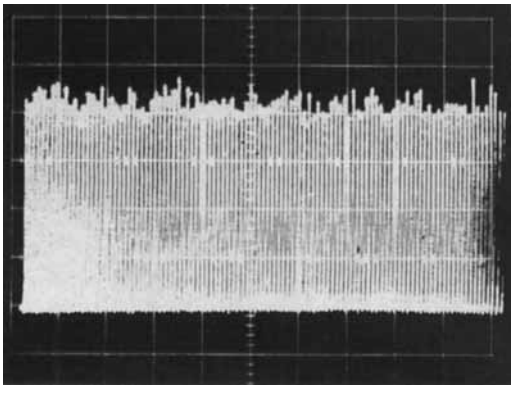

Fig. 12.—Photograph of oscilloscopic tracing of direct compaction mixture *C* compressed on $^1/_2$ BB2-27 IRTM with FFF at 700 TPM using 27–$^7/_{16}$ in. full oval punches. Calibration: upper trace (C.F.), one large division = 1065 lb.; lower trace (E.F.), not shown; sweep, 1 sec./large division, left to right.

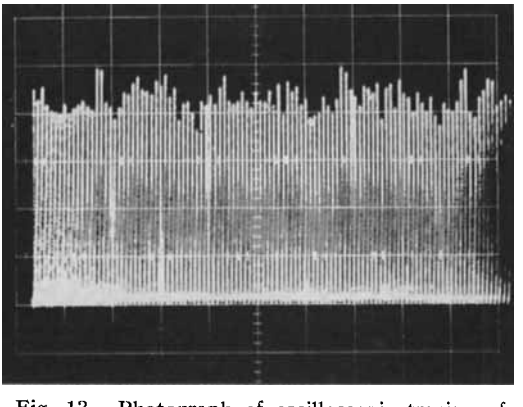

Fig. 13.—Photograph of oscilloscopic tracing of conventional granulation E compressed on $^1/_2$ BB2-27 IRTM with FFF at 700 TPM using 27–$^7/_{16}$ in. full oval punches. Calibration: upper trace (C.F.), one large division = 1065 lb.; lower trace (E.F.), not shown; sweep, 1 sec./large division, left to right. [The calibration differs between Figs. 12 and 13 and the other photographs shown for the $^1/_2$ BB2-27 presses. The sensitivity for these gauges was then 1 mv. of output per 1065 lb. of applied force with a 6 v. input (see *Footnote 8* in *Reference 1*).]

TABLE VI.—INFORMATION PERTAINING TO TABLETS PREPARED BY A DIRECT COMPACTION PROCESS

Run No.	Dry Binder Disinte-grant	Flow Promoting Agent	Force, lb., Comp.	Force, lb., Eject.	Friability, % Wt. Loss	Hardness, Strong-Cobb, Kg.	Dist. Time,[a] sec. w	Dist. Time,[a] sec. w/o
1	Yes	No	6000	125	.468	4.5	30	45
2	Yes	No	6000	125	1 of 20 capped	5.0	35	35
3	Yes	No	6000	125	.350	4.7	30	70
4	Yes	No	6100	125	.559	4.0	30	45
5	Yes	Yes	6100	125	.295	5.9	70	240
6	Yes	No	6100	125	.369	4.4	30	45
7	Yes	Yes	6000	125	.247	5.3	45	180
8	Yes	Yes	4500	100	.202	5.6	135	240
9	No	Yes	4500	100	.232	5.4	45	105
10	No	Yes	4500	100	.199	5.5	45	90
11	No	Yes	4500	100	.194	6.0	30	90
12	No	Yes	4500	100	.227	6.0	60	105
13	No	Yes	4500	100	.249	6.1	45	90
14	No	Yes	4500	100	.240	6.8	45	120
15L[b]	No	Yes	4500	...	.232	6.9	60	150
15R[b]	No	Yes	...	...	.220	6.3	45	120

[a] Total time for 6 tablets to disintegrate; w = with disks; w/o = without disks in deionized H_2O at 37°. [b] Dual compression BB2-27; only left side of press was instrumented; right side was set by comparing it to left.

formulas at constant and preselected compressional force levels. In this manner the real effects of an added ingredient or modified manufacturing procedure can be definitely determined (5, 7, 8, 12, 27). For example, during the development of a tablet prepared by a direct compaction process, the various formulations were compressed at the same force to determine the effects of both a dry binder-disintegrant and a flow promoting agent on the physical properties of the tablet. Some of the results obtained are shown in Table VI.

The flow promoting agent was so effective that the dry binder-disintegrant was no longer required because firmer tablets with acceptable disintegration times could be produced at lower compressional forces. Since there was less weight variation, there was also more uniformity in physical properties of the tablets; thus, individual units which were too soft or too hard were avoided. The mean ejectional force required was also reduced ~20% from 125 to 100 lb.

In another study involving a standard production granulation, it was found that by changing both the lubricating and disintegrating agents as well as their concentrations and using a finer mesh granule, the compressional force required to produce tablets with an acceptable hardness, friability, and dissolutive rate could be reduced from more than 5000 lb. to less than 2500 lb. The ejectional force required was concurrently reduced, too, not only because the compressional force level was decreased, but also because better lubricants were included.

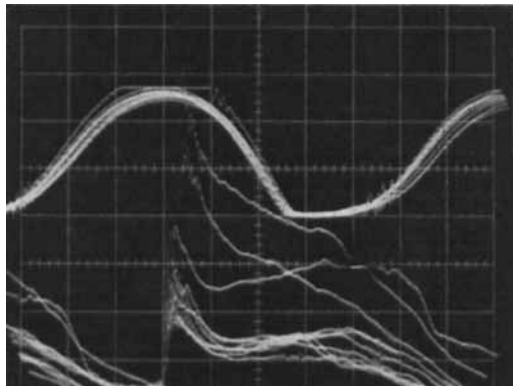

Fig. 15.—Photograph of oscilloscopic tracing of conventional granulation G compressed on 540-35 IRTM at 1500 TPM using 35-$^{15}/_{32}$ in. quarter oval punches. Calibration: upper trace (C.F.), one large division = 2700 lb.; lower trace (E.F.), one large division = 25 lb.; sweep, 10 msec./large division, left to right. Overload setting ~3.5 tons.

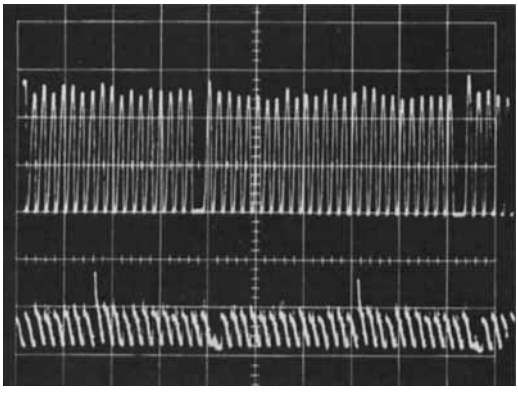

Fig. 14.—Photograph of oscilloscopic tracing of direct compaction mixture *B* compressed on $^1/_2$ BB2-27 IRTM with FFF at 600 TPM using 27-$^7/_{16}$ in. full oval punches (upper punch removed from station No. 1). Calibration: upper trace (C.F.), one large division = 3080 lb.; lower trace (E.F.), one large division = 125 lb.; sweep, 0.5 sec./large division, left to right.

Lower compressional and ejectional force requirements in rotary tablet machines mean reduced wear and longer life for punches, dies, and machine components, *e.g.*, cam tracks, compression rolls, and bearings (41, 42).

Production Activities.—(*a*) The IRTM has been useful in detecting and locating a malfunction of the machine or tools earlier than this can usually be accomplished by conventional methods. Observation of the oscilloscope gives an instantaneous picture of machine performance.

For example, as shown in Fig. 14, one punching

station was repeatedly responsible for an abnormally high ejectional force impulse (6). By removing the upper punch from station No. 1 and thus causing no tablet to be formed and no ejectional impulse to be produced for this station, the troublesome station was determined to be No. 16. Upon close examination, the lower punch in this station was found to have a deformed tip. When another lower punch was substituted, the high impulses disappeared.

Recently, an operator experienced difficulty in maintaining the desired tablet weight for a normally well-running granulation. Unfortunately, by using traditional methods, quite some time was required to trace the difficulty to a lower punch with a broken tip. Since the tip would not always drop completely, the die would not obtain the correct fill, and thus tablets with an incorrect weight were made at least two times per revolution. If, however, an IRTM had been in use, the trouble would have been located much more rapidly with less loss of granulation.

The use of the IRTM to detect a machine malfunction is illustrated in Fig. 15 where a combination of a time exposure and a rapid oscilloscopic sweep captured a number of successive compression and ejection events (23). Although one punch "unloaded," a relatively uniform compressional force level is shown. (Note the characteristic plateau in the compressional force curve for this tablet that is produced with the 540–35 IRTM.) The compressional forces ranged from 6500 to 7300 lb. (equivalent to pressures of 36,600 to 42,300 p.s.i.). However, there is an extreme variation in both initial peak height and shape of the ejectional force tracings for three of the tablets. The profiles for the other tablets are lower and relatively uniform. The complete profile of one of the "high peak" tracings (the lowest of the three) differs from the other two by the display of a plateau followed by a sharp drop to the baseline. This indicates that there was a considerable force required to keep the tablet (or punch) moving in the die until ejection occurred. For these tablets forces up to three to four times the "normal" ejectional force were observed. The normal ejectional pressure was about 290 p.s.i. while the binding tablets required pressures ranging from 870 to 1160 p.s.i. to eject them. The incidence of a higher impulse corresponded to a tendency for the punches to bind during the actual run.

Application of suitable amounts of lubricants to the lower punch shafts reduced the binding tendencies and eliminated the "high peak" tracings previously observed. Concurrently, the squeaking which had been heard was eliminated.

(*b*) Another use of an IRTM was to determine whether the ejectional forces required for a regular double press coated tablet (DPCT) made on a Manesty Bicota (triple turret) tablet press were actually excessive to the point of causing a breakdown of the press. Pictures of the ejectional force tracings were taken and compared for tablets compressed at 10 to 12 different thickness settings for six different standard production granulations (using only the

TABLE VII.—PERTINENT INFORMATION ABOUT SCALE-UP AND PILOT SIZE LOTS OF DIRECT COMPACTION FORMULA RUN ON IRTM'S

Run No.	Lot Size	Machine	Comp. Force, lb.	Thickness, in.	Hardness, S-C, Kg.	Friability,[a] % Wt. Loss	Disintegration Time,[b] sec. w	w/o
1	30 M	1/2 BB2	4500	.227–.232	6.0	.227	30	90
2	30 M	1/2 BB2	4500	.227–.232	6.1	.249	60	105
3	100 M	BB2	4500	.228–.233	L 6.0	.150	45	90
					R 5.9	.315	45	90
4	100 M	BB2	4500	.228–.233	L 5.1	.290	45	90
					R 5.6	.241	60	90
5	100 M	BB2	4500	.228–.233	L 5.7	.290	45	105
					R 5.6	.326	45	105
6	30 M	1/2 BB2	4500	.227–.232	6.0	.194	30	90
7	100 M	1/2 BB2	4500	.230	6.8	.240	45	120
8	400 M	BB2	4500	.228–.234	L 6.0	0/20	45	75
					R 5.7	0/20	45	75
9	100 M	1/2 BB2	4500	.228–.233	7.0	.261	60	90
10	100 M	1/2 BB2	4500	.228–.233	7.3	.270	60	120
11	100 M	1/2 BB2	4500	.228–.233	7.0	.242	60	105
12	1 $\bar{M}$	BB2	4500	.230	L 5.2	.216	45	75
					R 6.3	.198	45	90
12R	1 $\bar{M}$	BB2	4500	.230	L 7.2	0/20	45	75
					R 7.5	0/20	45	75
13	1 $\bar{M}$	BB2	4500	.230	5.6	.150	45	70
14	1.2 $\bar{M}$	BB2	4500	.229–.232	L 5.6	.150	45	90
					R 5.8	.180	60	150
15	1.2 $\bar{M}$	BB2	5000	.230	L 6.3	.180	60	120
					R 6.2	.290	60	120
16	1.2 $\bar{M}$	BB2	4500	.230	L 7.2	.238	60	90
					R 7.2	.165	60	90
17	1.2 $\bar{M}$	BB2	5500	.230	5.5	.250	60	120
18	1.2 $\bar{M}$	BB2	4500	.230	L 6.1	.218	45	90
					R 5.9	.238	45	90

[a] 0/20 means no capped tablets were found after 4 min. of friabilation. [b] Total time for 6 tablets to disintegrate in deionized H_2O at 37°; w = with disks; w/o = without disks.

third turret) as well as for the entire DPCT formula. Other formulations tested included granulations for three press coated tablet (PCT) coatings, a chewable vitamin, an APC combination, and a sulfonylurea compound.

It was concluded that the forces required to eject the entire DPCT or the tablets made of its coating granulation alone on the third turret were not any higher than those required to eject tablets of the same size made (on the third turret) of the other granulations studied at equivalent thickness settings. Thus, the lubrication of the DPCT formulation was apparently not responsible for the excessive wear to certain members of the Manesty press (3–5, 9–11, 13, 14, 17, 24).

(*c*) The IRTM was used to provide a type of "set-up" and "in-process" control for a specific product on a routine production basis (7, 43, 44). For this purpose a standard Stokes BB2–27 press was "instrumented" by transferring to it the strain gauged eyebolt from the 1/2 BB2–27 IRTM. The system was used to adjust the thickness control so that a preselected force level was obtained for consecutive laboratory scale-up and pilot lots of a new formulation. Previously, it had been found that tablets compressed at a force of 4500 lb. from a specific direct compaction mixture possessed certain desirable physical properties (Table VI). The runs were then monitored to maintain this compressional force level by using the IRTM.

The data in Table VII will attest to the success attained by controlling this variable more closely than heretofore has been possible.

The physical properties of the tablets for the various runs were very similar and exhibited no marked variation from lot to lot. Note that, for two of the large size lots, it was necessary to compress the tablets at somewhat higher force levels to obtain tablets with the desirable characteristics. These formulations were somewhat drier than the others reported and this may be the reason higher pressures were required to produce the necessary bond.

Not all of the tablets of run 12 were compressed when the stock was first prepared. The remaining material was compressed into tablets 2 months later. Note that the physical properties of the tablets (run 12R), except for the hardness values, are quite similar to those found initially in run 12 (Table VII).

If, during the run, the compressional force level shifted markedly from the norm, the tablet operator usually found that heavier or lighter than theoretical weight tablets were being prepared. Appropriate adjustments to the weight cam control brought the force level back to normal. Thus, the IRTM provided not only a way of reproducing batches of uniform tablets from consecutive runs, but also a means of in-process control. Perhaps it was equipment and methods like this that Swintosky, Kennon, and Tingstad visualized when they prophesied in their 1955 paper, "These data suggest that by (a) judicious selection of disintegrant, (b) suitable choice of tablet composition, and (c) *regulation of the compressional force to precise and known values, the rates of tablet disintegration and drug release may be controlled*," (7) (emphasis added).

As a result of these investigations, two conventional BB2-27 presses have been instrumented to measure both compressional and ejectional forces for both sides. These presses are now used routinely in the production of the new formula and are also available for other production oriented studies. (See Fig. 5, *Reference 1.*)

The foregoing examples have served to describe and demonstrate the use and usefulness of the IRTM at this time. These will in turn suggest many more studies in which the IRTM can be used to obtain valuable data to resolve other tableting problems. Some of these studies have already been carried out in this laboratory and future communications will cover in greater detail some of these as well as those only briefly mentioned in this report.

SUMMARY

The use and usefulness of instrumented rotary tablet machines to study a variety of tableting problems, both fundamental and applied, were described and discussed.

Using the two IRTM's and a variety of tablet formulations, fundamental information concerning the performance of rotary tablet machines was obtained. General relationships were established between the relative settings of the three press controls (speed, thickness, and weight cam) on the one hand, and certain physical characteristics of the tablets (thickness, weight, and density) and the compressional and ejectional forces generated in their preparation on the other.

General relationships between the force of compression and the physical properties of the tablets, such as weight, thickness, density, and the force required to eject them, were found to be relatively independent of the material being compressed and quite similar for all materials studied.

As others have found by using different methods to measure the variables, certain other physical characteristics of the tablets, such as hardness, friability, disintegration time, and dissolutive rates, were apparently affected by the nature of the formulations or the medicaments as well as by the compressional force involved in their preparation. Contrasting behaviors for different formulations were described.

It was concluded that the relatively large punch-to-punch and cycle-to-cycle variations in compressional and ejectional force levels, which were unexpectedly found to exist during tableting on rotary presses, were apparently due to unequal filling of the dies. When formulations were run which flowed well or which were induced to flow into the dies by mechanical means, compressional, and ejectional force impulse heights became more uniform and weight deviations decreased.

Descriptions of the manner in which the IRTM's were useful in studying typical development and production tableting problems included their use to: (*a*) screen and compare lubricants and lubricant concentrations; (*b*) determine and compare the relative flowabilities of tablet formulations; (*c*) compress experimental and developmental formulas at constant and preselected force levels; (*d*) detect and locate press or tool malfunction more rapidly than by conventional means; (*e*) determine whether the degree of lubrication for a given formulation was adequate to permit compression on a specific tablet press; (*f*) provide a means of "set-up"

and "in-process" control to obtain routinely both tablet-to-tablet and lot-to-lot uniformity for a production formulation.

REFERENCES

(1) Knoechel, E. L., Sperry, C. C., Ross, H. E., and Lintner, C. J., *J. Pharm. Sci.*, **56**, 109(1967).
(2) Brake, E. F., "Development of Methods for Measuring Pressures During Tablet Manufacture," M.S. Thesis, Purdue University, West Lafayette, Ind., 1951.
(3) Higuchi, T., Nelson, E., and Busse, L. W., *J. Pharm. Sci.*, **43**, 344(1954).
(4) Nelson, E., "Measurement of Force and Displacement Variables in the Tablet Compression Process," Ph.D. Dissertation, University of Wisconsin, Madison, Wis., 1954.
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(6) Nelson, E., Busse, L. W., and Higuchi, T., *ibid.*, **44**, 223(1955).
(7) Swintosky, J. V., Kennon, L., and Tingstad, J. E., *ibid.*, **44**, 112(1955).
(8) Raff, A. M., Arambulo, A. S., Perkins, A. J., and Deardorff, D. L., *ibid.*, **44**, 290(1955).
(9) Strickland, W. A., Jr., "A Study of the Fundamental Aspects of Tablet Lubrication and Quantitative Evaluation of Lubricants," Ph.D. Dissertation, University of Wisconsin, Madison, Wis., 1955.
(10) Strickland, W. A., Jr., Nelson, E., Busse, L. W., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 51(1956).
(11) Gagnon, L. P., "A Correlation of Physical Aspects in Tablet Production," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1957.
(12) Nelson, E., Arndt, J. R., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 257(1957).
(13) Markowski, S. L., "The Evaluation of Tablet Lubricants Using a Conventional Type Tablet Machine Instrumented with Strain Equipment," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1958.
(14) Strickland, W. A., Jr., Higuchi, T., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 35(1960).
(15) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **12**, Suppl. 87T(1960).
(16) *Ibid.*, **12**, 93T(1960).
(17) Daoust, R. G., "A Study of the Combined Effects of Binders, Lubricants, and Lubricant Concentration in Tablet Making," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1960.
(18) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **13**, Suppl. 144T(1961).
(19) Higuchi, T., Arnold, R. D., Tucker, S. J., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 93(1952).
(20) Higuchi, T., Rao, A. N., Busse, L. W., and Swintosky, J. V., *ibid.*, **42**, 144(1953).
(21) Higuchi, T., Elowe, L. N., and Busse, L. W., *ibid.*, **43**, 685(1954).
(22) Elowe, L. N., Higuchi, T., and Busse, L. W., *ibid.*, **43**, 718(1954).
(23) Nelson, E., *ibid.*, **44**, 494(1955).
(24) Munden, B. J., DeKay, H. G., and Banker, G. S., *Drug Std.*, **28**, 12(1960).
(25) Brown, C. L. M., *Quart. J. Pharm. Pharmacol.*, **12**, 489(1939).
(26) Berry, H., *ibid.*, **12**, 501(1939).
(27) Berry, H., and Ridout, C. W., *J. Pharm. Pharmacol.*, **2**, 619(1950).
(28) Burlinson, H., and Pickering, C., *ibid.*, **2**, 630(1950).
(29) Ramsey, R. A., *ibid.*, **10**, Suppl. 145T(1958).
(30) Filleborn, V. M., *Am. J. Pharm.*, **120**, 233(1948).
(31) Rehberg, W. F., "Analysis of the Variables Encountered in Compressed Tablets," M.S. Thesis, University of Wisconsin, Madison, Wis., 1953.
(32) Endicott, C. J., Lowenthal, W., and Gross, H. M., *J. Pharm. Sci.*, **50**, 343(1961).
(33) Shafer, E. G. E., Wollish, E. G., and Engel, C. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 144(1956).
(34) Schroeter, L. C., Tingstad, J. E., Knoechel, E. L., and Wagner, J. G., *J. Pharm. Sci.*, **51**, 865(1962).
(35) Schroeter, L. C., and Wagner, J. G., *ibid.*, **51**, 957 (1962).
(36) Arambulo, A. S., and Deardorff, D. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 690(1953).
(37) *Ibid.*, **42**, 692(1953).
(38) Nelson, E., *ibid.*, **44**, 435(1955).
(39) Hammerness, F. C., and Thompson, H. O., *J. Pharm. Sci.*, **47**, 58(1958).
(40) Gaskell, J. C., *Chemist Druggist*, **1960**, 403.
(41) Bobbitt, W. C., *Drug Cosmetic Ind.*, **85**, 468(1959).
(42) Swartz, C. J., Weinstein, S., Windheuser, J., and Cooper, J., *J. Pharm. Sci.*, **51**, 1181(1962).
(43) Nelson, E., "The Controlling and Testing of Tablets," reprint of oral presentation at a symposium of the Industrial Pharmacy Section, A.PH.A., Las Vegas meeting, March 1962, p. 4.
(44) Kovac, G. M., "The Tableting of Tomorrow," *ibid.*, 1962, p. 10.

Notes

Antagonism of Propoxyphene Poisoning in Albino Mice with Nalorphine HCl, Methylene Blue, and Tolonium Chloride

By DAVID E. MANN, JR.

The intraperitoneal administration of propoxyphene HCl to albino mice at a dosage level of 200 mg./Kg. ($LD_{99.5}$) was lethal to 98 per cent of the controls. The prophylactic subcutaneous administration of nalorphine HCl alone, or in combination with either methylene blue or tolonium chloride, significantly increased the number of survivors with the greatest degree of protection being afforded by the nalorphine–dye combinations.

HARPEL AND MANN (1) recently demonstrated the antidotal effectiveness of subcutaneously administered nalorphine HCl and methylene blue, alone and in combination, prior to lethal doses of propoxyphene HCl in mice. The greatest amount of protection against the lethality of the analgesic occurred when nalorphine and methylene blue were administered 5 min. before propoxyphene HCl.

The purpose of this investigation was to confirm the results previously obtained with nalorphine and methylene blue in mice and to determine whether tolonium chloride (toluidine blue), a dye chemically related to methylene blue, also possessed antidotal qualities.

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EXPERIMENTAL

Three hundred and fifty adult male albino mice (Huntingdon Farms, HTF strain), weighing between 20 and 25 Gm., were used in this study. Prior to treatment, the animals were caged in groups of 25 for several days and had access to laboratory chow (Purina) and water *ad libitum*.

The following solutions were prepared with distilled water: propoxyphene HCl, 2.0%, and methyl-

and "in-process" control to obtain routinely both tablet-to-tablet and lot-to-lot uniformity for a production formulation.

REFERENCES

(1) Knoechel, E. L., Sperry, C. C., Ross, H. E., and Lintner, C. J., *J. Pharm. Sci.*, **56**, 109(1967).
(2) Brake, E. F., "Development of Methods for Measuring Pressures During Tablet Manufacture," M.S. Thesis, Purdue University, West Lafayette, Ind., 1951.
(3) Higuchi, T., Nelson, E., and Busse, L. W., *J. Pharm. Sci.*, **43**, 344(1954).
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(5) Nelson, E., Naqvi, S. M., Busse, L. W., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 596(1954).
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(8) Raff, A. M., Arambulo, A. S., Perkins, A. J., and Deardorff, D. L., *ibid.*, **44**, 290(1955).
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(13) Markowski, S. L., "The Evaluation of Tablet Lubricants Using a Conventional Type Tablet Machine Instrumented with Strain Equipment," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1958.
(14) Strickland, W. A., Jr., Higuchi, T., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 35(1960).
(15) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **12**, Suppl. 87T(1960).
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(17) Daoust, R. G., "A Study of the Combined Effects of Binders, Lubricants, and Lubricant Concentration in Tablet Making," Ph.D. Dissertation, Purdue University, West Lafayette, Ind., 1960.
(18) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **13**, Suppl. 144T(1961).
(19) Higuchi, T., Arnold, R. D., Tucker, S. J., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 93(1952).
(20) Higuchi, T., Rao, A. N., Busse, L. W., and Swintosky, J. V., *ibid.*, **42**, 144(1953).
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EXPERIMENTAL

Three hundred and fifty adult male albino mice (Huntingdon Farms, HTF strain), weighing between 20 and 25 Gm., were used in this study. Prior to treatment, the animals were caged in groups of 25 for several days and had access to laboratory chow (Purina) and water *ad libitum*.

The following solutions were prepared with distilled water: propoxyphene HCl, 2.0%, and methyl-

TABLE I.—EFFECT OF SUBCUTANEOUS PRETREATMENT WITH NALORPHINE HCl, METHYLENE BLUE, AND TOLONIUM CHLORIDE ALONE AND IN COMBINATION, UPON THE DURATION AND INCIDENCE OF SURVIVAL FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 200 mg./Kg. OF PROPOXYPHENE HCl

Drug[a]	Dose, mg.	Mean Survival Time, sec.	Change in Mean Surv. Time, sec.	Survivors, No.	Survivors,[b] %
Control	...	583	...	1	2
Distilled water	...	580	−3	2	4
Nalorphine HCl	0.1	852	+269	6	12[c]
Methylene Blue	1.0	563	−20	3	6
Tolonium chloride	1.0	480	−103	4	8
Nalorphine HCl and Methylene blue	0.1 1.0	862	+279	19	38[d]
Nalorphine HCl and Tolonium chloride	0.1 1.0	1020	+437	15	30[d]

[a] Drug administered to groups of 50 mice 5 min. prior to propoxyphene HCl. [b] Animals which survived 24 hr. after propoxyphene HCl. [c] χ^2 test, $P < 0.05$. [d] χ^2 test, $P < 0.001$.

ene blue and tolonium chloride, 1.0%, respectively. Ampuls of nalorphine HCl, 5 mg./ml., were obtained from the manufacturer.

The experimental procedure has been previously described (1). In contrast to the former investigation, however, the end point of survival after propoxyphene HCl was extended from 17 min. to 24 hr.

The intraperitoneal LD_{50} of propoxyphene HCl, determined by the method of Litchfield and Wilcoxon (2), was formerly shown to be 118 (106 to 131) mg./Kg. (1). Based upon these data, a dose of 200 mg./Kg. was selected as the lethal amount to be used in future antidotal studies in mice with propoxyphene HCl because theoretically it should produce death in 99.5%.

The treatment schedule consisted of injecting subcutaneously either methylene blue or tolonium chloride (1.0 mg.) into the upper left abdomen, nalorphine HCl (0.1 mg.) or an equivalent volume of distilled water (0.02 ml.) into the upper right abdomen, or nalorphine HCl first (0.1 mg.) followed immediately with either dye (1.0 mg.). Five minutes after prophylactic treatment, propoxyphene HCl (200 mg./Kg.) was injected intraperitoneally and the mice were then placed in individual cages for close observation. Survival time extended from the moment of propoxyphene HCl injection to the cessation of respiration. To determine the precise time of death, the number of animals under observation at one time never exceeded four.

The data obtained from the seven groups of control and treated animals (50 per group) are reported in Table I.

The significance of the per cent survival in the treated group with respect to the control group was estimated by the χ^2 test (3). Probability values greater than 0.05 were considered nonsignificant.

RESULTS

The intraperitoneal administration of 200 mg./Kg. of propoxyphene HCl ($LD_{99.5}$) was fatal to 98% of the controls. The subcutaneous injection of distilled water in a volume equivalent to that employed for nalorphine HCl (0.02 ml.), followed in 5 min. with the intraperitoneal administration of 200 mg./Kg. of propoxyphene HCl, killed 96% of the animals, although the mean survival time (580 sec.) for this group closely approximated that of the controls (583 sec.).

The subcutaneous administration of nalorphine HCl (0.1 mg.) 5 min. prior to propoxyphene HCl resulted in survival of 12% of the mice and extended the mean survival time of the remainder to 852 sec. Interestingly enough, the mean survival time of the controls and nalorphine HCl in the earlier paper is considerably lower than that recorded in this study, yet the increase is approximately the same (256 and 269 sec., respectively). A decrease in the per cent survival after nalorphine HCl from 18.4 to 12 is to be expected because the criterion for survival was changed from 17 min. to 24 hr. These results confirm the earlier observation that definite antagonism of propoxyphene HCl by nalorphine HCl occurs in mice and are in agreement with similar reports in rats (4, 5).

The administration subcutaneously of either methylene blue or tolonium chloride (1.0 mg.) 5 min. prior to propoxyphene HCl did not increase significantly the per cent of survivors (6 and 8, respectively). On the contrary, tolonium chloride increased the convulsant episodes to such an extent that a marked decrease in mean survival time occurred (480 sec.). However, when nalorphine HCl (0.1 mg.) and either methylene blue or tolonium chloride (1.0 mg.) were given together 5 min. prior to propoxyphene HCl, the per cent of survival was significantly increased to 38 and 30, while the mean survival time increased 279 and 437 sec., respectively, beyond that of the controls.

The earlier observation that nalorphine HCl alone or in combination with methylene blue significantly protected mice against lethal doses of propoxyphene HCl has been confirmed under more stringent experimental conditions. In addition, tolonium chloride, a dye chemically related to methylene blue, when administered together with nalorphine HCl, has been shown to enhance the protective activity of the narcotic antagonist in a manner quantitatively similar to its congener.

REFERENCES

(1) Harpel, H. S., Jr., and Mann, D. E., Jr., *J. Pharm. Sci.*, **54**, 97(1965).
(2) Litchfield, J. T., and Wilcoxon, F. J., *J. Pharmacol. Expil. Therap.*, **96**, 99(1949).
(3) Simpson, G. G., Roe, A., and Lewontin, R. C., "Quantitative Zoology," Harcourt, Brace, and Co., New York, N. Y., 1960, pp. 176, 186.
(4) Robbins, E. B., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 497(1955).
(5) Chapman, J. E., and Walaszek, E. J., *Toxicol. Appl. Pharmacol.*, **4**, 752(1962).

Effect of a Pantothenic Acid-Deficient Diet on Monoamine Oxidase (MAO) and Deoxyribonucleic Acid (DNA) in Rat Liver

By KANTI G. BHANSALI* and JOHN L. LACH

Livers of rats maintained for 20 days on a pantothenic acid-deficient diet plus a molecular antagonist showed lower MAO activity and higher DNA content than those of the rats receiving a basic diet plus pantothenic acid. The biochemical changes reported are not understood.

DURING the course of investigation on MAO activity and DNA content in human liver the opportunity arose to assay MAO in the livers of Sprague-Dawley rats that had been maintained on a diet devoid of pantothenic acid (1). Pantothenic acid is an important constituent of coenzyme A (CoA); its deficiency in laboratory animals causes disorders of carbohydrate, fat, sterol, and steroid metabolism and produces lesions of the skin, adrenals, nerves, spinal cord, and entire gastrointestinal tract (2).

To assure that the effect of any indigenous pantothenic acid was eliminated, experimental rats received a molecular antagonist, ω-methyl pantothenic acid (3). Control rats were fed the basic diet plus pantothenic acid. The animals were sacrificed after 20 days and their livers immediately placed in the cold until analyzed. MAO activity was determined on liver homogenates by the method of Sjoerdsma *et al.* (4), a method based on the colorimetric determination of substrate (tyramine) disappearance. DNA content was assayed by the method of Webb and Levy (5), a colorimetric procedure based on the reaction of DNA with *p*-nitrophenylhydrazine. The data obtained from the analysis of the liver specimens are given in Table I. Statistical evaluation was by Student's *t* test.

TABLE I.—NORMAL AND PANTOTHENIC ACID-DEFICIENT RAT LIVERS

	MAO Units[a]	DNA, mcg./Gm. Liver
	Normal Livers	
1	3225	2008
2	2925	2681
3	3105	2146
4	3165	2597
5	3450	2666
6	3015	2239
Mean	3147.5	2389
S.D.	±182	±294
	Pantothenic Acid-Deficient Livers	
1	2385	3777
2	2115	3556
3	2220	3468
4	2145	3596
5	1860	3369
6	1650	3268
Mean	2062	3506
S.D.	±250	±179
	$P < 0.001$	$P < 0.001$

[a] One unit is amount of MAO causing the disappearance of 1 mcg. tyramine/Gm. tissue/hr.

The liver of the rat maintained on the pantothenic acid-deficient diet plus ω-methyl pantothenic acid shows a significantly lower MAO activity and significantly higher DNA content than the normal rat liver. This relationship of low MAO activity and high DNA content in normal and pathological human liver has been also observed (1). Under pantothenate deficient conditions, CoA of rat liver has been shown to be reduced to 35–40% of normal after 9 weeks and CoA of duck liver has been observed to be depleted 40% of normal after 5 days (6). Lipmann *et al.* (7) demonstrated that pantothenic acid is a constituent of CoA. The present data suggest that pantothenic acid might also be a part of MAO.

As the determination of MAO activity and DNA content is based on Gm. of liver tissue, the higher content of DNA might be attributed to the dehydration of pathological liver (that is, a greater number of cells/Gm. of liver tissue than is normal). However, if the above is true, MAO activity of pathological liver should be more than the normal. The data reported in the paper indicate that this is not so.

Since the DNA content per cell is known to remain constant (8), the increased DNA content suggests an increase in the number of liver cells, possibly as a natural defense mechanism. Increased numbers of liver cells have been observed in other pathological states, such as in inflammation.

Since MAO as yet has not been isolated in pure form, its chemical structure is unknown, and hence, the biochemical changes involved in the presently reported phenomenon are not understood.

Received April 25, 1966, from the College of Pharmacy, University of Iowa, Iowa City 52241.

Accepted for publication August 26, 1966.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

The authors thank Dr. James Clifton, College of Medicine, University of Iowa, Iowa City, for assistance during this investigation.

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REFERENCES

(1) Bhansali, K. G., Ph.D. Thesis, University of Iowa, Iowa City, Iowa, 1960.

(2) Williams, R. J., "Biochemical Individuality," John Wiley & Sons, Inc., New York, N. Y., p. 154.

(3) Bean, W. B., Lubin, R., and Daum, K., *J. Lab. Clin. Med.*, **46**, 793(1955).

(4) Sjoerdsma, A., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **89**, 36(1955).

(5) Webb, J. M., and Levy, H. B., *J. Biol. Chem.*, **213**, 107 (1955).

(6) Olson, R. E., and Kaplan, N. O., *ibid.*, **175**, 515(1948).

(7) Lipmann, F., *et al.*, *ibid.*, **186**, 235(1950).

(8) Leslie, I., in "The Nucleic Acids," vol. 2, Chargaff, E., and Davison, J. N., eds., Academic Press Inc., New York, N.Y., 1955, p. 24.

Gas Chromatographic Determination of Ethanol in Pharmaceuticals. Comparison of the Flame Ionization and Thermal Conductivity Detectors

By MILTON ELEFANT and JOSEPH M. TALMAGE

The use of a flame ionization detector has been investigated as a means of reducing the time necessary for the analysis of ethanol. Results obtained are in complete agreement with those found using a thermal conductivity detector.

GAS CHROMATOGRAPHY has been reported as an analytical tool for the determination of ethanol in wine (1), cosmetics (2), mixed solvents (3), and pharmaceuticals (4, 5). In a collaborative study sponsored by the Pharmaceutical Manufacturers Association (6), gas chromatography was found comparable in accuracy and precision to the official U.S.P. and N.F. distillation method for the determination of ethanol. Subsequently, the gas chromatographic procedure was adopted in the N.F. XII (7).

All of these methods specified a thermal conductivity detector which necessitated the elution of water from the column before injection of the next sample. Even with efficient columns which reduced or eliminated the tailing of water, most chromatograms required at least 12 min. It is interesting to note that in N.F. XII First Supplement (8) no detector is specified.

The use of a flame ionization detector would considerably reduce the analytical time, but the presence of water diminishes the flame detector's over-all sensitivity (9). The need to keep the flame ionization detector at constant sensitivity is the basis for obtaining results that would compare favorably with those using a thermal conductivity detector. By designing a column which gives efficient separation of acetone (internal standard) and ethanol while allowing the water to bleed off, it was found possible to use the flame ionization detector with constant sensitivity. This water bleeding phenomenon was probably responsible for the excellent results obtained by Parker *et al.* (10), in the determination of ethanol in blood using the flame ionization detector.

EXPERIMENTAL

Flame Ionization Detector

Apparatus.—Perkin-Elmer model 801 flame ionization gas chromatograph.

Column Packing.—A 6 ft., 1/4-in. o.d., copper column packed with 20% polyethylene glycol 4000[1] on Gas Pack RP 60/80 mesh (Applied Science Laboratory, State College, Pa.).

Operating Conditions.—Column temperature, 115°; injector temperature, 150°; detector temperature, 150°; He flow rate, 40 ml./min. Figure 1 illustrates a typical chromatogram.

Received June 9, 1966, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, N. J. 07950.
Accepted for publication August 24, 1966.

[1] Marketed as Carbowax by the Union Carbide Corp., New York, N. Y.

Thermal Conductivity Detector

Apparatus.—Perkin-Elmer model 154C gas chromatograph equipped with thermistor detector.

Column Packing.—A 6-ft., 1/4-in. o.d., copper column packed with 20% polyethylene glycol 4000 on 60/80 mesh Gas Pack F (a perfluorocarbon impregnated diatomaceous earth, Chemical Research Services, Inc., Addison, Ill.).

Operating Conditions.—Column temperature, 110°; injector temperature, 150°; He flow rate, 80 ml./min. Figure 2 illustrates a typical chromatogram.

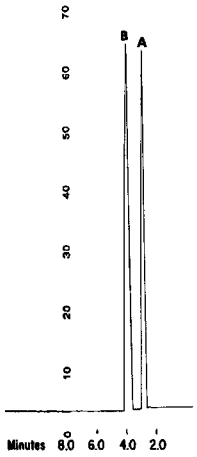

Fig. 1.—A typical chromatogram. (Flame ionization detector.) Key: A, acetone; B, ethanol.

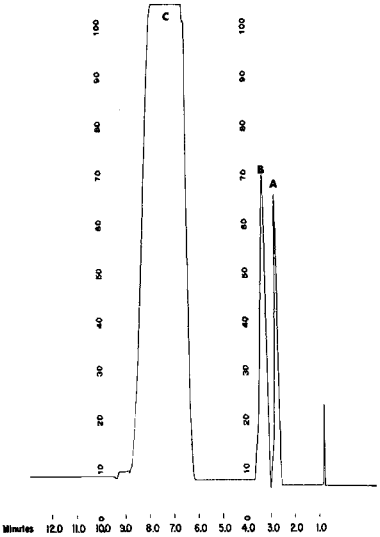

Fig. 2.—A typical chromatogram. (Thermal conductivity detector.) Key: A, acetone; B, ethanol; C, water.

Quantitation was performed, following the procedure outlined in the collaborative study of the Pharmaceutical Manufacturers Association (6).

RESULTS AND DISCUSSION

In order to demonstrate that the flame ionization detector technique was comparable to thermal conductivity detector technique, six repetitive analyses were done by each method on an experimental mouthwash product. The results obtained are summarized in Table I.

It is obvious from Table I that both methods give comparable results.

Previously during a 5-hr. period, 20 ethanol analyses could be done. Now, using a flame ionization detector, 60 analyses can be run in a 5-hr. period.

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Sample	Flame Ionization	Thermal Conductivity
1	13.0	13.1
2	13.2	13.2
3	13.1	13.1
4	13.0	13.0
5	12.9	13.0
6	13.0	13.0

REFERENCES

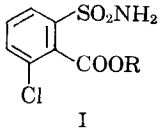
(1) Bouthelet, R. J., Caputi, A., Jr., and Ueda, M., *J. Assoc. Offic. Agr. Chemists*, **44**, 410(1961).
(2) *Ibid.*, **49**, 242(1966).
(3) Casazza, W. T., and Stellenkamp, R. J., *J. Gas Chromatog.*, **3**, 253(1965).
(4) Kawai, S., *Eisei Shikensho Hokoku*, **77**, 2733(1959).
(5) Wesselman, H. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 320(1960).
(6) "Pharmaceutical Manufacturers Association Yearbook," Pharmaceutical Manufacturers Association, Washington, D.C., 1964–1965, vol. 6, p. 571.
(7) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 432.
(8) *Ibid.*, 1st Suppl., p. 20.
(9) Hill, D. W., and Newell, H. A., *Nature*, **206**, 708 (1965).
(10) Parker, K. D., Fontan, C. R., Yee, J. L., and Kirk, P. L., *Anal. Chem.*, **34**, 1234(1965).

Anticonvulsants III. Alkyl Esters of 4-Bromo-2-sulfamoylbenzoic Acid and 4-Chloro-2-sulfamoylbenzoic Acid

By GLENN H. HAMOR and BERNARD L. REAVLIN

In addition to steric factors, electronic effects may also be important in the anticonvulsant activity of alkyl *o*-sulfamoylbenzoates and related compounds. To further explore the relationship between electronic properties and antielectroshock activity, three esters of 4-bromo-2-sulfamoylbenzoic acid and four esters of 4-chloro-2-sulfamoylbenzoic acid were prepared by the alcoholysis reaction of passing hydrogen chloride into a refluxing solution of 6-bromo- or 6-chlorosaccharin in the appropriate alcohol. The following alkyl 4-bromo- and 4-chloro-2-sulfamoylbenzoates were thus obtained: methyl; ethyl; *i*-propyl; and *sec*-butyl. In these compounds the bromine or chlorine atom is in the 4-position *para* to the alkoxycarbonyl group; thus, they do not possess the steric interactions between the large halogen atom and the ester moiety, which are believed to be necessary in the anticonvulsant activity of the related, potent *ortho*-substituted alkyl 6-chloro-2-sulfamoylbenzoates. Preliminary pharmacological results indicate that isopropyl 4-bromo-2-sulfamoylbenzoate lacks antielectroshock effects in mice.

RECENT WORK has shown that alkyl esters of 2-sulfamoylbenzoic acid, 4-amino-2-sulfamoylbenzoic acid, and 6-chloro-2-sulfamoylbenzoic acid (I) possess marked anticonvulsant activity as indicated by their prevention of the effect of strychnine or maximal electroshock in mice (1–5).



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Received April 25, 1966, from the School of Pharmacy, University of Southern California, Los Angeles 90007.
Accepted for publication September 9, 1966.
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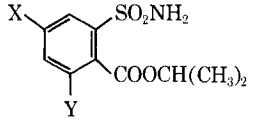
Previous paper: Hamor, G. H., and Farraj, N., *J. Pharm. Sci.*, **54**, 1265(1965).

TABLE I.—ANTICONVULSANT ACTIVITIES OF *ortho*- AND *para*-SUBSTITUTED ISOPROPYL SULFAMOYLBENZOATES[a]



Compd.	X	Y	Antielectroshock ED_{50}, Mice, mg./Kg.
1	NH_2	H	13
2	H	H	39
3	NO_2	H	240
4	Cl	H	Less active than compd. *2* above[b]
5	H	Cl	100[c]

[a] The pharmacological testing was performed by Smith Kline & French Laboratories, Philadelphia, Pa. [b] *Reference 1.* [c] Pharmacological testing was performed by Riker Laboratories, Northridge, Calif.

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In addition to steric factors, electronic effects may also be important in the anticonvulsant activity of alkyl *o*-sulfamoylbenzoates and related compounds. To further explore the relationship between electronic properties and antielectroshock activity, three esters of 4-bromo-2-sulfamoylbenzoic acid and four esters of 4-chloro-2-sulfamoylbenzoic acid were prepared by the alcoholysis reaction of passing hydrogen chloride into a refluxing solution of 6-bromo- or 6-chlorosaccharin in the appropriate alcohol. The following alkyl 4-bromo- and 4-chloro-2-sulfamoylbenzoates were thus obtained: methyl; ethyl; *i*-propyl; and *sec*-butyl. In these compounds the bromine or chlorine atom is in the 4-position *para* to the alkoxycarbonyl group; thus, they do not possess the steric interactions between the large halogen atom and the ester moiety, which are believed to be necessary in the anticonvulsant activity of the related, potent *ortho*-substituted alkyl 6-chloro-2-sulfamoylbenzoates. Preliminary pharmacological results indicate that isopropyl 4-bromo-2-sulfamoylbenzoate lacks antielectroshock effects in mice.

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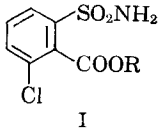

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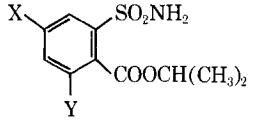

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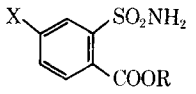
TABLE II.—ALKYL 4-BROMO- AND 4-CHLORO-2-SULFAMOYLBENZOATES[a,b]

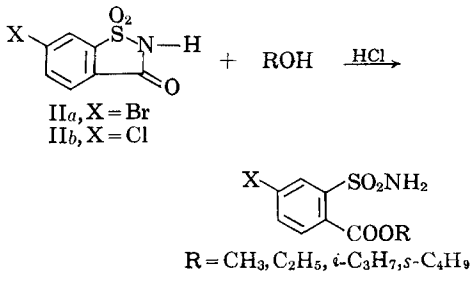


Compd.	X	R	Formula	M.p.,[c] °C.	Recrystallizing Solvent	Yield, %	Anal., % Calcd.	Found
6	Cl	CH_3	$C_8H_8ClNO_4S$	178–180	Methanol	60	C, 38.49 H, 3.20	38.51 3.34
7	Cl	C_2H_5	$C_9H_{10}ClNO_4S$	148–150	Ethanol	55	C, 41.04 H, 3.83	41.18 3.88
8	Cl	*i*-C_3H_7	$C_{10}H_{12}ClNO_4S$	125–127[d]	2-Propanol	31	C, 43.26 H, 4.36	43.44 4.45
9	Cl	*s*-C_4H_9	$C_{11}H_{14}ClNO_4S$	169–171	2-Butanol	28	C, 45.30 H, 4.80	45.26 5.03
10	Br	C_2H_5	$C_9H_{10}BrNO_4S$	152–154	Ethanol	70	C, 35.08 H, 3.24	35.06 3.38
11	Br	*i*-C_3H_7	$C_{10}H_{12}BrNO_4S$	151–153	2-Propanol	29	C, 37.29 H, 3.73	37.22 3.81
12	Br	*s*-C_4H_9	$C_{11}H_{14}BrNO_4S$	129–131	2-Butanol	29	C, 39.38 H, 4.16	39.31 4.31

[a] Analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. [b] Infrared analysis of selected compounds corresponded to the indicated structures. [c] Melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected. [d] Reported m.p. 142–144° (1).

It has been proposed that the activity of these sulfamoylbenzoates is related to their resistance to hydrolysis (5). To support this concept, data correlating anticonvulsant potency with increased chain branching of the alkyl portion of the ester were presented (5). Apart from the steric factors noted, a relationship also appears to exist between the anticonvulsant activity of these esters and the electronic effects of substituents attached to the benzene ring. The pharmacological properties reported in Table I for five previously synthesized isopropyl sulfamoylbenzoates show that the presence of electron-donating substituents *para* to the alkoxycarbonyl group tend to increase the activity, while electron-withdrawing substituents tend to decrease it. Thus, the *para* amino analog (compound *1*, Table I) possesses greater antielectroshock activity than does the desamino compound *2*, with the *para* nitro compound *3* being considerably less active.

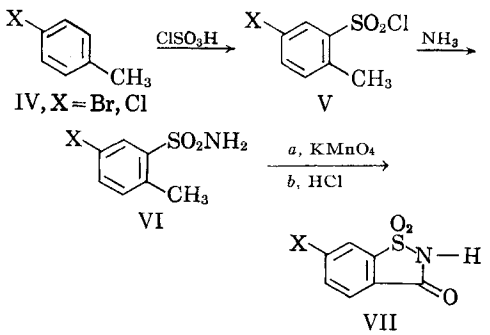
In an effort to further explore the relationship between electronic effects and anticonvulsant activity, a number of esters containing a bromine or a chlorine atom *para* to the alkoxycarbonyl group were prepared. The three alkyl 4-bromo-2-sulfamoylbenzoates and the four alkyl 4-chloro-2-sulfamoylbenzoates synthesized are listed and their properties given in Table II, and have the formula shown



IIa, X = Br
IIb, X = Cl

III

Scheme I



IV, X = Br, Cl

V

VI

VII

Scheme II

below (III). These compounds were prepared by the alcoholysis reaction of passing hydrogen chloride into a refluxing solution of 6-bromosaccharin or 6-chlorosaccharin in the appropriate alcohol, as pictured in Scheme I (4, 5).

6-Bromosaccharin (II*a*) and 6-chlorosaccharin (II*b*) were synthesized by the reaction sequence shown in Scheme II. Chlorosulfonation of the *p*-halotoluene (IV) by the procedure of Huntress (6) followed by treatment with aqueous ammonia gives 5-halo-2-methylbenzenesulfonamide (VI). Alkaline potassium permanganate oxidation of VI by the method of Noyes (7) gives the known 6-bromosaccharin (VII) (8) and 6-chlorosaccharin (VII) (8).

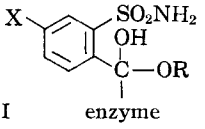
Preliminary pharmacological results indicate isopropyl 4-bromo-2-sulfamoylbenzoate,[1] (compound *11*, Table II), lacks antielectroshock effects when administered intraperitoneally, in suspension form, to mice at a dose of 100 mg./Kg. (0/10 mice protected). The compound was also inactive at doses of 200 mg./Kg. i.p. and 250 mg./Kg. p.o. The LD_{50} is greater than 800 mg./Kg. (0/10 mice i.p.). It is of interest to note that isopropyl 4-chloro-2-

[1] The authors thank Riker Laboratories, Northridge, Calif. for performing the pharmacological testing.

sulfamoylbenzoate has previously been reported to be less active than the deschlorocompound isopropyl 2-sulfamoylbenzoate, but no pharmacological data were given (1).

DISCUSSION

One possible explanation for the greatly reduced activity of the *p*-bromo-, *p*-chloro-, and *p*-nitro-substituted *o*-sulfamoylbenzoates when compared with the *p*-amino and desamino compounds may be obtained by considering that these esters are undergoing nucleophilic attack in a manner that approximates alkaline hydrolysis ($B_{AC}2$) in a chemical system (9). Alkaline hydrolysis of esters has been shown to be quite sensitive to the electronic effects of substituents on a benzene ring. One might then postulate that during the hydrolytic process these sulfamoylbenzoates are forming an enzyme–ester intermediate analogous to the tetrahedral intermediate demonstrated in $B_{AC}2$ hydrolysis (10). If this is the case, then electron-attracting substituents by increasing the polarization at the ester carbonyl group promote the formation of the enzyme–ester complex (VIII) and thus increase the rate of hydrolysis leading to pharmacologically inactive compounds.



VIII enzyme

Isopropyl 6-chloro-2-sulfamoylbenzoate (Table I, compound *5*), in which the chlorine atom is *ortho* to the ester moiety, has antielectroshock activity intermediate between that of the unsubstituted compound isopropyl *o*-sulfamoylbenzoate (compound *2*) and the compounds with electron-attracting group *para* to the alkoxycarbonyl group. The electron-withdrawing properties of the *ortho* chlorine atom, which would tend to labilize the ester to hydrolysis, are opposed by a steric hindrance to hydrolysis as a result of the steric effects of the chlorine atom. It may be of interest to note that correlation of antielectroshock activity of some 12 selected sulfamoylbenzoates with substituent effects by subjection to regression analysis according to the method of Hansch[2] (11), shows that electronic, steric, and hydrophobic factors are all important.

EXPERIMENTAL

4-Bromo- and 4-Chloro-2-sulfamoylbenzoic Acid Esters.—These esters were prepared by acidic alcoholysis of 6-bromosaccharin (II*a*) and 6-chlorosaccharin (II*b*). The known saccharins were synthesized by chlorosulfonation of the corresponding *p*-halotoluene (IV) (6), followed by treatment with aqueous ammonia which gave the 5-halo-2-methylbenzenesulfonamide (VI). The subsequent alkaline potassium permanganate oxidation of (VI) by the method of Noyes (7) yielded the desired 6-bromo- and 6-chlorosaccharins.

The esters synthesized are listed and their properties given in Table II. The alcoholysis procedure used will be illustrated by the following account of the preparation of isopropyl 4-bromo-2-sulfamoylbenzoate (compound *11*). Five grams (0.02 mole) of 6-bromosaccharin [m.p. 213–214°; reported m.p. 217° (12)] was placed in a two-necked flask fitted with a reflux condenser protected by a calcium chloride tube, and a gas inlet tube. To this was added 100 ml. of dry 2-propanol; the saccharin dissolved at the reflux temperature. The refluxing was continued while a continuous current of dry hydrogen chloride gas was passed through the solution for 2.5 hr. The passage of hydrogen chloride gas was discontinued and the reflux condenser removed; the solution was then heated for several minutes to expel hydrogen chloride. After partial evaporation, the cooled solution gave 1.8 Gm. (28.5%) of crystalline isopropyl 4-bromo-2-sulfamoylbenzoate (compound *11*), m.p. 151–153°.

REFERENCES

(1) Loev, B., and Kormendy, M., *J. Org. Chem.*, **27,** 1703(1962).
(2) *Ibid.*, **27,** 2177(1962).
(3) Park, A., and Bester, J. F., *J. Pharm. Sci.*, **54,** 474 (1965).
(4) Hamor, G. H., and Janfaza, M., *ibid.*, **52,** 102(1963).
(5) Hamor, G. H., and Farraj, N., *ibid.*, **54,** 1266(1965).
(6) Huntress, E. H., and Carten, F. H., *J. Am. Chem. Soc.*, **62,** 513(1940).
(7) Noyes, W. A., *Am. Chem. J.*, **8,** 167(1886).
(8) De Roode, R., *ibid.*, **13,** 217(1891).
(9) Bender, M. L., *Chem. Rev.*, **60,** 53(1960).
(10) Bender, M. L., *J. Am. Chem. Soc.*, **84,** 2582(1962).
(11) Hansch, C., and Fujita, T., *ibid.*, **86,** 1616(1964).
(12) Remsen, I., and Bayley, W. S., *Am. Chem. J.*, **8,** 229(1886).

[2] The authors are grateful to Dr. Corwin Hansch, Chemistry Department, Pomona College, Claremont, Calif., for performing this analysis.

Mouse Preparation for Pharmacodynamics Studies

By G. E. DEMAREE, R. L. MUNDY, M. H. HEIFFER, E. H. HERMAN, and D. P. JACOBUS

A method is described for recording an essentially undamped arterial pulse pressure in the mouse using methyl-2-cyanoacrylate physiological adhesive to secure a 22 gauge needle shaft in the carotid artery for cannulation. A miniaturized pneumotachograph screen for recording respiratory exchange in the mouse is described. Pulse pressure, ECG, and respiratory exchange tracings are shown.

PREVIOUS methods for recording directly the arterial pulse pressure of the mouse have been handicapped by low frequency response of the hydraulic and electronic systems utilized (1–6). Through the use of a large bore, hard plastic cannula, and a small displacement strain gauge pressure transducer in conjunction with rapid response electronics, an essentially undamped pulse pressure was obtained.

This report deals with simple techniques and instruments adapted for the direct recording of physiological measurements in the anesthetized mouse.

Preparation.—Female ICR mice from the Walter Reed colony weighing 25–35 Gm., aged 6–7 weeks, were used. Sodium pentobarbital (75 mg./Kg.) was administered intraperitoneally for anesthesia. The mouse was secured to the operating surface in a supine position using masking tape at each appendage and either side of the muzzle to secure the ears and whiskers to the table. The left femoral vein was exposed. A 27-gauge needle shaft, attached to a 15 μl. polyethylene cannula, was inserted into the vein and secured in position using methyl-2-cyanoacrylate monomer[1] as an adhesive. Drugs were introduced into this cannula in volumes of 10 μl. or less and flushed into circulation with 30 μl. of 0.9% NaCl solution. Additional doses of anesthetic (150–300 mcg.) were administered as needed through this cannula.

Cannulation of the Trachea.—The ventral neck area was exposed by a 3 cm. longitudinal incision. The strap muscles were exposed by separating the submaxillary glands by blunt dissection. (The fused ends of melting point capillaries worked well as dissecting probes.) The trachea was exposed by blunt dissection of the strap muscles. A tracheotomy was performed below the larynx, and a polyethylene tube large enough to fit snugly was inserted into the trachea.

Carotid Artery Cannulation.—The right carotid artery was visualized through the tissue and isolated to include the vagus nerve and as much fascia as possible using curved forceps for blunt dissection. The artery, nerve, and supporting fascia were stabilized using a single backtie which was secured to the table with beeswax. The exposed artery was supported by a melting point capillary. A 22-gauge needle shaft attached to a saline-filled vinyl cannula (B. D., No 442T) was inserted into the artery. The arterial wall was fixed to the cannula tip with the monomer adhesive.

All operative procedures were carried out with the aid of an illuminated magnifier.

ECG Leads.—Recording needle electrodes were implanted in the left hind leg and right foreleg; a needle electrode was implanted in the right hind leg as a ground lead.

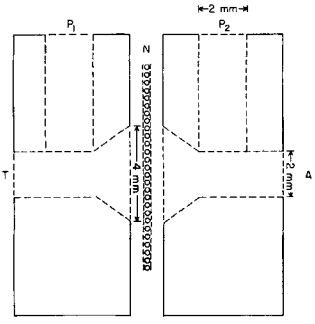


Fig. 1.—Diagram of pneumotachograph screen. Broken lines outline axes and size of holes bored in 4 mm. thick acrylic plastic. Nylon woven screen (N) in cemented between plastic halves. P_1 and P_2 lead to transducer. T leads to mouse tracheal cannula. A opens to ambient pressure. Over-all dimensions: 8 × 8 × 8 mm.

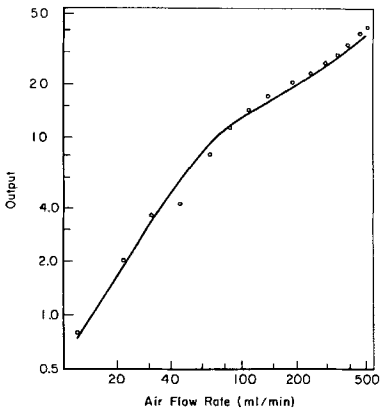


Fig. 2.—Calibration curve of pneumotachograph screen. Output voltage is in arbitary units.

Received July 22, 1966, from the Pharmacology Department, Walter Reed Army Institute of Research, Washington, D. C. 20012.

Accepted for publication August 15, 1966.

A portion of this work dealing with direct measurement of arterial pressure was reported in *Federation Proc.*, **24**, 365(1965).

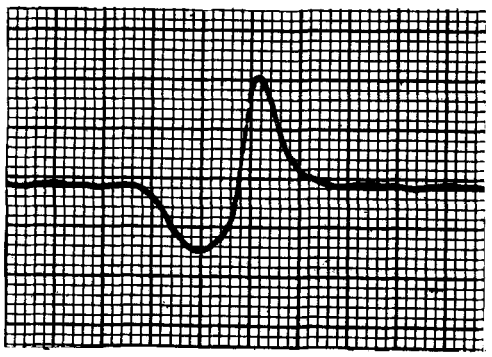
[1] Supplied through the courtesy of Ethicon, Inc., Somerville, N. J. For a description of the properties of this material see: Coover, H. W., Joyner, F. B., Shearer, N. H., and Wicker, T. H., *Soc. Plastics. Eng. J.*, **15**, 413(1959).

Respiratory Exchange Measurements.—The tracheal cannula was attached to a pneumotachograph screen which was made from a nylon screen cemented between two pieces of acrylic plastic. (See Fig. 1.) The two chambers of the pneumotachograph screen were connected by polyethylene tubing to the two sides of a Sanborn 270 differential gas pressure transducer which was activated by a Sanborn 350-1100 carrier preamplifier.

Arterial Pulse Pressure Measurements.—The arterial cannula was attached to a Sanborn 267B differential pressure transducer which was activated by a Sanborn 350-1100 carrier preamplifier.

ECG Recording.—The ECG impulses were amplified by a Phipps and Bird balanced biological preamplifier. The output of this first stage amplification was fed into a Sanborn 350-3200 ECG preamplifier.

All recordings were made on a Sanborn 964 polygraph at 50 mm./sec. and monitored on a fast sweep oscilloscope.



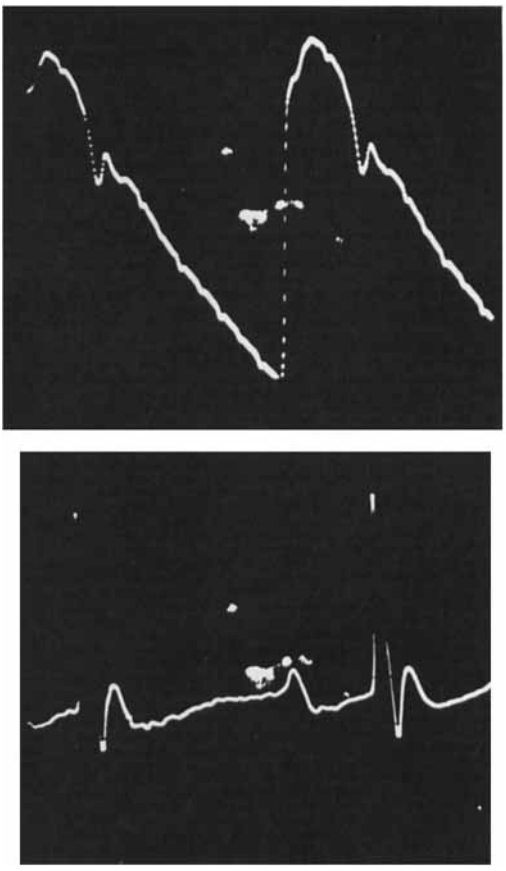


Fig. 3.—Tracings of physiological measurements of the mouse. Key: Bottom left, respiratory exchange cycle (chart speed: 50 mm./sec.); peak inspiration reached 55 ml./min.; peak expiration rate was 80 ml./min. Top right, pulse pressure curve (sweep time: 200 msec.); diastolic pressure is 60 mm. Hg; systolic pressure is 90 mm. Hg; heart rate is 550/min. Bottom right, ECG tracing (sweep time: 200 msec.).

The calibration curve of the improvised pneumotachograph screen-transducer assembly is shown in Fig. 2. A typical recording of respiratory cycle is shown in Fig. 3 (bottom left). Pulse pressure and ECG oscilloscope tracings are shown in Fig. 3, top right and bottom right, respectively.

REFERENCES

(1) McMaster, P. O., *J. Exptl. Med.*, **74**, 29(1941).

(2) Pradhan, S. N., Achinstein, B., and Shaw, M. J., *Federation Proc.*, **14**, 380(1955).

(3) Pradhan, S. N., Achinstein, B., and Shaw, M. J., *J. Pharmacol. Exptl. Therap.*, **113**, 44(1955).

(4) Pradhan, S. N., Achinstein, B., and Shaw, M. J., *Am. J. Physiol.*, **184**, 599(1956).

(5) Pradhan, S. N., Achinstein, B., and Shaw, M. J., *Arch. Intern. Pharmacodyn.*, **120**, 1(1959).

(6) Wu, H. C., and Visscher, M. B., *Am. J. Physiol.*, **153** 330(1948).

Isolation of Two Bisbenzylisoquinoline Alkaloids from the Rhizomes and Roots of *Xanthorhiza simplicissima*

By JOSEPH E. KNAPP*, FAWZY T. HUSSEIN†, JACK L. BEAL, RAYMOND W. DOSKOTCH, and TOSHIAKI TOMIMATSU‡

The alkaloids obamegine and oxyacanthine were isolated from the tertiary alkaloid fraction of the rhizomes and roots of *Xanthorhiza simplicissima*. The tertiary alkaloids were separated from the quaternary fraction using a chromatographic column of a polyamide (nylon 66). The mixture of obamegine and oxyacanthine was separated by partition chromatography.

DURING THE course of the investigation of the quaternary alkaloids of the rhizomes and roots of *Xanthorhiza simplicissima* Marsh., a quantity of tertiary alkaloid material was isolated (1). This investigation was undertaken to isolate and identify the alkaloids in the tertiary fraction.

EXPERIMENTAL[1]

Plant Material.—The rhizomes and roots of *X. simplicissima* Marsh. used in this investigation were obtained in granulated form from S. B. Penick and Co. (lot B-19753).

Extraction.—Twenty kilograms of the plant material was extracted with ethanol U.S.P. by percolation at room temperature. Extraction was continued until the extractive failed to give a positive test with Valser's solution (2). Evaporation of the ethanol *in vacuo* at 40° or less yielded 1.7 Kg. of residue which was dissolved in 5% hydrochloric acid solution and filtered. The filtrate was basified to pH 8–9 (indicated by pHydrion paper) with 10% ammonium hydroxide solution and extracted with ethylene dichloride. After drying over anhydrous sodium sulfate the ethylene dichloride was removed *in vacuo* to yield 19.6 Gm. of crude tertiary alkaloids. Thin-layer chromatography on Silica Gel G,[2] using a solvent system of benzene–acetone–ammonium hydroxide (32:32:1) indicated the presence of only two tertiary alkaloids in the mixture; a spot at the origin was also evident indicating the presence of quaternary alkaloids in the mixture. The alkaloid spots were detected with Dragendorff's spray reagent (3). By means of a modification of a spot test for phenols (4) it was possible to determine on the thin-layer chromatogram that both tertiary alkaloids were phenolic. This was performed by spraying the developed chromatogram with a 2% solution of phosphomolybdic acid in 50% aqueous acetone and then exposing the chromatogram to ammonia fumes. Phenolic compounds will give a blue to black color with this treatment.

Separation of the Quaternary from the Tertiary Alkaloids.—The small quantity of quaternary alkaloids present in the crude tertiary alkaloid fraction were removed by utilizing a chromatographic column prepared with a polyamide (nylon 66) powder.[3] Two glass chromatographic columns (4 × 40 cm.) each containing 200 Gm. of the polyamide powder were prepared. One column was prepared by the slurry method in water while the other by the slurry method in benzene. The crude alkaloid mixture (19.6 Gm.) was dissolved in chloroform and adsorbed onto about 5 Gm. of the polyamide powder followed by evaporation of the solvent. The powder was placed on the column prepared with water and the column eluted with water. Several colored, fluorescent bands were eluted and were shown by thin-layer chromatography to be traces of quaternary alkaloids. The column was next eluted with methanol and the effluent reduced in volume and again adsorbed onto about 5 Gm. of the polyamide powder as before. The resulting powder was placed on the column prepared with benzene and the column eluted with benzene until the eluate was alkaloid free (as indicated by Valser's test). Evaporation of the benzene from the effluent, *in vacuo*, yielded 12.3 Gm. of semicrystalline material. This material was shown by thin-layer chromatography to consist mainly of the two phenolic alkaloids.

Separation of the Tertiary Alkaloids by Partition Chromatography.—The two tertiary alkaloids were purified by partition chromatography according to the procedure of Brown and Kupchan (5). The solvent system consisted of methanol–water (5:1, v/v) as the stationary phase and ethylene dichloride–Skellysolve F (1:2, v/v) as the mobile phase with a diatomaceous earth[4] serving as the inert support. Bromothymol blue was incorporated into the stationary phase to detect the alkaloid bands as they moved through the column. A chromatographic column (2 × 90 cm.) was prepared as follows. The solvent mixture of Skellysolve F, 1600

Received April 26, 1966, from the College of Pharmacy, Ohio State University, Columbus 43210.

Accepted for publication September 27, 1966.

Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported by research grant GM-05640 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* American Foundation for Pharmaceutical Education Fellow. Sydnor Barksdale Penick Memorial Fellowship, 1965–1967.

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[1] Melting points were determined with a Thomas-Hoover Uni-Melt capillary melting point apparatus. The infrared spectra were taken in chloroform and as KBr pellets on a Perkin-Elmer model 237 infrared spectrophotometer. The ultraviolet spectra were determined on a Perkin-Elmer model 4000 Spectracord.

[2] Made by E. Merck (Darmstadt, West Germany); obtained through Brinkmann Instruments, Inc., Cantiague Road, Westbury, N. Y.

[3] Obtained as Polypenco Powder from the Polymer Corp., Pittsburgh, Pa.

[4] Marketed as Hyflo Super-Cel by Johns-Manville Corp., New York, N. Y. Purified by suspending in 4 *N* HCl, with occasional agitation, for 24 hr. It was then filtered on a Büchner funnel and washed successively with distilled water, methanol, chloroform, benzene, and finally methanol again. After air-drying it was dried in an oven at 105° and stored in an air-tight container. Skellysolve F is a petroleum ether fraction, b.p. 30–60°. Ethylene dichloride C.P., Union Carbide Co., methanol A.R.

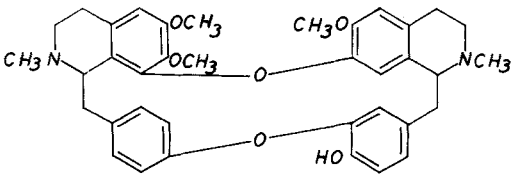
ml.; ethylene dichloride, 800 ml.; methanol, 300 ml.; and distilled water, 60 ml., was shaken well and allowed to equilibrate overnight. Two hundred grams of diatomaceous earth in a 1-L. glass-stoppered flask was treated with 132 ml. of the stationary phase (lower phase) of the solvent mixture, in which 60 mg. of bromothymol blue was dissolved. The flask was vigorously shaken to remove all lumps and the mixture then packed into the column in about 1-cm. increments. The mobile phase was then passed through the column until all the air had been displaced and the column was a uniform yellow color. A solution of 1.0 Gm. of the alkaloid mixture in 25 ml. of the mobile phase (upper phase) was added to the column. When the last portion of the alkaloid mixture had passed into the top of the column, elution was begun at a rate of about 100 ml./hr. The alkaloids were observed as two blue bands advancing down the column and were collected separately. The first band was labeled "alkaloid I" and the second "alkaloid II." Both bands were shown by thin-layer chromatography to consist of only one alkaloid. In this fashion, the remainder of the alkaloid mixture was separated.

Identification of Alkaloid I as Oxyacanthine.—The total effluent fractions containing alkaloid I were evaporated *in vacuo*. The residue was dissolved in ether and washed several times with a 10% sodium carbonate solution followed by distilled water. The ether solution was dried over anhydrous sodium sulfate followed by evaporation *in vacuo* to yield a residue of 4.75 Gm. which resisted all attempts of crystallization of the free base. The alkaloid was crystallized as the hydrochloride salt from a 5% hydrochloric acid solution. It was recrystallized from water, after saturation with sodium chloride, to give needles melting above 280° dec.

An aqueous solution of 1.4 Gm. of the hydrochloride salt was made basic with ammonia and extracted with chloroform. After drying over anhydrous sodium sulfate and removal of the chloroform *in vacuo*, a residue was obtained which crystallized from petroleum ether (b.p. 30–60°) as rosettes, m.p. 212–214°. $[\alpha]_D^{29}$ + 285.6° (c = 0.5, $CHCl_3$). The ultraviolet absorption spectrum was indicative of a benzylisoquinoline nucleus, having a $\lambda_{max.}^{MeOH}$ 283 mμ (log ε 3.95) (6). The infrared absorption spectrum in chloroform indicated a hydroxyl function ($\nu_{max.}$ = 3510 cm.$^{-1}$) in the molecule. The NMR spectrum in deuterochloroform gave indications of two *N*-methyl groups (7.40 and 7.45 τ, 3H each) and three *O*-methyl groups (6.25, 6.40, and 6.85 τ, 3H each) which suggested an oxyacanthine-type alkaloid with methoxyl groups at 6, 6′, and 7 positions, respectively, of the bisbenzylisoquinoline nucleus (7). The compound was identified to be oxyacanthine (I) on the basis of melting point, optical rotation, NMR data, and comparison of ultraviolet and infrared absorption spectra with an authentic sample of oxyacanthine.[5]

Identification of Alkaloid II as Obamegine.—The total effluent fractions containing alkaloid II were combined and treated in the same manner as for alkaloid I to yield 2.1 Gm. of residue. The material crystallized readily from benzene as colorless needles, m.p. 163–168° dec. $[\alpha]_D^{29}$ + 99° (c = 0.5, $CHCl_3$). The infrared spectrum in chloroform indicated the presence of hydroxyl function ($\nu_{max.}$ = 3525 cm.$^{-1}$). The ultraviolet spectrum gave $\lambda_{max.}^{MeOH}$ 280 mμ (log ε, 3.19).

The NMR spectrum[6] in deuterochloroform showed two *N*-methyl groups (7.6 and 7.75 τ, 3H each) and two *O*-methyl groups (6.3 and 6.4 τ, 3H each) which suggested a berbamine-type alkaloid with methoxyl groups at the 6 and 6′ positions of the molecule (7). The alkaloid was identified as obamegine (II) on the basis of melting point, optical rotation, NMR data, and comparison of infrared and ultraviolet absorption spectra with an authentic sample of obamegine.



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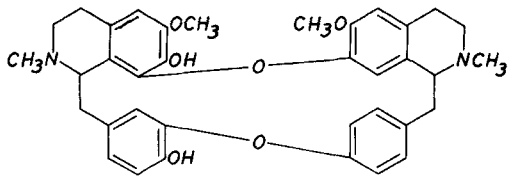

II

DISCUSSION

X. simplicissima is an example of a plant which when investigated with the advantage of more modern techniques such as chromatography was found to contain several more alkaloids than reported in the older literature. In 1862, Perrins (8) reported the isolation of berberine from the rhizomes and roots of the plant; Jones (9), in 1886 verified the presence of berberine and suggested the presence of a second alkaloid. Two additional quaternary alkaloids, jatrorrhizine and magnoflorine (1), were isolated in 1963. This investigation has shown that the rhizomes and roots contain the tertiary bisbenzylisoquinoline alkaloids oxyacanthine (1) and obamegine (II), thus bringing the total to five alkaloids reported to be isolated from *X. simplicissima* Marsh.

Oxyacanthine, first obtained in crystalline form by Spath and Kolbe in 1925 (10), has been found previously in eight species of the genus *Berberis* (*Berberidaceae*), seven species of the genus *Mahonia* (*Berberidaceae*), and in the species *Cocculus leaeba* (*Menispermaceae*) (11).

Obamegine was first isolated by Tomita and Kugo (12) in 1959 from *Berberis tschonoskyana* and recently by Tominatsu and Beal (13) from *Thalictrum rugosum* (*Ranunculaceae*). This is the third report of the isolation of this compound.

[5] The authors thank Dr. E. Fujita, Institute for Chemical Research, Kyoto University, Takatsuki, Osaku-fu, Japan, for an authentic sample of oxyacanthine.

[6] The authors thank Dr. David R. Dalton for determining the NMR spectra of obamegine and oxyacanthine. These were determined on a Varian A-60 NMR spectrometer.

REFERENCES

(1) Hussein, F. T., Beal, J. L., and Cava, M. P., *Lloydia*, **26**, 254(1963).
(2) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 1096.
(3) Block, R. J., Durrum, E. L., and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press Inc., New York, N. Y., 1958, p. 362.
(4) Platokovskaya, V. M., and Vatkina, S. G., *J. Appl. Chem. (U.S.S.R.)*, **10**, 202(1937); through *Chem. Abstr.*, **31**, 4232(1937).
(5) Brown, K. S., Jr., and Kupchan, S. M., *J. Chromatog.*, **9**, 71(1962).
(6) Sangster, A. W., and Stuart, K. L., *Chem. Rev.*, **65**, 84(1965).
(7) Bick, I. R. C., Harley-Mason, J., Shepard, N., and Vernengo, M. J., *J. Chem. Soc. (London)*, **1961**, 1896.
(8) Perrins, J. D., *Am. J. Pharm.*, **34**, 356(1862).
(9) Jones, S. S., *ibid.*, **58**, 161(1886).
(10) Grundon, M. F., in "Progress in Organic Chemistry," vol. 6, Cook, Sir J., and Carruthers, W., eds., Butterworths, Inc., Washington, D. C., 1964, pp. 38–85.
(11) Boit, H. G., "Ergnebissne der Alkaloid-chemie bis 1960," Akademie-Verlag, Berlin, Germany, 1961, p. 236.
(12) Tomita, M., and Kugo, T., *Yakugaku Zasshi*, **79**, 317 (1959).
(13) Tomimatsu, T., and Beal, J. L., *J. Pharm. Sci.*, **55**, 208(1966).

Ion-Exchange Separation and Ultraviolet Determination of Phenylephrine, Codeine, and Selected Antihistamines

By K. O. MONTGOMERY, P. V. JENNINGS, and M. H. WEINSWIG

A method for the isolation and determination of phenylephrine, codeine, or an antihistamine as a single active ingredient is presented. Also, a method is presented for the separation and determination of an antihistamine and codeine or an antihistamine and phenylephrine in pharmaceutical products containing these combinations. All of the amines are extracted with a strong cation-exchange resin, AG 50W-X4. The phenylephrine or codeine is eluted with 1 *N* hydrochloric acid in 60 per cent methanol in water. The antihistamine is then eluted with 3.5 *N* hydrochloric acid in 40 per cent methanol in water. The compounds are determined by subjecting the eluates to ultraviolet spectrophotometry. The assay is used successfully on several commercial products.

A LARGE NUMBER of pharmaceuticals are presently marketed for treatment of the common cold and other respiratory disorders. These products use a large variety of active ingredients but for the most part they are, chemically, basic amines. These amines are in the form of vasoconstrictors, antitussives, and antihistamines.

Phenylephrine is widely used as a vasoconstrictor in these products. The assay for this compound in liquid pharmaceuticals has been difficult because of the water solubility of phenylephrine even as the base. The U.S.P. (1) describes the time honored bromination procedure. This procedure is ineffective when oxidizable substances are present because of their reactions with bromine. Reducing sugars are a good example of this type of interference. The reactions of phenols with ferricyanide and 4-aminoantipyrine has been proposed by several workers (2, 3). Chafetz (4) oxidized phenylephrine and other phenylethanolamines to their aromatic aldehydes, extracted with organic solvents, and determined the ultraviolet absorption. Clark and Rosenberg (5) reported phenylephrine separation using a Levine column with acetylation on the column. The phenylephrine was determined in the eluates. Auerbach (6) used a diazotization technique where phenylephrine acted as the coupler. Kelly and Auerbach (7) utilized ion-exchange chromatography in a method similar to that presented in this paper.

Codeine, which is a very popular antitussive, has been the subject of extensive analytical study for many years because of its wide usage. Nonaqueous titrimetry has been utilized by several workers (8–10). Artamonov and Bugreeva (11) applied the techniques of high frequency titration for the determination of codeine.

Several colorimetric methods have been proposed (12, 13) as well as fluorimetry (14, 15), refractometry (16), and spectrophotometry (17, 18).

Various types of chromatographic procedures have been used for codeine analysis. Izmailov and co-workers (19) used paper chromatographic techniques to separate codeine from other poppy extract alkaloids. Adsorption columns of silicic acid were utilized by Andreeva and Figurovskii (20). The more recent techniques of gas chromatography (21) and thin-layer chromatography (22) have also been reported.

The common methods of assay for antihistamines include nonaqueous titrimetry (23, 24), ultraviolet spectrophotometry (25), and reineckate salt formation with subsequent colorimetric determination of the acetone solution (26). A widely used colorimetric method, specific for compounds containing the 2-pyridyl group, is based on the König reaction with cyanogen bromide and aniline. This was described by Jones and Brody (27) and later modified by Hudanick (28). Gas chromatography has been used successfully by Celeste and Turczan (29) and by Fontan and co-workers (30).

This paper utilizes strongly acidic cation-exchange resins to separate the amines from common dosage form ingredients. The amines are then eluted separately from the resin using specific concentrations of hydrochloric acid. The amines are deter-

Received August 18, 1966, from the College of Pharmacy, Butler University, Indianapolis, Ind. 46207.
Accepted for publication September 27, 1966.

REFERENCES

(1) Hussein, F. T., Beal, J. L., and Cava, M. P., *Lloydia*, **26**, 254(1963).
(2) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 1096.
(3) Block, R. J., Durrum, E. L., and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press Inc., New York, N. Y., 1958, p. 362.
(4) Platokovskaya, V. M., and Vatkina, S. G., *J. Appl. Chem. (U.S.S.R.)*, **10**, 202(1937); through *Chem. Abstr.*, **31**, 4232(1937).
(5) Brown, K. S., Jr., and Kupchan, S. M., *J. Chromatog.*, **9**, 71(1962).
(6) Sangster, A. W., and Stuart, K. L., *Chem. Rev.*, **65**, 84(1965).
(7) Bick, I. R. C., Harley-Mason, J., Shepard, N., and Vernengo, M. J., *J. Chem. Soc. (London)*, **1961**, 1896.
(8) Perrins, J. D., *Am. J. Pharm.*, **34**, 356(1862).
(9) Jones, S. S., *ibid.*, **58**, 161(1886).
(10) Grundon, M. F., in "Progress in Organic Chemistry," vol. 6, Cook, Sir J., and Carruthers, W., eds., Butterworths, Inc., Washington, D. C., 1964, pp. 38–85.
(11) Boit, H. G., "Ergnebissne der Alkaloid-chemie bis 1960," Akademie-Verlag, Berlin, Germany, 1961, p. 236.
(12) Tomita, M., and Kugo, T., *Yakugaku Zasshi*, **79**, 317 (1959).
(13) Tomimatsu, T., and Beal, J. L., *J. Pharm. Sci.*, **55**, 208(1966).

Ion-Exchange Separation and Ultraviolet Determination of Phenylephrine, Codeine, and Selected Antihistamines

By K. O. MONTGOMERY, P. V. JENNINGS, and M. H. WEINSWIG

A method for the isolation and determination of phenylephrine, codeine, or an antihistamine as a single active ingredient is presented. Also, a method is presented for the separation and determination of an antihistamine and codeine or an antihistamine and phenylephrine in pharmaceutical products containing these combinations. All of the amines are extracted with a strong cation-exchange resin, AG 50W-X4. The phenylephrine or codeine is eluted with 1 *N* hydrochloric acid in 60 per cent methanol in water. The antihistamine is then eluted with 3.5 *N* hydrochloric acid in 40 per cent methanol in water. The compounds are determined by subjecting the eluates to ultraviolet spectrophotometry. The assay is used successfully on several commercial products.

A LARGE NUMBER of pharmaceuticals are presently marketed for treatment of the common cold and other respiratory disorders. These products use a large variety of active ingredients but for the most part they are, chemically, basic amines. These amines are in the form of vasoconstrictors, antitussives, and antihistamines.

Phenylephrine is widely used as a vasoconstrictor in these products. The assay for this compound in liquid pharmaceuticals has been difficult because of the water solubility of phenylephrine even as the base. The U.S.P. (1) describes the time honored bromination procedure. This procedure is ineffective when oxidizable substances are present because of their reactions with bromine. Reducing sugars are a good example of this type of interference. The reactions of phenols with ferricyanide and 4-aminoantipyrine has been proposed by several workers (2, 3). Chafetz (4) oxidized phenylephrine and other phenylethanolamines to their aromatic aldehydes, extracted with organic solvents, and determined the ultraviolet absorption. Clark and Rosenberg (5) reported phenylephrine separation using a Levine column with acetylation on the column. The phenylephrine was determined in the eluates. Auerbach (6) used a diazotization technique where phenylephrine acted as the coupler. Kelly and Auerbach (7) utilized ion-exchange chromatography in a method similar to that presented in this paper.

Codeine, which is a very popular antitussive, has been the subject of extensive analytical study for many years because of its wide usage. Nonaqueous titrimetry has been utilized by several workers (8–10). Artamonov and Bugreeva (11) applied the techniques of high frequency titration for the determination of codeine.

Several colorimetric methods have been proposed (12, 13) as well as fluorimetry (14, 15), refractometry (16), and spectrophotometry (17, 18).

Various types of chromatographic procedures have been used for codeine analysis. Izmailov and co-workers (19) used paper chromatographic techniques to separate codeine from other poppy extract alkaloids. Adsorption columns of silicic acid were utilized by Andreeva and Figurovskii (20). The more recent techniques of gas chromatography (21) and thin-layer chromatography (22) have also been reported.

The common methods of assay for antihistamines include nonaqueous titrimetry (23, 24), ultraviolet spectrophotometry (25), and reineckate salt formation with subsequent colorimetric determination of the acetone solution (26). A widely used colorimetric method, specific for compounds containing the 2-pyridyl group, is based on the König reaction with cyanogen bromide and aniline. This was described by Jones and Brody (27) and later modified by Hudanick (28). Gas chromatography has been used successfully by Celeste and Turczan (29) and by Fontan and co-workers (30).

This paper utilizes strongly acidic cation-exchange resins to separate the amines from common dosage form ingredients. The amines are then eluted separately from the resin using specific concentrations of hydrochloric acid. The amines are deter-

Received August 18, 1966, from the College of Pharmacy, Butler University, Indianapolis, Ind. 46207.
Accepted for publication September 27, 1966.

mined in the eluate by ultraviolet spectrophotometry.

EXPERIMENTAL

Apparatus.—Glass column 20 cm. × 1 cm. with stopcock made of Teflon and containing built in needle valve for control of flow rate. The column is also fitted with a reservoir with a capacity of 250 ml.

A suitable recording ultraviolet spectrophotometer such as the Beckman DK-2A or Spectronic 505 which records in absorbance units.

Reagents.—Cationic exchange resin AG 50W-X4 100–200 mesh in hydrogen form available from Bio-Rad Laboratories, Richmond, Calif. Enough resin, about 3 Gm., is added in the form of a slurry to the glass column and rinsed with water.

Hydrochloric acid, 0.05 *N* in 50% methanol in water. Hydrochloric acid, 1.0 *N* in 60% methanol in water. Hydrochloric acid, 3.5 *N* in 40% methanol in water.

Standard Solutions.—Prepare the following standard solutions using U.S.P., N.F., or other suitable reference standards.

(*a*) Phenylephrine hydrochloride, 10 mg./200 ml., 1.0 *N* hydrochloric acid in 60% methanol in water. (*b*) Codeine phosphate, 20 mg./200 ml., 1.0 *N* hydrochloric acid in 60% methanol in water. (*c*) Chlorpheniramine maleate, 4 mg./200 ml., 3.5 *N* hydrochloric acid in 40% methanol in water. (*d*) Pheniramine maleate, 6 mg./200 ml., 3.5 *N* hydrochloric acid in 40% methanol in water. (*e*) Promethazine hydrochloride, 12.5 mg./200 ml., 3.5 *N* hydrochloric acid in 40% methanol in water. (*f*) Methapyrilene hydrochloride, 4 mg./200 ml., 3.5 *N* hydrochloric acid in 40% methanol in water.

Sample Treatment.—Use 10-ml. samples of each product except antihistamines *A*[1] (5 ml.), *B*[2] (20 ml.), and *C*[3] (20 ml.). Pipet the sample into the reservoir, rinse the pipet with distilled water, and add to the reservoir.

Add distilled water to the sample to make the volume approximately 100 ml. and mix well.

Allow the sample solution to flow through the resin bed at the rate of 2–3 ml./min. Wash the column by adding 100 ml. of distilled water and allow it to flow through the resin at the rate of 5 ml./min.

Traces of aromatic amines from flavors or coloring agents are removed by allowing 50 ml. of 0.05 *N* hydrochloric acid in 50% methanol in water to flow through the column at 5 ml./min.

For products containing phenylephrine or codeine as a single active ingredient, place a 200-ml. volumetric flask under the column and add 190 ml. of 1.0 *N* hydrochloric acid in 60% methanol in water to the reservoir. Allow this to flow through the column at the rate of 3 ml./min. The volume is adjusted with 1.0 *N* hydrochloric acid in 60% methanol in water.

For products containing an antihistamine as a single amine active ingredient, position a 200-ml. volumetic flask under the column and add 190 ml. of 3.5 *N* hydrochloric acid in 40% methanol in water to the reservoir. Allow this to flow through the column at the rate of 4 ml./min. The volume is adjusted with 3.5 *N* hydrochloric acid in 40% methanol in water.

For products containing either phenylephrine or codeine in addition to an antihistamine, follow the same procedures outlined above eluting the phenylephrine or codeine first with 1.0 *N* hydrochloric acid in 60% methanol in water, then eluting the antihistamine from the same column using 3.5 *N* hydrochloric acid in 40% methanol in water.

Determination.—Quantitatively dilute the sample eluates, if necessary, so that the theoretical concentrations are as close as possible to the standard concentrations. Record the ultraviolet spectra of the final sample dilutions and the standard solutions using a suitable spectrophotometer. Absorbance units should be used.

Using the base line technique, determine the absorbance for each sample and standard at the particular maximum. Calculate the amounts of each compound present in each sample from the values obtained for the standard solutions.

DISCUSSION AND RESULTS

Standard solutions, when subjected to the above procedures, gave the results shown in Table I. These results are derived from 10 determinations for each compound. These data indicate that the method is accurate to within 1% of the true values and are reproducible to ±1.5% at 95% confidence limits. The data for the analyses of marketed products are shown in Tables II and III.

A slightly raised base line was noted with some products where a small portion of the coloring agent was held and eluted with the sample. This did not cause any problem since the base line technique was used in the calculations.

When the maleate salt of a compound is used for a standard, there is a small difference in the base line caused by ultraviolet absorbance of the maleic acid

Table I.—Accuracy, Reproducibility, and Precision Analysis Data

Std. Material	Concn.	Means ($\bar{X}$) of Results of 10 Determinations in % of Theoretical	S.D. of 10 Determinations in % of Theoretical
Phenylephrine HCl	20 mg./10 ml.	99.4	±0.61
Codeine phosphate	20 mg./10 ml.	99.9	±0.64
Chlorpheniramine maleate	4 mg./10 ml.	100.4	±0.44

Table II.—Analysis of Products Containing a Single Amine Component

Ingredient	Label Claim	Found % of Label Claim
Phenylephrine HCl[a]	5 mg./5 ml.	101.2
Codeine phosphate[b]	1 Gm./fl. oz.	102.5
Codeine[c]	0.91 Gm./fl. oz.	98.5
Promethazine HCl[d]	6.25 mg./5 ml.	98.1
Methapyrilene HCl[e]	4 mg./ml.	98.9

[1] Marketed as Histadyl Syrup by Eli Lilly & Co., Indianapolis, Ind.

[2] Marketed as Demazine Syrup by Schering Corp., Union, N. J.

[3] Marketed as Novahistine Elixir by Pitman-Moore, Indianapolis, Ind.

[a] Neo-Synephrine Elixir, Winthrop Laboratories, New York, N. Y. [b] Cheracol, The Upjohn Co., Kalamazoo, Mich. [c] Elixir Terpin Hydrate and Codeine, Parke-Davis and Co., Detroit, Mich. [d] Phenergan Syrup, Wyeth Laboratories, Philadelphia, Pa. [e] Histadyl Syrup, Eli Lilly and Co., Indianapolis, Ind.

TABLE III.—ANALYSIS OF PRODUCTS CONTAINING TWO AMINE COMPONENTS

Ingredients	Label Claim	Found % of Label Claim
Codeine phosphate[a]	10 mg./5 ml.	98.7
Pheniramine maleate[a]	7.5 mg./5 ml.	103.3
Phenylephrine HCl[b]	2.5 mg./5 ml.	98.5
Chlorpheniramine maleate[b]	1.25 mg./5 ml.	100.3
Phenylephrine HCl[b]	5 mg./5 ml.	99.4
Chlorpheniramine maleate[c]	1 mg./5 ml.	100.2

[a] Robitussin AC, A. H. Robins Co., Richmond, Va. [b] Demazine Syrup, Schering Corp., Union, N. J. [c] Novahistine Elixir, Pitman-Moore Co., Indianapolis, Ind.

radical. This also is overcome for the most part by the use of the base line technique.

One deviation from the described procedures was necessary for antihistamine *D*[4]. Since terpin hydrate is precipitated on dilution with water, the sample dilution prior to column application and the washing of the column were performed using 50% methanol in water instead of distilled water.

The proposed method was successful in the presence of normal syrup and elixir components in addition to other materials such as menthol, glyceryl guaiacolate, terpin hydrate, ammonium chloride, tartar emetic, potassium guaiacolsulfonate, and chloroform.

SUMMARY

A method of assay for liquid dosage forms containing phenylephrine, codeine, or an antihistamine has been presented. Also included was an assay method for combinations of phenylephrine or codeine with an antihistamine. The procedures have been used successfully on commonly available products with accurate and reproducible results.

[4] Marketed as Elixir Terpin Hydrate with Codeine by Parke-Davis & Co., Detroit Mich.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) Hiskey, C. F., and Levin, N., *J. Pharm. Sci.*, **50**, 393 (1961).
(3) Koshy, K. T., and Mitchner, H., *ibid.*, **52**, 802(1963).
(4) Chafetz, L., *ibid.*, **52**, 1193(1963).
(5) Clark, W. R., and Rosenberg, L. A., *J. Assoc. Offic. Agr. Chemists*, **48**, 579(1965).
(6) Auerbach, M. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **39**, 50(1950).
(7) Kelly, C. A., and Auerbach, M. E., *J. Pharm. Sci.*, **50**, 490(1961).
(8) Vida, L., *Acta Pharm. Hung.*, **33**, 3(1963); through *Chem. Abstr.*, **59**, 5213*f*(1963).
(9) Chatten, L. G., *Titrimetric Methods, Proc. Symp. Cornwall Ont., Can.*, **1961**, 129–170.
(10) Konstantinova, E., and Boichinov, K., *Farmatsiya (Sofia)*, **13**, 30(1963); through *Chem. Abstr.*, **60**, 2721*e*(1964).
(11) Artamonov, B. P., and Bugreeva, E. V., *Med. Prom. SSSR*, **16**, 30(1962); through *Chem. Abstr.*, **58**, 10039*a*(1963).
(12) Bose, P. C., Singh, R. K., Ray, G. K., and Mukerji, B., *J. Sci. Ind. Res. (India)*, **210**, 386(1962); through *Chem. Abstr.*, **58**, 3270*g*(1963).
(13) Thilmans, Y., and Smeyers, R., *Ann. Pharm. Franc.*, **20**, 719(1962); through *Chem. Abstr.*, **58**, 6646*f*(1963).
(14) Brandt, R., Ehrlich-Rogosinsky, S., and Cheronis, N. D., *Microchem. J.*, **5**, 215(1961); through *Chem. Abstr.*, **60**, 11849*f*(1964).
(15) Claeys, P., Delcambre, J., and Traisnel, M., *Bull. Soc. Chim. France*, **1962**, 1654; through *Chem. Abstr.*, **58**, 3269*h*(1963).
(16) Rapaport, L., and Solyanik, G., *Farmatsevt. Zh. (Kiev)*, **18**, 31(1963); through *Chem. Abstr.*, **59**, 11187*f*(1963).
(17) Genest, K., and Farmilo, C. G., *Anal. Chem.*, **34**, 1464 (1962).
(18) Milos, C., *J. Assoc. Offic. Agr. Chemists*, **48**, 607 (1965).
(19) Izmailov, N. A., Mushinskaya, S., and Danelyants, V. A., *Med. Prom. SSSR*, **17**, 47(1963); through *Chem. Abstr.*, **59**, 12594*c*(1963).
(20) Andreeva, L. G., and Figurovskii, N. A., *Zh. Analit. Khim.*, **17**, 105(1962); through *Chem. Abstr.*, **57**, 6027*d* (1962).
(21) Yamaguchi, S., Seki, I., Okuda, S., and Tsuda, K., *Chem. Pharm. Bull. (Tokyo)*, **10**, 755(1962).
(22) Mary, N. Y., and Brochmann-Hanssen, E., *Lloydia*, **26**, 223(1963); through *Chem. Abstr.*, **60**, 7871*h*(1964).
(23) Clair, E. C., and Chatten, L. G., *J. Pharm. Sci.*, **50**, 848(1961).
(24) Mainville, C. A., and Chatten, L. G., *ibid.*, **53**, 154 (1964).
(25) Banes, D., *J. Assoc. Offic. Agr. Chemists*, **34**, 703 (1951).
(26) Bandelin, F. J., Slifer, E. D., and Pankratz, R. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **39**, 277(1950).
(27) Jones, H. M., and Brody, E. S., *ibid.*, **38**, 579(1949).
(28) Hudanick, J., *J. Pharm. Sci.*, **53**, 332(1964).
(29) Celeste, A., and Turczan, J., *J. Assoc. Offic. Agr. Chemists*, **46**, 1055(1963).
(30) Fontan, C. R., Smith, W. C., and Kirk, P. L., *Anal. Chem.* **35**, 591(1963).

Double Isotopic Labeling in Toxicological Analysis I. Strychnine in Blood—A Preliminary Report

By R. A. WILEY* and J. L. METZGER

Initial results in the development of toxicological assays based on double isotopic labeling methodology are presented. It has been found that strychnine can be analyzed more accurately by this method than by more conventional procedures.

Among accepted analytical procedures for strychnine in biological fluids and tissues, probably the most commonly used is the spectrophotometric method, which ordinarily follows ethylene dichloride extraction (1). This method appeared to be the best available, but suffered from the serious disadvantage that recovery of strychnine from samples containing known amounts was never higher than 75%, and was most commonly in the 40–60% range. Since very sensitive radiochemical assays using double isotopic labeling have been reported (2) for aldosterone and other steroids, the authors were prompted to investigate the applicability of these methods to strychnine analysis.

In the steroid procedures, it is usual to add a quantity of the ^{14}C-labeled steroid to be assayed, allow the total sample to react with a tritium labeled reagent, purify the steroid derivative of interest chromatographically, and count the ^{14}C and tritium. Using ^{14}C recovery to evaluate losses during purification, it is then possible to calculate the total tritium activity, thus the concentration of the desired steroid, in the sample.

Since ^{14}C-labeled strychnine is not readily available, it was necessary to prepare a suitable ^{14}C-labeled derivative of strychnine. The planned assay envisioned allowing strychnine present in the sample to react with methyl iodide, so it was obvious that the derivative of choice would be ^{14}C-labeled strychnine methiodide. This would be added to the sample at the outset, following which the total sample would be allowed to react with ^{3}H-labeled methyl iodide. The resulting double-labeled strychnine methiodide would be purified chromatographically, and strychnine estimated by calculating the total tritium content of the sample as in the steroid case.

Thin-layer chromatography seemed well suited to the planned assay, due to its speed and simplicity. Numerous reports of recovery of radioactive substances from thin-layer plates can be found, but in this case strychnine methiodide was so firmly bound to the adsorbent that effective elution and counting were not possible. Paper chromatography was next investigated, and a number of experiments indicated that, although strychnine methiodide could not be eluted from the paper, immersion of small pieces of paper containing the radioactive material into standard fluor solutions enabled ^{14}C and ^{3}H to be counted with efficiencies of 60.3 and 21.4%, respectively. Counting efficiency was reasonably reproducible with all samples of paper used. In counting double-labeled samples, the authors found that it is a simple matter to insure that all activity due to tritium is confined to the tritium channel. However, it is never possible completely to eliminate activity due to ^{14}C from the tritium channel, so that a correction for this factor must be applied. It was found that the ratio ^{14}C c.p.m. in ^{3}H channel/^{14}C c.p.m. in ^{14}C channel exhibited sufficient variation with different pieces of filter paper that it was not possible to calculate a single correction factor for use in all assays. Instead, it was necessary to apply a spot of ^{14}C-labeled strychnine methiodide to each chromatogram and to calculate this factor independently for each run. Observed radioactivity appeared to be independent of the positioning of filters in the counting vials.

Suitable chromatographic procedures having been found, it was necessary to establish conditions ensuring 100% reaction between strychnine and methyl iodide-^{3}H. Initial attempts, using a 100:1 *M* excess of methyl iodide-^{3}H and making no particular effort to remove the water from the reaction mixture, indicated that the reaction had proceeded to about 30% completion. On increasing the molar ratio of methyl iodide to about 1000:1 and removing most of the water initially present by evaporation in the presence of a large excess of ethanol, quantitative reaction was achieved. Results, including complete calculations, for a typical assay of strychnine in aqueous solution are presented in Table I, where it is seen that recovery is essentially quantitative.

Table I.—Assay Results for Strychnine in Water

		Blank	Sample 1	Sample 2
^{14}C	c.p.m. (obs.)	1116	500	360
	dpm[a]	1850	829	600
	recovery, %[b]	19.3	8.64	6.22
^{3}H c.p.m. (obs.)		619	1120	775
^{14}C in ^{3}H, c.p.m.[c]		513	210	151
^{3}H c.p.m. (corr.)		106	910	624
^{3}H dpm[a]	obs.	495	4250	2920
	total	2560	49,200	46,900
^{3}H dpm due to strychnine			46,600	44,300
Strychnine, mg.[d]			0.34	0.32
Strychnine recovery, %[e]			104	100

[a] Counting efficiency: ^{14}C, 60.3%; ^{3}H, 21.4%. [b] ^{14}C added: 9600 dpm. [c] ^{14}C c.p.m. ratio: ^{3}H channel/^{14}C channel = 0.42 (this run). [d] Strychnine specific activity: 140,000 dpm/mg. [e] Strychnine added: 0.32 mg.

No particular difficulty was encountered in applying the method to analysis of strychnine in blood, for which results from one run are shown in Table II. Blood was selected as the biological specimen to be used because in our experience it is the most difficult fluid from which to obtain good analytical results, since it contains many compounds of all chemical types. As can be seen in Table II, blood yielded

Received July 25, 1966, from the Department of Medicinal Chemistry, School of Pharmacy, The University of Kansas, Lawrence 66045.

Accepted for publication September 30, 1966.

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TABLE II.—ASSAY RESULTS FOR STRYCHNINE IN BLOOD

		Sample		
		Blank	1	2
^{14}C	c.p.m. (obs.)	350	290	109
	dpm[a]	580	481	181
	recovery, %[b]	6.04	5.01	1.89
^{3}H c.p.m. (obs.)		1330	1561	569
^{14}C in ^{3}H, c.p.m.[c]		161	133	50
^{3}H c.p.m. (corr.)		1179	1428	519
^{3}H dpm[a]	obs.	5509	6670	2425
	total	91,200	133,200	128,300
^{3}H dpm due to strychnine			42,000	37,100
Strychnine, mg.[d]			0.30	0.27
Strychnine recovery, %[e]			94	84

[a] Counting efficiency: ^{14}C, 60.3%; ^{3}H, 21.4%. [b] ^{14}C added, 9600 dpm. [c] ^{14}C c.p.m. ratio: ^{3}H channel/^{14}C channel = 0.46 (this run). [d] Strychnine specific activity: 140,000 dpm/mg. [e] Strychnine added: 0.32 mg.

quite high blank values, but the analytical results show that the method is capable of yielding much better recoveries than the spectrophotometric procedure, despite this complication.

It is believed that the results presented here are sufficient to establish the feasibility of the method. Although an insufficient number of assays has been run to present the usual estimates of accuracy and precision over a range of concentrations, results obtained in several duplicate assays lead the authors to believe recoveries of at least 80% may be routinely achieved. In principle, the sensitivity of the method is limited only by the specific activity of the reagents. As strychnine concentrations decrease, it will probably be necessary to perform two or more chromatographic separations on each sample in order adequately to purify the strychnine methiodide for counting. In these initial studies, relatively substantial quantities of strychnine were employed in order to minimize the required specific activity of the reagents. The authors will continue to explore the method with respect to accuracy, precision, sensitivity, and applicability to other substances of toxicological interest which are assayed only with difficulty by other methods.

EXPERIMENTAL

Preparation of Strychnine Methiodide-^{14}C.—Strychnine (430 mg.) was dissolved in 50 ml. of absolute ethanol, and a twofold molar excess of methyl iodide-^{14}C in absolute ethanol was added. The mixture was heated under reflux for 2 hr., at which time a thin-layer chromatogram indicated the reaction to be complete. On cooling the solution in dry ice, a precipitate appeared, which was collected, washed, and dried, affording 170 mg. of strychnine methiodide. Evaporation of the solvent yielded an additional 270 mg. of product. Both fractions were again checked by TLC and found to be homogeneous. The specific activity of the strychnine methiodide was found to be 20.9 μc./mmole.

Preparation of Strychnine Methiodide-^{3}H.—In order accurately to determine the specific activity of the methyl iodide-^{3}H to be used, strychnine methiodide-^{3}H was prepared in a similar fashion from 105 mg. of strychnine. Its specific activity was found to be 12.6 μc./mmole.

Preparation of Strychnine Stock Solution.—Strychnine (8 mg.) was added to 5 ml. water, and 5 drops of 0.1 *N* HCl was added to dissolve the alkaloid. Five drops of 0.1 *N* NaOH was then added, and the solution made to a volume of 25 ml. Its pH was found to be 8.2, and the solution was stored in a refrigerator for not more than 1 week.

Assay Procedure.—Strychnine stock solution and/or water was added to 1 ml. human citrated blood to make a total volume of 2 ml., followed by addition of 0.2 ml. (9600 dpm) of a solution of strychnine methiodide-^{14}C in absolute ethanol. After addition of 2 ml. of absolute ethanol, the mixture was evaporated at 60° on a rotary evaporator under water pump vacuum nearly to dryness. After addition of about 20 ml. of ethanol, the mixture was again evaporated nearly to dryness. One milliliter of a solution of methyl iodide-^{3}H in absolute ethanol (0.1 Gm. or about 30 μc./ml.) was then added, and the mixture was sealed in a glass tube. After heating at 60° for 4 hr., the tube was opened, and excess methyl iodide and ethanol evaporated in an efficient hood, until the final volume of solution was approximately 0.1 ml. This was transferred to Whatman No. 1 chromatography paper. Two control spots were also applied to each chromatogram. First, in order to be sure of the location of strychnine methiodide, "cold" strychnine methiodide was spotted. After development of the chromatogram, the edge on which this spot had run was cut off and sprayed with potassium iodoplatinate reagent to locate the strychnine methiodide. A band of the chromatogram corresponding to the R_f displayed by this strychnine methiodide was used for counting. Second, a spot containing a total of 2400 dpm of strychnine methiodide-^{14}C was applied in order to be sure of quenching corrections, as previously detailed. The chromatogram was developed by the descending technique with ethyl acetate–absolute methanol–28% NH_4OH (100:10:5). The band of the chromatogram found to contain the strychnine methiodide from each spot was then cut out and inserted in a counting solution consisting of 10 ml. of a toluene fluor solution containing 0.5% PPO and 0.04% dimethyl POPOP and 5 ml. of toluene. The amount of ^{14}C and ^{3}H contained in each spot was then determined, using a Beckman liquid scintillation spectrometer. Using the percentage recovery of ^{14}C to correct for losses during workup, the amount of strychnine methiodide-^{3}H, thus the amount of strychnine, in the original sample was calculated.

REFERENCES

(1) "Toxicology: Mechanisms and Analytical Methods," Stewart, C. P., and Stolman, A., eds., Academic Press Inc., New York, N. Y., 1960.
(2) Kliman, B., and Peterson, R. E., *J. Biol. Chem.*, **235**, 1639(1960).

Baeocystin, a Mono-Methyl Analog of Psilocybin from *Psilocybe baeocystis* Saprophytic Culture

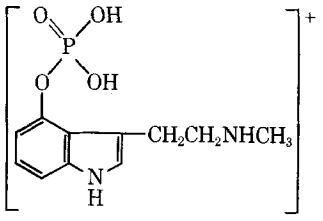
Sir:

The occurrence of psilocybin and/or psilocin in *Psilocybe baeocystis* Singer and Smith has been reported (1–3). During the authors' investigations of the indole derivatives of this fungus, an unknown compound was observed by thin-layer chromatography (3) in extracts of wild carpophores and of mycelial pellets formed in submerged culture. The compound, named baeocystin, exhibited the same color reactions with Ehrlich's reagent and with a modified phosphate reagent (4) as did psilocybin. The R_f values of psilocybin and baeocystin were similar but the compounds were separable using a two-dimensional technique (3). Baeocystin was not detected in extracts of carpophores of *Psilocybe strictipes* Smith, *Ps. caerulipes* Peck, *Ps. pelliculosa* Smith, or *Ps. atrobrunnea* (Lasch) Gillet.

The organism was grown in submerged culture, the mycelial tissue freeze-dried, and extracted as described previously (3). The extract was evaporated to dryness and chromatographed on a powdered cellulose column using 1-butanol saturated with water as the eluant. Fractions richest in psilocybin and baeocystin were combined, concentrated to dryness, and the residue was crystallized from hot methanol. This crystalline material was rechromatographed on a silicic acid column using 1-propanol–5% ammonium hydroxide (5:2) as the eluant. Fractions containing psilocybin and those containing baeocystin were concentrated to dryness and the residues were crystallized from hot methanol. From 23 Gm. of freeze-dried tissue, 10 mg. of psilocybin and 6.5 mg. of baeocystin were isolated.

Baeocystin (m.p. 254–258° dec.) showed a U.V. spectrum identical with psilocybin, indicating it to be a 4-substituted indole derivative (5), yet different from psilocin (6). The I.R. spectrum exhibited primary or secondary amine absorption bands at 3275 cm.$^{-1}$ and 1640 cm.$^{-1}$ which are absent in the I.R. spectrum of psilocybin (6).

The mass spectrum[1] showed a weak molecular ion peak at m/e = 270, corresponding to I, a



I

strong peak at m/e = 190, which corresponds to the dephosphorylated species, and a strong peak at m/e = 44 [$CH_2{=}\overset{+}{N}HCH_3$]. The mass spectrum of psilocybin showed a weak molecular ion peak at m/e = 284 and both psilocybin and psilocin showed strong peaks at m/e = 204 and m/e = 58 [$CH_2 = \overset{+}{N}(CH_3)_2$].

Apart from these differences, all three compounds showed similar fragmentation patterns with prominent peaks at m/e = 160, 159, 146, 130, and 117 in their mass spectra, the structures of which have been postulated.

Thin-layer chromatography and color reactions, U.V., I.R., and mass spectra indicate that baeocystin is the monomethyl analog of psilocybin. This report constitutes the first on its isolation from nature. The pharmacology and clinical applications in mental disease await future investigations.

Details of the isolation and characterization procedures will be reported at a later date.

(1) Benedict, R. G., Brady, L. R., and Tyler, V. E., Jr., *J. Pharm. Sci.*, **51**, 393(1962).
(2) Benedict, R. G., Brady, L. R., Smith, A. H., and Tyler, V. E., Jr., *Lloydia*, **25**, 156(1962).
(3) Leung, A. Y., Smith, A. H., and Paul, A. G., *J. Pharm. Sci.*, **54**, 1576(1965).
(4) Venker, P., and Schmidt, G., *J. Chromatog.*, **9**, 121 (1962).
(5) Stoll, A., Troxler, F., Peyer, J., and Hofmann, A., *Helv. Chim. Acta*, **38**, 1452(1955).
(6) Hofmann, A., Heim, R., Brack, A., and Kobel, H. *Experientia*, **14**, 107(1958).

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Received October 17, 1966.
Accepted for publication November 3, 1966.

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[1] The authors are indebted to Dr. M. Grostic, Upjohn Co., Kalamazoo, Mich., and to Dr. F. Vane, Hoffmann-LaRoche, Inc., Nutley, N. J., for the mass spectral data.

A New Antifungal and Antibacterial Agent, Methyl 5(*or* 4)-(3,3-dimethyl-1-triazeno)imidazole-4(*or* 5)-carboxylate

Sir:

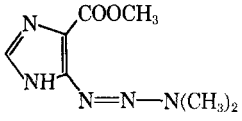
The relative scarcity (1–3) of useful antifungal drugs in comparison with the many antibacterial agents of microbial and synthetic origins emphasizes the potential importance of new compounds and structures that display potent antifungal activity. The need for more and better antifungal agents has become more urgent because of the increasing incidence and severity of fungal infections associated with debilitating diseases, such as neoplasia, and with the administration of antibiotics, corticosteroids, and antineoplastic drugs (4–8). The few medicinal antifungal agents, such as nystatin, amphotericin B, and griseofulvin, are essentially devoid of antibacterial activity; and, conversely, antibacterial drugs are generally ineffective against pathogenic fungi. A new compound, methyl 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxylate (I, NSC-87982), with pronounced activity *in vitro* against a broad spectrum of both fungi and bacteria is reported here. This compound is structurally related to 5(or 4)-(3,3-dimethyl-1-triazeno)- and 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide, both of which have antileukemic activity (9, 10).

The triazenoimidazole methyl ester (I) was prepared by diazotizing methyl 5(or 4)-aminoimidazole-4(or 5)-carboxylate with sodium nitrite and hydrochloric acid and then introducing a 25% aqueous solution of dimethylamine into the diazotization mixture. The product (I) was extracted with chloroform and, after evaporation of the chloroform, was twice dissolved in methanol and treated with activated carbon. Evaporation of methanol after the second decolorization gave I as a white solid that was homogeneous by thin-layer chromatography (silica gel, 9:1 chloroform–methanol), m.p. 163–165° dec. $\lambda_{max.}^{methanol}$ in mμ 233 (ε 10,500) and 328 (ε 16,700).

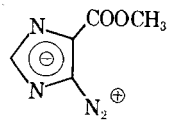
Anal.—Calcd. for $C_7H_{11}N_5O_2$: C, 42.64; H, 5.63; N, 35.52. Found: C, 42.56; H, 5.64; N, 35.23.

By extracting the diazotization reaction mixture with chloroform, the intermediate, methyl 5-diazoimidazole-4-carboxylate (II), may also be isolated as a crystalline solid, m.p. 95–96°, $\lambda_{max.}$ in mμ, 314 (ε 9400) and 270 (sh) in phosphate buffer (pH 7); $\bar{\nu}$ in cm.$^{-1}$: 2180 (N_2^+), 1700 (C=O).

Anal.—Calcd. for $C_5H_4N_4O_2$: C, 39.48; H, 2.65; N, 36.84. Found: C, 39.81; H, 2.98; N, 36.51.



I (NSC–87982)



II

Antimicrobial evaluation of NSC-87982 *in vitro* was performed by the paper disk–agar diffusion method previously described (11). NSC-87982 inhibits the growth of numerous strains of various microorganisms; the data shown in Table I illustrate its inhibitory action against representative yeasts, filamentous fungi, and Gram-positive and Gram-negative bacteria. Zones of inhibition produced by NSC-87982 in most of the fungal tests were comparable to, or greater than, those given by amphotericin B or by nystatin under similar conditions (12). It is less toxic to mice than certain 1,2,5-selenadiazoles that also have antifungal and antibacterial activity (13).

NSC-87982 significantly increased the lifespan of BDF_1 mice bearing lymphatic leukemia L1210. The average survival time of leukemic mice receiving chronic, intraperitoneal treatment (q.d. 1–30 days or to death) with NSC-87982 at 31 mg./Kg./day was approximately 160% of that of untreated leukemic mice. Inhibitory

TABLE I.—INHIBITION OF VARIOUS MICROORGANISMS BY NSC-87982[a]

Microorganisms	mcg./Disk of NSC-87982 1	10	100
Fungi			
Candida albicans ATCC 752	0	31	44
Hansenula wingei NRRL Y-2340	0	25	43
Saccharomyces carlsbergensis ATCC 9080	19	36	45
Rhodotorula sp. SRI 263	0	18	40
Venturia inequalis ATCC 11096	34	50	>60
Penicillium roqueforti ATCC 6987	0	16	45
Phycomyces nitens ATCC 9984	0	0	20
Aspergillus flavus ATCC 9643	0	0	28
Bacteria			
Pseudomonas aeruginosa ATCC 8709	tr	18	22
Salmonella typhimurium ATCC 7823	tr	17	21
Shigella sonnei ATCC 9290	0	13	19
Bacillus subtilis ATCC 10744	14	20	30
Staphylococcus aureus ATCC 6538	0	15	28
Mycobacterium phlei ATCC 354	23	32	44

[a] Numbers in table refer to the diameters in millimeters of the zones of inhibition of growth surrounding and including the disks. Diameter of disk is 12.7 mm. No inhibition is indicated by 0; tr = trace.

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Chemical Data Book. 2nd ed. Edited by G. H. Aylward and T. J. V. Findlay. John Wiley & Sons, Pty. Ltd., Sydney, Australia, 1966. viii + 88 pp. 15 × 24.5 cm. Paperbound. Price $2.95.

This small handbook serves as a source of data covering most of the chemical systems encountered by senior high school and first year college students. Included are properties of elements, inorganic, and organic compounds, some crystal forms, thermochemical data, and small tables of various constants and properties using only a few selected compounds.

NOTICES

Essentials of Biological Chemistry. 2nd Ed. By J. L. Fairley and G. L. Kilgour. Reinhold Publishing Corp., 430 Park Ave., New York, N. Y. 10022, 1966. xvii + 314 pp. 15.5 × 23.5 cm. Price $9.

Biochemistry: An Introduction to Dynamic Biology. By Ernest R. M. Kay. The Macmillan Co., 866 Third Ave., New York, N. Y. 10022, 1966. ix + 374 pp. 16 × 24 cm. Price $7.95.

Chemical Kinetics in Homogeneous Systems. By M. Ritchie. John Wiley & Sons, Inc., 605 Third Ave., New York, N. Y. 10016, 1966. viii + 115 pp. 14 × 21.5 cm. Price $2.95. Paperbound.

Outlines of Biochemistry. 2nd Ed. By Eric E. Conn and P. K. Stumpf. John Wiley & Sons, Inc., 605 Third Ave., New York, N. Y. 10016, 1966. x + 468 pp. 15.5 × 23.5 cm. Price $9.50.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF **Pharmaceutical Sciences**

February 1967 volume 56, number 2

Review Article

Medicinal Chemistry of the Mesoionic Compounds

By LEMONT B. KIER and EDWARD B. ROCHE*

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THE MESOIONIC COMPOUNDS possess structural features which have been of considerable interest to these laboratories as well as to other medicinal chemists. Their potential value as biologically active substances is found in their planar aromatic character, their relatively small size, the variation in electron density around the ring, and the possibility of selecting different patterns of electron density by selecting different mesoionic systems. Their highly charged, yet net neutral electrical character, and some of their chemical properties are also valuable assets in their potential usefulness as medicinal agents. Some biological studies have already been made or initiated with several mesoionic systems. It is, therefore, the purpose of this review to summarize the properties and the known biological activities of these systems and to discuss the relationships observed between the two.

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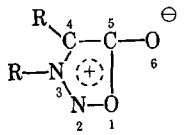
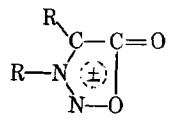
* College of Pharmacy, University of Nebraska, Lincoln.

MESOIONIC COMPOUNDS

The term "mesoionic" was first suggested by Simpson (83) to describe a type of molecule which defied covalent representation. Schönberg (82) was the first to have recognized the existence of such compounds in his consideration of the reagent "nitron." The generality of this term became apparent when the structure of the sydnones (I) became known.



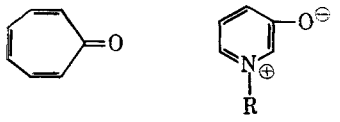
Ia



Ib

The definition of the term, given by Baker and Ollis (7) is as follows. (*a*) A five or six-membered heterocyclic aromatic ring which is not capable of being represented by a covalent structure. (*b*) All atoms of the ring furnish π-electrons to form a delocalized sextet. (*c*) The ring has a partial positive charge which is balanced by a corresponding negative charge on an exocyclic atom or group. (*d*) The ring must be planar or nearly so and must possess considerable resonance energy.

The definition precludes tropone (II) since a covalent representation is possible.



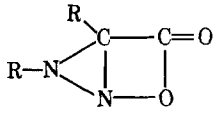
II III

Also the betaines (III) are not so classified since, in these cases, a high degree of charge fixation exists and the compound may be satisfactorily represented by the dipolar structure. Conventionally, mesoionic systems are depicted as in I*a* or I*b*. The atoms thus far encountered in rings have been carbon, oxygen, nitrogen, and sulfur. Bieber (12) has calculated that this combination of atoms could give possibly 288 five-membered mesoionic ring systems. The number of 5-membered rings actually synthesized to date is less than 20.

In this review, the authors will consider only those mesoionic ring systems for which any biological studies have been reported. The chemistry of mesoionic compounds has been reviewed by Baker and Ollis (7) and more recently by Ohta and Kato (75) in Japanese. Our objective is to discuss only the chemical and physical properties which may relate to, or may shed some light on, reported biological activity.

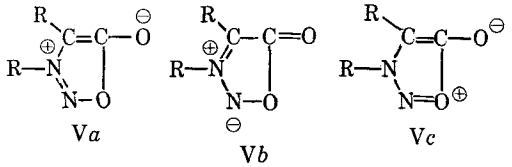
SYDNONES

Chemistry.—The first sydnone was prepared in 1935 by Earl and Mackney (33), who suggested structure IV for the product. Baker and Ollis

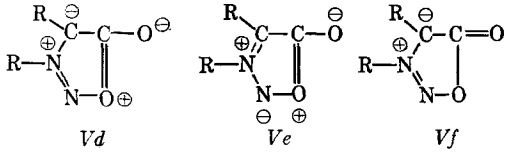


IV

(3) regarded this structure as unacceptable and proposed that the sydnones were resonance hybrids of numerous dipolar and tetrapolar forms (V).



Va *Vb* *Vc*



Vd *Ve* *Vf*

The proper evaluation of the contribution of the resonance forms to the structure is extremely difficult, pointing out an inherent defect in classical resonance or valence bond theory. The possibility that molecular orbital theory might be more illuminating as far as the sydnone electronic structure led Hill, Sutton, and Longuet-Higgins (50), Orgel, Cottrell, Dick, and Sutton (76), and later Bochvar and Bagaturyants (14) and Paoloni and Givmanini (99) to perform simple Hückel molecular orbital (MO) calculations. More recently, Kier and Roche (67) have performed MO calculations on the sydnones using the improved Hückel procedure known as the ω-technique, which takes into consideration some electron correlation. The parameters used were derived from ionization potentials and modified to give self-consistent values which would reproduce meaningful charge densities and relative energies (63, 64). From these values, the dipole moments of 3-phenyl- and 3-methylsydnone were calculated quite closely, and the ultraviolet absorption maxima were correlated with suitable MO calculated energies (67). The structures calculated are shown in Figs. 1 and 2.

Several observations can be made from these ground state structures.

(*a*) The exocyclic oxygen atom bears a high negative charge density. In comparison with the comparable oxygen atom of butyrolactone, the sydnone oxygen atom has a charge density higher by 0.15 of an electron. This indicates a more polarized carbonyl group, but the bond

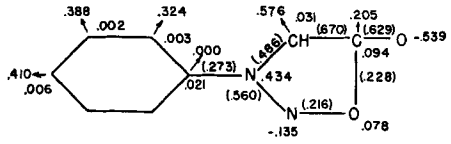


Fig. 1.—Molecular orbital calculated properties of 3-phenylsydnone from *Reference 67*. (The numbers in parentheses are bond orders, the numbers at the arrowheads are free valencies, and the other numbers are charge densities.)

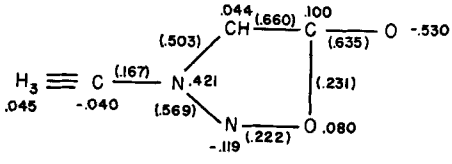


Fig. 2.—Molecular orbital calculated properties of 3-methylsydnone from *Reference 67*. (The numbers in parentheses are bond orders, and the remaining numbers are charge densities.)

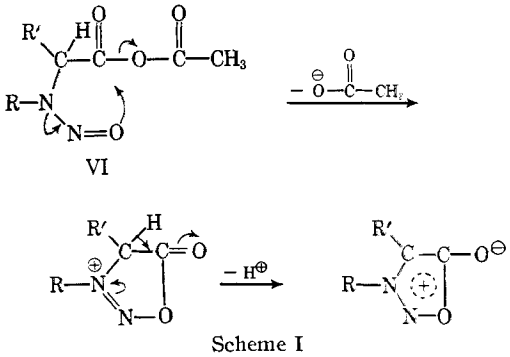
order suggests more double bond character than single bond character. This is consistent with X-ray diffraction bond length measurements by Bärnighausen, Jellinck, and Vos (8, 9), who found the C—O bond length to be 1.20 Å., close to a normal carbonyl bond length.

(*b*) The No. 3 nitrogen atom bears a high positive charge. The highest ring bond order is between the two ring carbon atoms while low bond orders exist between the ring O—N bond and the ring O—C bond. This suggests a significant contribution of canonical form V*a* to the structure of the sydnone, in a valence bond consideration.

(*c*) The sydnone ring is an electron-withdrawing substituent on the phenyl ring in 3-phenylsydnone, deactivating it at all positions. Inasmuch as ground state properties mirror excited state reactivities, this indicates that electrophilic substitution would be retarded on the benzene ring. This is borne out by experiment. Substitution reactions on 3-phenylsydnone invariably favor the 4-position of the sydnone ring (7).

The sydnones are derivatives of the 1,2,3-oxadiazole ring system, hence were named by Baker, Ollis, and Poole (4) as Ψ-5-keto-3-methyl-3,5-dihydro-1-oxa-2,3-diazole, using 3-methylsydnone as an example. The symbol Ψ reflects the uncertain electron distribution—hence, the inability to write formal covalent or single polar structures. As an alternative, Katritsky (59) has proposed a name based on a theoretical betaine representation; hence 3-methylsydnone becomes anhydro-5-hydroxy-3-phenyl-1-oxa-2,3-diazolinium hydroxide. In the opinion of the authors, the former name is superior since it does not attempt to invoke the use of a single canonical or polar form but is adaptable to a new electronic pattern in a heterocycle, one of uncertain electron distribution. Even within variously substituted sydnones, the electronic distribution would be quite variable depending on the substituents in the 3 and/or 4-position. *Chemical Abstracts* employs the use of the trivial name "sydnones" for these compounds, a name taken from the University of Sydney, where they were first made (33).

The original method of synthesis, described by Earl and Mackney (33), has not been significantly improved upon. It involves the treatment of an *N*-nitroso-α-amino acid with an anhydride such as acetic anhydride or trifluoroacetic anhydride (5). The latter reagent results in a completed reaction in seconds, while with acetic anhydride, standing for days may be necessary. Thionyl chloride (5) and phosgene (45) have also been used to close the ring. The possible substituents are alkyl or aryl but not hydrogen on the 3-position, and aryl, alkyl, or hydrogen on the 4-position. Baker, Ollis, and Poole proposed a mechanism for the formation, involving an intermediate mixed anhydride (VI) which they independently synthesized (5). (Scheme I.)



VI

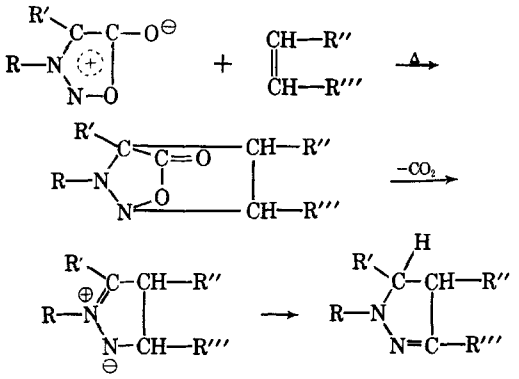
Scheme I

The chemistry of the sydnone ring has been studied extensively and reviewed recently by Nöel (72), Stewart (87), and Ohta and Kato (75). It is the authors' intention to discuss briefly only those reactions having any possible biological significance.

An argument for the aromatic character of the sydnone ring is its stability and ability to undergo electrophilic substitution at the No. 4 carbon atom (7). Examples include halogenation (11) with chloride (5), bromine (4, 58), and iodine (71), mercuration (71), deuteration (95), nitration (45), acylation (98), formylation (90), and sulfonation (94). The 4-bromo derivative has been used as an intermediate to 3-phenyl-4-carboxysydnones (58) and 3-alkyl-4-carboxysydnones (62). Attempts to put electron-donating groups in the 4-position have been unsuccessful to date (58, 96).

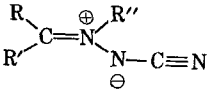
Baker, Ollis, and Poole (5) observed the unreactivity of the phenyl ring of 3-phenylsydnone to electrophilic substitution. This is predicted by the MO calculations of Kier and Roche (67) discussed earlier. On the other hand, it has been observed that a phenyl group in the 4-position of the sydnone is disposed to electrophilic attack, such as nitration (84).

An interesting reaction of the sydnones is their ability to add alkenes, alkynes, and nitriles to give pyrazole or pyrazoline derivatives with elimination of CO_2 (92, 54). Huisgen proposed Scheme II as the mechanism for the reaction (54). Huisgen (55) has compared this reaction of the



Scheme II

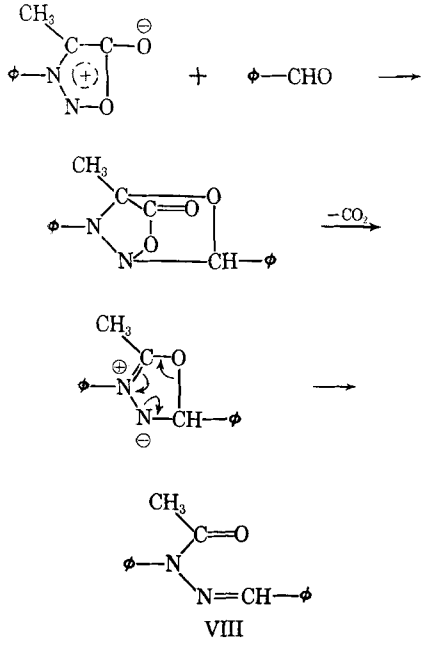
sydnones with the behavior of the azomethine imines (VII) which undergo similar addition reactions quite readily.



VII

The proposal has been made that resonance contributions (*Vb* and *Vf*) are more prominent in the ultimate structure of the sydnone than had been previously supposed (85). Addition 1,3 of a carbonyl group has also been observed (55), but a different sequence is followed to give an α-acyl hydrazine azomethine (VIII). (Scheme III.)

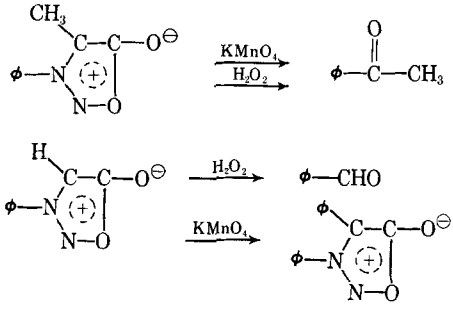
An extremely interesting reaction with an



VIII

Scheme III

as yet unresolved mechanism is the oxidation of sydnones. Hashimoto and Ohta (46) observed the products shown in Scheme IV.



Scheme IV

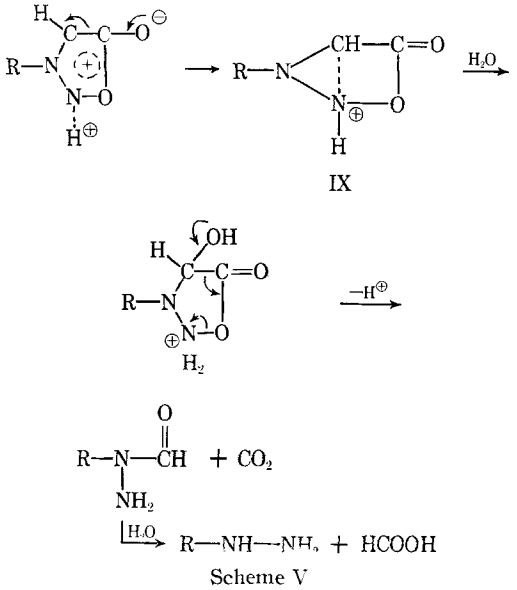
They suggested the formation of a phenyl radical from decomposition of the ring, followed by attack of the radical on an intact sydnone ring at position 4 to produce the products shown. Molecular orbital calculations show the value for the free valence at position 4 to be quite favorable for such a radical attack. (See Fig. 1.)

Acid hydrolysis of sydnones yield a substituted hydrazine, a carboxylic acid, and CO_2 (32, 33). Garrett (40) has studied the kinetics of this hydrolysis and found the order of decreasing rate in a series of 3-alkyl sydnones to be *tert*-butyl > isopropyl > methyl. He proposed, on the basis of his calculated entropies of activation, that the observed order was due to a decreasing negative charge on the ring due to a decreasing alkyl inductive effect. However, recent molecular orbital calculations (68) indicate that the alkyl groups cause insignificant perturbations in the electron distribution in the ring, an observation borne out by the fact that all of the 3-alkyl sydnones absorb at essentially the same wavelength (44, 60).

Garrett proposed a mechanism of acid hydrolysis (40) based on initial protonation of the nitrogen at position 2. This position was also predicted as the most energetically favorable position for protonation from molecular orbital calculations of protonation energies (68). The proposed mechanism of Garrett's further included the formation of an "incipient 2,4 bond or a quasidiazomethane" (IX). (Scheme V.)

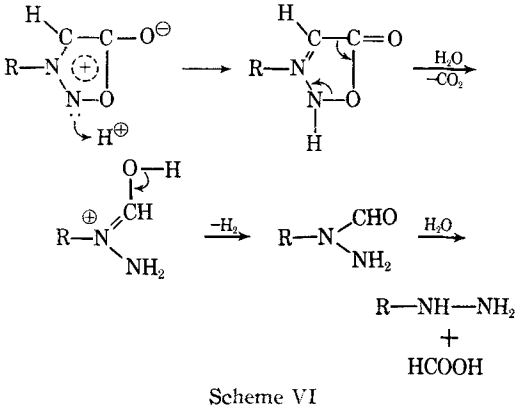
An alternate mechanism proposed by Garrett (40) involved the unlikely addition of H_2O across the 2,4 atoms, involving attack of $OH^{\ominus}$ at the N_2 position.

These two proposals seem unlikely on the basis of steric and electronic considerations. The quasidiazomethane ring structure was eliminated early in the sydnone studies (3). The attack by $OH^{\ominus}$ on the No. 2 nitrogen is predicted to



Scheme V

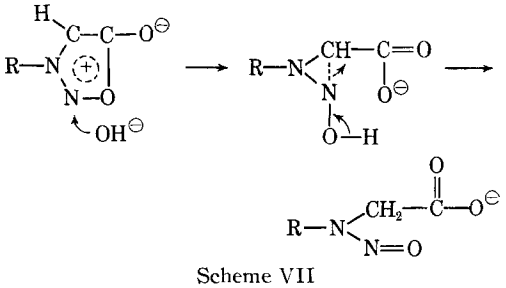
be, energetically, the least likely point of attack based on molecular orbital calculations of anion localization energies (68). A likely mechanism of acid hydrolysis would be Scheme VI (68), which is an expansion of the mechanism proposed by Baker and Ollis (7).



Scheme VI

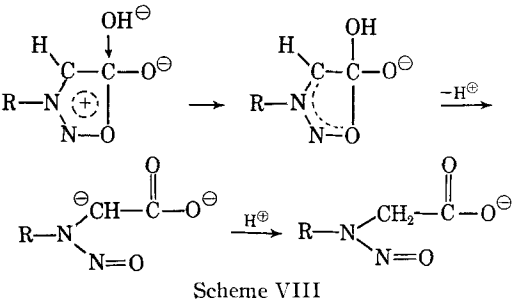
Acid hydrolysis of sydnone derivatives has become a general method for the synthesis of monoalkyl hydrazines that are difficult to obtain from more direct means (96).

Base-catalyzed hydrolysis of sydnones has been reported to regenerate the starting *N*-nitrosoaminoacid salt (32). Garrett (40) studied the kinetics of this reaction, and found the decreasing order of the rate of hydrolysis of 3-alkyl sydnones to be methyl > propyl > isopropyl > *tert*-butyl. The proposed mechanism of this hydrolysis (40) involved initial attack by $OH^{\ominus}$ on the No. 2 nitrogen, followed again by the formation of the intermediate "incipient 2,4 bond or quasidiazomethane." (Scheme VII.)



Scheme VII

This mechanism appears quite unlikely from the standpoint of valence bond reasoning. Furthermore, anion localization energies from molecular orbital calculations argue against this point of attack by $OH^{\ominus}$ (68). Energetically, the carbonyl carbon is the preferred point of attack by a reagent with a pair of electrons. Localization energies of the 3-alkyl sydnones predict the experimental order of hydrolytic rates and suggest the mechanism shown in Scheme VIII (68).

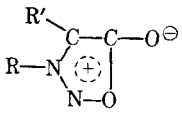


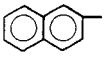
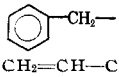
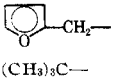
Scheme VIII

Physical Properties.—The assignments of carbonyl and carbon-hydrogen stretching frequencies in the infrared spectra of numerous sydnones have been made. A number of representative compounds are listed in Table I. The ν CO assignment of 1720–1770 cm.$^{-1}$ is consistent with the γ-lactone CO absorption at 1740 cm.$^{-1}$. The C—H absorption at 3190–3140 cm.$^{-1}$ is quite characteristic for this bond and has been used to detect the absence of a 4-substituent in sydnones (86).

The presence of an intense absorption band in the ultraviolet spectra of sydnones at 290–340 mμ ($\epsilon \sim$ 6000–8000) has been used as evidence of the aromatic character of the ring (7). Conjugation of the ring results in displacement to lower energy, *i.e.*, higher wavelength. Thus, 3-phenylsydnone absorbs at 310 mμ (44), 3-methyl-4-phenylsydnone at 317 mμ (86), and 3,4-diphenylsydnone at 340 mμ (35). (See Table II.) Kier and Roche (67) have calculated

TABLE I.—INFRARED ABSORPTION OF REPRESENTATIVE SYDNONES



R	R′	νCO (cm.⁻¹)	νCH (cm.⁻¹)	Ref.
$CH_3CH_2CH_2CH_2$—	H	1768	3185	(39)
—CH_2—CH_2— (bis)	H	1735	3190	(24)
	H	1766		(36)
	Br	1756		(39)
	H	1752		(36)
	H	1768	3140	(39)
CH_2=CH—CH_2—	H	1740		(60)
$(CH_3)_3C$—	H	1725		(60)
	H	1710		(60)
$(CH_3)_3C$—	CH_3	1735		(31)
CH_3CH_2—CH_2—	CH_3	1725		(31)
CH_3CH_2—CH(—CH_3)—	COOH	1690		(30)
	H		3150	(70)

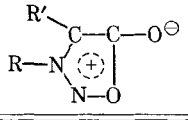
the energy levels by MO methods for 10 substituted sydnones with $\lambda_{max.}$ values ranging from 290 to 340 mμ. They have shown a correlation between the frequency maxima of the longest wavelength high intensity absorption and the energy difference between the highest filled and lowest empty MO's, characteristic of a $\pi \rightarrow \pi^*$ electronic transition.

The dipole moments of the sydnones are quite large compared to most organic compounds. Hill and Sutton (51, 52) and Earl, Leake, and LeFevre (34) studied a large number of sydnone moments and interpreted them. The moment is directed toward the exocyclic oxygen atom, and support was derived from these studies in the formulation of these compounds as mesoionic. An attempt to reproduce the dipole moment by calculation from MO charge densities led Orgel, Cottrell, Dick, and Sutton (76) to a total moment that was high by a factor of 1.6. Coulson (23) scaled the charge densities calculated by Hill, Sutton, and Longuet-Higgins (50) for phenylsydnone to conform to the experimental dipole moment. Calculations by Kier and Roche (67), using MO procedures and parameters previously described (63, 64), produced charge densities from which dipole moments for 3-methyl- and 3-phenylsydnone were calculated. These values corresponded quite closely to experimental values. (See Table III.)

Two studies on the proton magnetic resonance spectra of sydnones have appeared in the literature. Stewart and Danieli (85) observed an unexpectedly high field for the ring proton of several sydnone derivatives. They concluded that this occurrence was difficult to reconcile with the mesoionic formulation since the positive charge associated with the ring nitrogen adjacent to this position should markedly deshield this proton. A partially delocalized azomethineimine system was proposed in which some negative charge could be generated at the sydnone 4 carbon to contribute some diamagnetic shielding to the attached proton.

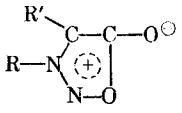
Lawson, Brey, and Kier (69) found essentially the same chemical shift for the sydnone ring proton. However, they concluded that this is a downfield shift from the normal resonances for olefinic hydrogens due either to the anisotropic effect of the aromatic sydnone ring, or to

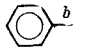
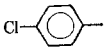
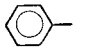
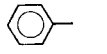
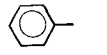
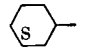
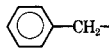
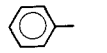
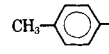
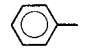
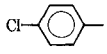
TABLE II.—ULTRAVIOLET ABSORPTION MAXIMA OF REPRESENTATIVE SYDNONES



R	R′	$\lambda_{max.}$ (EtOH)	ε	Ref.
CH_3	H	290	6600	(44)
$CH_3(CH_2)_3$—	H	289.5	6450	(44)
	H	310	5650	(44)
	H	312.5	4450	(44)
	H	310	4700	(44)
	H	300	6500	(44)
	CH_3CH_2—	307	6200	(44)
CH_3—		317	7700	(86)
$(CH_3)_3C$—	H	290	5900	(60)
$CH_3(CH_2)_3$—	CH_3—	295	6440	(31)
	H	315		(35)
		340		(35)
	CH_3—C(=O)—	324	6340	(43)

TABLE III.—DIPOLE MOMENTS OF REPRESENTATIVE SYDNONES



R	R′	Exptl. (μ Benzene, 25°)	Ref.
CH_3[a]	H	7.31 D	(67)
 [b]	H	6.48	(51)
CH_3	H	6.89	(51)
	H	5.01	(51)
	Cl	6.49	(51)
	Br	6.42	(51)
	CH_3	6.57	(51)
	H	6.7	(51)
	H	7.27	(51)
		6.55	(52)
		7.15	(52)
	NO_2	6.18	(52)
		4.61	(52)

[a] Calculated from MO charge densities (67), 7.06 D. [b] Calculated from MO charge densities (67), 6.47 D.

the electron withdrawing effect of the positive nitrogen. The resonance location of the ring proton is not sufficient evidence to support or discard theories concerning the aromaticity of the sydnone ring. (Table IV.)

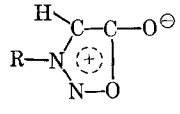
A further look at the chemical shifts of the hydrogens attached to the carbon α to the sydnone ring in 3-alkyl sydnones uncovered some interesting evidence dealing with the electronic structure of the sydnone ring (69). These protons show downfield shifts of about 1.8-2.1 τ units from their normal resonance positions in alkyl benzene derivatives and alkyl amines. The downfield shifts from their normal aliphatic positions are about 3.2 τ units. The strong deshielding of these protons has been interpreted (69) as being due to the combined effects of the ring current in the sydnone ring and the electronegativity of the positively charged 3 nitrogen of the sydnone nucleus which bears these substituents.

Another interesting phenomenon discovered by Lawson, Brey, and Kier (69) is the concentration dependency of the chemical shift of the sydnone ring proton in $CDCl_3$ solution. As the concentration of sydnone decreases in these solutions, the sydnone proton resonance shifts upfield. This has been attributed by these authors (69) to aggregation of the sydnone in concentrated solutions. The intermolecular interaction of sydnone rings will withdraw electron density and cause the proton to appear at comparably low field. As the concentration of solvent ($CDCl_3$) increases relative to that of the sydnone, hydrogen bonds could be formed presumably with the exocyclic oxygen atom, and thus bring about the break-up of the aggregates. The interaction of the sydnone with solvent would result in less electron withdrawal than sydnone–sydnone interaction; therefore, the ring hydrogen shifts upfield. This mechanism is supported by the fact that the solvent peak for sydnone concentrations of from 0–50% in $CHCl_3$ shifts from 2.74 to 2.25 τ. The indication here is that the solvent is forming hydrogen bonds with the solute.

There is also evidence that sydnone derivatives with larger side chains have their aggregates disrupted more easily than those derivatives with smaller side chains. This concentration effect is not nearly so pronounced in acetone solution due to the fact that the solvent cannot form the hydrogen bond necessary to cause the destruction of the sydnone aggregates.

The complexing ability of sydnones was first observed by Yamada and Kazima (93), who observed a carbonyl infrared absorption shift with iodine which was found to form a 1:1 complex with the sydnone, and the conclusion was drawn

TABLE IV.—PROTON MAGNETIC RESONANCE OF SYDNONES

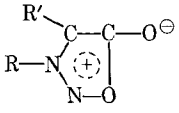


R	τ Sydnone	τ Alkyl αH	τ Alkyl αH on R-Benzene	τ Alkyl αH on R-NH_2	Ref.
CH_3—	3.62	5.90	7.70	7.80	(69)
CH_3—CH_2—	3.64	5.66	7.38	7.50	(69)
$(CH_3)_2CH$—	3.68	5.29		7.13	(69)
$(CH_3)_3C$—	3.72				(69)
	3.24	2.12		3.40	(69)
	3.22	2.30			(85)
	3.81	4.64			(85)
CH_3—(4-ϕ)		5.87			(85)

that the carbonyl oxygen was involved in the complex. Lawson, Brey, and Kier (69) observed a tendency to aggregate in solution with a series of 3-alkyl sydnones as was described in the NMR discussion. Gentile and Mao (41) studied metal halide complexes with a large number of sydnones and found complexation with strong Lewis acids such as $TiCl_4$, $SbCl_5$, $SnCl_4$, $FeCl_3$, $ZnCl_2$, and $TeCl_2$. They found that bonding occurred with the exocyclic oxygen in a 1:1 ratio in the solid state. These complexes dissociated completely in solution.

Biological Activity.—The earliest reported consideration of sydnones as biologically important agents was made by Brookes and Walker (17) in 1957 when they prepared several 3-methyl-4-alkyl sydnones as potential amino acid antagonists. Their compounds were tested against several microorganisms, including *Streptococcus haemolyticus*, *Staphylococcus aureus*, *B. coli*, *L. mesenteroides*, and filarial infections. No activity was observed. Davis, Becker, and Rogers (29) pursued the concept of the sydnones being analogs of amino acids. They tested a number of 3-substituted sydnones, mostly 3-aryl, against two fungal infections of plants. (See Table V.) The observed disease reduction appears not to be a function of where the phenyl ring substituent is, but rather what it is, in the case of controlling wheat rust. Specifically, the presence of chlorine at any position usually imparts activity to some extent. The same generalization can be made for the control of bean rust with the addition that 3-phenyl-4-substituted sydnones with no substituent on the phenyl ring also showed activity. Interestingly, the sydnones studied did not inhibit the fungal growth *in vitro*. This suggests that the sydnones perhaps function by increasing resistance to disease by a mechanism which renders the host tissue unfavorable to disease development. This is the role that the sydnones as possible amino acid antagonists might play (29).

Table V.—Activity of Sydnones Against Wheat and Bean Rust at 500 p.p.m. 3 Days After Inoculation[a]

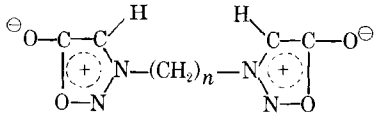


R	R′	Disease Reduction[b] Wheat Rust	Disease Reduction[b] Bean Rust
Phenyl	H	++	++++
o-Chlorophenyl	H	++++	+++
m-Chlorophenyl	H	+++	+++
p-Chlorophenyl	H	+++	—
2,4-Dichlorophenyl	H	+++	+++
3,4-Dichlorophenyl	H	+	—
p-Bromophenyl	H	—	+
p-Nitrophenyl	H	—	—
p-Tolyl	H	—	+
p-Methoxyphenyl	H	—	—
o-Ethylphenyl	H	—	++++
Phenyl	Methyl	—	+++
Phenyl	Nitro	—	
Phenyl	Chloro	+	++++
Phenyl	Bromo	—	++++
p-Chlorophenyl	Chloro	—	—
Methyl	H	—	+
Benzyl	H	—	—
Cyclohexyl	H	—	+

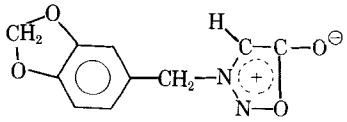
[a] Results taken from *Reference 29*. [b] ++++, 76–100% reduction, few lesions; +++, 76–100%, many lesions; ++, 51–75% reduction; +, 10–50% reduction.

Pütter and Wolfrom (78), in a series of patents, claimed ascaridal activity for a number of substituted 3-phenylsydnones. The claim was made that the compounds were particularly effective against mites resistant to phosphorus-containing insecticides. Tien and Hunsberger (90) synthesized *N*-(3-pyridyl)sydnone and subjected the compound to a general screen against tuberculosis, several bacteria, cancer, and other chemotherapy assays. The results reported were all negative.

A number of sydnones have been examined in the search for anticancer agents. Daeniker and Druey (24) synthesized a number of polymethylene-bis-sydnones, and reported some antitumor activity for the ethylene homolog.



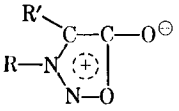
In a more recent antitumor study of sydnones and sydnonimines, Greco, Nyberg, and Cheng (42) reported activity against carcinoma 755 in mice with 3-(*p*-methoxybenzyl)sydnone. The same compound was inactive against sarcoma 180 and leukemia 1210 systems. An analog of this compound, 3-piperonylsydnone (X), was reported by Nyberg and Cheng (73) to be an active agent in preliminary antimalarial evaluation. At a dose of 10 mg./Kg. it was found to



X

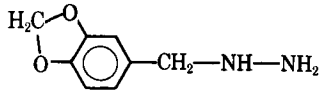
be active against *Plasmodium berghei* in mice. It was active either orally or subcutaneously and was found to be nontoxic at a dose of 500 mg./Kg. The acid and base hydrolysis products, piperonylhydrazine (XI) and *N*-nitroso-*N*-piperonylglycine (XII), respectively, were also tested and found to be inactive, suggesting that the intact ring is the active substance.

TABLE VI.—ALKYLSYDNONE PHYSICAL AND PHARMACOLOGICAL DATA[a]

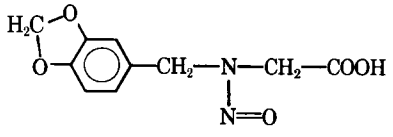


R	R′	$P_c\left(\frac{AMOH}{H_2O}\right)$	CD_{50}, mmole/Kg.	LD_{50}, mmole/Kg.
$(CH_3)_2CH-$	H	2.3	2.96	3.75
$CH_3(CH_2)_2-$	H	2.5	1.60	1.87
$(CH_3)_3C-$	H	4.9	0.87	1.30
$CH_3CH_2CH(CH_3)-$	H	6.4	1.13	1.55
$CH_3-C(CH_3)_2-CH_2-C(CH_3)_2-$	H	8.0	0.25	0.31
	CH_3-	1.85	0.24	
CH_3CH_2-	CH_3-	2.05		
$CH_3(CH_2)_2-$	CH_3-	2.8	0.34	
$CH_3(CH_2)_3-$	CH_3-	2.9	0.13	
$CH_3(CH_2)_3-$	CH_3CH_2-	4.1	0.19	
$(CH_3)_3C-$	CH_3	6.7	0.71	

[a] Taken from *References 60* and *31*.



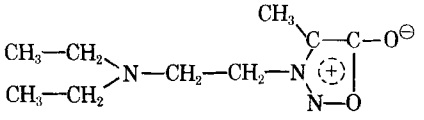
XI



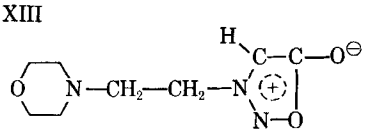
XII

A number of 3-alkyl sydnones were found by Kier and co-workers (61) to be potent central nervous stimulants. The 3-*sec*-butylsydnone was found to be a powerful respiratory stimulant in a heavily barbitalized dog. The stimulation appeared to be predominantly central, as there was little decrease of activity after vagotomy or denervation of the carotid sinus. The respiratory activity was superior to that produced by the same dose of pentylenetetrazol.[1] Kier and Dhawan (31, 60) studied the relationship between the partition coefficients of several mono and dialkyl substituted sydnones. It was observed that there was a correlation between partitioning into an oil phase and convulsive potency in the case of the 3-alkyl sydnones. (See Table VI.) In the case of the 3,4-dialkyl sydnones, the convulsive potency was found to be greater and independent of the partition coefficient. It is possible that in the case of the 3-alkyl sydnones, the absolute lipid solubility is sufficiently low so that the partition coefficient becomes a limiting factor concerning the amount of drug reaching the CNS. With the disubstituted sydnones, the absolute lipid solubility may be appreciably high so that in spite of a less favorable partition coefficient in some compounds, there is a sufficient concentration in the biolipid phase to permit an active level of sydnone to reach the site of action. Another explanation is that the intrinsic activity of the 3,4-dialkyl sydnones is significantly higher than that of the 3-alkyl sydnones, so that a less favorable partition coefficient would not mitigate against a high level of activity (31). The basic hydrolysis products of these sydnones did not elicit the same activity. Bruzzese and co-workers (18) synthesized a number of 3-aminoalkyl sydnones and noted essentially the same CNS stimulation previously observed (60), although a few were mild depressants. They also observed an analgesic action in most cases, particularly with the 3-diethylaminoethyl-4-methylsydnone (XIII) and 3-morpholinylethylsydnone (XIV). They also noted a hypoglycemic activity for several compounds. Bruzzese and his co-workers (19) hydrolyzed the 3-aminoalkyl sydnones to the corresponding hydrazines and subjected these products to the same tests. They found a dif-

[1] Marketed as Metrazole by Knoll Pharmaceutical Co., Orange, N. J.



XIII

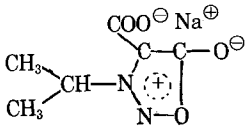


XIV

ferent pattern of activities from the sydnone precursors in most cases.

It is noteworthy that products of acid or base hydrolysis of sydnones failed to give the biological activities seen for the intact sydnone, suggesting that it is the ring itself, rather than an *in vivo* degradation product, which is the active species. This would tend to minimize the early suggestion of an amino acid antagonist (17, 29).

Fregly, Kier, and Dhawan (38) studied the diuretic and hypotensive properties of 3-*sec*-butylsydnone, 3-butyl-4-ethylsydnone, and 3-*iso*-propyl-4-sodium carboxylate sydnone (62) (XV). With comparable doses, 3-butyl-4-ethylsydnone was the most active with respect to urinary sodium output. (See Fig. 3.)



XV

The 3-*sec*-butylsydnone reduced rat blood pressure 5 to 10% over a 10 to 20 mg./Kg. dose range. The sydnone acid was inactive against blood pressure while the dialkyl sydnone was convulsive at low enough doses so that

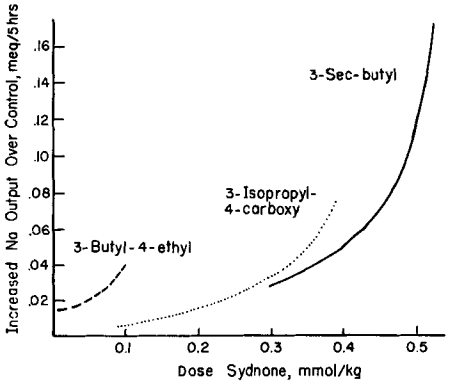


Fig. 3.—Comparison of urinary Na output by the three sydnones. (*Reference 33.*)

blood pressure measurements were interfered with.

Oehme and co-workers (74) conducted an extensive pharmacological study of a large number of 3-phenylsydnones. They found that these compounds were significantly less toxic than the 3-alkyl sydnones (60) though the toxic manifestations were quite similar. (See Table VII and compare with Table VI.) They noted that at subtoxic doses in the rat a marked change occurred in the blood picture characterized by diminution of erythrocytes as well as formation of methemoglobin. They noted an analgesic effect $^1/_{30}$ of the effectiveness of morphine with the 3-(*o*-tolyl)- and the 3-(*m*-tolyl)sydnones.

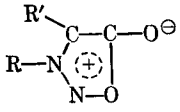
The sydnones studied were found not to have hypnotic effect themselves, but they did influence hexobarbital narcosis even in small doses. Particularly effective were the 3-(*o*,*m*, and *p*-tolyl)-sydnones, the 3-(*p*-methoxyphenyl)-, and the 3-phenyl-4-isopropylsydnones, which more than doubled the sleeping time. Substituents in the 4-position generally diminished this effect. They noted, in the case of the 3-(*m*-tolyl)sydnone, that the hexobarbital level in the rat brain, after sydnone pretreatment, was doubled. This suggested an interference with the microsomal decomposition process of the barbiturate as the cause of prolongation of the hexobarbital narcosis. This conclusion was reached when it was observed that 3-(*m*-tolyl)sydnone did not prolong sleeping time due to ethylbarbital, which is not metabolized by the rat.

In contrast to the convulsive activity found for the 3-alkyl sydnones (61), Oehme and co-workers (74) found considerable anticonvulsive activity for the 3-aryl sydnones. The 3-(*o*-tolyl)sydnones were the most active of the compounds tested. (See Table VII.) On isolated rat duodenum and guinea pig ileum, the sydnones studied had no effect. Finally, they observed that 3-methylsydnone did not inhibit glutamic acid decarboxylase. Furthermore, 3-methylsydnone and a number of 3-aryl sydnones did not inhibit monoamine oxidase, suggesting that conversion of the sydnones to the corresponding hydrazines is not involved.

In some recent work on four 3-phenyl-4-acyl sydnones (43), a modest hypotensive activity was noted when administered i. v. in dogs. There was quantitatively little differences between the compounds tested (acetyl, propionyl, isobutyryl, butyryl), the average blood pressure drop being about 10% at 10 mg./Kg. The action was of short duration.

It is evident that the 3-aryl sydnones generally

TABLE VII.—PHARMACOLOGY OF SYDNONES[a]



R	R′	LD_{50}, mmole/Kg.	ED_{50}, mg./Kg. Anticonvulsive: Electro-shock	ED_{50}, mg./Kg. Anticonvulsive: Pentylene-tetrazol	mm. Hg Rat, b.p. Lowering[b]
m-Tolyl	H	2.8	42	35	26
p-Tolyl	H	4.25	176	176	14
p-Tolyl	Isopropyl	2.8	218	218	21
p-Tolyl	Chloro	5.0	211	211	Lethal
p-Tolyl	Phenyl	—	—	252	11
p-Tolyl	Methyl	4.2	190	190	26
o-Tolyl	H	1.85	48	62	19
o-Tolyl	Nitro	3.3	221	221	14
m-Methoxyphenyl	H	5.0	80	140	15
p-Methoxyphenyl	H	2.4	192	192	8
o-Methoxyphenyl	H	5.0	165	192	16
p-Ethoxyphenyl	H	5.0	205	140	11
Phenyl	Phenyl	5.0	238	238	3
p-Chlorophenyl	Phenyl	2.8	273	273	24
Methyl	H	5.0	100	100	4
Phenyl	Methyl	2.9	140	176	10
Phenyl	Isopropyl	1.7	102	204	9
p-Carboxyphenyl	H	5.0	206	206	8
m-Tolyl	Methyl	2.4	190	95	15
m-Tolyl	Isobutyl	2.5	232	232	11

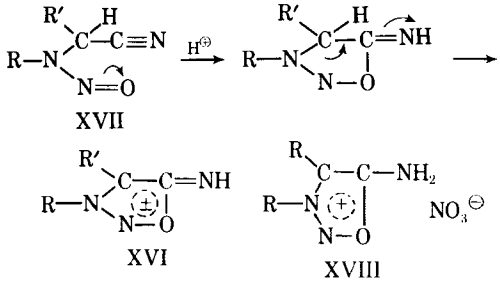
[a] From *Reference 74*. [b] 0.025 mmole/Kg.

exert a depressant type of CNS response while the 3-alkyl sydnones exert a convulsive response. This dichotomy of action is not unlike barbiturates and hydantoins, depending on the presence of or absence of certain substituents on the nitrogen atoms of these compounds. This analogy has been noted and commented upon (61). A glance at the ground state electronic structures of 3-phenyl- and 3-methylsydnones (see Figs. 1 and 2) reveals some differences in the charge density patterns around the rings of these two modifications. These differences are possibly involved in determining the type of CNS action elicited.

SYDNONIMINES

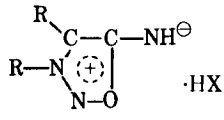
Chemistry and Physical Properties.—Brookes and Walker (17) first reported the synthesis of a sydnonimine (XVI) from the reaction of an *N*-methyl-*N*-nitroso-α-amino-nitrile (XVII) with nitric acid. (Scheme IX.) The salt in this case was the nitrate (XVIII). The sydnonimines have also been reported to be formed with hydrogen chloride (25, 57, 91) and dilute hydrochloric acid (25). The free sydnonimine (XVI) does not exist and removal of the acid causes ring opening (17).

The structure of sydnonimine salts has not been settled. Structure XVIII is usually used to represent the imine salt; however, the infrared



Scheme IX

spectrum of this compound shows absorption at 1588–1606 cm.$^{-1}$ and 1671–1770 cm.$^{-1}$, which Yashunskii (97, 98) ascribes to a $=\overset{\oplus}{N}H_2$ and a C=N group, respectively. This raises the possibility that a better representation for a sydnonimine salt may be structure XIX with a closely associated molecule of acid. Ohta and Kato (26) point out that structure XVIII does not give the usual amino group reactions such as diazotization or alkylation; hence, this structure



XIX

may not be an adequate model for the sydnonimine salt. Other derivatives of the imine salt can be formed such as the *N*-nitroso (57), *N*-nitro

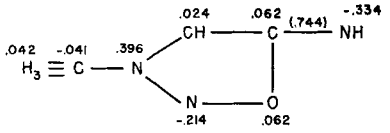
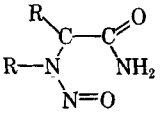


Fig. 4.—Molecular orbital calculated properties of 3-methylsydnonimine from *Reference 67*. (Number in parenthesis is a bond order. The remaining numbers are charge densities.)

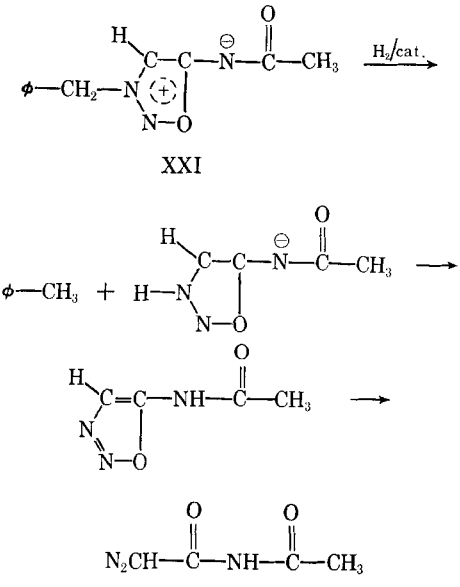
(17), *N*-acyl (57), *N*-phenyl-carbamoyl (26), and *N*-benzene-sulfonyl (27). The aromatic nature of the ring is illustrated by the fact that the 3-phenyl-*N*-acetyl-sydnonimine can be brominated and mercurated in the 4-position. Also, the NMR spectrum of the proton at position 4 supports ring aromaticity (26).

A molecular orbital treatment (68) (Fig. 4) lends support to structure XIX as being more truly representative of the sydnonimine structure. The exocyclic NH group bears a distinct negative charge density, while the C—NH bond is decidedly more of a double bond than the C—NH bond depicted in structural representation XVIII. The trisubstituted ring nitrogen bears a high positive charge density, though it is lower than the corresponding atom in methylsydnone.

Sydnonimines, unlike sydnones, are stable to acid (26). In alkali they suffer ring opening to give the nitrosoamides (XX). Prolonged exposure to hot concentrated acid cleaves the ring (25) but does not give a hydrazine derivative, in contrast to the behavior of the sydnones. Catalytic hydrogenation of sydnonimines and derivatives gives aliphatic amides with the exception of the 3-benzyl-*N*-acetylsydnonimine (XXI) which debenzylates to give α-diazoacetylacetamide (XXII) (28). Ohta (75) reported the same product from the treatment of the hydrochloride salt with acetic anhydride. (Scheme X.)



XX



XXI

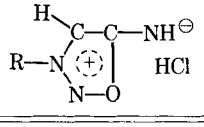
XXII

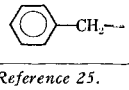
Scheme X

The ultraviolet absorption of the sydnonimines closely resembles that of the sydnones. The alkyl derivatives absorb at about 290 mμ (ε ~ 8500), while the aryl derivatives are shifted to lower energy, *i.e.*, 306 mμ (ε ~8100) for 3-phenylsydnonimine HCl, and 323 mμ (ε ~ 8700) for 3,4-diphenylsydnonimine HCl (25). Molecular orbital calculations on 3-methylsydnonimine (68) predict an absorption maxima, using the correlation equation of Kier and Roche (68), of 300 mμ in fair agreement with the experimental value.

The NMR spectra of sydnonimines (25) exhibits a low field singlet for the proton on C-4. (See Table VIII.) The position of this peak is very strong evidence of π-electron delocalization in this sytem. The induced ring current causes a magnetic anisotropic shift of the proton to lower field than benzene protons. It is further interesting to note the very large downfield shift of the C-4 proton in the 3-phenyl derivative. This may be due to a combined anisotropic effect of the two aromatic rings. The magnitude of the downfield shift for the ring proton in the 3-alkyl sydnonimines also appears to be greater by more than 1 τ than the corresponding chemical shift in 3-alkyl sydnones.

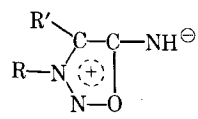
TABLE VIII.—CHEMICAL SHIFT OF SYDNONIMINES[a]

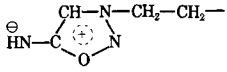
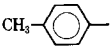
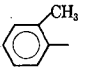


R	τ Ring H
CH_3	2.45
	1.97
	2.55

[a] From *Reference 25*.

TABLE IX.—PHARMACOLOGY OF SYDNONIMINES[a]



R	R′	LD_{50}, mmole/Kg.	Rat Blood Pressure Lowering, mm. Hg
$CH_3CH_2CH_2CH_2$—	H	0.54	13
			1
$CH_3CH_2CH_2CH_2$—	H	1.30	4
	H	1.23	8
	H	0.86	28
	H	1.50	13
		1.35	12
	H	2.38	22

[a] From *Reference 74*.

Biological Activity.—Interest in the potential biological activity of sydnonimines stems from the first reported testing of salts of 3-methyl-4-isopropyl and 3-methyl-4-isobutylsydnonimines (17). They showed no activity in experimental filarial infections. Daeniker and Druey (26) and subsequent patents (10) described the analgesic, antipyretic, anti-inflammatory, antiallergenic, and hypotensive effect of sydnonimines, with the 3-benzylsydnonimine salt being particularly active. Greco, Nyberg, and Cheng (42) synthesized a number of 3-alkyl sydnonimine salts for anticancer screening and reported no activity.

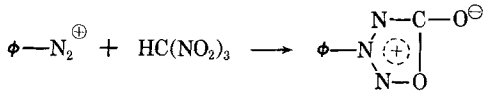
Oehme and co-workers (74) made a detailed pharmacological study of a number of sydnonimine salts. They found the sydnonimine salts studied to be more toxic than a series of sydnones studied at the same time. (See Table IX and compare with Table VII.) Deaths occurred with marked convulsions. The analgesic effects were not significant. The sydnonimines have no hypnotic effect themselves but a number of them did potentiate hexobarbital sleeping time. (See Table IX.) The compounds unsubstituted at position 4 were the most active, a situation comparable to the sydnones. The sydnonimines had no effect as anticonvulsants against pentylenetetrazol or electroshock at 0.1–0.4 mmole/Kg.

The sydnonimines tested by Oehme (74) showed spasmolytic effects in the rat duodenum and the guinea pig ileum. They noted a decrease in acetylcholine and histamine-induced contractions. They also noted a decrease in barium-induced spasms, with 3,4-diphenyl- and 3-*p*-tolyl-4-phenylsydnonimine salts. The strength of the effect corresponded to that of papaverine. The sydnonimines were hypotensive only in high doses, the effect not being pronounced (74). They did not inhibit the enzyme glutaminic acid decarboxylase *in vitro*.

There appear to be some fundamental differences in the activities of the sydnonimines and the sydnones. While the sydnones appear to act more by central mechanisms, the sydnonimines act more strongly on isolated organs.

PSEUDOÖXATRIAZOLES

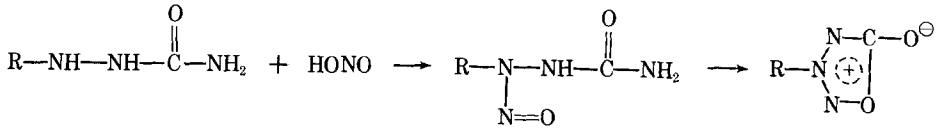
Chemistry and Physical Properties.—The Ψ-oxatriazoles (XXIII) are a group of mesoionic compounds formed by replacing a ring carbon atom in the sydnone with a nitrogen. The nitrogen atom donates one electron to the 6 π-electron sextet, hence the two classes of heterocycles are isoconjugate. Quilico (79) and Ponzio (77) reported the synthesis of this ring system when they observed the reaction of phenyldiazonium salt with nitroform. (Scheme XI.)



XXIII

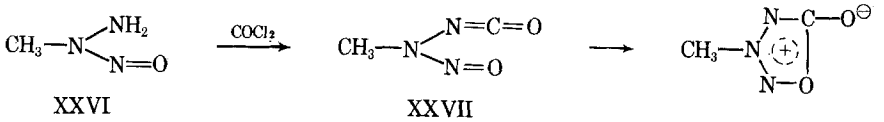
Scheme XI

They prepared the phenyl and *p*-nitrophenyl derivatives. Boyer and Cantor (15) reported the synthesis of the first alkyl-Ψ-oxatriazole using the appropriately substituted semicarbazide (XXIV) as the starting material. Boyer and Cantor proposed as the intermediate in the nitrosation step, the *N*-nitrososemicarbazide (XXV) a proposal subsequently confirmed by Boyer and Hernandez (16), who isolated the intermediate nitrososemicarbazide at low temperature. (Scheme XII.) Hashimoto and Ohta (49) reported the synthesis of methyl pseudoöxatriazole from the reaction of phosgene on *N*-nitroso-*N*-methylhydrazine (XXVI). They proposed an isocyanate intermediate (XXVII) although the carbamylchloride (XXVIII) is a possibility. (Scheme XIII.) Farrar (37) has prepared the phenyl derivatives by reacting potassium diazomethanedisulfonate (XXIX) with phenyldiazonium salt to form potassium hydrazono-

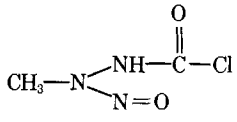


XXIV XXV

Scheme XII



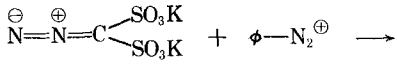
XXVI XXVII



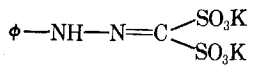
XXVIII

Scheme XIII

methanedisulfonate (XXX). (Scheme XIV.) Reaction of this compound with nitrous acid gave phenyl-Ψ-oxatriazole.



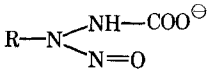
XXIX



XXX

Scheme XIV

Practically nothing is known about the chemistry of this mesoionic heterocycle. Boyer and Hernandez (16) noted that cyclohexyl-Ψ-oxatriazole resisted dilute acid, but with 80% sulfuric acid at 75° gave cyclohexanol, CO_2, and NH_3. The Ψ-oxatriazoles are considerably less stable in base (68) yielding a base-soluble product which gives a positive nitroso test. A likely structure for this compound is XXXI. This would be analogous to the base hydrolysis product of the sydnones.



XXXI

Molecular orbital calculations (68) give the ground state charge densities and bond orders shown in Fig. 5. Using the localization energy technique (81) the predicted point of protonation is the No. 2 nitrogen atom. It is interesting to note that the charge density of the Ψ-oxatriazole exocyclic oxygen is lower than that calculated for the corresponding atom of 3-methylsydnone. By contrast, the No. 3 nitrogen atom of methyl-Ψ-oxatriazole is substantially higher in positive charge then the corresponding atom of 3-methylsydnone.

Using the correlation equation derived from sydnones by Kier and Roche (67) relating U. V. absorption frequency with the MO calculated energy difference between the highest filled and lowest empty molecular orbitals (corresponding to a $\pi \rightarrow \pi^*$ spectral transition), the calculated $\lambda_{max.}$ for methyl-Ψ-oxatriazole is found to be 264 mμ, which agrees very closely with the experimental value of 260 mμ (68). The calculated value for phenyl-Ψ-oxatriazole is 277 mμ, which is very close to the experimental value of 275 mμ (37).

The infrared absorption band due to the carbonyl group lies between 1780 and 1800 cm.$^{-1}$. The ultraviolet absorption maximum is at 260 mμ ($\epsilon \sim 9000$) for alkyl derivatives and at 275 mμ ($\epsilon \sim 13{,}500$) phenyl (37).

Biological Activity.—Kier (65, 66) studied a number of alkyl substituted Ψ-oxatriazoles in the hope of finding an enhanced hypotensive activity previously noted for the isoconjugate sydnones (38). The compounds studied (see Table X) were free from toxicity at doses of 200 mg./Kg. in mice. Doses of 20 mg./Kg. i. v. in anesthetized dogs gave very prompt depressor effects in each case. The more potent hypo-

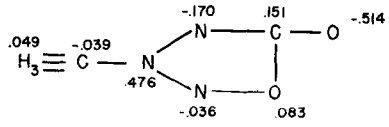
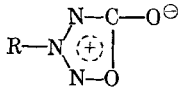


Fig. 5.—Molecular orbital calculations for methyl Ψ-oxatriazole from *Reference 68*, showing charge densities.

TABLE X.—BLOOD PRESSURE EFFECT OF Ψ-OXATRIAZOLES AT VARIOUS INTERVALS IN ANESTHETIZED DOGS[a]



R (20 mg./Kg.)	% of Blood Pressure After Injection					
	T + 10 sec.	T + 5 min.	T + 10 min.	T + 15 min.	T + 30 min.	T + 60 min.
CH_3	81	91	92	90		
$CH_3—CH_2—$	80	88	88	84		
$(CH_3)_2CH—$	66	75	70		69	76
$CH_3—CH_2$ $\quad\quad\diagdown$ $\quad\quad CH—$ $\quad\quad\diagup$ $CH_3—CH_2$	69	72	68		75	67
$CH_3—CH_2$ $\quad\quad\diagdown$ $\quad\quad CH—$ $\quad\quad\diagup$ $\quad CH_3$	43	64	54		59	64
$\quad CH_3$ $\quad\quad\vert$ $CH_3—C—$ $\quad\quad\vert$ $\quad CH_3$	46	66	57		47	43

[a] From *Reference 66*.

tensive compounds exhibited prolonged activity, in some cases lasting several hours. Other signs such as respiration, EKG pattern, heart rate, and gross and general appearance remained normal during this period of hypotension. The hypotensive effect ranged from approximately a 10% drop in blood pressure for methyl-Ψ-oxatriazole to about a 40% decrease for *tert*-butyl-Ψ-oxatriazole. Hypotensive activity was observed in the spinal cat, indicating that activity in the cat is not centrally mediated. Pretreatment in dogs with atropine or tripelennamine did not block the hypotensive effects, indicating that cholinergic effects or histamine release are unlikely causes of the hypotensive effects. Pressure responses in the dog to DMPP and epinephrine or norepinephrine were not reduced by the Ψ-oxatriazoles, indicating that ganglionic or α receptor blockade are also unlikely causes. On isolated rabbit ileum and rat ileum or uterus, the Ψ-oxtriazoles produced a prompt inhibition of smooth muscle activity in low concentrations. The hypotensive activity of these compounds may well be due to the relaxation of smooth muscle at the periphery. The order of potency found by Kier (66) was *tert*-butyl > *sec*-butyl $\cong$ 3-pentyl $\cong$ isopropyl > ethyl > methyl. This is in the order of increasing mesomeric contribution to the ring, suggesting that a minimization of π electron contribution enhances hypotensive activity.

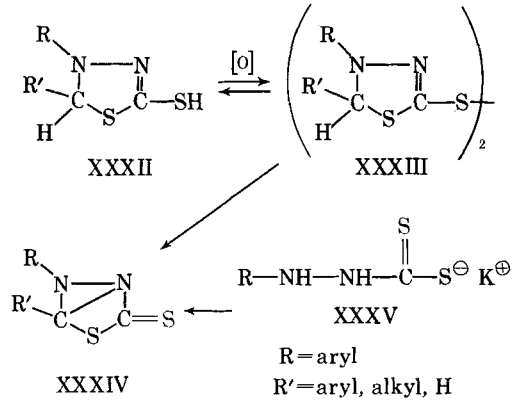
A comparison of three groups of mesoionic compounds which have shown hypotensive activity—namely, the Ψ-oxatriazoles (66), the 4-acyl sydnones (43), and the alkyl sydnones (38)—revealed the decreasing order of hypotensive potency as Ψ-oxatriazoles > 4-acyl sydnones > 3-alkyl sydnones. A comparison of the π-charge densities of the No. 3 nitrogen atom of each series reveals a decreasing positive charge, +.476, +.448, +.421, respectively. Thus, the involvement of this portion of the ring as a cationic moiety with a receptor was suggested (66). It is interesting to note that the calculated charge density of the No. 3 nitrogen of phenyl-Ψ-oxatriazole is +.484 (68) which, if the previous correlation holds, should lead to the prediction that it is a more potent hypotensive agent than the alkyl Ψ-oxatriazoles. No tests have been reported on this compound.

MESOIONIC 1,3,4-THIADIAZOLES

Chemistry and Physical Properties.—In 1895, Busch (20) noted that a thiadiazolinethiol (XXXII), oxidized to its disulfide (XXXIII), underwent a disproportionation to give the original compound (XXXII) plus a new compound postulated as XXXIV. Busch later synthesized the same compound by reaction of the potassium salt of an aryldithiocarbazinic acid (XXXV) with an acid chloride (21). (Scheme XV.) In this latter procedure, the compound in which R′ = H was made by using ethyl

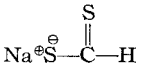
$CH_3—CH_2—O—CH{=}NH \cdot HCl$

XXXVI



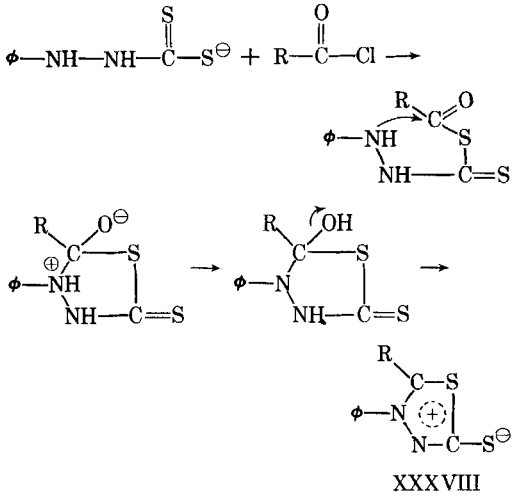
Scheme XV

formamidate hydrochloride (XXXVI) as the reagent (22). A more recent synthesis of this compound utilizes sodium dithioformate (XXXVII) with XXXV (6). The mechanism



XXXVII

of the reaction forming the mesoionic thiadiazoles is unknown. Baker (6) suggested a mixed anhydride as an intermediate. Stewart and Kier (89) proposed a mechanism employing this mixed anhydride intermediate. (Scheme XVI.)



Scheme XVI

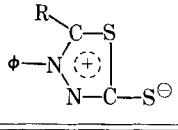
In 1938, Schönberg (82) suggested that the compound (XXXIV), synthesized by Busch, would be better represented by a hybrid structure involving several charged forms. These are now known to be a member of the mesoionic family of aromatic heterocycles and are conventionally represented as XXXVIII.

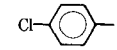
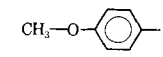
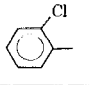
The question of proper nomenclature is still unsettled. According to Katritzky (59) these compounds would be named anhydro - 4,5-disubstituted - 2 - thio - 1 - thia - 3,4 - diazolinium thiol, and according to Baker (6), Ψ - 4,5 - disubstituted - 2,4 - dihydro - 2 - thio - 1 - thia - 3,4 - diazole. As a matter of expediency, we have referred to these compounds as mesoionic 1,3,4-thiadiazoles in print or as "pseudothiadiazoles" in conversation.

These compounds are high melting, yellow to orange solids. They are not dethionated by HgO in boiling benzene but at elevated temperatures and pressures the diphenyl derivative decomposes to several products including *N*-, *N*′-dibenzophenylhydrazine (22). With HgO in boiling ethanol, the ring cleaves to give a dithiocarbazinic acid (XXXV) (22). Oxidation with potassium permanganate results in the formation of benzanilide from the diphenyl compound. In hot base, the ring opens to reform the dithiocarbazinic acid salt (XXXV).

The diphenyl and the 4-phenyl-5-methyl compounds have dipole moments of 8.8 D in benzene (56) characteristic of mesoionic compounds. Stewart and Kier (89) reported an absorption band at 1325–1355 cm.$^{-1}$ for a number of mesoionic thiadiazoles, which they assigned to the thiocarbonyl group. (See Table XI.) In the same study they reported a U. V. absorption maximum of high molar absorptivity at about

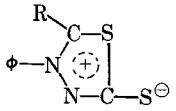
Table XI.—Mesoionic 1,3,4-Thiadiazole I.R. and U. V. Absorption[a]

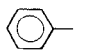
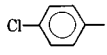
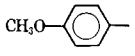
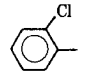


R	ν (C—S$^{\ominus}$) cm.$^{-1}$	λmax.	ε
H	1335	380	1800
CH_3—	1335	353	3000
CH_3CH_2—	1345	356	3100
$(CH_3)_2CH$—	1355	358	3400
$CH_3(CH_2)_2$—	1345	358	3400
$(CH_3)_3C$—	1355	357	3700
CH_3—$(CH_3)_3$—	1345	358	3400
$CH_3(CH_2)_4$—	1345	358	3400
	1335	405	4600
	1325	410	5000
	1335	403	4400
	1325	396	3700

[a] From *Reference 89*.

TABLE XII.—ANTIBACTERIAL TESTING OF MESOIONIC 1,3,4-THIADIAZOLES[a]



R	Sensitivity to *S. aureus*[b]	Microorganisms *D. pneumoniae*[b]	Hypotensive Effect	Pulse Rate Decrease	Pulse Pressure Increase
H—	++	—	—		
CH_3	++++	+++	+		
CH_3CH_2—	++++	+++	—		
$(CH_3)_2CH$—	++++	+++	+		+
CH_3—$(CH_2)_2$—	++++	+++	—		
$(CH_3)_3C$—	+	+	+	+	+
CH_3—$(CH_2)_3$—	+	+	+	+	+
CH_3—$(CH_2)_4$—	0	0	—		
	+	+	+		
	+	+			+
	+	+	+		
	0	0	—		
Control	0	0			
Penicillin G	++++	++++			

[a] From *References 88, 89.* [b] +, relative radius of inhibition zone on agar plates. The compound was placed on the paper disk by evaporation of a DSMO solution.

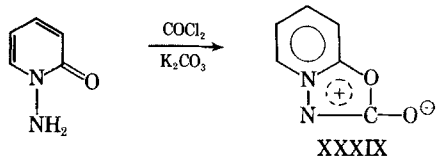
358 mμ for the 4-phenyl-5-alkylthiadiazoles and an absorption at about 400 mμ for the diaryl derivatives. (See Table XI.) It is interesting to note that the presence of an *ortho* substituent on the 5-phenyl group (4-phenyl-5-*o*-chlorophenylthiadiazole) must provide sufficient steric bulk to twist the ring out of coplanarity with the mesoionic ring, thereby isolating the two from maximal conjugation. This results in a shift of the $\lambda_{max.}$ to higher energy (lower wavelength). (See Table XI.)

Biological Activity.—Kier and Stewart (88, 89) tested a series of 4-phenyl-5-substituted mesoionic thiadiazoles against Gram-positive *Staphylococcus aureus*, *Diplococcus pneumoniae*, and Gram-negative *Escherichia coli*. Activity was observed with the 5-methyl, 5-ethyl, 5-isopropyl, and 5-propyl derivatives. Compounds with longer chains or greater bulk in this position had lower activity. The compounds were not active against the Gram-negative *E. coli*.

In other pharmacological tests, Stewart (88) observed that the LD_{50} exceeded 500 mg./Kg. i. p. in mice with each of the compounds in Table XII. The 5-alkyl compounds produced moderate convulsions in rats at higher doses. In anesthetized dogs, a number of compounds produced a fairly rapid lowering of blood pressure with a varying effect on the pulse rate. (See Table XII.) A direct cardiac effect or a peripheral vasodilation was assumed to be implied by the increase in pulse pressure with the 5-*p*-chlorophenyl compound as well as the production of extra systoles with the onset of the hypotensive effect.

ISOSYDNONES

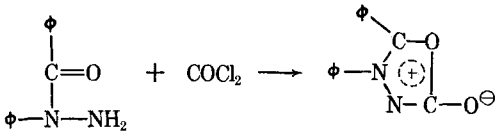
Chemistry and Physical Properties.—A modification of the sydnone ring, in which the lactone moiety is reversed, has been referred to as an isosydnone (XXXIX) (1). Hashimoto and Ohta (48) have named these Ψ-2,4-dihydro-4,5-disubstituted 2-keto-1-oxa-3,4-diazoles, while *Chemical Abstracts* indexed the compounds as 5-hydroxy-2,3-disubstituted-1,3,4-oxadiazolium hydroxide inner salts. This ring system was first reported by Hoegerle (53), who synthesized a number of bicyclic isosydnones (XXXIX). (Scheme XVII.) Using this



XXXIX

Scheme XVII

same method, Hashimoto and Ohta synthesized the first monocyclic isosydnone (48) (XL) from an α-acylhydrazine (XLI). (Scheme XVIII.)

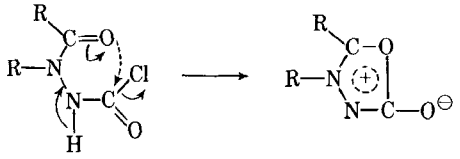


XLI XL

Scheme XVIII

The same authors attempted the synthesis of 4-methyl-5-phenylisosydnone but were not able to isolate enough of their products to fully characterize it. Ainsworth (1) succeeded in synthesizing and characterizing the 4-methyl-5-phenyl compound using phosgene in dioxane. Two 4,5-dialkyl isosydnones were first reported by Roche and Kier (80) using essentially the same synthesis, thus establishing the stability of a completely nonaromatic substituted isosydnone.

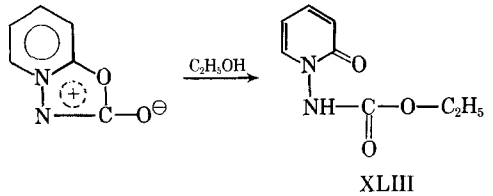
Ainsworth (1) has suggested the mechanism of formation starting with chloracylation of the hydrazine (XLII) followed by ring closure.



XLII

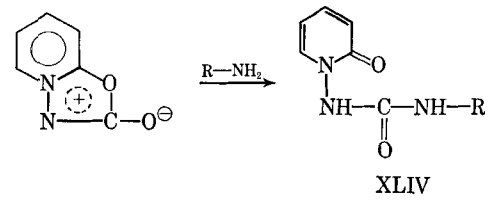
Scheme XIX

(Scheme XIX.) Hoegerle (53) noted that the ring was unstable when warmed in ethanol, yielding the substituted ethylurethane (XLIII). (Scheme XX.) Similarly, he noted the formation of the substituted urea XLIV, upon warming with NH_3 or an aliphatic amine. (Scheme XXI.) This behavior was confirmed by Hashimoto and Ohta (48) in the monocyclic isosydnone. Hashimoto and Ohta (48) observed that the product of acid hydrolysis of diphenylisosydnone was the starting material *N*-benzoyl-*N*-phenylhydrazine. They proposed a mechanism for the reaction. (Scheme XXII.) They observed that alkaline hydrolysis of the same compound led to phenylhydrazine and benzoic acid. They proposed the intermediate *N*-carboxy-*N'*-benzoyl-*N'*-phenylhydrazine (XLV) in the reaction.



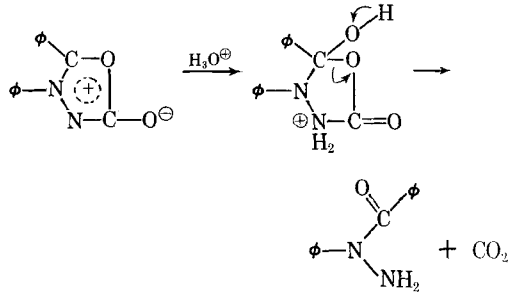
XLIII

Scheme XX

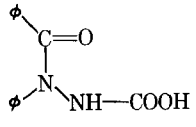


XLIV

Scheme XXI



Scheme XXII



XLV

The isosydnones possess high dipole moments, characteristic of the high charge separations found in mesoionic compounds. Ainsworth (1) reported a dipole moment of 7.58 ± 0.06 D for 4-methyl-5-phenylisosydnone in benzene at 25°. Bishop (13) in Ollis' laboratory reported dipole moment values for several aryl isosydnones, all at least 1 D higher than similarly substituted sydnones.

The infrared spectrum revealed a band at 1750–1760 cm.$^{-1}$ (1, 47, 53) which undoubtedly is due to the exocyclic C—O group. This is quite close to the typical value for the same group in the sydnones. The ultraviolet absorption maximum occurs (1, 53) at 290–325 mμ with high molar absorptivity. This is probably due to the isosydnone ring and is again comparable to the sydnone ring absorption.

Molecular orbital calculations using the methods described by Kier and Roche (67) give some insight into the electronic structure of the isosydnones. (See Fig. 6 for the calculation of 4-methyl-5-phenylisosydnone.) The charge den-

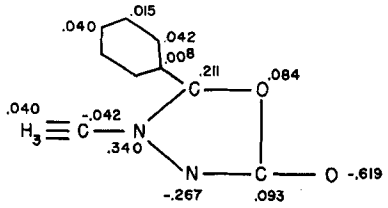


Fig. 6.—Molecular orbital calculation of 4-methyl-5-phenylisosydnone from *Reference 68*. (The numbers refer to charge densities.)

sity on the exocyclic oxygen atom is almost 0.1 electron higher than on the corresponding atom of the sydnone. This higher charge density indicates a higher calculated dipole moment than for sydnones, a prediction confirmed by the value of Ainsworth (1) of 7.58 D for 4-methyl-5-phenylisosydnone. The positive charge on the trisubstituted ring nitrogen is substantially lower than that of the sydnones, while the No. 5 ring carbon bears a much higher positive charge than the similarly placed ring carbon atom of the sydnone. Using the correlation equation derived from sydnones by Kier and Roche (67) relating U. V. absorption frequency with the energy difference between the highest filled and lowest vacant molecular orbital (corresponding to a $\pi \rightarrow \pi^*$ spectral transition), the calculated $\lambda_{max.}$ for 4-methyl-5-phenylisosydnone is found to be 296 mμ which corresponds very well to Ainsworth's experimental value of 294 mμ (1).

Biological Activity.—Interest in the isosydnones arose because of their isomeric relationship with the sydnones. In some preliminary studies Ainsworth reported (2) that 4-methyl-5-phenylisosydnone was inactive in an antibiotic screen at 25 mcg./ml. against 19 organisms representing animal pathogens, saprophytes, and bacterial and fungal plant pathogens. It was also inactive in entomology screening at 500 p.p.m. In a mouse behavior screening test, the pattern of activity suggested that the compound may be a cholinergic agent. The 4,5-diphenylisosydnone was found to give the same results against microorganisms.

SUMMARY

Much remains to be explored concerning the biological activity of mesoionic compounds. The work done to date clearly indicates significant activity and a potential as useful medicinal agents in several compounds. That the mesoionic compounds possess marked activity is not surprising, in the view of the authors. These ring systems are composed of delocalized π electrons which have been appreciably perturbed by the cores from which they arise. This perturbation is sufficient to deny the use of conventional, classical structural representations, and leads to the definition and depiction of a new class of heterocycle, the mesoionics. This perturbation results in a common structural feature found in the systems just reviewed and in many other mesoionic systems, that is, an oppositely charged dipolar segment at the extrema of a four-atom chain.

$$\overset{+\delta}{\text{—N—X—Y—}}\overset{-\delta}{\text{O}}$$

The extent of these partial charges is not obvious from classical resonance considerations, but emerges only after molecular orbital treatments, pointing out the tremendous value and utility of this technique to the medicinal chemist.

This charged four-atom segment is the hallmark of many pharmacologically active classes of drugs. Its significance perhaps lies in its ability to electrostatically interact with two complementary partially charged positions on a receptor macromolecule such as a protein helix.

A second structural feature of the mesoionic systems which suggests potential value is the fact that, although the molecules are internally charged and possess high dipole moments, they are over-all electrically neutral, therefore soluble to a much greater extent in nonpolar or lipoid solvents. Thus, *in vivo*, we have the capability of crossing lipoid barriers with a molecule which internally is appreciably ionic. This circumvents the problem found with quaternary salts and molecules rich in polar groups such as carbohydrates, which are usually constrained in their action to a water compartment, being generally unable to pass through a lipoid barrier.

Finally, the mesoionic rings are comparatively small in size, about 16 Å.^{2} for methylsydnone, and are planar, thus eliminating conformational problems and permitting the relatively close approach of all ring atoms to a receptor surface.

As the chemistry and electronic structures of more of these ring systems are studied and elucidated, more obvious relationships between these properties and their biological activities will be revealed, and the direction of future research on these compounds will be indicated.

REFERENCES

(1) Ainsworth, C., *Can. J. Chem.*, **43**, 1607(1965).
(2) Ainsworth, C., Eli Lilly & Co., personal communication.
(3) Baker, W., and Ollis, W. D., *Nature*, **158**, 703(1946).
(4) Baker, W., Ollis, W. D., and Poole, V. D., *J. Chem. Soc.*, **1949**, 307.

(5) *Ibid.*, **1950,** 1542.
(6) Baker, W., Ollis, W. D., Phillips, A., and Strawford, T., *ibid.*, **1951,** 289.
(7) Baker, W., and Ollis, W. D., *Quart. Rev.*, **1957,** 15.
(8) Bärnighausen, H., Jellinck, F., and Vos, A., *Proc. Chem. Soc.*, **1961,** 120.
(9) Bärnighausen, H., Jellinck, F., Munnick, J., and Vos, A., *Acta Cryst.*, **16,** 471(1963).
(10) Belg. pat. 618, 822; 618, 823; 618, 824(December 12, 1962).
(11) Bellas, M., and Sustritzky, H., *J. Chem. Soc.*, **1966,** 189.
(12) Bieber, T. I., *Chem. Ind. (London)*, **1955,** 910.
(13) Bishop, R. J., Ph.D. Dissertation, Sheffield University, England, 1966.
(14) Bochvar, D. A., and Bagaturyants, A. A., *Zh. Fiz. Khim.*, **39,** 1631(1965).
(15) Boyer, J. H., and Cantor, F. C., *J. Am. Chem. Soc.*, **77,** 1280(1955).
(16) Boyer, J. H., and Hernandez, J. A., *ibid.*, **78,** 5124 (1956).
(17) Brookes, P., and Walker, J., *J. Chem. Soc.*, **1957,** 4409.
(18) Bruzzese, T., Casadia, S., Marazzi-Uberti, E., and Turbo, C., *J. Pharm. Sci.*, **54,** 1042(1965).
(19) Bruzzese, T., Casadia, S., Coppi, G., and Marazzi-Uberti, E., *ibid.*, **54,** 1056(1965).
(20) Busch, M., *Ber.*, **28,** 2635(1895).
(21) Busch, M., and Mumker, H., *J. Prakt. Chem.*, **60,** 217(1899).
(22) Busch, M., Kamphansen, W., and Schneider, S., *ibid.*, **67,** 201(1903).
(23) Coulson, C. A., "Valence," Oxford Press, London, England, 1963, p. 387.
(24) Daeniker, H. U., and Druey, J., *Helv. Chim. Acta*, **40,** 918(1957).
(25) *Ibid.*, **45,** 2426(1962).
(26) *Ibid.*, **45,** 2441(1962).
(27) *Ibid.*, **45,** 2462(1962).
(28) *Ibid.*, **46,** 805(1963).
(29) Davis, D., Becker, H. J., and Rogers, E. F., *Phytopathology*, **49,** 821(1959).
(30) Dhawan, D., Ph.D. Dissertation, University of Florida, Gainesville, Fla., 1963.
(31) Dhawan, D., and Kier, L. B., *J. Pharm. Sci.*, **53,** 83 (1964).
(32) Eade, R. A., and Earl, J. C., *J. Chem. Soc.*, **1946,** 591.
(33) Earl, J. C., and Mackney, A. W., *ibid.*, **1935,** 899.
(34) Earl, J. C., Leake, E. M. W., and LeFevre, R. J. W., *ibid.*, **1948,** 2269.
(35) Earl, J. C., LeFevre, R. F. W., and Wilson, I. R., *ibid.*, **1949,** 5103.
(36) Earl, J. C., LeFevre, R. J. W., Pulford, A. G., and Walsh, A., *ibid.*, **1951,** 2207.
(37) Farrar, W. V., *ibid.*, **1964,** 906.
(38) Fregly, M. J., Kier, L. B., and Dhawan, D., *Toxicol. Appl. Pharmacol.*, **6,** 529(1964).
(39) Fugger, J., Tien, J. M., and Hunsberger, I. M., *J. Am. Chem. Soc.*, **77,** 1843(1955).
(40) Garrett, E. R., *J. Pharm. Sci.*, **53,** 42(1964).
(41) Gentile, P. S., and Mao, T. L., *J. Inorg. Nucl. Chem.*, **27,** 867(1965).
(42) Greco, C. V., Nyberg, W. H., and Cheng, C. C., *J. Med. Pharm. Chem.*, **5,** 851(1962).
(43) Greco, C. V., Tobias, J., Franco, J. M., and Kier, L. B., *J. Med. Chem.*, to be published.
(44) Hammick, D. L., and Voaden, D. J., *J. Chem. Soc.*, **1961,** 3303.
(45) Hashimoto, M., and Ohta, M., *J. Chem. Soc. Japan*, **78,** 181(1957).
(46) Hashimoto, M., and Ohta, M., *Bull. Chem. Soc. Japan*, **31,** 1048(1959).
(47) Hashimoto, M., Ph.D. Dissertation, Tokyo Institute of Technology, Tokyo, Japan, 1961.
(48) Hashimoto, M., and Ohta, M., *Bull. Chem. Soc. Japan*, **34,** 668(1961).
(49) Hashimoto, M., and Ohta, M., *J. Chem. Soc., Japan*, **35,** 766(1962).
(50) Hill, R. A. W., Sutton, L. E., and Longuet-Higgins, H. C., *J. Chim. Phys.*, **46,** 244(1949).
(51) Hill, R. A. W., and Sutton, L. E., *J. Chem. Soc.*, **1949,** 746.
(52) *Ibid.*, **1953,** 1482.
(53) Hoegerle, K., *Helv. Chim. Acta*, **41,** 548(1958).
(54) Huisgen, R., Gotthardt, H., and Grasbey, R., *Angew. Chem.*, **74,** 30(1962).
(55) Huisgen, R., *ibid.*, **75,** 604(1963).
(56) Jensen, K. A., and Friedliger, A., *Kgl. Danske Videnskab. Selskab, Mat.-Fys. Medd.*, **20,** 1(1943).
(57) Kato, H., Hashimoto, M., and Ohta, M., *Nippon Kagaku Zasshi*, **78,** 707(1957).
(58) Kato, H., and Ohta, M., *Bull. Chem. Soc. Japan*, **32,** 282(1959).
(59) Katritsky, A. R., *Chem. Ind. (London)*, **1955,** 521.
(60) Kier, L. B., and Dhawan, D., *J. Pharm. Sci.*, **51,** 1058(1962).
(61) Kier, L. B., Fox, L. E., Dhawan, D., and Waters, I. W., *Nature*, **195,** 817(1962).
(62) Kier, L. B., Dhawan, D., and Fregly, M. J., *J. Pharm. Sci.*, **53,** 677(1964).
(63) Kier, L. B., *Tetrahedron Letters*, **1965,** 3273.
(64) Kier, L. B., *J. Pharm. Sci.*, **55,** 98(1966).
(65) Kier, L. B., Al-Shamma, A., Campbell, D., Patil, P., and Tye, A., *Nature*, **210,** 742(1966).
(66) Kier, L. B., Al-Shamma, A., Hahn, R., and Tye, A., *J. Pharm. Sci.*, **55,** 1467(1966).
(67) Kier, L. B., and Roche, E. B., *ibid.*, **55,** 807(1966).
(68) Kier, L. B., unpublished data.
(69) Lawson, K. D., Brey, W. S., and Kier, L. B., *J. Am. Chem. Soc.*, **86,** 463(1964).
(70) Metz, F. I., Servass, W. C., and Welsh, F. E., *J. Phys. Chem.*, **66,** 2446(1962).
(71) Nakahara, K., and Ohta, M., *Nippon Kagaku Zasshi*, **77,** 1306(1956).
(72) Nöel, Y., *Bull. Soc. Chim. France*, **1964,** 163.
(73) Nyberg, W. H., and Cheng, C. C., *J. Med. Chem.*, **8,** 531(1965).
(74) Oehme, P., Gores, E., Schwarz, K., Pelsch, G., Faulhaber, H. D., and Lange, P., *Acta Biol. Med. German*, **14,** 369(1965).
(75) Ohta, M., and Kato, H., *Nippon Kagaku Zasshi*, **86,** 661(1965).
(76) Orgel, L. E., Cottrell, T. L., Dick, W., and Sutton, L. E., *Trans. Faraday Soc.*, **47,** 113(1951).
(77) Ponzio, G., *Gazz. Chim. Ital.*, **63,** 471(1933).
(78) Pütter, R. W., and Wolfrom, G., Brit. pat. 823,001 (November 4, 1959); Ger. pat. 1,057,124(May 14, 1959); Ger. pat. 1,069,633(November 26, 1959).
(79) Quilico, A., *Gazz. Chim. Ital.*, **62,** 912(1932).
(80) Roche, E. B., and Kier, L. B., *J. Pharm. Sci.*, **54,** 1700(1965).
(81) Roche, E. B., and Kier, L. B., to be published.
(82) Schönberg, A., *J. Chem. Soc.*, **1938,** 824.
(83) Simpson, J. C. E., *ibid.*, **1946,** 95.
(84) Stewart, F. H. C., *Chem. Ind. (London)*, **1962,** 1718.
(85) Stewart, F. H. C., and Danieli, N., *ibid.*, **1963,** 1926.
(86) Stewart, F. H. C., *J. Chem. Soc.*, **1963,** 701.
(87) Stewart, F. H. C., *Chem. Rev.*, **64,** 129(1964).
(88) Stewart, T. G., Ph.D. Dissertation, University of Florida, Gainesville, Fla., 1964.
(89) Stewart, T. G., and Kier, L. B., *J. Pharm. Sci.*, **54,** 731(1965).
(90) Tien, J. M., and Hunsberger, I. M., *J. Am. Chem. Soc.*, **83,** 178(1961).
(91) Vasileva, V. F., and Yashunskii, V. G., *Khim. Nauka Promy.*, **4,** 679(1959).
(92) Vasileva, V. F., Yashunskii, V. G., and Shchukina, M. N., *Zh. Obshch. Khim.*, **30,** 698(1960).
(93) Yamada, H., and Kazima, K., *J. Am. Chem. Soc.*, **82,** 1543(1960).
(94) Yashunskii, V. G., Vasileva, V. F., and Sheinker, Y. N., *Zh. Obshch. Khim.*, **29,** 2712(1959).
(95) Yashunskii, V. G., and Vasileva, V. F., *Dokl. Akad. Nauk SSSR*, **130,** 350(1960).
(96) Yashunskii, V. G., Vasileva, V. F., Naphlin, Y. G., Sycheve, T. P., and Shchukina, M. N., USSR pat. 148,062 (June 2, 1962).
(97) Yashunskii, V. G., and Sheinker, Y. N., *Zh. Obshch. Khim.*, **32,** 1681(1962).
(98) Yashunskii, V. G., Peresleni, E. M., and Sheinker, Y. N., *Invest. Akad. Nauk SSSR Ser. Fiz.*, **26,** 1295(1962).
(99) Paoloni, L., and Givmanini, A. G., *Gazz. Chim. Ital.*, **96,** 291(1966).

Computer Program for a Double Exponential Equation to Determine Biological Constants

By WERNER LOWENTHAL and BARBARA L. VITSKY

A new computer program in Fortran II (with Format) for the equation, $C = \frac{a_0 k_a}{V_d(k_a - k_d)} \cdot (e^{-k_d t} - e^{-k_a t})$, has been written. This program overcomes the problems of the previous programs published. A grid method was used to search for the constants and a method to handle exponential overflow was included. Applicability of the equation and the computer program was demonstrated on the IBM 1410 computer system with eight different sets of data from the literature. The data represented a variety of drugs, *i.e.*, sulfaethylthiadiazole, aspirin alone or with additives, oxazepam, penicillin salts, and trimeprazine, in a variety of dosage forms. The conclusions of the authors were verified by the calculated constants. In some instances the conclusions of the originators of the data could be expanded by the use of the calculated constants.

As our techniques become more sophisticated and our understanding of biological systems becomes greater, the mathematical interpretation of the data can become more refined.

Teorell's work (1) stands as a landmark in the field of kinetics of drug absorption, distribution, and excretion. Teorell's equation 25 is a simplification of the kinetics of drug absorption, distribution, and excretion. It describes the kinetics of drug appearance in the blood and its disappearance from the blood when the drug is administered by any route other than i.v. The constant k_d is a combination of various rate constants due to tissue absorption, drug release from tissues, metabolism, and excretion by all organs.

$$C = \frac{k_a a_0}{V_d(k_a - k_d)} (e^{-k_d t} - e^{-k_a t}) \quad \text{(Eq. 1)}$$

a_0 = drug dose in mg./Kg.,
C = blood drug concentration in mcg./ml.,
t = time in hours,
k_a = the apparent first-order absorption rate constant in hr.$^{-1}$,
k_d = the apparent first-order drug disappearance rate constant from the blood in hr.$^{-1}$,
V_d = the specific apparent volume of drug distribution in L./Kg.

Equation 1 also neglects any effect due to excretion of the drug in the bile and reabsorption by the intestine. Teorell assumed apparent first-order kinetics for drug appearance and disappearance in the blood, which has been shown many times before to be a valid assumption. The absorption and excretion of certain drugs and dosage forms may not follow apparent first-order kinetics, *e.g.*, some sustained-release dosage forms or when metabolism of the drug is the slowest step.

In spite of the limitations of this equation, it may yield useful information about the rate of drug absorption, indirectly about the process of absorption, and the rate of drug disappearance from the blood.

It is the purpose of this paper to describe a new computer program used to solve Eq. 1, and to illustrate the application of this equation and computer program.

This program is different from the one previously reported (2) because a simpler, more rugged search routine is used to determine the constants, a method is included to handle exponential overflow, and the number of search grids is not limited. In addition, one less parameter is calculated, increasing speed and accuracy. A value for a_0/V_d is determined rather than for V_d only.

COMPUTER PROGRAM

Using the following:

$$\frac{a_0}{V_d} = \gamma, \text{ and } k_a - k_d = \delta$$

Received August 4, 1966, from the Department of Pharmacy and the Department of Biometry, Medical College of Virginia, Richmond 23219.
Accepted for publication October 14, 1966.
This investigation was supported in part by grant P07-FR-00016-04 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The suggestions and assistance given by Mr. Samuel E. Ketner, Department of Biometry, concerning the grid search routine and its program, are acknowledged.
The flow diagram for the program shown in Fig. 2 is available from the authors on request.

Eq. 1 may be rewritten as:

$$C = \frac{\gamma k_a}{\delta} (e^{-k_d t} - e^{-k_a t}) \qquad \text{(Eq. 2)}$$

Rearranging

$$C = \frac{\gamma}{\delta} k_a e^{-k_a t} (e^{\delta t} - 1) \qquad \text{(Eq. 3)}$$

Taking natural logs

$$\ln C - \ln(e^{\delta t} - 1) = \ln \left(\frac{\gamma}{\delta} k_a\right) - k_a t \quad \text{(Eq. 4)}$$

Equation 4 may be calculated by means of the least squares procedure. The program calculates the best values for the constants by finding the curve with the minimum variance.

The program in Fortran II (with Format) is divided into three main parts: (*a*) the data input statements 1–70, (*b*) the search for minimum variance, statements 75–360, and (*c*) the printing of the final results, statements 360–480.

The first card of the input data contains the title of the data (up to 80 positions). The second card is the number of observations (N) and the print control k_1. If the value of the print control k_1 is anything other than a zero or blank, the minimum variance and corresponding k_a, k_d, and k_d/k_a will be printed after each grid search. This was included in the program to give greater versatility if one is interested in seeing the results of each search. The third card contains the estimated values of k_a and for the ratio k_d/k_a. These estimates were made from literature values for similar drugs or just estimated. These estimates could vary by a factor of 10 or more and still obtain a proper solution to the equation. If the initial estimates of k_a and k_d differ from the final calculated values by a factor greater than 10, the computations would take about 45 min. The computer will continue to compute until it has calculated the best values. The fourth card begins the observations, one time value, and the corresponding concentration per card.

The second portion of the program begins using the input to obtain a variance. The initial estimates of k_a and k_d/k_a become the center for a 2^3 factorial ($\times$ in Fig. 1). The initial incrementing value of 0.1 is added or subtracted from the estimates of k_a and k_d/k_a giving eight new estimates for which variances are computed. A new factorial is built around the minimum variance of the eight computed. The current estimates of k_a and k_d/k_a are incremented and decremented in steps of 0.1 until the current minimum variance computed is larger than the previous one, or until k_a or k_d/k_a become zero or less than zero by subtraction of 0.1. The 0.1 incrementing value is then reduced to 0.05. The grid search routine continues alternately reducing the increment values by 0.005 each time the currently computed minimum variance exceeds the previous minimum variance, until the value of k_a increment reaches zero. The values for k_a and k_d/k_a are essentially incremented and decremented independently.

In this manner, the program converges on the minimum variance, which is assumed when the k_a increment reaches zero.

A simplified grid search routine is presented in Fig. 1.

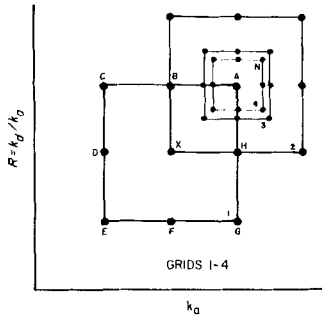


Fig. 1.—Grid search routine. Key: $\times$, initial intersection, the first estimates of k_a and k_d/k_a; A–H, first eight values to calculate variances. First minimum variance at A. The eight variances in grid 2 were greater than the one at A, so that the increments were reduced to 0.05 giving grid 3. For the purpose of this example both k_a and k_d/k_a were reduced simultaneously. In the program they are incremented and decremented independently. In grid 3 the new values of k_a and k_d/k_a gave larger variances than in A, so that the 0.05 increments were reduced by 0.005 (new increment = 0.045), giving grid 4. Again for simplification both constants were reduced simultaneously. A new minimum variance was found at N. The eight values in a grid using grid 1 as an example are: A: R + increment, k_a + increment; B: R + increment, k_a; C: R + increment, k_a − increment; D: R, k_a − increment; E: R − increment, k_a − increment; F: R − increment, k_a; G: R − increment, k_a + increment; and H: R, k_a + increment.

The final portion of the program computes and prints calculated concentrations at 15-min. intervals through the maximum time of the observed data. Following this, the values for δ, a_0/V_d, k_a, k_d, k_d/k_a, and the variance are printed.

A routine is set up in the program to avoid exponential overflow. This is done by checking the value of $-k_a t$. The exponential value ($e^{-k_a t}$) approaches zero as time increases. Absorption should be complete after many hours (*e.g.*, 24 hr.), so that if the value of $k_a t$ is greater than 50, the rate of absorption into the blood is negligible and the effect due to absorption is set to zero.

The approach using input of k_d/k_a is taken rather than using a constant for k_d because it is more accurate to work with this ratio.

The program was written for an IBM 1410 computer system, and due to the small core usage, it may be adopted to almost any computer system using Fortran. This program is fairly lengthy, dependent in part on the accuracy of the initial estimates for k_a and k_d/k_a and in part due to running the program concurrently with an interruptible telecommunications monitor. The procedure takes from 5 min. to 45 min. per set of data.

The complete program for Fortran II (with Format) is presented in Fig. 2.

RESULTS AND DISCUSSION

Most blood concentration *versus* time data in the literature could not be fitted by this equation because of: (*a*) poor spacing of time intervals, (*b*)

insufficient number of data, and (*c*) data were not collected over a sufficiently long period of time. These shortcomings of the data usually meant that the experiments were not designed for kinetic studies. Most of the usable data were found in the pharmaceutical literature where experiments were often designed for rate studies.

If the data showed an initial "lag phase" and only two points on the ascending portion of the curve, then the program may fail. Dropping the data for the lag phase and solving the equation would reduce the variance but may still give a poor fit due to lack of data. If the observed data give a high, sharp peak, the program would calculate a line below the peak and give a relatively poor estimate of the concentrations representing the peak.

Obviously, if the data do not follow apparent first-order kinetics, the equation would not fit the

```
C
C     CALCULATION OF KINETIC CONSTANTS FROM BLOOD LEVELS OF DRUGS
C     SEARCH ROUTINE FOR BEST REGRESSION FIT
C
C           DATA INPUT
C     THE FIRST CARD CONTAINS AN EIGHTY POSITION TITLE
C     THE SECOND CARD CONTAINS THE NUMBER OF OBSERVATIONS TO FOLLOW AND
C     THE PRINT CONTROL
C     THE THIRD CARD CONTAINS THE ESTIMATED VALUE FOR KA AND THE VALUE
C     FOR R, WHERE R= KD/KA
C     FOLLOWING THIS ARE THE VALUES FOR THE EXPERIMENTAL DATA.  ONE TIME
C     VALUE AND THE CORRESPONDING OBSERVED CONCENTRATION PER CARD
C
      DIMENSION COBS(25),TIME(25),TRY(8),SKA(8),SR(8),TITLE(8),SGAM(8)
    1 READ 10, ((TITLE(I), I=1,8)
   10 FORMAT(8A10)
      READ 20, N, K1
   20 FORMAT(2I2)
      XN=N
      READ 30, XKA, R
   30 FORMAT(2F10.0)
C
C     N= NUMBER OF OBSERVATIONS.  K1 = PRINT CONTROL.  IF K1 IS ANYTHING
C     THAN ZERO OR BLANK, THE MINIMUM VARIANCE AND CORRESPONDING KA AND
C     KD WILL BE PRINTED AFTER EACH GRID SEARCH
C     KA = FIRST ORDER ABSORPTION CONSTANT.     R = KD/KA, WHERE KD IS
C     THE APPARENT FIRST ORDER DISAPPEARANCE CONSTANT
C
      NCOUNT =0
      TKA=XKA
      TR=R
      JN=1
      XINC=0.1
      RINC=0.1
      DEC=0.05
      RDEC=0.05
      SUMX=0.
      J9=0
      NPAGE=0
C
C     SET INITIAL CONDITIONS INTO TEMPORARY MINIMUM POSITION
C     SET UP VALUES FOR INCREMENTS AND SET UP J9 AS CONTROL FOR WHICH
C     INCREMENT IS TO BE CHANGED FIRST
C
      NPAGE=NPAGE+1
      PRINT 40,(TITLE(I), I=1,8),NPAGE
   40 FORMAT(1H1,5X,70HSEARCH FOR CALCULATION OF KINETIC CONSTANTS FROM
     1BLOOD LEVELS OF DRUGS,5X,4HPAGE,I3//7X,8A10)
      PRINT 50
   50 FORMAT(//32X,17HEXPERIMENTAL DATA//29X,10HOBSERVED C,8X,4HTIME//)
      DO 70 J= 1,N
      READ 30, TIME(J),COBS(J)
      PRINT 60, COBS(J),TIME(J)
   60 FORMAT(27X,F12.5,6X,F8.3/)
   70 SUMX=SUMX+TIME(J)


  270 XSIG = TRY(1)
      XKA = SKA(1)
      R = SR(1)
      GAMMA = SGAM(1)
  280 CONTINUE
C
C     TEST PRINT CONTROL.  IF SET, PRINT MINIMUM VARIANCE AND
C     CORRESPONDING CONSTANTS ON GRID
C
      IF(K1)281,289,281
  281 XKA=XKA*R
      NCOUNT=NCOUNT-1
      IF(NCOUNT)282,282,283
  282 NPAGE =NPAGE +1
      PRINT 40,(TITLE(I),I=1,8),NPAGE
      NCOUNT = 41
  283 PRINT 285,XSIG,XKA,XKD,R
  285 FORMAT(/5X,5HVAR =,F12.5,5H KA= ,F12.5,5H KD= ,F12.5,4H R= ,F12.5)
C
C     IS THIS MINIMUM LESS THAN PREVIOUS MINIMUM?
C     IF SO, CONTINUE SEARCH AROUND CURRENT POINT
C     IF NOT, REDUCE INCREMENT AND CONTINUE SEARCH AROUND TEMPORARY
C     MINIMUM
C
  289 IF(XSIG-TEMP)290,335,335
  290 TEMP = XSIG
      TGAM=GAMMA
      TKA =XKA
      TR = R
C
C     R CAN NEVER BE ZERO, KA CAN NEVER BE LESS THAN OR EQUAL TO ZERO
C
  300 IF(R-RINC)310,335,310
  310 IF(XKA-XINC)335,335,320
  320 XKA = XKA + XINC
      GO TO 75
C
C     FOR FIRST POINT OF KA AND R
C
  330 TEMP = SIGMA
      TGAM = GAMMA
      JN = 8
      GO TO 300
C
C     REDUCE THE VALUE OF THE INCREMENTS ALTERNATELY
C
  335 IF(J9-1)340,350,340
  340 XINC = XINC - DEC
      DEC =0.005
      XKA = TKA
      R = TR
      J9 =1
C
C     IF KA INCREMENT IS ZERO ASSUME MINIMUM VARIANCE HAS BEEN FOUND
C


   75 DO 240 I=1,IN
      XKD=XKA*R
      DELTA=XKA-XKD
C
C     KD MAY NEVER BE = OR GREATER THAN KA
C
      IF(DELTA)190,190,80
   80 SUMY=0.
      DO 90 J=1,N
   90 SUMY=SUMY + LOGF(COBS(J)/(EXPF(DELTA*TIME(J))-1.))
      IF ACCUMULATOR OVERFLOW, 190, 100
  100 GAMMA = EXPF(SUMY/XN + XKA*SUMX/XN)*DELTA/XKA
C
C     GAMMA= AO/VD
C
      VAR=0.
      DO 160 J= 1,N
C
C     ROUTINE TO AVOID EXPONENTIAL OVERFLOW
C
      IF(-XKA*TIME(J) + 50.)110,110,150
  110 CALC=(GAMMA*XKA/DELTA)*(EXPF(-XKD*TIME(J)))
      GO TO 160
  150 CALC= (GAMMA*XKA/DELTA)*(EXPF(-XKA*TIME(J)))*(EXPF(DELTA*TIME(J))-
        1.)
  160 VAR=VAR + (COBS(J)-CALC)**2
      IF(IN-1)180,170,180
  170 SIGMA = VAR/(XN-3.)
      GO TO 330
  180 TRY(I) = VAR/(XN-3.)
      SKA(I)=XKA
      SR(I) = R
      SGAM(I) = GAMMA
  190 GO TO (200,210,210,220,220,230,230,240),I
C
C     SIGMA = INITIAL AND FINAL VARIANCES
C     STATEMENT 190 CAUSES COMPUTATION OF THE EIGHT POINTS ON THE GRID
C
  200 R = R-RINC
      GO TO 240
  210 XKA = XKA-XINC
      GO TO 240
  220 R = R + RINC
      GO TO 240
  230 XKA = XKA + XINC
  240 CONTINUE
C
C     SEARCH FOR MINIMUM VARIANCE ON GRID
C
      XSIG = TRY(1)
      XKA = SKA(1)
      R = SR(1)
      GAMMA = SGAM(1)
      DO 280 I = 2,8
      IF(XSIG-TRY(I))280,280,270



      IF(XINC)360,360,300
  350 RINC = RINC -RDEC
      RDEC = 0.005
      XKA = TKA
      R = TR
      J9 = 0
      GO TO 300
C
C     MINIMUM VARIANCE HAS BEEN FOUND.  PRINT FINAL RESULTS
C
  360 GAMMA = TGAM
      XKA = TKA
      R = TR
      SIGMA = TEMP
      XKD = XKA*R
      DELTA = XKA-XKD
      NPAGE = NPAGE +1
      PRINT 40,(TITLE(I),I = 1,8),NPAGE
      PRINT 370
  370 FORMAT(///32X,8HBEST FIT///15X,12HCALCULATED C,8X,4HTIME//)
      NCOUNT = 41
      JN=TIME(N)+4.
C
C     CALCULATE CONCENTRATION AT FIFTEEN MINUTE INTERVALS
C
      XTIME=0.
      DO 450 J=1,JN
      XTIME=XTIME+.25
C
C     ROUTINE TO AVOID EXPONENTIAL OVERFLOW
C
      IF(-XKA*XTIME+50.)380,380,420
  380 CALC=(GAMMA*XKA/DELTA)*(EXPF(-XKA*XTIME))*(EXPF(-XKD*XTIME))
      GO TO 430
  420 CALC=(GAMMA*XKA/DELTA)*(EXPF(-XKA*XTIME))*(EXPF(DELTA*XTIME)-1.)
  430 NCOUNT = NCOUNT - 1
      IF(NCOUNT)440,440,450
  440 NAPGE = NPAGE +1
      PRINT 40,(TITLE(I),I=1,8), NPAGE
      PRINT 370
      NCOUNT = 41
  450 PRINT 460,CALC,XTIME
  460 FORMAT(15X,F12.5,4X,F8.3/)
      IF(NCOUNT-18)470,470,480
  470 NPAGE = NPAGE +1
      PRINT 40,(TITLE(I),I=1,8),NPAGE
  480 PRINT 490,DELTA,GAMMA,XKA,XKD,R,SIGMA
  490 FORMAT(//20X,7HDELTA =,4X,F12.5//20X,7HAO/VD =,4X,F12.5//20X,4HKA
     1=,7X,F12.5//20X,4HKD =,7X,F12.5//20X,7HKD/KA =,4X,F12.5//20X,11HVA
     2RIANCE = ,F12.5)
      PAUSE
      GO TO 1
      END
```

Fig. 2.– Computer program in Fortran II (with Format) for double exponential equation.

observed data. Symmetrical curves on rectangular coordinates are an indication of non-first-order rates.

If the data for each subject were used instead of averages, then additional steps may be added to the program to calculate the variation about each point on the observed curve. With the present program separate tests must be made to determine the goodness of fit of the calculated curve. The χ^2 test,

$$\chi^2 = \frac{(C_{obs.} - C_{calcd.})^2}{C_{calcd.}}$$

can be used. The χ^2 values obtained are compared with the table of χ^2 values. It was found that the computer calculated variance was just as good an indicator of goodness of fit as the χ^2 test.

The following is a brief discussion of several sets of data obtained from the literature. The constants are presented in Table I.

The disappearance rate constant for sulfaethylthiadiazole (SETD) calculated by Swintosky *et al.* (3) was verified. Using averages of the published data, the k_d calculated by the computer is 0.0856 hr.$^{-1}$ and that reported was 0.073 hr.$^{-1}$ (average of four values). The k_a calculated is 2.68 hr.$^{-1}$, indicating a much more rapid absorption than disappearance from the blood. The a_0 was calculated from the 2.0-Gm. dose divided by the average weight of the subjects.

Table I.—Computer Calculated Constants from Literature Data

Ref.	k_a hr.$^{-1}$	k_d hr.$^{-1}$	V_d L./Kg.
(3)	2.675	0.0856[a]	0.62
(4)			
1.0 Gm.	0.500	0.438	0.0718
2.0 Gm.	0.455	0.417	0.0895
3.0 Gm.	0.285	0.221	0.120
4.0 Gm.	0.495	0.370	0.117
(5)			
Plain tablet	0.970	0.0909[b]	0.189
Sustained-release	0.955	0.0604[c]	0.271
(6)			
D	2.07	0.246	0.146
E	0.535	0.461	0.0826
L	0.635	0.400	0.0955
(7)	0.295	0.0723	0.906
(8)			
Fasting:			
penicillin acid	2.495	0.986	5.643[d]
cal. penicillin V	3.315	1.061	7.112[d]
pot. penicillin V	3.225	0.903	7.908[d]
sod. penicillin V	2.210	0.641	3.312[d]
Std. meal:			
penicillin acid	0.820	0.816	2.274[d]
cal. penicillin V	0.990	0.985	2.903[d]
pot. penicillin V	1.035	1.025	3.809[d]
sod. penicillin V	0.940	0.931	1.367[d]
(9)			
Buffered ASA	1.310	0.116	0.142
138	0.155	0.136	0.228
152	0.195	0.120	0.165
134 B	Poor fit		
134 C	0.435	0.154	0.129
(10)			
5 mg.	0.465	0.0860	0.000457
15 mg.	0.865	0.0800	0.000533
5 mg. t.i.d.	0.100	0.0835	0.000520
15-mg. sustained-release	0.0950	0.0931	0.000528

[a] 0.073 hr.$^{-1}$ reported. [b] 0.09 hr.$^{-1}$ reported for 1 patient. [c] 0.05 reported. [d] a_0/Vd values are not given because patient weights were not reported.

Later Swintosky *et al.* (4) reported blood concentrations from SETD given in 1.0, 2.0, 3.0, and 4.0-Gm. doses. These data could not be fitted by Eq. 1 using the previous program (2). There is an apparent "lag time" in the initial absorption of the drug before absorption becomes an exponential process, so that the concentration at 20 min. was omitted. This resulted in better fitting the observed data by the calculated data, and the variance was decreased by about one-half. The omission of the data at 20 min. resulted in only small increases in the values on the constants. The dose a_0 was calculated as indicated above. The present equation would require an additional term to fit the initial "lag time." This "lag time" is important in determining length of time for absorptive process to occur.

The constants calculated from these data differ considerably from those calculated from the first set of data described above. There is essentially no difference in the k_a for 1.0, 2.0, and 4.0-Gm. doses, and for k_d for 1.0 and 2.0-Gm. doses. The V_d for 1.0 and 2.0-Gm. doses are similar, as are those for 3.0 and 4.0-Gm. doses. The k_a and k_d for the 3.0-Gm. dose are smaller than those for the other doses. Some of the discrepancies are due to the fact that the calculated data cannot reproduce the high peaks in the 1.0 and 2.0-Gm. doses, so the computer-calculated k_a could be smaller than those reported by Swintosky *et al.* (3) in their earlier work. Also, if the data had been collected for longer than 4 hr., a different k_d might have been obtained. The k_d for the 3.0-Gm. dose may be only an estimate because of the lack of data to properly calculate the k_d. For the 4.0-Gm. dose, the calculated concentrations did not go so high as the observed concentrations. In the other three cases the calculated data appear to give a good approximation of the experimental data. Since the 3.0-Gm. dose gives different constants, the 4.0-Gm. dose a relatively small V_d, and the V_d for the 3.0 and 4.0-Gm. doses differ from the 1.0 and 2.0-Gm. doses, it is possible that different rate-limiting steps become important at the 3.0-Gm. dose and greater.

Nicholson *et al.* (5) compared blood levels due to SETD in plain tablets and as sustained-release tablets. The data were averaged and the computer calculated constants found in Table I verify the constants for rate of disappearance from the blood reported by Nicholson and co-workers. The k_a did not vary significantly between the plain tablets and the sustained-release tablets, although the rates of absorption vary. The decrease in k_d for the sustained-release tablets is accompanied by an increase in V_d indicating wider distribution or more SETD in the fluids which would be expected from Eq. 1. The smaller k_d indicates slower disappearance from the blood.

Liberman and Wood (6) compared three different aspirin formulations and reported serum salicylate levels up to 2.5 hr. The three formulations

were: *D*, aspirin, dihydroxyaluminum glycinate, and magnesium carbonate; *E*, commercial aspirin–starch granulation; and *L*, a commercial aspirin tablet. The constants for formula *E* could not be calculated by the previous program (2). The data showed higher and earlier peak of salicylate in serum with formula *D*. This can be seen from the k_a which is 3 to 4 times greater than the k_a for the other two preparations, a smaller k_d and a larger V_d, indicating a more rapid absorption and a slower loss from the blood.

The constants for the data of Walkerstein *et al.* (7) could not be calculated by the previous program but were satisfactorily calculated by the new program. Plasma levels of ^{14}C-oxazepam given orally to dogs were reported from 0.5–120 hr. The calculated constants are shown in Table I.

Juncher and Raaschou (8) gave 400,000 units of penicillin acid and various penicillin salts to 10 subjects, either fasting or after a standard meal. Blood levels were measured from 0.25 to 6 hr. The fasting subjects had higher blood levels and the calcium and potassium salts of penicillin V gave the highest blood levels. The authors' conclusions are verified by the calculated constants. The data from subjects which had fasted gave much higher k_a, indicating higher rates of absorption than the data from subjects who ate a standard meal. The rate of decline in the blood levels was about the same for all preparations.

Wood and Syarto (9) compared buffered aspirin to aspirin coated with different ratios of ethyl- and methylcellulose; for formula 138 the ratio was 100:1; for 152, 82.5:17.5; for 134B, 75:25; and for 134C, 25:75. The workers did not calculate any rate constants, but stated plots of cumulative *in vivo* release fit apparent first-order better than zero-order kinetics. The same amount of aspirin was used in all the formulas. Blood levels were obtained from 0.5 to 24 hr. The constants shown in Table I indicate that the absorption rate for buffered aspirin is much greater than for the other preparations. The rate of decrease in blood for all the preparations is similar; also V_d does not change very much with formulation changes. As the ethylcellulose content of the coatings decreases, the rate of aspirin absorption increases and the apparent specific body volume decreases. Therefore, the amount of ethylcellulose in the coating affects the rate of aspirin absorption.

Johnson and Masters (10) gave ^{35}S-labeled trimeprazine tartrate orally to patients. Serum levels up to 48 hr. were used to compare a 5-mg. dose, a 15-mg. dose, a 5-mg. dose given 3 times a day, and a 15-mg. dose sustained-release preparation. These workers reported the characteristic differences expected between a 5-mg. dose and a 15-mg. dose, and that 5 mg. given 3 times a day and the 15-mg. sustained-release product gave similar blood levels. The constants shown in Table I indicate that the rate of disappearance of the drug from the blood given by the various dosage regimens and the V_d closely agree. The V_d for the 5-mg. dose is smaller because there is only one-third the amount to be distributed in the body compared to the other 15-mg. doses. The V_d for the other three preparations are similar. The k_a for the 15-mg. dose is about 1.86 times greater than the k_a for the 5-mg. dose; assuming instantaneous availability for both dosages, this could indicate k_a is dose dependent. The more drug exposed to absorbing surfaces, the more rapid the absorption. The k_a for the 5-mg. dose given 3 times a day and the 15-mg. sustained-release preparation appear the same, the k_d are similar, and the V_d appear the same, indicating the divided dose therapy and the sustained-release preparation are similar.

REFERENCES

(1) Teorell, T., *Arch. Intern. Pharmacodyn.*, **57**, 205 (1937).
(2) Borzelleca, J. F., and Lowenthal, W., *J. Pharm. Sci.*, **55**, 151(1966).
(3) Swintosky, J. V., Robinson, M. J., Foltz, E. L., and Free, S. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 399(1957).
(4) Swintosky, J. V., Foltz, E. L., Bondi, A., Jr., and Robinson, M. J., *ibid.*, **47**, 136(1958).
(5) Nicholson, A. E., Tucker, S. J., and Swintosky, J. V., *ibid.*, **49**, 40(1960).
(6) Liberman, S. V., and Wood, J. H., *J. Pharm. Sci.*, **53**, 1492(1964).
(7) Walkerstein, S. S., Wiser, R., Gudmundsen, C. H., Kimmel, H. B., and Corradino, R. A., *ibid.*, **53**, 1181(1964).
(8) Juncher, H., and Raaschou, F., *Antibiot. Med. Clin. Therap.*, **4**, 497(1957).
(9) Wood, J. H., and Syarto, J., *J. Pharm. Sci.*, **53**, 877 (1964).
(10) Johnson, P. C., and Masters, Y. F., *J. Lab. Clin. Med.*, **59**, 993(1962).

Isolation of Cantharidin from *Epicauta pestifera*

By WILBERT G. WALTER and JOHN F. COLE

Epicauta pestifera Werner (*Meloidae*) is a species of blister beetle which is found in North America and contains a powerful irritant and vesicant. This paper reports the isolation, quantitative determination, and identification of the active principle responsible for this activity. Employing standard practices of analysis, which involved infrared and NMR spectroscopy, thin-layer chromatography, elemental analysis, and determination of physical constants, the substance was identified as cantharidin.

THIS PAPER reports the isolation and quantitative determination of cantharidin from a species of blister beetle, *Epicauta pestifera* Werner (*Coloptera, Meloidae*) which is found in North America and occurs commonly in Mississippi. This particular specie of blister beetle has not been previously investigated for its vesicating principle. Cantharidin, as it was determined in this investigation, is the substance responsible for the strong irritating and vesicating effects. This compound has also been isolated from several other species of beetles of the genera *Epicauta* of Asia, Indonesia, and South America.

Investigators have reported previously that cantharidin occurs in *E. pilmus* Porter (1), which is found in Chile, in concentrations ranging from 1.7% to 3.5%; in *E. gorhami* Marseul (2), from 0.4% to 1.75%; in *E. waterhousei* (3), which is found in Formosa, from 1.02% to 1.26%; and *E. ruficeps* Ill. (4), which is found in Java, and *E. odspersa* (5), found in Brazil, in concentrations of 2.5%. Shimano *et al.* (2) showed that the substance is present in *E. gorhami* in the head (2.5%), thorax (1.2%), abdomen (94%), and legs (2%), which is contrary to general belief that it is confined principally to the abdomen where it may be secreted from between the segments of the underside. The cantharidin content varies considerably from one genera and species to another but is found usually in concentrations of approximately 1% and has been reported as high as 3.5% in *Epicauta gorhami* (2). In this investigation, cantharidin was found to be present in *E. pestifera* in the amount of 1.09% by weight.

Received April 25, 1966, from the Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University.

Accepted for publication September 29, 1966.

Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

The authors acknowledge support of this work by the Research Institute of Pharmaceutical Sciences, Natural Product Division, School of Pharmacy, University of Mississippi.

The authors express their appreciation to Dr. Richard B. Selander, Department of Entomology, University of Illinois, Chicago, who identified the beetle, for his encouragement and assistance.

DISCUSSION

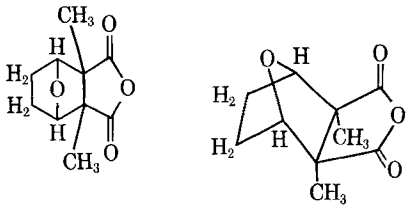
E. pestifera is, in the United States, often referred to as the common margined blister beetle of potatoes. It has been called *E. solani* Werner and *E. marginata* Fabr. The latter may sometimes be used in reference to *E. cinera* Forster. *E. pestifera* is widely scattered throughout North America and the U. S. but occurs principally from the Atlantic Coast to the Rocky Mountains. The seasonal range of the adult beetles is July to September. They are regarded as garden pests and are known to attack garden vegetables in addition to the potato plant. However, Werner (6) points out that the margined blister beetle of potatoes does have a grey form and that they occur on *Clematis* species of plants rather than potatoes, and furthermore, that they are actually an *Epicauta* species distinct from that found on potatoes. This and related species have been found on plants of the *Ranunculaceae, Solanaceae, Leguminosae,* and *Compositae* familes. One is referred to studies conducted by Werner (6–9) and Selander (10–13) on the *Meloidae*, genera *Epicauta*, for plants from which particular species were collected.

The *Meloidae*, or blister beetle family, are centered mostly in the arid and semiarid areas of the world, and at the present time, this family comprises approximately 75 genera and some 2000 described species (14). More than three-fourths of the North American entomological literature on the *Meloidae* deals with members of *Epicauta*. Werner (6–9) and Selander (15, 16) have made major contributions to the collection and entomological data of the genus *Epicauta* and the *Meloidae* in North America and Mexico, while MacSwain (14) and Blackwelder (17) have compiled comprehensive classification check lists on the Colepterous insects.

Because of its toxicity and irritation properties, cantharidin is little used in human medicine, although it was once used extensively as a vesicant. Today, its use is confined to veterinary medicine as an irritant and stimulant for hair growth. Among the uses that have been reported for this substance are: aphrodisiac, anticarcinogenic, antihypertensive, herbicide, insecticide, vesicant, hair growth stimulant, diagnostic agent in leprosy, and agent to remove warts. Several cases (18–21) of poisoning from cantharidin have been reported.

Cantharidin (cantharidine) (exo-1,2-*cis*-dimethyl-3,6 - epoxyhexahydrophthalic anhydride) has been assigned structure I by Gadamer (22) and his collaborators (23). It is optically inactive,

nonresolvable, and exists naturally as the *cis*-isomer.



I

Further proof of this structure was made by Woodward and associates (24), who synthesized desoxycantharidine (cantharidin lacking the oxide bridge). Stork and his associates (25) reported the first total synthesis of cantharidin in 1952. However, other investigators also contributed significantly toward the chemistry and synthesis of this compound; worthy of mention, they are Schenck *et al.* (26), Paranjape (27), Pai *et al.* (28), Iyer *et al.* (29), and Ziegler *et al.* (30). Physical and chemical characteristics of cantharidin may be found in the literature (31, 32). In this investigation, 1 Gm. of cantharidin was found to be soluble in 50 ml. of dimethylsulfoxide at room temperature (28°).

The isolation of cantharidin from blister beetles has been studied extensively, particularly in an attempt to improve upon previous methods of separation. It occurs partly free and partly combined in the insect; the nature of the combination is unknown. This unknown complex adds to the difficulty of extraction and quantitative determination. In general, the methods employed for separation utilize continuous extraction with chloroform, acetone, or ethanol, alone or in the presence of an acid (HCl or HNO_3). The extract is concentrated *in vacuo* and treated with petroleum ether or benzene to remove lipid material, then crystallized from a mixture of petroleum ether and ethanol (1:9). Further purification may be obtained by repeated recrystallization or sublimation.

In this study, cantharidin was extracted from *E. pestifera* in a Soxhlet extractor using acetone, cooled, the insoluble material collected, the extract evaporated *in vacuo*, then submitted directly to sublimation. Pure cantharidin was obtained. The quantitative analysis was conducted using the procedure outlined in the "United States Pharmacopeia XI" (33). Infrared and NMR spectra, elemental analysis, physical constants, and thin-layer chromatography of isolated cantharidin when compared to the pure substance showed the two compounds to be identical.

EXPERIMENTAL

Approximately 200 Gm. of *E. pestifera* beetles were collected for this study during July and August of 1965 near Hattiesburg, Miss., and stored in a refrigerator (10°) in a closed jar to which a few drops of chloroform were added to prevent microbial deterioration. They were found on the ornamental vine, *Clemenatis paniculata*.

Isolation of Cantharidin.—The beetles (100 Gm.) were placed in a Waring blender and 150 ml. of acetone was added. The mixture, after being blended for 5 min., was decanted into a Soxhlet thimble and extracted for 18 hr. with acetone. The olive colored solution, on cooling, yielded a heavy dark brown precipitate and a white flocculant precipitate which settled on top of the brown syrupy material. The white substance (0.22 Gm.) was easily decanted from the heavy gummy material and sublimed directly at 110° with vacuum using a sublimation apparatus (McCarter, Nester Faust). The crystalline needle-like sublimate gave a melting point of 217–218° uncorrected. A mixed melting point with pure cantharidin showed no change. The infrared spectrum of the isolated cantharidin was obtained using a Perkin Elmer spectrophotometer in KBr pellets (Fig. 1) and was identical to the pure material.

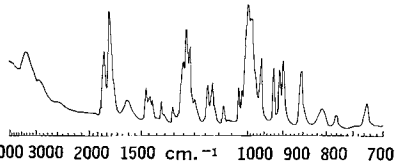


Fig. 1.—Infrared spectrum of cantharidin in KBr pellet.

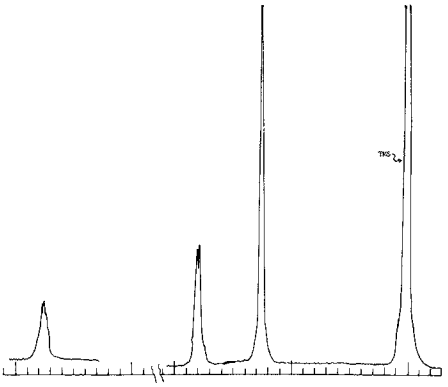


Fig. 2.—NMR spectrum of cantharidin in $CDCl_3$. Solvent, $CDCl_3$; temperature, 37°; filter bandwidth, 4 c.p.s.; R. F. field, 0.04 mg.; sweep time, 250 sec.; sweep width, 500 c.p.s.; sweep-offset, 0 c.p.s.; spectrum amp, 25; 10; integral amp, 625 →; remarks, 500 ←.

NMR Spectroscopy.[1]—Nuclear magnetic resonance (NMR) spectra were obtained with a Varian A-60A spectrometer and were in agreement with the expected chemical shifts, multiplicity, and intensities. Samples were prepared as 10% solutions in $CDCl_3$ and were not degassed. Tetramethylsilane (TMS) was used as internal reference standard. The NMR spectra of pure cantharidin and the isolated substance (Fig. 2) consisted of the following: (*a*) a sharp singlet at $\tau = 8.74$, relative intensity 3, assigned to the methyl protons of the methyl groups *cis* to the oxide bridge; (*b*) a doublet, $J = 2$ c.p.s., centered near $\tau = 8.21$ with relative intensity of 2 assigned to the adjacent pairs of protons on the cyclohexane ring; (*c*) a broad, low field absorption

[1] Acknowledgment is made to Mr. J. L. McClanahan, and Mrs. J. M. Settine, Chemistry Department, University of Mississippi, for their assistance in measuring and interpreting the spectra.

centered near τ = 5.25, relative intensity 1, assignable to tertiary protons of the type at the oxide bridge. The absorption peaks at τ = 2.70 are assigned to a $CHCl_3$ impurity in $CDCl_3$ and at τ = 10 to the reference standard, TMS.

Anal.—Calcd. for $C_{10}H_{12}O_4$: C, 61.21; H, 6.17; O, 32.62. Found: C, 61.29; H, 6.27; O, 33.52.

Thin-Layer Chromatography.—Thin-layer chromatography was conducted on acetone solutions of cantharidin prepared from *E. pestifera* and of the pure substance. Chromagram sheets (Eastman, K301R2) and prepared Silica Gel G plates were used in the tests with comparable results. Shimano (2) has reported the use of paper chromatography for analyzing mixtures containing cantharidin.

Several detection systems were tested with satisfactory results: iodine; bromocresol green, 0.1% in isopropanol with NaOH added until appearance of blue color; 50% aqueous sulfuric acid solution with a trace of chromium trioxide. In the case of iodine the chromatogram was placed in a closed container containing iodine crystals. Cantharidin with bromocresol green gave a yellow coloration. A contrasting white spot revealed the presence of cantharidin when sprayed with the sulfuric acid solution.

Development of the chromatogram can be made with either acetone, chloroform, or an acetone–benzene (1:9) mixture. All systems showed relative movement of cantharidin: acetone (R_f = 0.74–0.75); chloroform (R_f = 0.61 to 0.59); acetone–benzene mixture (R_f = 0.50).

The R_f values and chemical response to the indicators for pure cantharidin and isolated cantharidin were sufficiently close to conclude these data as further confirmation that the isolated substance was indeed cantharidin.

Quantitative Determination.—A quantitative estimation of cantharidin and *E. pestifera* was determined by the method outlined in the U.S.P. XI (33). The dried beetles (15 Gm.) were ground to a coarse powder and placed in a round-bottom flask. A mixture (150 ml.) of benzene and petroleum ether (2:1) which contained 2 ml. of HCl was added. The flask was stoppered, shaken, and allowed to stand for 24 hr. at room temperature (28°). It was then heated to 40° and maintained at this temperature for 3 hr. with frequent shaking. After cooling, a portion of the mixture, 100 ml. representing 10 Gm. of beetles, was decanted and filtered into a tared beaker. The solution was evaporated to a volume of approximately 5 ml. using a stream of nitrogen gas and 5 ml. of chloroform added. The solution was evaporated to dryness. Ten milliliters of a wash mixture of equal volumes of petroleum ether and absolute ethanol which had previously been saturated with cantharidin was added to the residue to remove coloring and lipid materials. This mixture was allowed to stand for 15 min., then decanted through a pledget of purified cotton. This step was repeated until approximately 40 ml. of the wash solution had been used. The crystals which had collected on the cotton were then washed in the same manner with 25 ml. of petroleum ether which had been saturated with cantharidin. The crystals were white and free of coloring and lipid materials. Chloroform (10-ml. portions) was passed over the cotton to remove any cantharidin adhering to the cotton. This washing was returned to the tared beaker. The solvent was evaporated, and the crystalline residue dried for 0.5 hr. at 60°. The 100 ml. of original extract, representing 10 Gm. of beetles, yielded 0.1092 Gm. of cantharidin or approximately 1.09% by weight of dried beetles.

Further work is being conducted on the structure–activity relationship of the cantharidin molecule and the specific structure of certain derivatives of which past investigators have expressed some reservation.

REFERENCES

(1) Pfister, A., *Anales Quim. Farm. (Chile)*, **1940**, 26; through *Chem. Abstr.*, **35**, 6061(1941).
(2) Shimano, T., Nizuno, M., and Bato, T., *Ann. Proc. Gifu. Coll. Pharm.*, **3**, 44(1953).
(3) Chen, M.-Y., and Liang, T.-T., *J. Agr. Assoc. China (Taipei) (N. S.)*, **19**, 30(1957).
(4) Zyp, Van C., *Pharm. Weekblad*, **54**, 295(1917).
(5) Berwatzik-Vogl, *Lehrb. J. Arzneimittellehre, 3 aufl*, **1910**, 542.
(6) Werner, F. G., *Bull. Mus. Compar. Zool.*, **95**, 421 (1945).
(7) Werner, F. G., *Psyche*, **50**, 65(1943).
(8) *Ibid.*, **48**, 75(1945).
(9) *Ibid.*, **60**, 105(1953).
(10) Selander, R. B., *J. Kansas Entomol. Soc.*, **27** (3), 84(1954).
(11) Selander, R. B., *Entomol. Soc. Washington*, **61** (5), 205(1959).
(12) Selander, R. B., *The Coleopterists Bulletin, VIII*, 1954, p. 1.
(13) Selander, R. B., *Ann. Entomol. Soc. America*, **50**, 88 (1957).
(14) MacSwain, J. W., *Angeles*, **1956**, 182.
(15) Selander, R. B., *Illinois Biol. Monog.*, No. 28, 1960.
(16) Selander, R. B., *Proc. U. S. National Museum, III*, (No. 3428), 197(1960).
(17) Blackwelder, F. E., *U. S. National Museum Bull. 185*, **I–IV**, 343(1945).
(18) Wyosocki, K., *Wienr Klin. Wochschr.*, **45**, 964(1932).
(19) Stary, Z., *Samml. Vergiftungsfallen 7, Abstr. A*, **1936**, 117.
(20) Teare, D., "What's New," Abbott Laboratories, North Chicago, Ill., 1962, No. 229.
(21) Kusche, E., *Med. Monatsschr.*, **7**, 252(1953).
(22) Gadamer, J., *Pharm. Post.*, **46**, 821(1914).
(23) Danckwortt, P. W., *Arch. Pharm.*, **252**, 632(1914); Gadamer, J., *ibid.*, **252**, 636(1914); Rudolph, V. W., *ibid.*, **254**, 454(1916).
(24) Woodward, R. B., and Loftfield, R. B., *J. Am. Chem. Soc.*, **63**, 3167(1941).
(25) Stork, G., Van Tamelen, E. E., Friedman, L. J., and Burgstahler, A. W., *ibid.*, **75**, 384(1953).
(26) Schenck, G. O., and Wirtz, R., *Naturwissenschaften*, **40**, 581(1953).
(27) Paranjape, K. D., *et al.*, *Proc. Indian Acad. Sci.*, **19A**, 385(1944).
(28) Pai, V. N., and Guha, P. C., *J. Indian Chem. Soc.*, **11**, 231(1934).
(29) Iyer, B. H., and Guha, P. C., *J. Indian Inst. Sci.*, **23A**, 159(1941).
(30) Ziegler, K., *Ann.*, **551**, 1(1942).
(31) "The Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 204.
(32) Garrido, J., *Acta Cryst.*, **1**, 159(1948).
(33) "United States Pharmacopeia," 11th rev., Mack Publishing Co., Easton, Pa., 1936, p. 105.

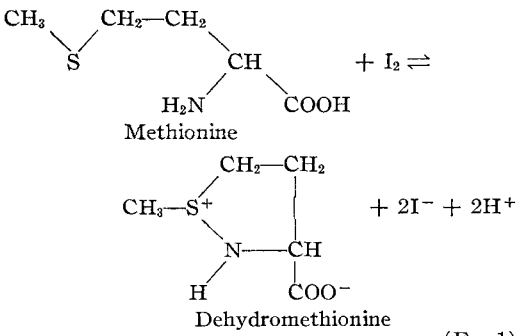
Kinetic Investigation of Reversible Reaction Between Methionine and Iodine

Improved Iodometric Determination of Methionine

By KARL-HEINZ GENSCH* and TAKERU HIGUCHI

Rate studies of reactions discovered by Lavine involving reversible oxidation of methionine to dehydromethionine with iodine are presented. The data suggest that the initial iodine attack may occur either on the nitrogen or on the sulfur. The hydrolytic rate of dehydromethionine to the corresponding sulfoxide appears to be sufficiently fast to cause some inherent errors in the original method of assay for the amino acid.

ALTHOUGH methionine has been determined by a number of iodometric methods (1–5), the chemistry involved has not been clearly established. A method of assay was proposed by Lavine (1) in 1943 based on the following reaction:



(Eq. 1)

Methionine reacts reversibly with iodine to form a cyclic sulfonium compound which Lavine called dehydromethionine (1). According to the method, methionine is allowed to react in a buffered neutral solution with an excess of iodine for 20 min. After removal of the unreacted iodine by careful addition of sodium thiosulfate solution, sufficient potassium iodide and hydrochloric acid (to pH 0.5 to 1.0) are added to reverse the reaction. The liberated iodine is finally titrated with a standardized sodium thiosulfate solution from which the amount of methionine can be calculated. This method suffers from two important disadvantages: (*a*) it is time consuming because of several operations, and (*b*) it cannot theoretically yield stoichiometrically correct results as will be shown later. Several modifications of the original Lavine method have been proposed, but they also require relatively long times for analysis (2–5).

The present study was undertaken to elucidate the microscopic chemistry of this interesting reaction and to provide possible leads for improvement in the method. As parts of these investigations the dependences of the reaction rates in both directions on pH, iodide-ion concentration, and iodine concentration have been determined. The hydrolysis of the cyclic oxidation product has also been studied. A possible improved method of analysis of the acid is proposed as a result of this investigation.

Received June 23, 1966, from the School of Pharmacy, University of Wisconsin, Madison 53706.

Accepted for publication July 27, 1966.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This study was supported in part by grants from Smith Kline & French Laboratories, Philadelphia, Pa., and from the Research Committee of the Graduate School from funds supplied by Wisconsin Alumni Research Foundation.

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RESULTS AND DISCUSSION

Rate of Oxidation of Methionine.—*Forward Reaction.*—The rate of oxidation of methionine with iodine to form dehydromethionine can be conveniently followed spectrophotometrically at 353 mμ, the absorbance peak of I_3^-. From pseudo first-order rate constants obtained in presence of excess methionine and essentially constant iodide and hydrogen-ion concentrations, observed second-order rate constants for the forward reaction, $k_{f(obs.)}$ can be readily calculated by dividing by the concentration of the amino acid. Or

$$-\frac{d[I_2]_T}{dt} = k_{f(obs.)}\,[Me]_T[I_2]_T \quad \text{(Eq. 2)}$$

where Me represents methionine, and the subscript, T, designates the total concentration of both the titratable iodine and methionine.

In Fig. 1 the observed second-order rate constants obtained at pH 7 in this manner have been plotted against the reciprocal of the iodide concentration. It is evident from the observed linear relationship that the forward rate is inversely proportional to the iodide concentration in the system. This behavior can be best rationalized on the basis that free iodine is involved in this mechanism since, according to the equilibrium in Eq. 3, the concen-

$$I_2 + I^- \underset{}{\overset{K_I}{\rightleftharpoons}} I_3^- \quad \text{(Eq. 3)}$$

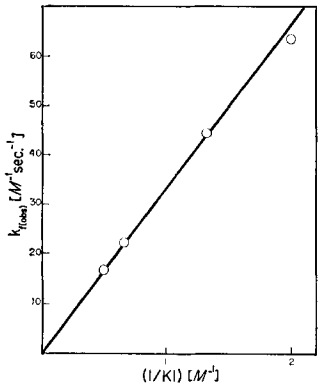


Fig. 1.—Observed second-order rate constants *vs.* (1/KI) for the oxidation of methionine with iodine at pH 7.2. Initial $C_5H_{11}NO_2S$ = 6.82 × 10^{-4} *M*; initial total I_2 = 10^{-4} *M*; phosphate buffer = 0.02 *M*; ionic strength = 2.05 (adjusted with KCl); temperature = 25.0°.

tration of free iodine is inversely proportional to that of iodide at higher iodide concentrations.

In Fig. 2 the product of observed second-order rate constants obtained in the above fashion and the iodide concentration for each run have been plotted as a function of the pH of the system. Different iodide concentrations were used to adjust the observed rate to a convenient range. From the profile, it is evident that the rate is essentially inversely proportional to the hydrogen concentration in the range between pH 4 and 8. Above pH 8 the curve levels off, the observed second-order rate constant at pH 10 being approximately twice that at pH 9. Since the pKa_2 of methionine in this system is approximately 9, the observed pH dependence strongly suggests that the free amino group is involved in this reaction.

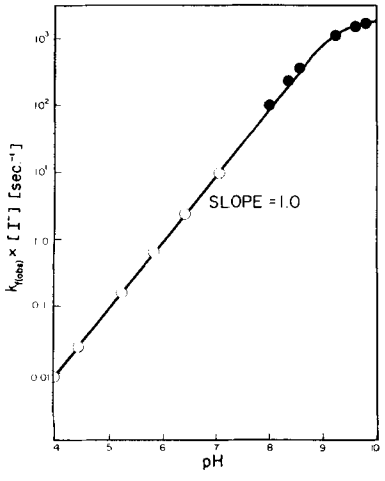


Fig. 2.—Products of observed second-order rate constant and iodide concentration *vs.* pH for the oxidation of methionine with iodine. Initial $C_5H_{11}NO_2S$ = 5 × 10^{-4} *M*; initial total I_2 = 10^{-4} *M*; phosphate or phthalate buffer = 0.02 *M*; ○ = $k_{f(obs.)}$ measured in 0.1 *M* KI, ● = $k_{f(obs.)}$ measured in 1 *M* KI; temperature = 25.0°.

On the basis of the results presented in Figs. 1 and 2 the following rate law can be written:

$$\frac{-d[I_2]_T}{dt} = k_{f(obs.)}\,[Me]_T\,[I_2]_T$$

$$= k_f[Me^-]\,[I_2] \quad \text{(Eq. 4)}$$

where Me^- represents the anionic methionine species and k_f is the second-order rate constant for the species Me^- and I_2 of the forward reaction. Considering Eq. 2 and the dissociation of methionine as expressed by

$$Ka_2 = \frac{[Me^-]\,[H^+]}{[Me]} \quad \text{(Eq. 5)}$$

we can write the forward rate as

$$\frac{-d[I_2]_T}{dt} = \frac{k_f Ka_2\,[Me]_T[I_2]_T}{([H^+] + Ka_2)\,(1 + K_I[I^-])} \quad \text{(Eq. 6)}$$

and for the observed second-order rate constant as follows

$$k_{f(obs.)} = \frac{k_f Ka_2}{([H^+] + Ka_2)\,(1 + K_I[I^-])} \quad \text{(Eq. 7)}$$

From Eq. 7 and the data as shown in Figs. 1 and 2, a mean value for k_f at 25° was determined to be 6.5 × 10^5 M^{-1} sec.$^{-1}$.

Rate of Reduction of Dehydromethionine.—*Reverse Reaction.*—The reduction of the cyclic species by iodide was readily followed spectrophotometrically by the appearance of the I_3^- species. Pseudo first-order rate constants, for the reverse reaction, $k_{r(obs.)}$, were obtained from such data in either of two ways. For fast reaction at constant pH and excess iodide concentration, pseudo first-order rate constants were obtained by plotting log $(A_\infty - A)$ against time. If the rate was expected to be slow, a relatively high concentration of dehydromethionine was used, and the initial rate of formation was measured. Based on previously determined values for the molar absorptivity of iodine in the particular system, the initial rate of reaction was converted to pseudo first-order rate constants by dividing through by the initial dehydromethionine concentration.

Pseudo first-order rate constants obtained in this fashion at different pH values are shown plotted *versus* pH in Fig. 3. From the resulting pH profile it is evident that above pH 4.5 the rate is directly proportional to the hydrogen-ion concentration, while in the more acidic region the order of the over-all reaction with respect to the hydrogen concentration is larger than 1.

The dependence of the reverse pseudo first-order rate constant on the iodide concentration at pH of 5.02 is evident from the log-log data plotted in Fig. 4. The line which closely fits the experimental points was drawn with a slope of 2. On this basis, it would appear that the kinetic dependence with respect to iodide is 2. Similar determinations at pH 2.48, however, yielded a log-log plot with a slope of 1.52 also shown in Fig. 4. From these studies it would appear that the responsible reaction pathway in the acidic region may differ at least in part from that operating at neutral pH. If we assume that the protonated dehydromethionine requires an attack by only a single iodide anion in the rate-determining step while the

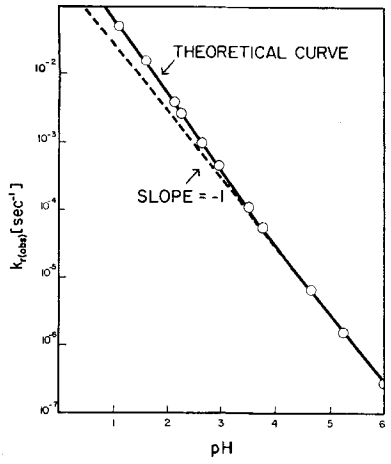


Fig. 3.—Observed first-order rate constant *vs.* pH for the reduction of dehydromethionine. KI = 0.1 *M*; $C_5H_9NO_2S$ = 8.3 × 10^{-5} to 10^{-2} *M*; phosphate or acetate buffer = 0.016 *M*; the theoretical curve was calculated from Eq. 10. The constants were determined by trial and error to be k'_r = 27 [M^{-3} sec.$^{-1}$], k''_r = 3 × 10^{-3} [M^{-2} sec.$^{-1}$], Ka = 3 × 10^{-3} [*M*] at 25.0°.

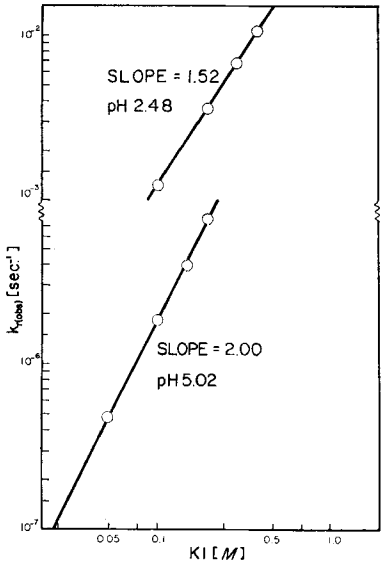


Fig. 4.—Log-log plot of observed first-order rate constant *vs.* potassium iodide for the reduction of dehydromethionine. At pH 5.02: succinate buffer = 0.1 *M*; $C_5H_9NO_2S$ = 0.01 *M*; ionic strength = 0.35 (adjusted with KCl). At pH 2.48: phosphate buffer = 0.01 *M*; $C_5H_9NO_2S$ = 5 × 10^{-5} *M*; ionic strength = 0.41 (adjusted with KCl); temperature = 25.0°.

neutral species requires two, the following rate law can be written:

$$\frac{d[I_2]_T}{dt} = k_{r(obs.)}[De]_T = k_r'[De][H^+][I^-]^2 + k_r''[DeH^+][H^+][I^-] \quad \text{(Eq. 8)}$$

where $[De]_T$, [De], and $[DeH^+]$ represent the concentration of the total, unprotonated, and protonated dehydromethionine, respectively. The constants k_r' and k_r'' are fourth and third-order rate constants, respectively, for these different mechanisms. Since the dissociation constant of the protonated dehydromethionine is

$$Ka = \frac{[De][H^+]}{[DeH^+]} \quad \text{(Eq. 9)}$$

from Eq. 8 the following rate law can be derived:

$$k_{r(obs.)} = \frac{k_r'Ka[H^+][I^-]^2}{[H^+] + Ka} + \frac{k_r''[H^+]^2[I^-]}{[H^+] + Ka} \quad \text{(Eq. 10)}$$

The constant k'_r can be easily determined from the pH profile in Fig. 3, the apparent pseudo first-order rate constant being, above pH 4.5,

$$k_{r(obs.)} \cong k_r'[H^+][I^-]^2 \quad \text{(Eq. 11)}$$

A mean value of k_r', the rate constant for the reverse reaction in essentially neutral system, was calculated on this basis to be 27 [M^{-3} sec.$^{-1}$] at 25°.

From the shape of the pH profile and from Eq. 10, we can estimate the dissociation constant of the protonated dehydromethionine and the rate constant for the reverse reaction in acid solution to be Ka = 3 × 10^{-3} [*M*] and k_r'' = 7 [M^{-2} sec.$^{-1}$].

Although the dissociation constant was not measured directly, the obtained pKa value of 2.52 appears reasonable on another basis. From the structure point of view, a pKa value not too different from that of methionine (pKa = 2.22) appears to be likely because the positive sulfur in dehydromethionine may be expected to increase the dissociation of the carboxyl group as the protonated amino group in methionine does. Furthermore, the pH profile of the hydrolysis of dehydromethionine to methionine sulfoxide (Fig. 10), as will be discussed later, corresponds to the same value based on the assumption that the protonated dehydromethionine hydrolyzes with a different rate than the neutral species.

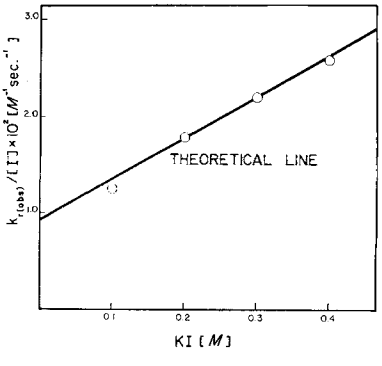


Fig. 5.—According to Eq. 12, observed first-order rate constants obtained at pH 2.48 (see Fig. 4) for the reduction of dehydromethionine divided by the iodide concentration are shown plotted against the iodide concentration. The theoretical curve was calculated from Eq. 12 using the constants showing best fit for pH profile in Fig. 3.

The constants k_r', k_r'', and Ka can also be shown to be internally consistent with the observed rate dependence on the iodide concentration. From Eq. 10 the linear relationship

$$\frac{k_{r(obs.)}}{[I^-]} = \frac{k_r'Ka[H^+][I^-]}{[H^+] + Ka} + \frac{k_r''[H^+]^2}{[H^+] + Ka} \qquad \text{(Eq. 12)}$$

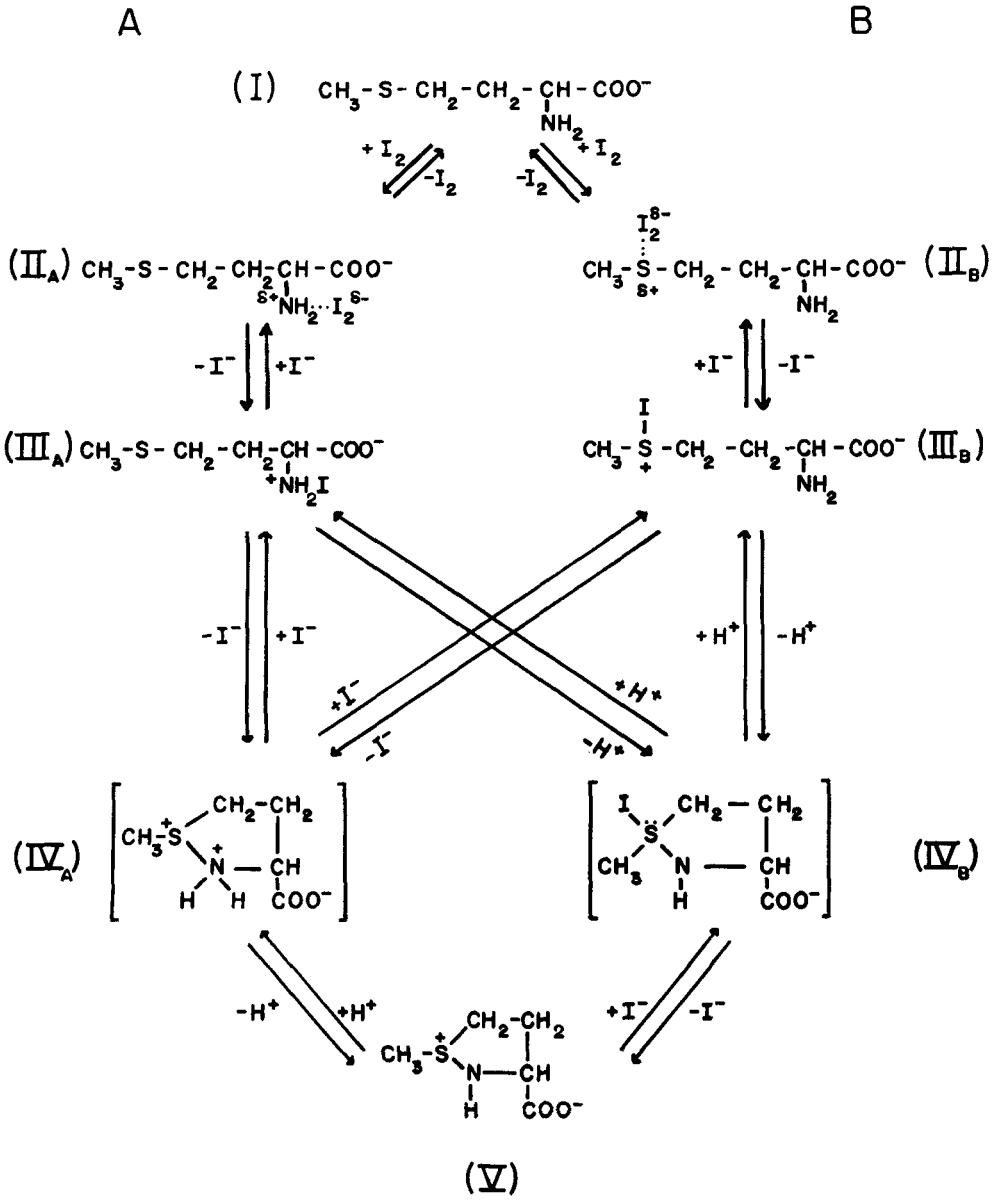
can be derived.

By plotting $k_{r(obs.)}/[I^-]$ against the iodide concentration of a particular system for the reduction of dehydromethionine, the data can be expected to fall on a straight line with a slope of $k_r''Ka[H^+]/([H^+] + Ka)$ and an intercept of $k_r''[H^+]^2/([H^+] + Ka)$. Such a plot is presented in Fig. 5 for data obtained at various iodide concentrations at pH 2.48. The reasonable fit of the theoretical line calculated from Eq. 12 and the constants obtained from Fig. 3 suggest that the proposed mechanism can account for the observed fractional iodide dependence at lower pH.

GENERAL DISCUSSION

Since free iodine has been shown and would be expected to interact with both amines (6) and alkyl sulfides (7), both sites of initial interaction must be considered. Possible reaction schemes based on these routes are diagrammed in Scheme I.

Pathway A considers an attack of iodine on the amino group by which the iodine amine complex



Scheme I

II_A might be formed. Dissociation to III_A seems to be very likely; the further pathway, however, might go either through the species IV_A or IV_B, both of which must be regarded as suitable. Considering the iodination of the amino group to be the rate-determining step, the rate law for the forward reaction would agree with this assumption. Also, the rate law for the reverse reaction is in agreement with pathway A; however, the formation of IV_A in neutral solution and further attack by iodide to form III_A appears to be not very likely. Also, the alternative in the mechanism going from IV_B to III_A cannot be easily accepted because it would require a fast migration of iodine from sulfur to nitrogen for which no support can be presented.

If an iodine attack on sulfur is assumed, as shown in pathway B, the reverse reaction could be more easily explained; the forward reaction, however, would only fit the rate law if additional assumptions were made. Studies with simple aliphatic thioether have revealed (8) that the rate of oxidation by iodine forming sulfoxide is inversely proportional to the square of the iodide concentration. For this reaction, the mechanism in Scheme II is proposed.

$$I_2 + I^- \rightleftharpoons I_3^-$$

$$R{-}S{-}R' + I_2 \rightleftharpoons R{-}\overset{+}{S}(I){-}R' + I^-$$

$$R{-}\overset{+}{S}(I){-}R + OH^- \rightarrow R{-}S(=O){-}R' + H^+ + I^-$$

Scheme II

If in the forward reaction also an iodosulfonium-cation of methionine is formed, the over-all rate, too, should be inversely proportional to the square of the iodide concentration. However, this has not been observed. This contradiction might be removed if one assumes that the dissociation from II_B to III_B is relatively slow or occurs with about the same rate as the cyclization of III_B. The reverse reaction may go through a nucleophilic attack of dehydromethionine by iodide, forming IV_B which on protonation goes under ring opening to the iodosulfonium species III_B. By a second attack of iodide, as the rate law requires, methionine and iodine are formed, the latter is greatly complexed with I^- to form I_3^-. Although pathway V $\rightarrow$ $IV_A \rightarrow III_B$ is also possible, it seems unlikely in neutral solution.

Some support for a mechanism based on attack of iodine on sulfur is evident in the behavior of the forward reaction in the presence of very low iodine, very low methionine, and very low iodide concentration and at pH values not higher than 5.50. The observed second-order rate constants obtained in these studies multiplied by $(1 + K_I [I^-])(1 + [H^+]/Ka_2)$ are shown in Fig. 6 against the reciprocal of the iodide concentration ranging from 3.3×10^{-4} to 1.66×10^{-3} *M* K_I. If Eq. 6 would be the true rate law under these conditions, a rate corrected rate constant should be independent of iodide. The graph, however, shows straight lines with intercepts approximately equal to k_f and with slopes which increased with increasing hydrogen-ion concentration. Although this behavior is not very well understood, it appears likely that at lower pH, where the concentration of Me^- is very low, positive iodosulfonium species is involved in this reaction.

Hydrolysis of Dehydromethionine.—Besides the reduction reaction discussed above, in the presence of iodide and hydrogen ions, dehydromethionine appears to undergo hydrolysis to methionine sulfoxide which is more difficult to reduce than dehydromethionine itself. A critical investigation of the Lavine method must, therefore, also include a kinetic study of this competitive hydrolysis in the absence and in the presence of buffer species.

The rates of hydrolysis in the absence and in the presence of different buffer species have been determined by withdrawing periodic samples from a stock solution of dehydromethionine and titrating the liberated iodine with sodium thiosulfate solution after addition of potassium iodide and hydrochloric acid. Plots of log (ml. 0.01 *N* $Na_2S_2O_3$) *versus* time yielded

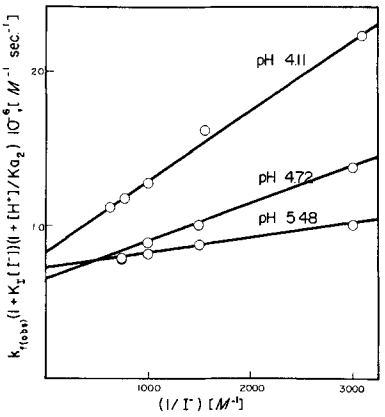


Fig. 6.—Plot showing the change of the dependence of the forward rate on the iodide concentration at different pH values. Initial total iodine = 5×10^{-6} *M*; initial total methionine = 4×10^{-4} *M*; succinate buffer = 0.002 *M*; temperature = 25.0°.

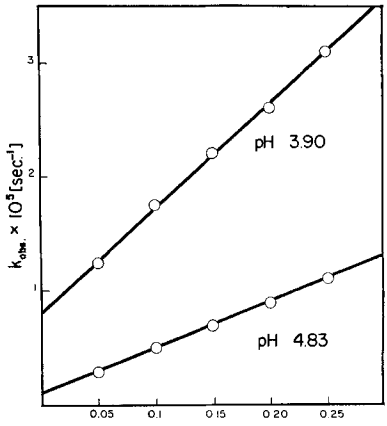


Fig. 7.—Observed first-order rate constants *vs.* total acetate buffer concentration for the hydrolysis of dehydromethionine (measured titrimetrically). Buffer ratio = 0.3 mole CH_3COO^-/0.7 mole CH_3COOH at pH 3.90; 0.7 mole CH_3COO^-/0.3 mole CH_3COOH at pH 4.83. $C_5H_9NO_2S$ = 0.01 *M*; ionic strength = 0.875 (adjusted with KCl); temperature = 25.0°.

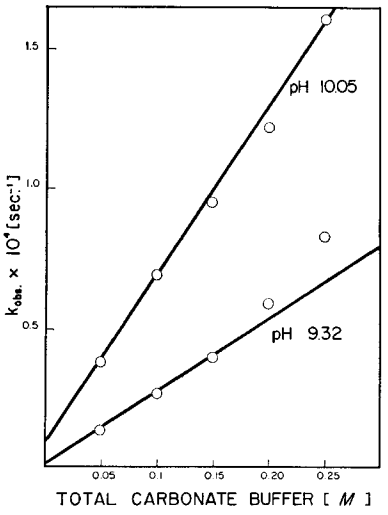


Fig. 8.—Observed first-order rate constants *vs.* total carbonate buffer concentration for the hydrolysis of dehydromethionine (measured titrimetrically). Buffer ratio = 0.3 mole HCO_3^-/0.7 mole $CO_3^=$ at pH 10.05; 0.7 mole HCO_3^-/0.3 mole $CO_3^=$ at pH 9.32. $C_5H_9NO_2S$ = 0.01 *M;* ionic strength = 0.875 (adjusted with KCl); temperature = 2.50°.

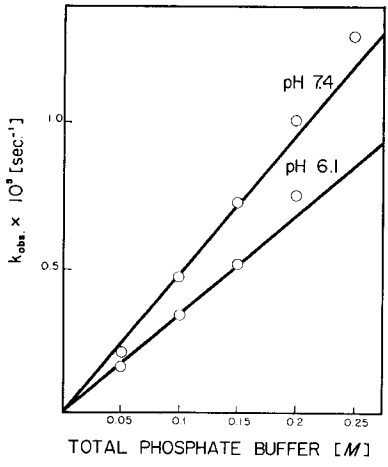


Fig. 9.—Observed first-order rate constants *vs.* total phosphate buffer concentration for the hydrolysis of dehydromethionine (measured titrimetrically). Buffer ratio: 0.15 mole $H_2PO_4^-$/0.85 mole $HPO_4^=$ at pH 7.4; 0.7 mole $H_2PO_4^-$/0.3 mole $HPO_4^=$ at pH 6.1. $C_5H_9NO_2S$ = 0.01 *M;* ionic strength = 0.875 (adjusted with KCl); temperature = 25.0°.

straight lines from which observed pseudo first-order rate constants could be calculated. In strongly acidic solutions, the rate was also measured spectrophotometrically by following the decrease in absorbance at 243 mμ.

Figures 7, 8, and 9 show the influence of the different buffer species on the rate of hydrolysis of dehydromethionine. In these graphs the observed pseudo first-order rate constants obtained in presence of acetate, carbonate, and phosphate buffer are shown plotted against the total buffer concentration. For most plots the experimental points fall essentially on a straight line, the intercepts representing pseudo first-order rate constants at the particular pH in absence of any buffer and the slopes the specific second-order rate constants for the buffer species involved.

$$k_{obs.} = k_{H_2O} + k_T\,[\text{buffer}]_T \quad \text{(Eq. 13)}$$

Since each buffer mixture consisted of an acid and a basic species, the following equation for the slope can be written

$$k_T = k_{base} \times F_{base} + k_{acid} \times F_{acid} \quad \text{(Eq. 14)}$$

where F represents the fraction of the basic or acidic species, *i.e.*, $F_{base} + F_{acid} = 1$. Since for each buffer system two series of experiments with different buffer ratios were carried out from the slopes of these two plots and the particular fractions, the catalytic second-order rate constant for the acidic species and the basic species was calculated. These rate constants are summarized in Table I.

The rate of hydrolysis in absence of any buffer has also been studied, mainly at low and at high pH, the hydrogen-ion concentration was kept constant by repeatedly adding sodium hydroxide solution. The results of these studies and the extrapolated values for zero buffer concentration of Figs. 7, 8, and 9 are presented as the pH profile in Fig. 10 showing that dehydromethionine hydrolyzes easily in presence of hydrogen ions and hydroxide ions and is very stable at neutral pH ($t_{1/2} \approx 600$ days).

As can be seen in Fig. 10, the rate of hydrolysis in strong acid solution is about only a third of the rate which could be expected by extrapolation from the observed values at pH 5. This decrease of the observed rate constants may be due to the slower rate of reaction of the protonated species relative to the free form. On the basis of this assumption, the following rate law for the hydrolysis of dehydromethionine in acidic solution and in the absence of any buffer species can be written:

$$\frac{-d[\text{De}]_T}{dt} = k_{obs.}\,[\text{De}]_T =$$

$$k_{H^+}[\text{De}]\,[H^+] + k'_{H^+}\,[\text{DeH}^+]\,[H^+] \quad \text{(Eq. 15)}$$

Considering Eq. 9, the observed first-order rate constant can be expressed as

$$k_{obs.} = \frac{k_{H^+}\,\text{Ka}\,[H^+] + k'_{H^+}\,[H^+]^2}{[H^+] + \text{Ka}} \quad \text{(Eq. 16)}$$

From Eq. 16 and the experimental data of Fig. 10, the constants were determined to be $k_{H^+} = 6 \times 10^{-2}$ [M^{-1} sec.$^{-1}$], $k'_{H^+} = 1.7 \times 10^{-2}$ [M^{-1} sec.$^{-1}$], and Ka = 3×10^{-3} [*M*] which agrees with the value obtained from Fig. 3. The theoretical curve of

TABLE I.—CATALYTIC CONSTANT FOR HYDROLYSIS OF DEHYDROMETHIONINE FOR SEVERAL BUFFER SPECIES FROM FIGS. 7, 8, AND 9 AT 25°

Catalytic Active Species	k [M^{-1}sec.$^{-1}$] × 10^4
CH_3COOH	1.31
CH_3COO^-	0.0013
$H_2PO_4^-$	0.25
$HPO_4^=$	0.5
HCO_3^-	0.09
$CO_3^=$	8.6

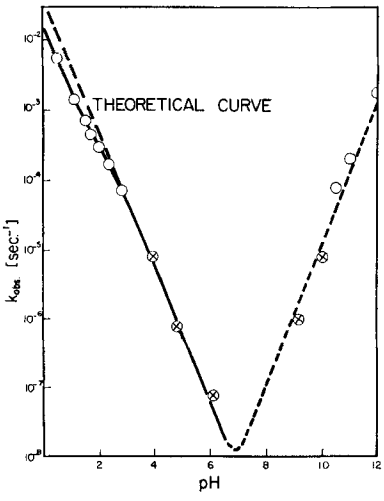


Fig. 10.—Observed first-order rate constants *vs.* pH for the hydrolysis of dehydromethionine (measured titrimetrically and spectrophotometrically). Key: ○, determined directly; ⊗, determined by extrapolation from Figs. 7, 8, and 9. $C_5H_9NO_2S$ = 0.01 *M*; ionic strength = 0.875 (adjusted with KCl); temperature = 25.0°. The theoretical curve was calculated from Eq. 16, derived from the assumption of different rates for unprotonated and protonated dehydromethionine, values of k_{H^+}, k'_{H^+}, and Ka of Eq. 16 were chosen to fit best the experimental points.

Fig. 10 is calculated from Eq. 16 and is in good agreement with the experimental results.

The mechanism of the hydrolysis of dehydromethionine in alkali solution seems to be best rationalized by assuming a nucleophilic attack of hydroxide ion or anionic buffer species on the positive sulfur, followed by ring opening and protonation of the nitrogen. In acidic solution protonation of the imino group probably occurs first, followed by a water attack on sulfur. The relatively high catalytic activity of acetic acid compared to the acetate species might be caused through a nucleophilic attack of the carbonyl oxygen on the sulfur. Protonation of the imino group occurs then through general acid catalysis of the attached carboxyl group.

The influence of iodide ions on the rate of hydrolysis of dehydromethionine as mentioned by Lavine (1) was not observed. It seems to be likely that Lavine was not aware of the catalytic activity of phosphate buffer.

Application of Kinetics Leading to Improvement of Assay Method.—In the original method proposed by Lavine, dehydromethionine stands for 20 min. in contact with 0.2 *M* phosphate buffer at pH 7. It is evident from the data given above on the hydrolysis of dehydromethionine that a significant portion will be observed to form a sulfoxide species which would not undergo reversible reduction back to the amino acid. The extent of loss on the basis of our experiment would be of the order of 1%. Another small error of about 0.2% in the presence of 1 *M* potassium iodide solution and at pH 1 can be calculated from Eqs. 10 and 16 because the reduction and the hydrolysis of dehydromethionine are competitive reactions.

From the relative high value of the specific second-order rate constant $k_f = 6.5 \times 10^5$ $[M^{-1}$ sec.$^{-1}]$ of the forward reaction, it could be expected that at about pH 9 and in presence of low potassium iodide concentration methionine can be directly titrated with iodine. This possibility has already been mentioned by Lavine (1); no result, however, has been presented. The following procedure was tested with the same lot of methionine.

About 150 mg. methionine, previously dried at 60° in vacuum for 2 hr., was accurately weighed and dissolved in 50 ml. of water, with 1 Gm. of sodium borate. After addition of 1 ml. of 1% starch solution, it was titrated with 0.1 *N* iodine until a slightly blue color remained for at least 1 min.

The results are given in Table II for 9 separate analyses and compared with 9 independent determinations of the same sample according to the procedure of N.F. XII.

Both methods have practically the same precision judged by the standard deviation. The differences of about 0.5% between the mean values seems to be partially due to the different ways by which the end point is approached. In the direct iodometric titration a slight excess of iodine is added to produce the starch–iodine complex, while for the back titration of iodine with sodium thiosulfate, a slight excess of the titrant is unavoidable. Therefore, the difference of about 0.5% includes the sum of the titration errors of the two methods. Both methods were also carried out in the presence of glycine (equivalent to 10% of the applied amount of methionine). No higher values were observed.

The value obtained by direct titration can also be checked by continuing with an indirect determination of methionine in the same solution.

The slight excess of iodine is carefully removed by sodium thiosulfate solution, 12 Gm. of potassium iodide and 10 ml. of 25% hydrochloric acid are added, and the liberated iodine is titrated with 0.1 *N* sodium thiosulfate solution. Since in a solution of 1 *M* potassium iodide at low pH the iodide species can easily be oxidized by air, a blank is performed by preparing a solution of 1 Gm. sodium borate, 11 Gm. potassium iodide, and 10 ml. of 25% hydrochloric acid in 70 ml. of water and titrating the liberated iodine after 1 to 2 min. of stirring.

The mean value of the indirect determination and its standard deviation is also given in Table II and shows very good agreement with the direct titration. Since this indirect procedure, however, shows no advantages, the direct iodometric method appears to be sufficient and also superior to the N.F. XII assay, because it can be carried out in much shorter time.

TABLE II.—RESULTS OF A NEW DIRECT AND INDIRECT IODOMETRIC METHOD OF ASSAY OF METHIONINE COMPARED WITH THE DETERMINATION IN "THE NATIONAL FORMULARY," 12TH ED.

Procedure	No. of Determinations	Mean Value, %	S. D., %
Direct	9	100.17	±0.22
Indirect	9	100.00	±0.25
N.F. XII	9	99.68	±0.24

EXPERIMENTAL

Reagents.—Methionine (Eastman-Kodak, white label) was recrystallized from 40% ethanol. All other reagents were analytical grade. Water was purified by distilling tap distilled water from a potassium permanganate solution acidified with sulfuric acid.

The rate of oxidation of methionine with iodine and the rate of reduction of dehydromethionine by iodide forming iodine have been determined spectrophotometrically with a Cary model 11 or 15 by measuring the change in absorbance at 353 mμ, the absorption peak of I_3^-.

For the forward reaction, in general 5 ml. of a methionine solution was added to 45 ml. of a solution of buffer, potassium iodide, and iodine to yield 50 ml. of a solution with concentration as given for each figure. Very fast reactions were measured by injecting 1 ml. of methionine solution through the cover of the cell compartment into 2 ml. of solution containing buffer, iodide, and iodine while the instrument was operating.

For studying the reverse reaction, a stock solution of 0.025 *M* dehydromethionine was prepared by adding an equivalent amount of iodine and sodium hydroxide solution to a methionine solution, the pH was kept between pH 5 and 7. Iodide was removed with silver nitrate, the filtrate was brought to the calculated volume. Pseudo first-order rate constants were obtained as described above in the section for the reverse reaction.

The rate of hydrolysis of dehydromethionine was determined in 0.01 *M* solution of the cyclic species in the presence and in the absence of the buffer species. In both cases 10-ml. samples were withdrawn from the thermostated stock solution, 3 Gm. potassium iodide and 10 ml. 0.5 *N* hydrochloric acid were added, and the liberated iodine was titrated with 0.01 *N* sodium thiosulfate solution. From a plot of log (ml. 0.01 *N* $Na_2S_2O_3$) *versus* time, pseudo first-order rate constants were obtained.

Since dehydromethionine hydrolyzes under acid formation, the rate of hydrolysis in the absence of any buffer was carried out under simultaneous addition of sodium hydroxide solution. For this purpose the reaction solution was kept in a water jacket container closed with a ground-glass plate. Through the holes in the glass plate, electrodes and the tip of a microburet filled with 1 *N* sodium hydroxide solution were immersed into the solution stirred by a magnetic stirrer. Since for most cases a half-life of more than 10 min. was expected, the pH could be easily controlled manually.

In strong acidic solution the rate of hydrolysis was also determined spectrophotometrically by following a decrease in absorbance at 243 mμ.

REFERENCES

(1) Lavine, T. F., *J. Biol. Chem.*, **151**, 281(1943); Lavine, T. F., *Federation Proc.*, 4, 96(1945).
(2) "New and Nonofficial Remedies," J. B. Lippincott Co., Philadelphia, Pa., 1952, p. 627.
(3) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 378.
(4) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 255.
(5) "Deutsches Arzneibuch 6. Ausgabe, Dritter Nachtrag," R. V. Decker's, Verlag-G. Schenck G.m.b.H, Hamburg-Berlin-Bonn, 1959, p. 116.
(6) Yada, H., Tanaka, J., and Nagakura, S., *Bull. Chem. Soc. Japan*, **33**, 1660(1960).
(7) Tamres, M., and Searles, S., Jr., *J. Phys. Chem.*, **66**, 1099(1962).
(8) Higuchi, T., and Gensch, K.-H., *J. Am. Chem. Soc.*, to be published.

Coumarins IV

Coumarins of *Pteryxia terebinthina*. Structures of Two New Coumarins, Isopteryxin and Calipteryxin

By B. EICHSTEDT NIELSEN* and T. O. SOINE

The ether extract of the root of *Pteryxia terebinthina* var. *californica* (Coult. and Rose) Mathias, in addition to osthol, anomalin (I), and pteryxin (III), afforded two new coumarins: 3′(*S*),4′(*S*)-3′-angeloyloxy-4′-acetoxy-3′,4′-dihydroseselin (IV) and 3′(*S*),4′(*S*)-3′-angeloyloxy-4′-senecioyloxy-3′,4′-dihydroseselin (V). These coumarins are provisionally named isopteryxin and calipteryxin, respectively.

In keeping with an interest in naturally occurring coumarins (1) and prompted by the recent isolation of anomalin (I) from *Angelica anomala* Lall by Hata *et al.*, and its structure determination as the 3′,4′-diangelate ester of 3′,4′-dihydroxy-3′,4′-dihydroseselin (II), the present paper reports the isolation of I and several related coumarins from *Pteryxia terebinthina* var. *californica* (Coult. and Rose) Mathias.[1] In particu-

Received July 5, 1966, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis.
Accepted for publication August 29, 1966.
Presented to the Divisional Meeting, Section 8, No. 4, Eleventh Pacific Science Congress, Tokyo, Japan, August 1966.
* The Royal Danish School of Pharmacy, Copenhagen, Denmark. Recipient of a grant from the Danish Government Fund for Scientific and Industrial Research for research at the University of Minnesota.
This investigation was supported by grant HE 07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[1] The authors are indebted to Dr. T. G. Call, California State Polytechnic College, San Luis Obispo, Calif., for collection and identification of the plant material. Preliminary experiments by Dr. Call had shown the presence of osthol and a new coumarin which has since been identified as isopteryxin. Suitable specimens of the plant material have been placed in the herbarium of the Botany Department, University of Minnesota.

EXPERIMENTAL

Reagents.—Methionine (Eastman-Kodak, white label) was recrystallized from 40% ethanol. All other reagents were analytical grade. Water was purified by distilling tap distilled water from a potassium permanganate solution acidified with sulfuric acid.

The rate of oxidation of methionine with iodine and the rate of reduction of dehydromethionine by iodide forming iodine have been determined spectrophotometrically with a Cary model 11 or 15 by measuring the change in absorbance at 353 mμ, the absorption peak of I_3^-.

For the forward reaction, in general 5 ml. of a methionine solution was added to 45 ml. of a solution of buffer, potassium iodide, and iodine to yield 50 ml. of a solution with concentration as given for each figure. Very fast reactions were measured by injecting 1 ml. of methionine solution through the cover of the cell compartment into 2 ml. of solution containing buffer, iodide, and iodine while the instrument was operating.

For studying the reverse reaction, a stock solution of 0.025 *M* dehydromethionine was prepared by adding an equivalent amount of iodine and sodium hydroxide solution to a methionine solution, the pH was kept between pH 5 and 7. Iodide was removed with silver nitrate, the filtrate was brought to the calculated volume. Pseudo first-order rate constants were obtained as described above in the section for the reverse reaction.

The rate of hydrolysis of dehydromethionine was determined in 0.01 *M* solution of the cyclic species in the presence and in the absence of the buffer species. In both cases 10-ml. samples were withdrawn from the thermostated stock solution, 3 Gm. potassium iodide and 10 ml. 0.5 *N* hydrochloric acid were added, and the liberated iodine was titrated with 0.01 *N* sodium thiosulfate solution. From a plot of log (ml. 0.01 *N* $Na_2S_2O_3$) *versus* time, pseudo first-order rate constants were obtained.

Since dehydromethionine hydrolyzes under acid formation, the rate of hydrolysis in the absence of any buffer was carried out under simultaneous addition of sodium hydroxide solution. For this purpose the reaction solution was kept in a water jacket container closed with a ground-glass plate. Through the holes in the glass plate, electrodes and the tip of a microburet filled with 1 *N* sodium hydroxide solution were immersed into the solution stirred by a magnetic stirrer. Since for most cases a half-life of more than 10 min. was expected, the pH could be easily controlled manually.

In strong acidic solution the rate of hydrolysis was also determined spectrophotometrically by following a decrease in absorbance at 243 mμ.

REFERENCES

(1) Lavine, T. F., *J. Biol. Chem.*, **151**, 281(1943); Lavine, T. F., *Federation Proc.*, 4, 96(1945).
(2) "New and Nonofficial Remedies," J. B. Lippincott Co., Philadelphia, Pa., 1952, p. 627.
(3) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 378.
(4) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 255.
(5) "Deutsches Arzneibuch 6. Ausgabe, Dritter Nachtrag," R. V. Decker's, Verlag-G. Schenck G.m.b.H, Hamburg-Berlin-Bonn, 1959, p. 116.
(6) Yada, H., Tanaka, J., and Nagakura, S., *Bull. Chem. Soc. Japan*, **33**, 1660(1960).
(7) Tamres, M., and Searles, S., Jr., *J. Phys. Chem.*, **66**, 1099(1962).
(8) Higuchi, T., and Gensch, K.-H., *J. Am. Chem. Soc.*, to be published.

Coumarins IV

Coumarins of *Pteryxia terebinthina*. Structures of Two New Coumarins, Isopteryxin and Calipteryxin

By B. EICHSTEDT NIELSEN* and T. O. SOINE

The ether extract of the root of *Pteryxia terebinthina* var. *californica* (Coult. and Rose) Mathias, in addition to osthol, anomalin (I), and pteryxin (III), afforded two new coumarins: 3′(*S*),4′(*S*)-3′-angeloyloxy-4′-acetoxy-3′,4′-dihydroseselin (IV) and 3′(*S*),4′(*S*)-3′-angeloyloxy-4′-senecioyloxy-3′,4′-dihydroseselin (V). These coumarins are provisionally named isopteryxin and calipteryxin, respectively.

In keeping with an interest in naturally occurring coumarins (1) and prompted by the recent isolation of anomalin (I) from *Angelica anomala* Lall by Hata *et al.*, and its structure determination as the 3′,4′-diangelate ester of 3′,4′-dihydroxy-3′,4′-dihydroseselin (II), the present paper reports the isolation of I and several related coumarins from *Pteryxia terebinthina* var. *californica* (Coult. and Rose) Mathias.[1] In particu-

Received July 5, 1966, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis.
Accepted for publication August 29, 1966.
Presented to the Divisional Meeting, Section 8, No. 4, Eleventh Pacific Science Congress, Tokyo, Japan, August 1966.
* The Royal Danish School of Pharmacy, Copenhagen, Denmark. Recipient of a grant from the Danish Government Fund for Scientific and Industrial Research for research at the University of Minnesota.
This investigation was supported by grant HE 07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

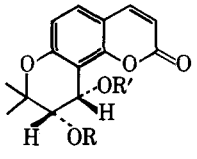
[1] The authors are indebted to Dr. T. G. Call, California State Polytechnic College, San Luis Obispo, Calif., for collection and identification of the plant material. Preliminary experiments by Dr. Call had shown the presence of osthol and a new coumarin which has since been identified as isopteryxin. Suitable specimens of the plant material have been placed in the herbarium of the Botany Department, University of Minnesota.

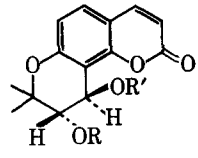
lar, in addition to the isolation of I, pteryxin (III), and osthol, two new coumarins, provisionally named isopteryxin (IV) and calipteryxin (V),[2] have been obtained. Spectral evidence further indicates the probability that a coumarin fraction, thus far unresolved, consists of approximately equal parts of lomatin angelate and senecioate or their isomers.

DISCUSSION

The structure of I, determined prior to the communication of Hata *et al.*, was elucidated by treatment of the isolated coumarin with methanolic potassium hydroxide to yield (+)-*cis*- and (−)-*trans*-methylkhellactones (VI and VII). The acidic hydrolytic component was shown to be only angelic acid by conversion to the *p*-phenylphenacyl ester. The conclusion that I was the 3′,4′-diangeloyloxy derivative of 3′,4′-dihydroseselin was substantiated completely by the nuclear magnetic resonance (NMR) spectrum which, in turn, was in complete accord with that described by Hata *et al.* (2) for I. Similarly, all physical constants were virtually identical, providing a secure basis for assuming the identity of the two coumarins from different sources.

The structure of V was ascertained by alkaline hydrolysis in dioxane to yield the (+)-*cis*- and (−)-*trans*-khellactones (VIII and IX), together with angelic and acetic acids identified by conversion to the *p*-phenylphenacyl ester derivatives. The NMR spectrum showed the 4 and 3 protons, respectively, as the usual pair of doublets at τ 2.38 and τ 3.79 (J = 9.5 c.p.s.) and *ortho* protons in the benzene ring as a pair of doublets at τ 2.62 and τ 3.20 (J = 8.5 c.p.s.). Other signals were noted as doublets (1H each) at τ 3.42 and τ 4.61 (J = 5 c.p.s.), at τ 3.87 (1H, multiplet), at τ 8.55 and τ 8.57 (singlets, 3H each), a multiplet centering at about τ 8.07 (6H), and a singlet at τ 7.90 (3H). The doublets at τ 3.42 and 4.61 are characteristic of the chemical shifts and pattern found for the 4′ and 3′ protons, respectively, in the diesters of *cis*-khellactone (3). The angelate character of one of the ester moieties is evident from the one-proton multiplet centering at τ 3.87 and the six-proton multiplet centering at about τ 8.07, whereas the three-proton singlet at τ 7.90 is characteristic of acetate methyl. The *gem*-dimethyl character of the compound at the 2′-position is evident from the pair of three-proton singlets at τ 8.55 and 8.57. With very minor differences, this spectrum was identical with that of pteryxin (III), the known *cis*-3′-acetoxy-4′-angeloyloxy-3′,4′-dihydroseselin (4). Thus, the weight of evidence indicates that IV is the correct structure. To confirm the placement of the angelate moiety at the 3′-position, advantage was taken of the well-known fact (4,5) that the ester moiety in the benzylic 4′-position is exceedingly labile under fixed base hydrolytic conditions, whereas the ester moiety at the 3′-position is much less reactive. Furthermore, the ester group at the 4′-position undergoes solvolytic *O*-alkyl cleavage and, finally, incorporation of the solvent into the system. Thus, with alcoholic base saponification, the final group in the 4′-position is determined by the alcohol, *i.e.*, methoxy, ethoxy, *etc.* When IV was subjected to ethanolic potassium hydroxide saponification for a short time the workup of the product provided small amounts of *cis*- and *trans*-ethylkhellactones (X and XI) but, more important, also yielded a large quantity of a noncrystalline blue fluorescent compound (XII), apparently identical to the compound obtained by Hata *et al.* (2) in a similar fashion from anomalin (I). The NMR spectrum of this compound showed the usual pattern, integration and splitting constants for the 3,4,5, and 6 protons of the parent compound and showed the usual multiplet pattern and integration expected for the angelate methyls and vinyl proton of a single angelate moiety. The acetate methyl of the parent spectrum was missing but the typical pattern for an ethoxy group was evident with the methyl protons being observed as a triplet at τ 8.67 (3H, J = 7.5 c.p.s.) and the methylene protons as a quartet at τ 5.93 (2H, J = 7.5 c.p.s.). The doublets corresponding to the 3′ and 4′ protons were now found at τ 4.80 and 5.50 (J = 2 c.p.s.) indicating the *trans*-configuration expected on the basis of reasons discussed in an earlier paper (4). These results provide strong evidence for the structure of isopteryxin as 3′-angeloyloxy-4′-acetoxy-3′,4′-dihydroseselin.





I, R = R′ = Angeloyl
II, R = R′ = H
III, R = Acetyl; R′ = Angeloyl
IV, R = Angeloyl; R′ = Acetyl
V, R = Angeloyl; R′ = Senecioyl
VI, R = H; R′ = CH_3
VIII, R = R′ = H
X, R = H; R′ = C_2H_5

VII, R = H; R′ = CH_3
IX, R = R′ = H
XI, R = H; R′ = C_2H_5
XII, R = Angeloyl; R′ = C_2H_5

The structure of V was established by a short hydrolysis of the compound with ethanolic potassium hydroxide which yielded principally XII, unreacted V, and small amounts of X and XI. The acidic hydrolytic components were identified as principally senecioic acid and, in much smaller amount, angelic acid through the *p*-phenylphenacyl esters. The NMR spectrum of V was remarkably similar to that of I with the major difference being the signals from the senecioyl group at τ 8.05 and 7.78 (3H each) and τ 4.37 (1H). In addition, the signals from the angelate moiety integrated for only one-*half* the values found for I. The decision as to the location of the senecioyl group at 4′ was obvious from the isolation of XII, identical to that obtained from IV in the same way. These results provide conclusive

[2] Hata *et al.* (9) have recently reported on a new coumarin, peuformosin, from the root of *Peucedanum formosanum* Hayata which appears to be structurally identical to calipteryxin. Peuformosin, inexplicably, has a somewhat higher melting point (155–156°) than calipteryxin but has a roughly equal {$[\alpha]D^{27}$ + 67.3° ($CHCl_3$)}, although opposite rotation. Since they have chosen to utilize a name indicating its botanical origins we choose to retain the name calipteryxin to identify this enantiomeric form and thus to preserve its botanical origin. If peuformosin, as seems likely, is the mirror image of calipteryxin it is possible to assign the 3′(*R*), 4′(*R*) 3′-angeloyloxy-4′-senecioyloxy-3′,4′-dihydroseselin nomenclature to it.

evidence for the structure of calipteryxin as 3′-angeloyloxy-4′-senecioyloxy-3′,4′-dihydroseselin.

The NMR spectra of I, IV, and V all show the same coupling constant of $J_{3',4'}$ = 5.0 c.p.s. Furthermore, the signals arising from the two methyl groups in the chroman ring differ by only 0.02 p.p.m. As pointed out by Lemmich *et al.* (6) these spectral features are typical of khellactone derivatives possessing the relative *cis*-configuration. From the observation of a $J_{3',4'}$ = 2.0 c.p.s. and of well-separated *gem*-dimethyl signals (Δ = 0.7 p.p.m.) in the NMR spectrum of XII it is, in turn, quite safe to deduce the relative *trans*-configuration for this compound, bearing out the expectations based on chemical grounds.

The absolute configuration of all previously isolated diesters of 3′,4′-dihydroxy-3′,4′-dihydroseselin has been shown to be 3′(*S*),4′(*S*) (4,7). Since the alkaline degradation products from anomalin (I) and isopteryxin (IV) are shown to be mixtures, respectively, of VI and VII and of VIII and IX the configuration 3′(*S*) may be assigned to I and IV. By optical comparison of XII obtained from IV with that obtained from V the configuration 3′(*S*) may be assigned to calipteryxin (V) also. Since I, IV, and V are all of the *cis*-configuration it follows, therefore, that they all possess the configuration 3′(*S*), 4′(*S*).

EXPERIMENTAL

Melting points were determined in capillary tubes in a Thomas-Hoover melting point apparatus checked for accuracy against a set of standard samples. Infrared spectra were determined on a Perkin-Elmer 237B grating infrared spectrophotometer or a Perkin-Elmer spectrophotometer, model 21. Values of [α] were determined on a Perkin-Elmer 141 polarimeter. NMR spectra were determined on a Varian Associates A-60 instrument through the courtesy of Dr. William B. Schwabacher, School of Chemistry, University of Minnesota. Analyses were performed by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, or by A. Bernhardt, Mülheim, Germany.

Material.—Dried roots of *P. terebinthina* var. *californica* (Coult. and Rose) Mathias, collected at an elevation of 7200 ft. on June 26, 1965, in basalt rocks the first mile after leaving Slinkard Creek and ascending toward Monitor Pass, Alpine County, Calif.

Isolation of the Coumarins.—The dried and ground root (500 Gm.) was extracted with diethyl ether. Upon evaporation of the solvent, 88 Gm. of a viscous oil remained. This residue was dissolved in 90% methanol, defatted with petroleum ether, and evaporated. The residue (60 Gm.) was chromatographed on silica gel (J. T. Baker Co., No. 3405, 450 Gm.) activated at 120° and impregnated with 10% of water. Upon elution with benzene, benzene–chloroform, and, subsequently, chloroform–ethyl acetate, the following fractions were collected.

(*A*) A blue-fluorescent, noncrystalline material (71 mg.). Thin-layer chromatography on silica gel (Merck) using different solvent systems gave only one spot with the same R_f value as jatamansin.[3] The NMR spectrum, however, indicated that it was a mixture of two monoesters, possibly lomatin angelate (*i.e.*, jatamansin) and the isomeric senecioate. The fraction has not been investigated further.

(*B*) Osthol, 10.2 Gm., m.p. 84.5–85.0°. This was identified by its infrared and NMR spectra and also by a mixed melting point determination with an authentic sample of osthol.

(*C*) A fraction (1.3 Gm.) which was rechromatographed on silica gel (J. T. Baker Co., No. 3405, 100 Gm.) activated at 120° and impregnated with 3% of water. The eluant was a mixture of methylene chloride–carbon tetrachloride (2:1) to which increasing amounts of ethyl acetate were added. The following substances were eluted: (*a*) 307 mg. of a blue-fluorescent compound (I), recrystallized from ether, m.p. 173.0–174.0°, $[\alpha]_D^{25}$ −48.5° (*c* 0.5, chloroform), $[\alpha]_{546}^{25}$ − 53° (*c* 0.5, chloroform).

Anal.—Calcd. for $C_{24}H_{26}O_7$: C, 67.59; H, 6.15. Found: C, 67.87; H, 6.15.

(*b*) A 314-mg. quantity of a blue-fluorescent compound (V), recrystallized from ether, m.p. 147.5–148.0°, $[\alpha]_D^{25}$ −55° (*c* 0.8, chloroform), $[\alpha]_{546}^{25}$ −62° (*c* 0.8, chloroform).

Anal.—Calcd. for $C_{24}H_{26}O_7$: C, 67.59: H, 6.15. Found: C, 67.55; H, 6.06.

(*D*) Further elution yielded a fraction (5.8 Gm.) which was rechromatographed using the same conditions as mentioned for fraction (*C*). The following substances were obtained: (*a*) 130 mg. of a blue-fluorescent compound, m.p. 86.0–86.5°, $[\alpha]_D^{23}$ +12.5° (*c* 2.0, ethanol, 95%), $[\alpha]_D^{25}$ − 4° (*c* 1.1, chloroform). The infrared spectrum was identical with that of an authentic sample of pteryxin (III).

(*b*) A 3.5-Gm. quantity of a blue-fluorescent compound (IV), recrystallized from ethanol and cyclohexane, m.p. 135.0–135.5°, $[\alpha]_D^{24}$ − 39° (*c* 2.0, ethanol, 95%), $[\alpha]_D^{25}$ −45° (*c* 1.3, chloroform), $[\alpha]_{546}^{25}$ −52° (*c* 1.3, chloroform).

Anal.—Calcd. for $C_{21}H_{22}O_7$: C, 65.27; H, 5.74. Found: C, 65.29; H, 5.82.

(*E*) Elution with ethyl acetate to which increasing amounts of methanol were added yielded a mixture of several blue-fluorescent compounds. This mixture is currently under investigation.

Treatment of I with Methanolic Potassium Hydroxide.—Compound I (175 mg.) was dissolved in 10 ml. of methanol containing 1.5 Gm. of potassium hydroxide and the mixture refluxed for 6 min. Upon cooling, the reaction mixture was acidified with 4 *N* sulfuric acid, water (20 ml.) was added, and after standing for 30 min. adjusted to pH 8 with sodium carbonate and finally extracted with chloroform. The chloroform extract, after drying and evaporation, was chromatographed on silica gel (Merck, 20 Gm.) activated at 120° and impregnated with 5% of water. The eluant was benzene to which increasing amounts of ethyl acetate were added. The following substances were obtained: (*a*) 4 mg. (+)-*cis*-methyl khellactone (VI), recrystallized from petroleum ether, m.p. 126.5–127.0°, $[\alpha]_D^{25}$ + 74° (*c* 0.15, chloroform), $[\alpha]_{546}^{25}$ + 90° (*c* 0.15, chloroform). The infrared spectrum was identical with that of an authentic sample of (+)-*cis*-methyl khellactone. (*b*) A 74-mg. quantity of (−)-*trans*-methyl khellactone (VII), recrystallized from ether–petroleum ether, m.p. 162.5°, $[\alpha]_D^{25}$ − 30° (*c* 1.0, chloroform). The infrared spectrum was identical with that of an authentic sample of (−)-*trans*-methyl khellactone.

The aqueous phase (pH 8) was evaporated, acidified with 4 *N* sulfuric acid, and extracted with ether.

[3] Supplied by Dr. S. C. Bhattacharyya, National Chemical Laboratory, Poona, India.

TABLE I.—OPTICAL ROTATIONS OF COMPOUND XII OBTAINED FROM COMPOUNDS IV AND V

Compd. from	Concn. in $CHCl_3$	$[\alpha]_D^{25}$	$[\alpha]_{578}^{25}$	$[\alpha]_{546}^{25}$	$[\alpha]_{436}^{25}$	$[\alpha]_{364}^{25}$
IV	0.4	−10.2	−9.6	−9.3	+15.4	+69.2
V	0.2	−11.1	−10.0	−10.0	+11.7	+75.9

Upon addition of dicyclohexylethylamine (100 μl.) the dried ether extract was evaporated. The residue was converted to the *p*-phenylphenacyl ester according to the method described by Stodola (8). A chloroform extract, containing a mixture of this ester and unreacted *p*-phenylphenacyl bromide, was evaporated and chromatographed on silica gel (Merck, 10 Gm.), activated at 120°, and impregnated with 10% of water. The eluant was benzene–petroleum ether (2:1) to which increasing amounts of benzene were added. *p*-Phenylphenacyl angelate (92 mg.), m.p. 88.5–89.0°, was obtained. The identity was established by infrared spectroscopy and by mixed melting point determination with an authentic sample.

Treatment of IV with 1 N Potassium Hydroxide.—A solution of IV (500 mg.) in 5 ml. of dioxane and 15 ml. of 1 *N* potassium hydroxide was left at room temperature overnight. The reaction mixture was acidified with 2 *N* sulfuric acid and, after standing for 45 min., was adjusted to pH 8 with sodium carbonate and extracted with chloroform. The dried extract was evaporated and the residue chromatographed on silica gel (J. T. Baker Co., No. 3405, 15 Gm.) activated at 120° and impregnated with 10% of water. The eluant was chloroform to which increasing amounts of ethyl acetate were added. Besides unreacted IV (81 mg.) the following compounds were obtained: (*a*) 60 mg. (+)-*cis*-khellactone (VIII), recrystallized from benzene, m.p. 172.0–173.0°, $[\alpha]_D^{22}$ + 80° (*c* 0.5, chloroform). The identity was established by infrared spectroscopy. (*b*) A 96-mg. quantity of (−)-*trans*-khellactone (IX), recrystallized from benzene–chloroform, m.p. 184.5–186.0°, $[\alpha]_D^{22}$ − 17.5° (*c* 1.1, chloroform). The identity was established by infrared spectroscopy.

The aqueous phase (pH 8) when worked up as described above for compound I yielded *p*-phenylphenacyl angelate, m.p. 87.5–89.0°, and *p*-phenylphenacyl acetate, m.p. 110.5–111.0°.

Treatment of IV with Ethanolic Potassium Hydroxide.—A solution of IV (300 mg.) in 12 ml. of ethanol was mixed with 12 ml. of 1 *N* ethanolic potassium hydroxide, left at room temperature for 45 sec., and acidified with 4 *N* sulfuric acid. Upon standing for 45 min., 30 ml. of water was added and the mixture extracted with chloroform. The dried extract was evaporated and chromatographed on silica gel (Merck, 20 Gm.) activated at 120° and impregnated with 5% of water. The eluant was methylene chloride–carbon tetrachloride (2:1). XII (221 mg.) was obtained. Further elution with methylene chloride–carbon tetrachloride (2:1) to which increasing amounts of ethyl acetate were added yielded small amounts of (+)-*cis*- and (−)-*trans*-ethylkhellactones (X and XI) which were identified by co-chromatography with authentic samples.

Treatment of V with Ethanolic Potassium Hydroxide.—A solution of V (169 mg.) in 8 ml. of ethanol was mixed with 8 ml. of 1 *N* ethanolic potassium hydroxide, left at room temperature for 1 min., acidified with 4 *N* sulfuric acid, and then diluted with 25 ml. of water. Upon standing for 30 min. the mixture was adjusted to pH 8 with sodium carbonate and extracted with chloroform. The extract, after drying and evaporation, was chromatographed as described above for IV. XII (78.5 mg.) was obtained and, as in the previous case, was found to be an uncrystallizable oil. Further elution yielded 60.7 mg. of unreacted V and small amounts of X and XI (identified by co-chromatography with authentic samples). The water phase (pH 8) was worked up as described for I to yield 0.8 mg. of *p*-phenylphenacyl angelate and 26 mg. of *p*-phenylphenacyl senecioate, m.p. 143.5–145.0°. The identity of these esters was established by infrared comparison with authentic samples.

Comparison of Optical Activity of XII from IV and V.—The noncrystalline compound XII obtained from isopteryxin (IV), distilled at 0.1 mm. Hg (160–180°) when compared with the same compound from calipteryxin (V) obtained directly by chromatography gave the values shown in Table I.

REFERENCES

(1) Soine, T. O., *J. Pharm. Sci.*, **53**, 231(1964).
(2) Hata, K., Kozawa, M., and Ikeshiro, Y., *Chem. Pharm. Bull.*, **14**, 94(1966).
(3) College of Pharmacy, University of Minnesota, Minneapolis, Minn., unpublished data.
(4) Willette, R. E., and Soine, T. O., *J. Pharm. Sci.*, **51**, 149(1962).
(5) Smith, E., Hosansky, N., Bywater, W. G., and van Tamelen, E. E., *J. Am. Chem. Soc.*, **79**, 3534(1957).
(6) Lemmich, J., Lemmich, E., and Nielsen, B. E., *Acta Chem. Scand.*, to be published.
(7) Schroeder, H. D., Bencze, W., Halpern, O., and Schmid, H., *Chem. Ber.*, **92**, 2338(1959).
(8) Stodola, F. H., *Microchem. J.*, **7**, 389(1963).
(9) Hata, K., Kozawa, M., and Yen, K., *Chem. Pharm. Bull.*, **14**, 442(1966).

Negative Thixotropy in Flocculated Clay Suspensions

By JOSEPH C. SAMYN and WAN YIN JUNG

The flocculated clay system is shown to possess negative thixotropic flow properties. Low and high shear measurements are performed to demonstrate this phenomenon. The variations in floccule sizes and interfloccule contacts are accepted as the cause of this effect. Sediment volume and filtration rate measurements are presented to demonstrate the floccule size-agitation intensity relationship. These reversible changes in apparent viscosity are also shown to occur more readily with low-intensity agitation than with standing. Several commercial suspensions containing this system did not display appreciable negative thixotropy. However, the influence of negative thixotropy in the control of concentrated clay slurries is still considered important.

THIS PAPER reports the negative thixotropic behavior of the flocculated system: clay[1] plus added salts. Flocculated systems displaying negative thixotropy are characterized by a reversible transformation from a more viscous state achieved by high shear to a less viscous state obtained by low shear. The only previous report of negative thixotropy in a pharmaceutical system was that of Chong *et al.* (1) for milk of magnesia.

The differences between negative thixotropy and dilatancy should be emphasized since these two types of flow can be confused. The negative thixotropic systems are necessarily flocculated as reported by Medalia and Hagopian (2). In addition, they are of relatively low concentrations. The flocculated clay systems cited in this report are in the 1–3% range while the milk of magnesia is a 7–8.5% suspension. The dilatant systems are deflocculated and of extremely high solid content, usually 40–50% (3). Chong *et al.* (1) also point out that the negative thixotropic systems are dependent on duration and rate of shear, while the dilatant systems are dependent on rate of shear only.

It will be shown that the negative thixotropic behavior of these clay slurries is observable with either high or low shear viscometers. Selected sediment volume and filtration rate measurements will be presented to further substantiate the flocculation theory for negative thixotropy. A phenomenon analogous to rheopexy, therefore termed negative rheopexy, has also been observed with the clay systems as well as with milk of magnesia. Finally, the practical significance of negative thixotropy will be considered.

EXPERIMENTAL

Apparatus.—The Brookfield viscometer model LVT (Brookfield Engineering Laboratories, Stoughton, Mass.) was used for the single-point viscosity measurements. The spindle, speed, and time of measurement were kept constant for each system and are listed in each table of data. The suggested conversion factors were used to express the apparent viscosities in Brookfield units.

A Hercules Hi-Shear viscometer (Martinson Machine Co., Kalamazoo, Mich.) was used for the multipoint measurements. The unit was modified to permit the use of a single cup-bob combination for all measurements. A variable lever arm was coupled to a Statham 1-oz. GI-1-350 strain gauge (Statham Instruments, Inc., Puerto Rico). An Ellis Bridge amplifier (Ellis Associates, Pelham, N. Y.) model BAM-1, provided the power, and data were read from the scale of this unit. Speed changes were manual and performed in a procedure as standard as possible. Although provision for thermostating was available, it was not employed since its effectiveness was largely unknown and the temperature effect, if any, would probably be small and opposite to the negative thixotropic effect.

The sedimentation and filtration measurements were made in standard laboratory glassware. S & S (Schleicher and Schuell) No. 588 fluted filter papers were used for the filtration according to the procedure of LaMer (4). The time of filtration of the second 10 ml. of filtrate for each sample was measured.

Materials.—The clay, sodium benzoate, and polysorbate 40[2] were used as received from the suppliers. The milk of magnesia U.S.P. (Parke, Davis & Co., Detroit, Mich.) was a commercial sample.

Sample Preparation.—The clay slurries were hydrated overnight at RT in distilled water using an Equipoise shaker (Fisher Scientific Co.). Rotation of samples was accomplished by end-over-end tumbling at 14 r.p.m. for the prescribed times. A model GT21 mixer (Gerald K. Heller Co., Las Vegas, Nev.) at 1500 r.p.m. was used for the high speed mixing.

RESULTS AND DISCUSSION

The viscosity measurements with the high shear viscometer were only performed on rotated samples. Since the actual process of measurement resulted in viscosity increase due to the high shear, it was unnecessary to measure additional sheared samples. The low shear measurements, on the other hand,

Received April 28, 1966, from the Product Development Department, Parke, Davis and Co., Detroit, Mich.
Accepted for publication September 19, 1966.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

[1] Marketed as Veegum HV by R. T. Vanderbilt Co., New York, N. Y.

[2] Marketed as Tween 40 by Atlas Chemical Co., Wilmington, Del.

were performed on samples prepared by rotation and by high shear since the measurements did not alter the samples in the above manner.

Milk of Magnesia and Dispersed Clays.—The data in Table I on milk of magnesia were obtained with a Brookfield viscometer after low speed rotation and high speed mixing. These data clearly show that the negative thixotropy of this system is measurable with this procedure. The reproducibility of the measurements as well as the reversible nature of the effect can also be noted.

Table II reports the viscosity values for three dispersed clay slurries after low and high shear mixing. The thixotropic effect, rather than the negative phenomena, is to be noted in these data. The high speed mixing gives the reduced viscosity values for these dispersed systems. These data on disperse systems are presented in order that the contrast in behavior between it and the data on the flocculated systems may be completely obvious.

Clay–Salt Systems.—Figure 1 was prepared from data on clay slurries at two concentrations, each at several salt levels. Each sample was rotated overnight, apparent viscosity measured, stirred at 1500 r.p.m. for 5 min., and then remeasured. At the lower salt levels, the usual thixotropic behavior is evident. In this region, the high shear destroys the particle-to-particle contact and results in reduced apparent viscosity. At the higher salt concentrations, the negative thixotropic effect is noticed. In this region, slow rotation of the samples has facilitated the build-up of relatively large compact floccules. This leads to a lower apparent viscosity because of the increase in free fluid between the floccules and a decrease in contacts between these larger floccules. The application of high shear to these systems disperses these floccules and increases interparticle contact and the apparent viscosity.

In many of the samples prepared by rotation, it was noticeable that some coagulation of the clay particles had occurred on the inner surface of the glass bottle or the cap liner. It seemed possible that this material on redispersion with high shear would explain the apparent viscosity rise with high shear. The data in Table III indicate that this was not the case with these samples. Three flocculated systems were studied by three procedures. In the usual procedure the rotation, measurements, and high shear were performed in a single container for each system. In the second procedure, the clay–salt slurry was transferred to a clean container for measurements and high shear treatment after the rotation step. In this instance, any coagulated clumps of clay adhering to glass and cap surfaces were effectively removed from the system. In the third treatment, the glass containers used were Siliclad[3] treated. No adhering clay was noted in these samples. The data in Table III demonstrate that the negative thixotropy reported for these systems is not affected by the amount of material adhering to the glass and cap surfaces. It should be noted, however, that at much higher clay–salt ratios, slow sample rotation has resulted in almost all the clay adhering to the glass and cap surfaces. In such instances, the viscosity measurements would obviously be affected. It is also worth noting that these coagulated clay particles are sometimes observable as a "gummy

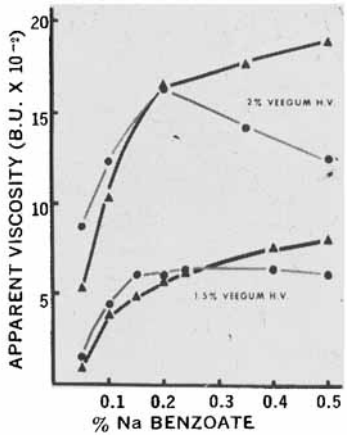


Fig. 1.—Clay–salt slurries. The influence of increasing salt concentrations. Key: ▲, mixed; ●, rotated.

TABLE I.—MILK OF MAGNESIA U.S.P.—APPARENT VISCOSITY READINGS AFTER HIGH AND LOW SHEAR TREATMENTS

Treatment	Brookfield Units[a]
Mix (1500 r.p.m.), 5 min.	720
Mix (1500 r.p.m.), 5 min. more	720
Rotation, 3 days	450
Mix (1500 r.p.m.), 5 min.	610
Mix (1500 r.p.m.), 5 min. more	690

[a] No. 2 spindle, 30 r.p.m., 3 min., readings × 10.

TABLE II.—CLAY SLURRIES—APPARENT VISCOSITY READINGS AFTER HIGH AND LOW SHEAR TREATMENT

System	Treatment: Rotate Overnight	Treatment: Mix 5 min. (Brookfield Units)[a]
3% Clay	260	40
4% Clay	1500	560
5% Clay	3070	2410

[a] No. 3 spindle, 30 r.p.m., 3 min., readings × 40.

TABLE III.—CLAY–SALT SLURRIES INFLUENCES OF COAGULATED CLAY PARTICLES ON THE APPARENT VISCOSITY VALUES

Systems	Procedure	Treatment: Overnight Rotation (Brookfield Units)[d]	Treatment: Mix 5 min. (Brookfield Units)[d]
1% Clay	[a]	160	430
0.5% Na benzoate	[b]	180	430
	[c]	190	430
1% Clay	[a]	100	400
1% Na benzoate	[b]	100	410
	[c]	100	410
1% Clay	[a]	90	380
1.5% Na benzoate	[b]	90	390
	[c]	80	380

[a] All measurements in one container. [b] Material transferred after rotation. [c] Silicone treated container. [d] No. 2 spindle, 30 r.p.m., 3 min., readings × 10.

[3] Clay-Adams, Inc., New York, N. Y.

ring" in suspensions containing flocculated clay particles.

Table IV illustrates negative thixotropy of a clay–salt system as measured with the high shear viscometer. Curve *1* was obtained beginning at the lowest shear rate, increasing to a maximum, and then at progressively decreasing rates. Curve *2* was repeated in the same manner immediately after the completion of curve *1* on the same sample. The negative thixotropic effect is clearly seen in curve *1* by the increase in shearing stress values at comparable shear rates. Curve *2* indicates little additional change.

Clay–Salt–Surfactant Systems.—The addition of a surfactant to the clay–salt system results in a degree of redispersion. This is seen in the high shear data of Table V for increasing concentrations of polysorbate 40. The two lower concentrations exhibit the negative thixotropy associated with flocculation, while thixotropy is noted in the 0.2% polysorbate sample. Additives such as surfactants or protective colloids tend to reduce the flocculation and explain to some extent why negative thixotropy is not observed in the complete formulations containing these ingredients.

Table IV.—Negative Thixotropy in System of 3% Clay and 3% Sodium Benzoate—High Shear Data[a]

Shear Rate, sec.$^{-1}$	Shearing Stress, dyne cm.$^{-2}$ Curve *1*		Curve *2*	
	Begin ↓			
140	130	290	Repeat ↓ 280	
250	210	310	310	300
360	230	320	330	320
470	260	340	340	...
590	280	350	360	340
1200	360	410	430	400
1760	430	460	470	460
2340	500		510	

[a] See text for general procedure of obtaining the data.

Negative Rheopexy.—The phenomenon of rheopexy is often associated with thixotropy. The term itself derives from the Greek *pectos*, meaning "curdled" or "solidified" (5). It is generally applied to thixotropic systems which set more rapidly by gently rolling or tapping than by standing. It is reasoned that the orientation of particles is facilitated by the gentle motion induced by these treatments.

For the negative thixotropic systems described in this paper, an analogous effect was noted. Here, too, the gentle rolling resulted in a more rapid change of the system than did the standing. However, since the system "thinned" rather than jelled, the term negative rheopexy was used to describe this effect in a manner analogous to the use of the term rheopexy for the thixotropic systems. The data in Fig. 2 illustrate the negative rheopexy of milk of magnesia and a clay–salt system. For the milk of magnesia samples, it was necessary to rotate the standing samples once by hand prior to measurement because of some internal settling. A single rotation of this nature had no effect on the other rotated or stirred samples.

Floccule Size.—Sediment volume and filtration rate measurements on selected systems were performed to further substantiate that the phenomenon of negative thixotropy was related to floccule size. Although only systems in a limited concentration range could be measured by sediment volume and filtration rate techniques, the data gathered support

Table V.—Clay–Salt–Surfactant Systems High Shear Measurements

System	Rate of Shear, sec.$^{-1}$	Shearing Stress, dyne cm.$^{-2}$ Curve *1*		Curve *2*		Curve *3*	
2% Clay	140	140	210		220		240
0.5% Na benzoate	250	150	230	230	240	240	260
0.05% Polysorbate 40	360	170	240	240	250	260	270
	470	190	250	260	260	270	280
	590	200	260	270	280	290	300
	1200	290	320	320	330	340	350
	1760	360	370	380	380	390	400
	2340	420	420	430	430	440	450
	2960	470	470	480	480	490	500
	3550	520		530		540	
2% Clay	140	110	210		240		
0.5% Na Benzoate	250	130	220	230	260		
0.1% Polysorbate 40	360	150	240	250	270		
	470	160	250	260	290		
	590	180	260	280	300		
	1200	200	320	340	350		
	1760	330	380	390	410		
	2340	400	430	450	480		
	2960	460	480	500	520		
	3550	530		560			
2% Clay	140	80	110				
0.5% Na Benzoate	250	120	120	120	120		
0.2% Polysorbate 40	360	140	140	130	130		
	470	160	150	150	140		
	590	180	170	160	150		
	1200	280	240	230	170		
	1760	350	310	300	300		
	2340	410	380	360	360		
	2960	460	450	430	430		
	3550	520		480			

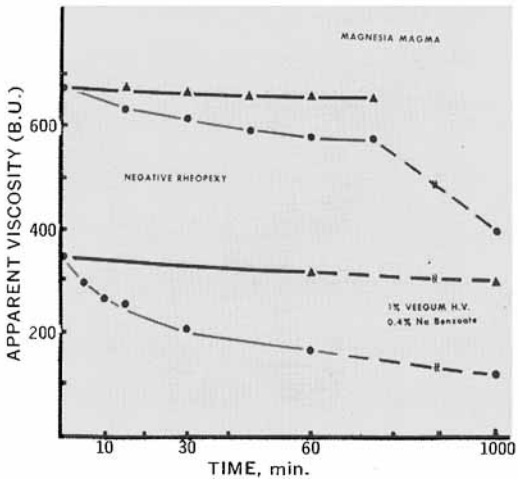


Fig. 2.—Negative rheopexy for milk of magnesia and a clay–salt system. Key: ▲, standing; ●, rotation.

the theory that the floccule size varies with sample treatment.

The process of slow rotation results in the formation of relatively large, compact floccules. This yields more free fluid in the system and reduces floccule-to-floccule contacts. Consequently, these rotated samples filter and sediment more readily. The sheared samples present the reverse situation. The large floccules are destroyed and smaller flocs with greater floccule-to-floccule contacts are produced. These sheared samples settle and filter more slowly, both characteristics of the smaller effective particle size. The sedimentation data are shown in Table VI. Sediment volume is expressed as per cent of total volume, 1 day after the shear treatment.

First attempts to demonstrate floccule size by the vacuum filtration procedures of LaMer (6) were unsuccessful. The pressure differential appeared to destroy the floccule structure. However, gravity filtration procedures as used earlier by LaMer (4) were quite successful and gave additional supporting evidence for the floccule theory of negative thixotropy. The system, 1% clay and 0.4% sodium benzoate, selected earlier as an example of negative rheopexy (Fig. 2), was studied. Filtration rate measurements were performed on this system after stirring and after rotation for different time intervals. The data are shown in Table VII. The gradual decrease in filtration times and the apparent viscosities of these systems with rotation is interpretable as negative thixotropy.

Table VI.—Sediment Volumes for Clay–Salt Systems

Systems		Sediment Vol., Expressed as % of Total Slurry Vol.—After 24 hr. Standing	
% Clay	% Na Benzoate	Rotated Sample	Mixed Sample
0.5	0.5	60	80
0.8	0.5	80	100
0.9	0.5	80	100
1.0	0.5	90	100

Table VII.—Clay–Salt System Filtration Rate Measurement after Various Sample Treatment

System	Treatment	Time of Filtration, sec.
1% Clay 0.4% Na benzoate	Stirring 5 min.	830
	Rotation, 15 min.	780
	Rotation, 30 min.	690
	Rotation, 1 hr.	520
	Rotation 6 hr.	360
	Rotation overnight	360

Significance of Negative Thixotropy.—The clay–salt systems described are used as part of the formulas of many suspensions. Since the clay is generally flocculated to induce the viscosity increase, the negative thixotropic properties which have been described might be of importance in the complete suspensions.

The coagulation of clay particles to the inner glass and cap surfaces has already been suggested as being similar to the "gummy ring" encountered in some of these systems. Silicone treatment of the container is suggested from these studies.

In the testing of a series of eight commercial suspensions containing the clay–salt systems, only two samples exhibited higher apparent viscosities under high shear than under low shear. In both cases, the magnitude of the effect was small compared to the negative thixotropy observed in the clay–salt systems. Therefore, other than milk of magnesia, negative thixotropy in these complete formulations would not appear to contribute much confusion to apparent viscosity measurements.

In any attempts to control or correlate complete suspension apparent viscosities with the apparent viscosities of the concentrated clay slurries, negative thixotropy would be expected to cause considerable confusion. A control and knowledge of the shear histories of these concentrated clay–salt slurries would be vital to a correct interpretation of these data.

SUMMARY

The flocculated clay system has been shown to possess negative thixotropic properties. Measurements on these systems as well as on milk of magnesia have been made by both single point and high shear viscometers. In an analogous fashion to negative thixotropy, the negative rheopexy of both the flocculated clays and milk of magnesia has been demonstrated. Some information on the floccule nature has been gained by sediment volume and filtration rate measurements. The importance of the negative thixotropic behavior of the flocculated clay systems has been considered.

REFERENCES

(1) Chong, C. W., Eriksen, S. P., and Swintosky, J. V., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 547(1960).
(2) Medalia, A. I., Hagopian, E., *Ind. Eng. Chem. Prod. Res. Develop.*, **3**, 120(1964).
(3) Fischer, E. K., "Colloidal Dispersions," John Wiley & Sons, New York, N. Y., 1950, p. 195.
(4) LaMer, V. K., and Smellie, R. H., Jr., *J. Colloid Sci.*, **11**, 710(1956).
(5) Weiser, H. B., "Colloid Chemistry," 2nd ed., John Wiley & Sons, New York, N. Y., 1949, p. 320.
(6) LaMer, V. K., Kane, J. C., and Linford, H. B., *J. Phys. Chem.*, **67**, 1977(1963).

3-Cyanopyrrolidines

By J. SWIDINSKY, J. KERVENSKI, and B. B. BROWN

A series of 1-alkyl-3-cyanopyrrolidines was prepared by the reaction of 1-alkyl-3-chloropyrrolidines with sodium cyanide in dimethylsulfoxide. One of these compounds, 1-methyl-3-cyanopyrrolidine, was used as a model to study the chemical properties of this type of substance. It was converted into 1-methyl-3-aminomethylpyrrolidine by hydrogenation, hydrolyzed to 1-methyl-3-pyrrolidine carboxylic acid (ethyl ester and *N*-methylamide), reacted with phenyl magnesium bromide to give 1-methyl-3-pyrrolidinyl phenyl ketone, and alkylated to a series of 1-methyl-3-alkyl-3-cyanopyrrolidines with alkyl halides and sodium amide.

CONSIDERABLE interest has been shown in 3-substituted pyrrolidines because of their biological activity. Compounds derived from 1-R-3-pyrrolidinol and 1-R-3-pyrrolidinylmethanol have been found to possess a wide range of physiological activity as antihistaminics (1), local anesthetics (2), cholinergic and parasympathetic depressants (3–5), ataractic agents (4), adrenergic blocking agents (6), muscle relaxants (7, 8), antispasmodics (9), antiallergenics (10, 11), anti-inflammatory agents (12), and central nervous system stimulants (13). 3-Cyanopyrrolidines, because of the reactivity of the CN group, may be the starting point for a large number of 3-pyrrolidine derivatives.

Up to now, no new 3-cyanopyrrolidines have been prepared by direct substitution on the ring. It has now been found that 1-alkyl-3-cyanopyrrolidines can be conveniently prepared from 1-alkyl-3-chloropyrrolidines by reaction with sodium cyanide in dimethylsulfoxide, a method recommended for the conversion of alkyl chlorides into the corresponding nitriles (14). The required 1-alkyl-3-chloropyrrolidines were prepared in 55–90% yield by reaction of the 1-alkyl-3-pyrrolidinols with thionyl chloride in benzene (3, 13, 15).

The chloro compounds were usually obtained as the free bases by addition of strong alkali to the reaction mixture. However, with the 1-benzyl derivative, a good yield of the hydrochloride was obtained directly from the reaction mixture. The bases were purified by vacuum distillation in order not to expose them to extreme temperatures. The lower members could, however, be isolated by distillation at ordinary pressures without much decomposition. The 1-alkyl-3-chloropyrrolidines were colorless liquids which slowly darkened on storage, depositing a solid formed by the self condensation of the chloro group with the tertiary amine. Storage at low temperature minimized this decomposition.

Received June 8, 1966, from the Research Laboratories, S. B. Penick Co., Orange, N. J. 07050.
Accepted for publication September 26, 1966.

DISCUSSION

3-Chloropyrrolidine (V) was obtained as its hydrochloride by hydrogenolysis of 1-benzyl-3-chloropyrrolidine hydrochloride (IV, HCl) in isopropyl alcohol over palladium on charcoal. However, the free base, liberated by treatment with alkali, was found to be less stable than the 1-substituted 3-chloropyrrolidines. In one run, V decomposed suddenly while it was being isolated by distillation at a bath temperature of slightly higher than 100°.

In order to obtain a more reactive halide for use in the alkylation of 1-methyl-3-cyanopyrrolidine (see below), 1-methyl-3-bromopyrrolidine (VI) was prepared in 43% yield by the reaction of a large excess of phosphorus tribromide with 1-methyl-3-pyrrolidinol in benzene (11). Like the chloro derivatives, it could be isolated as the free base by vacuum distillation. Compound VI decomposed rather rapidly on storage at room temperature, forming a crystalline solid in a few hours. At zero or below, this decomposition, presumably a self quaternization, was considerably delayed. However, after several months at −10°, one sample had solidified completely. The corresponding chloro compounds stored at 5° remained liquid and undiscolored.

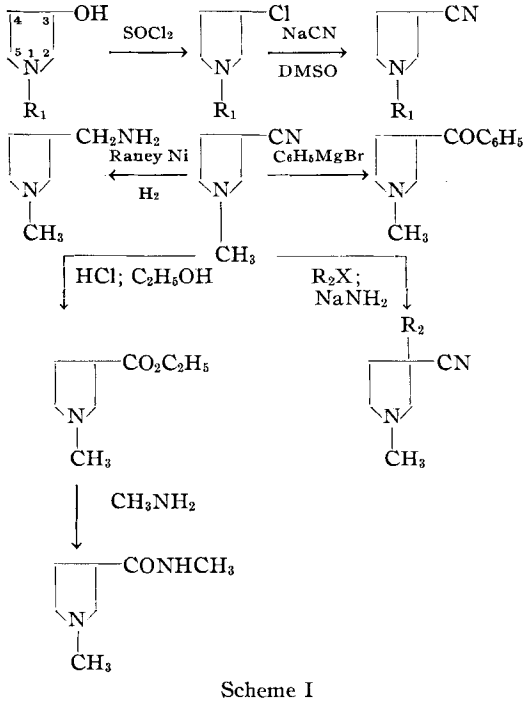
The 1-alkyl-3-chloropyrrolidines were converted to the corresponding 3-cyanopyrrolidines by reaction with sodium cyanide in dimethylsulfoxide (14). The reaction was effected by adding the 3-chloropyrrolidine to a slurry of sodium cyanide in dimethylsulfoxide at 90–100°. The reaction mixture was then heated at 155–170° for 30 min. The yields of the 1-alkyl-3-cyanopyrrolidines varied from 48–71%.

3-Chloropyrrolidine (V) failed to give pure 3-cyanopyrrolidine (XI) when it was treated in the same manner. A substance was obtained which appeared to contain about 72% of XI according to vapor–liquid chromatography analysis. An alternate approach to XI by hydrogenolysis of 1-benzyl-3-cyanopyrrolidine (X) failed.

A preliminary investigation of the chemical properties of these nitriles was made with 1-methyl-3-cyanopyrrolidine (VII) as a model (Scheme I, Table I).

Hydrogenation of VII over Raney nickel in acetic anhydride in the presence of sodium acetate (16) resulted in the formation of 1-methyl-3-acetylaminomethylpyrrolidine from which the free diamine, 1-methyl-3-aminomethylpyrrolidine (XII), was liberated by hydrolysis of the acetyl group (17).

Hydrolysis of VII with concentrated hydrochloric acid gave 1-methyl-3-pyrrolidine carboxylic



Scheme I

acid (18) which was esterified directly to ethyl-1-methyl-3-pyrrolidine carboxylate (XIII) (19). Compound XIII with methylamine gave the *N*-methylamide (XIV). Compound VII and phenyl magnesium bromide gave 1-methyl-3-pyrrolidinyl phenyl ketone (XV). A similar reaction with X gave 1-benzyl-3-pyrrolidinyl phenyl ketone (XVI). The intermediate ketimine magnesium halide of XVI was found to be resistant to the action of boiling concentrated hydrochloric acid. It was converted into the ketone by treatment with sodium hydroxide. The corresponding salt of XV was readily cleaved with ammonium chloride.

Compound VII was readily alkylated with alkyl halides and sodium amide. To effect the alkylation, VII was first added to a slurry of sodium amide in liquid ammonia followed by the alkyl halide. Toluene was then added to the mixture and the ammonia was allowed to evaporate. The toluene was then heated to reflux until the black color of the sodium amide disappeared. The 3-alkylated derivatives were isolated by distillation (20).

EXPERIMENTAL[1]

3-Halopyrrolidines

The 1-alkyl-3-pyrrolidinols used in the preparation of the 1-alkyl-3-halopyrrolidines were prepared according to the method of Franko and Lunsford (3) from 1,4-dichloro-2-butanol (21, 22) and the appropriate monoalkylamine.

[1] All infrared spectra were made with a Perkin-Elmer Infracord spectrophotometer. All melting points are uncorrected. The microanalyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Mich., and by the S. B. Penick Quality Control Laboratories.

1 - Methyl - 3 - chloropyrrolidine (I).—The method given below for the preparation of I was also used for 1-ethyl and 1-isopropyl-3-chloropyrrolidines (II, III).

To a solution of 50.5 Gm. (0.5 mole) of 1-methyl-3-pyrrolidinol in 500 ml. of dry benzene was added 119 Gm. (1 mole) of thionyl chloride in 100 ml. of benzene at such a rate that the mixture warmed up to 75°. The mixture was refluxed for 1 hr., 300 ml. of benzene distilled off and replaced with 300 ml. of fresh benzene, cooled, and made strongly alkaline with 2 *N* sodium hydroxide solution. The benzene layer was separated, washed once with water, and distilled through a short Vigreaux column to yield 46.3 Gm. (79.5%) of I, b.p. 55–60° at 50 mm. [Lit. value (15) 55–58° at 50 mm.]

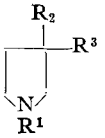
1 - Ethyl - 3 - chloropyrrolidine (II).—From 115.2 Gm. (1 mole) of 1-ethyl-3-pyrrolidinol and 180 Gm. (1.5 moles) of thionyl chloride, 105 Gm. (78.8%) of II was obtained; b.p. 157–159° at 760 mm. [Lit. value (13) 155° at 760 mm.]

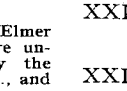
1 - Isopropyl - 3 - chloropyrrolidine (III) (13).—A yield of 99.5 Gm. (67.4%) of III was obtained from 129.2 Gm. (1 mole) of 1-isopropyl-3-pyrrolidinol and 180 Gm. (1.5 moles) of thionyl chloride; b.p. 93–94° at 47 mm.

Anal.—Calcd. for $C_7H_{14}ClN$: neutralization equivalent, 148. Found: 154.

1 - Benzyl - 3 - chloropyrrolidine (IV) (13).—Similarly, 177.4 Gm. (1 mole) of 1-benzyl-3-pyrrolidinol and 180 Gm. (1.5 moles) of thionyl chloride were reacted in 600 ml. of benzene. However, after the reaction was over, a solid crystallized on

TABLE I.—3-SUBSTITUTED PYRROLIDINES



Compd.	R^1	R^2	R^3
I	CH_3	H	Cl
II	C_2H_5	H	Cl
III	*i*-C_3H_7	H	Cl
IV	$C_6H_5CH_2$	H	Cl
V	H	H	Cl
VI	CH_3	H	Br
VII	CH_3	H	CN
VIII	C_2H_5	H	CN
IX	*i*-C_3H_7	H	CN
X	$C_6H_5CH_2$	H	CN
XI	H	H	CN
XII	CH_3	H	CH_2NH_2
XIII	CH_3	H	$CO_2C_2H_5$
XIV	CH_3	H	$CONHCH_3$
XV	CH_3	H	COC_6H_5
XVI	$C_6H_5CH_2$	H	COC_6H_5
XVII	CH_3	CH_3	CN
XVIII	CH_3	*n*-C_3H_7	CN
XIX	CH_3	*i*-C_3H_7	CN
XX	CH_3	*n*-C_4H_9	CN
XXI	CH_3		CN
XXII	CH_3	$C_6H_5CH_2$	CN
XXIII	CH_3		CN
XXIV	CH_3	$(CH_3)_2NCH_2CH_2$	CN

cooling. This substance was filtered and washed free of color with benzene to yield 204.5 Gm. (86.0%) of IV hydrochloride, m.p. 140–142°. [Lit. value (13) 131–132°.]

Anal.—Calcd. for $C_{11}H_{15}Cl_2N$: neutralization equivalent, 232. Found: 236.

To liberate the free base, the hydrochloride was suspended in 1000 ml. of ether and treated with 400 ml. of 1.5 *N* sodium hydroxide. The ether phase was separated, dried over sodium sulfate, and distilled to give 166 Gm. (84.7%) of IV, b.p. 87–89° at 0.4–0.8 mm.

3 - Chloropyrrolidine (V).—A suspension of 38.0 Gm. (0.16 mole) of IV hydrochloride in 150 ml. of isopropyl alcohol was hydrogenated (Parr shaker) at 15–30 p.s.i. and 60–70° over 1.5 Gm. of 15% palladium on charcoal. The theoretical amount of hydrogen was absorbed in 5 hr. After filtration from the catalyst, the solution was concentrated and the product crystallized to yield 19.7 Gm. (83.4%) of crude V hydrochloride, m.p. 106–110°. The product was purified by recrystallization from isopropyl alcohol; yield, 15.4 Gm. (68.1%), m.p. 111–113°.

Anal.—Calcd. for $C_4H_9ClN^+ Cl^-$: Cl^-, 24.96. Found: 25.03.

The free base was liberated by treating a suspension of 15.0 Gm. (0.106 mole) of V hydrochloride in 100 ml. of ether with 20 ml. of 10 *N* sodium hydroxide. Distillation of the ether yielded 8.0 Gm. (72.1%) of V, b.p. 86–88° at 100 mm. 3-Chloropyrrolidine was a colorless liquid which rapidly darkened on storage. In another preparation, the substance suddenly decomposed upon distillation forming a solid black tar. Freshly prepared V was used in attempts to prepare 3-cyanopyrrolidine (XI).

1 - Methyl - 3 - bromopyrrolidine (VI) (11).—To a solution of 8.7 Gm. (0.087 mole) of 1-methyl-3-pyrrolidinol in 100 ml. of dry benzene cooled to below 10°, there was added 28.3 Gm. (0.105 mole) of phosphorus tribromide. The mixture was refluxed for 2 hr., cooled, and treated with 120 ml. of 25% potassium hydroxide. The benzene solution was separated and distilled to give 7.0 Gm. (49.1%) of VI, b.p. 78° at 40 mm. Compound VI was a colorless liquid which soon started to deposit a solid upon standing at room temperature. No attempt was made at analysis. It was used as rapidly as possible for the preparation of 1-methyl-3 - (1 - methyl - 3 - pyrrolidinyl) - 3 - cyanopyrrolidine (XXIII).

3-Cyanopyrrolidines

The following method, illustrated by the preparation of 1-methyl-3-cyanopyrrolidine (VII), was used to prepare all of the 3-cyanopyrrolidines.

1-Methyl-3-cyanopyrrolidine (VII).—To a suspension of 165 Gm. (3.2 moles) of sodium cyanide in 775 ml. of dimethylsulfoxide (dried over calcium hydride) heated to 90–100° was added, with stirring over a 25-min. period, 309 Gm. (2.58 moles) of I. The mixture was heated to 165–170° for 30 min., cooled to 25°, and diluted with 1300 ml. of chloroform. The mixture was poured into 4000 ml. of saturated salt solution. The chloroform phase was separated and the aqueous layer extracted with chloroform. The combined chloroform solution was washed several times with saturated salt to remove residual dimethylsulfoxide and distilled to yield 190 Gm. (67.1%) of VII, b.p. 92–93° at 23 mm. A picrate was prepared in alcohol, m.p. 204–205°.

Anal.—Calcd. for $C_{12}H_{13}N_5O_7$: N, 20.64. Found: 20.61, 20.58.

1 - Ethyl - 3 - cyanopyrrolidine (VIII).—From 26.7 Gm. (0.2 mole) of II and 12.0 Gm. (0.25 mole) of sodium cyanide, 12.0 Gm. (48.5%) of VIII, b.p. 102–104° at 23 mm., was obtained; picrate m.p. 140–141°.

Anal.—Calcd. for $C_{13}H_{15}N_5O_7$: N, 19.82. Found: 20.18.

1 - Isopropyl - 3 - cyanopyrrolidine (IX).—From 81.2 Gm. (0.55 mole) of III and 36.0 Gm. (0.7 mole) of sodium cyanide was obtained 40.0 Gm. (52.6%) of IX, b.p. 102–104° at 10 mm.

Anal.—Calcd. for $C_8H_{14}N_2$: neutralization equivalent, 138. Found: 140. A picrate, m.p. 136–137°, was prepared in alcohol.

Anal.—Calcd. for $C_{14}H_{17}N_5O_7$: N, 19.07. Found: 19.08.

1 - Benzyl - 3 - cyanopyrrolidine (X).—The same procedure was used except the reaction temperature was lowered to 155–160° from 165–170°. Thus, 265.0 Gm. (1.35 moles) of IV and 100.0 Gm. (2.05 moles) of sodium cyanide yielded 179.0 Gm. (71.3%) of X, b.p. 120–124° at 0.5–0.6 mm.

Anal.—Calcd. for $C_{12}H_{14}N_2$: neutralization equivalent, 186. Found: 188.

3-Cyanopyrrolidine (XI).—The procedure for X was used. From 10.6 Gm. (0.1 mole) of V and 6.4 Gm. (0.13 mole) of sodium cyanide, only 1.4 Gm. of a product, b.p. 82–84° at 5 mm., was isolated.

Anal.—Calcd. for $C_5H_8N_2$: neutralization equivalent, 96; C, 62.48; H, 8.38; N, 29.14. Found: neutralization equivalent, 107; C, 59.52; H, 8.20; N, 26.51.

Vapor phase chromatography[2] showed that one component comprised about 72.5% of the above product and three others the remainder. The infrared absorption spectrum of the product showed a distinct CN maximum at 4.48 μ and one for NH at 3.0 μ. It was therefore concluded that XI was the major component of the mixture.

An attempt was made to prepare XI by hydrogenolysis of X hydrochloride. The latter, prepared by neutralizing a solution of X in isopropyl alcohol, was shaken with hydrogen over palladium on charcoal (Parr shaker). More than the theoretical amount of hydrogen was absorbed. Only a tarry product could be isolated. Compound X did not take up hydrogen under the same conditions.

Products from 1-Methyl-3-cyanopyrrolidine

1 - Methyl - 3 - aminomethylpyrrolidine (XII).—A mixture of 11.0 Gm. (0.1 mole) of VII, 120 ml. of acetic anhydride, 12 Gm. of anhydrous sodium acetate, and 3 Gm. of Raney nickel (previously washed and suspended in acetic anhydride) was shaken at 50° at 15–25 p.s.i. of hydrogen (Parr shaker). Approximately 2 moles of hydrogen per mole of VII was absorbed in 1 hr. The reaction

[2] The authors thank Mr. F. A. E. Schilling of these laboratories for performing the analysis on a Perkin-Elmer 154 instrument. A 2-M. 1/4 in. i.d. stainless steel column filled with 20% Silicone SE 52 on Chromosorb W (45/60 mesh) and operated at 150° with 15 p.s.i. of helium at a rate of 33 ml./min. was used to effect the separation.

mixture was filtered from the catalyst and the acetic anhydride was removed by vacuum distillation. The semisolid residue was dissolved in benzene and filtered from the insoluble sodium acetate. A yield of 11.6 Gm. (75%) of what apparently was the acetyl derivative of XII was obtained by distillation of the filtrate, b.p. 104–106° at 0.3 mm. This substance discolored rapidly on standing. To liberate the free diamine, 7.3 Gm. (0.064 moles) of the above product was refluxed in 20 ml. of 7.5 *N* sodium hydroxide until all went into solution. The solution was cooled, neutralized with hydrochloric acid, and evaporated to dryness in vacuum. The residue was treated with excess 50% sodium hydroxide and extracted with ether. The ether extracts were dried over sodium hydroxide pellets and distilled to give 4.5 Gm. (87%) of XII, b.p. 72–76° at 30 mm. A dipicrate was prepared and recrystallized from alcohol, m.p. 206–207°. [Lit. value (17) 208–211°.]

Ethyl - 1 - methyl - 3 - pyrrolidine Carboxylate (XIII) (19).—A mixture of 11.0 Gm. (0.1 mole) of VII and 80 ml. of concentrated hydrochloric acid was refluxed overnight. The resulting orange solution was vacuum stripped to dryness and the residue dissolved in 150 ml. of anhydrous alcohol. The insoluble salt was filtered. Weight, 5.0 Gm. Calcd. for ammonium chloride: 5.2 Gm. The filtrate was saturated with hydrogen chloride and refluxed for 3 hr. The excess hydrogen chloride and alcohol were removed by vacuum distillation. The residue was dissolved in water and extracted with ether. The aqueous solution was made alkaline and extracted 7 times with ether. Distillation of the ether extracts gave 6.2 Gm. (39.3%) of XIII, b.p. 107–109° at 40 mm.

Anal.—Calcd. for $C_8H_{15}NO_2$: neutralization equivalent, 157. Found: 159.

1 - Methyl - 3 - pyrrolidine - N - methylcarboxamide (XIV).—A solution of 3.9 Gm. (0.025 mole) of XIII in 65 ml. of 40% aqueous methylamine was allowed to stand at room temperature for 1 week. The excess methylamine and water were removed and the residue distilled to give 2.4 Gm. of XIV, b.p. 90–94° at 0.2 mm. The liquid distillate soon solidified to give a waxy solid melting at about 35°.

Anal.—Calcd. for $C_7H_{14}N_2O$: N, 19.70. Found: 19.59.

1 - Methyl - 3 - pyrrolidinyl Phenyl Ketone (XV).—To a solution of phenyl magnesium bromide prepared from 62.8 Gm. (0.4 mole) of bromobenzene and 9.7 Gm. (0.4 atoms) of magnesium turnings in 100 ml. of ether, was added 11.0 Gm. (0.1 mole) of VII at such a rate that a gentle reflux was maintained. The reaction mixture was then stirred and refluxed for 5 hr. It was then cooled and treated with 50 ml. of saturated ammonium chloride solution. The ether layer was decanted from the semisolid aqueous phase. The latter was diluted with water to dissolve the solid and extracted with ether. The combined ether solutions were dried over sodium sulfate and distilled to give 7.8 Gm. (41.0%) of XV, b.p. 112–118° at 0.3 mm. A picrate was prepared and recrystallized from benzene, m.p. 146–147°.

Anal.—Calcd. for $C_{18}H_{18}N_3O_8$: N, 13.39. Found: 13.29.

The infrared spectrum of XIV showed a maximum at 5.9 μ corresponding to the C=O group and a doublet at 3.4 and 3.6 μ which characterized the spectra of most of the 1-alkyl-3-substituted pyrrolidines.

1 - Benzyl - 3 - pyrrolidinyl Phenyl Ketone (XVI).—In a manner similar to the procedure for XV, 18.6 Gm. (0.1 mole) of X was reacted with a solution of phenyl magnesium bromide prepared from 18.8 Gm. (0.12 mole) of bromobenzene and 2.7 Gm. (0.12 atoms) of magnesium turnings in 90 ml. of ether. The cold reaction mixture was decomposed with 70 ml. of 8 *N* hydrochloric acid to give, on filtration, 25 Gm. of a crystalline solid. This substance, apparently an acid resistant ketimine magnesium halide, gave an ash on fusion and was recovered unchanged after refluxing overnight with concentrated hydrochloric acid. Suspension in 300 ml. of toluene and agitation at 60° for 30 min. with 160 ml. of 4 *N* sodium hydroxide hydrolyzed it to the ketone. Filtration of the reaction mixture gave some solid magnesium hydroxide. The toluene layer was separated, dried over sodium sulfate, and distilled to yield 19.6 Gm. (74.0%) of XVI, b.p. 179–180° at 0.3 mm.

Anal.—Calcd. for $C_{18}H_{19}NO$: neutralization equivalent, 265. Found: 266.

A hydrochloride, m.p. 120–122°, was prepared by neutralizing a tetrahydrofuran solution of XVI with concentrated hydrochloric acid.

Anal.—Calcd. for $C_{18}H_{20}ClNO$: N, 4.64. Found: 4.25.

The infrared spectrum of XVI showed a maximum at 5.9 μ and a doublet at 3.35 and 3.58 μ.

Alkylation of 1-Methyl-3-cyanopyrrolidine

The following method illustrated in the preparation of XVIII was used for all of the 1-methyl-3-alkyl-3-cyanopyrrolidines.

1,3-Dimethyl-3-cyanopyrrolidine (XVII).—To a suspension of sodium amide prepared from 2.3 Gm. (0.1 atom) of sodium in 150 ml. of liquid ammonia (23) was added 11.0 Gm. (0.1 mole) of VII. The mixture was stirred in refluxing ammonia for several minutes and 17.0 Gm. (0.12 mole) of methyl iodide followed by 150 ml. of dry toluene were added. The ammonia was allowed to evaporate off, the mixture heated to reflux until the black color of the sodium amide disappeared (3–4 hr.), cooled, and mixed with 100 ml. of water. The aqueous layer was separated and extracted with toluene. The combined toluene solution was dried over sodium sulfate and distilled to yield 7.8 Gm. (63.2%) of XVII, b.p. 88–89° at 24 mm. A picrate was prepared in alcohol, m.p. 211–213°.

Anal.—Calcd. for $C_{13}H_{15}N_5O_7$: N, 19.82. Found: 19.89, 19.81.

1 - Methyl - 3 - n - propyl - 3 - cyanopyrrolidine (XVIII).—Compound XVIII, 9.6 Gm. (63.3%), b.p. 112–113° at 23 mm., was obtained in a similar manner from 20.4 Gm. (0.12 mole) of *n*-propyl iodide and the same quantities of sodium amide and VII; picrate, m.p. 163–164°.

Anal.—Calcd. for $C_{15}H_{19}N_5O_7$: N, 18.36. Found: 18.49.

1 - Methyl - 3 -i-propyl - 3 - cyanopyrrolidine (XIX).—Similarly, 7.1 Gm. (46.7%) of XIX, b.p. 76–79° at 5 mm., was obtained from 20.4 Gm. (0.12 mole) of *i*-propyl iodide; picrate, m.p. 221–223°.

Anal.—Calcd. for $C_{15}H_{19}N_5O_7$: N, 18.36. Found: 18.32.

1 - Methyl - 3 - n - butyl - 3 - cyanopyrrolidine (XX).—Similarly, 8.1 Gm. (48.5%) of XX, b.p. 136° at 25 mm., was obtained from 18.4 Gm. (0.1 mole) of *n*-butyl iodide; picrate, m.p. 158–160°.

Anal.—Calcd. for $C_{16}H_{21}N_5O_7$: N, 17.71. Found: 17.78, 17.62.

1 - Methyl - 3 - cyclopentyl - 3 - cyanopyrrolidine (XXI).—Similarly, 17.9 Gm. (0.12 mole) of cyclopentyl bromide yielded 3.3 Gm. (18.7%) of XXI, b.p. 106–108° at 5 mm.; picrate, m.p. 204–206°.

Anal.—Calcd. for $C_{17}H_{21}N_5O_7$: N, 16.78. Found: 17.10, 17.21.

1 - Methyl - 3 - benzyl - 3 - cyanopyrrolidine (XXII).—Similarly, 11.5 Gm. (57.6%) of XXII, b.p. 134–136° at 2 mm., was obtained from 12.7 Gm. (0.1 mole) of benzyl chloride; picrate, m.p. 176–177°.

Anal.—Calcd. for $C_{19}H_{19}N_5O_7$: N, 16.31. Found: 16.12, 16.38.

1 - Methyl - 3 - (1 - methyl - 3 - pyrrolidinyl)-3 - cyanopyrrolidine (XXIII).—Similarly, 19.7 Gm. (0.12 mole) of freshly prepared VI yielded 2.7 Gm. (13.8%) of XXIII, b.p. 120–126° at 8 mm.; dipicrate, m.p. 232–233° dec.

Anal.—Calcd. for $C_{23}H_{25}N_9O_{14}$: N, 19.35. Found: 19.60, 19.52.

1 - Methyl - 3 - (2 - dimethylaminoethyl) - 3-cyanopyrrolidine (XXIV).—Similarly, with double the amount of sodium amide used for the preceding compounds, 28.0 Gm. (0.12 mole) of 2-dimethylaminoethylbromide hydrobromide yielded 2.9 Gm. (16.2%) of XXIV, b.p. 110–112° at 6 mm.; dipicrate, m.p. 148–150° (opaque liquid), clear at 155–160°.

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REFERENCES

(1) Lunsford, C. D., Ward, J. W., Pallota, A. J., Tusing, T. W., Rose, E. K., and Murphey, R. W., *J. Med. Pharm. Chem.*, **1**, 73(1959).
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(6) Lunsford, C. D., U. S. pat. 2,878,264(1959).
(7) Winder, C. V., Wax, J., Serrano, B., Scott, L., Stackhouse, S. P., and Wheelock, R. H., *J. Pharmacol. Exptl. Therap.*, **133**, 117(1961).
(8) Parke, Davis and Co., Brit. pat. 907,424(1962).
(9) Wu, Y.-H., Feldkamp, R. F., Corrigan, J., and Rhodes, H. J., *J. Org. Chem.*, **26**, 1524(1961).
(10) Wu, Y.-H., and Feldkamp, R. F., *ibid.*, **26**, 1529(1961).
(11) Schuler, W. A., U. S. pat. 2,784,185(1957).
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(13) Lunsford, C. D., Cale, A. D., Jr., Ward, J. W., Franko, B. V., and Jenkens, H., *J. Med. Chem.*, **7**, 302 (1964).
(14) Smilely, R. A., and Arnold, C., *J. Org. Chem.*, **25**, 257(1960).
(15) Ames, D. E., *J. Chem. Soc.*, **1960**, 2780.
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(21) Reppe, W., *et al.*, *Ann.*, **596**, 141(1955).
(22) Abruzov, Yu. A., and Ovchinikov, Yu., *Dokl. Akad. Nauk SSSR*, **117**, 813(1957).
(23) Greenlee, K. W., and Henne, A. L., "Inorganic Synthesis," vol. 2, Fernelius, W. S., ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1946, p. 128.

Separation and Quantitative Determination of Adrenaline Using Thin-Layer Chromatography

By N. H. CHOULIS

The separation of adrenaline from noradrenaline and dopamine and its quantitative determination using thin-layer chromatography have been studied. A complete spot separation of the above amines can be achieved by the use of cellulose thin layers and a phenol–water solvent system. For the quantitative determination of the separated adrenaline two methods were used, namely (*a*) a method studying the weight/area relationship of the spots and (*b*) a method employing elution of the spots and measurement of the ultraviolet absorption of the eluents.

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Received April 25, 1966, from the School of Pharmacy, Texas Southern University, Houston 77004.
Accepted for publication October 19, 1966.
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A complication in the interpretation of chromatograms of catecholamines (on paper or thin-layer chromatography) may result from the presence of multiple spots which are sometimes produced during chromatography of pure substances

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REFERENCES

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(19) Cavalla, J. F., *J. Chem. Soc.*, **1959**, 851.
(20) Cope, A. C., Holmes, H. L., and House, H. O., "Organic Reactions," vol. 9, Adams, R., Blatt, A. H., Cope, A. C., Curtin, D. Y., McGrew, F. C., and Nieman, C., eds., John Wiley & Sons, Inc., New York, N. Y., 1957, pp. 107–331.
(21) Reppe, W., *et al.*, *Ann.*, **596**, 141(1955).
(22) Abruzov, Yu. A., and Ovchinikov, Yu., *Dokl. Akad. Nauk SSSR*, **117**, 813(1957).
(23) Greenlee, K. W., and Henne, A. L., "Inorganic Synthesis," vol. 2, Fernelius, W. S., ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1946, p. 128.

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Thin-layer chromatographic methods have often been used for the separation of various components of a mixture. The importance of this method and the ease with which it is carried out have been generally recognized since Stahl (3–5) introduced it as an analytical tool.

A complication in the interpretation of chromatograms of catecholamines (on paper or thin-layer chromatography) may result from the presence of multiple spots which are sometimes produced during chromatography of pure substances

(6–11). Multiple spots are formed when catecholamines are chromatographed in the presence of acids (*i.e.*, dichloro- or trichloroacetic, picric, etc.) and also when pure samples of salts of catecholamines (tartrate, hydrochloride) are chromatographed in *n*-butanol–HCl or *n*-butanol–acetic acid–water solvent systems (2, 12).

Adrenaline and noradrenaline have been previously chromatographed on buffered silica gel plates, using a 70% solution of ethanol as solvent system (13). Waldi (14) has also achieved the separation of adrenaline, noradrenaline, and serotonin and other catecholamines on Silica Gel G plates, but after conversion of the amines to their acetyl derivatives.

For quantitative thin-layer chromatography a number of methods have been used. Seher (15) has suggested that in thin-layer chromatography the weight of the material and the spot are proportional. Purdy and Truter (16) found that quantitation is based on a linear relationship existing between the square root of the area of a component after chromatographing, and the logarithm of the weight of the applied sample; according to them Seher's data as well as that of Stahl (17) and Breuner and Niederwieser (18) fit the relationship for loads of 1–80 mcg. per spot. Pelka and Metcalf (19) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Recently Morrison and Orr (20), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms, by using thin-layer chromatography, obtained quantitative results from the developed chromatograms. A linear relationship between the spot area, in sq. mm., and the weight of the spot was observed.

The method was applicable for concentrations up to 250 mcg. However, this method requires a satisfactory spray reagent, otherwise its use for quantitative work is doubtful.

Ganshirt *et al.* (21) used the thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (22) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (23) described other techniques for quantitative thin-layer chromatography using furoic and hydroxymethylfuroic acids as model compounds. An application of thin-layer chromatography for quantitative assay of tryptamine was suggested by Eble and Brooker (24).

The purpose of the present paper is to provide, by means of thin-layer chromatography, a rapid method of separation and quantitative determination of catecholamines.

EXPERIMENTAL

Materials.—Adrenaline, dopamine, and noradrenaline were used. Adrenaline and noradrenaline were liberated from the hydrochloride and bitartrate monohydrate salts, respectively, by the addition of a dilute ammonium hydroxide solution containing a trace of sodium metabisulfite. The precipitates were filtered off, washed with water, methanol, ether, and dried at low temperature. Thin-layer chromatography, melting point determinations, and infrared spectra analysis demonstrated that each compound had a high degree of purity.

Solvent Systems.—The following solvent systems were used: (*1*) *n*-butanol–acetic acid–water, 4:1:5 v/v (the liquids were shaken together and set aside overnight; the organic layer was separated and used as the running solvent). (*2*) *n*-Amyl alcohol–acetic acid–water, 4:1:5 v/v. (*3*) Methanol–acetone–triethylamine, 50:50:1 v/v. (*4*) Dimethylformamide. (*5*) Phenol–water, 8:2 w/w.

Detection.—The spots were visualized by spraying the plates with a solution of 0.6 Gm. of potassium ferrocyanide and 0.5 Gm. of sodium hydroxide in 100 ml. of water (25).

An ultraviolet lamp was used also to locate the spots in cases where spraying reagents had to be avoided.

Thin-Layer Plates.—All plates used were 20 × 20 cm.

Using MN 300G cellulose powder (Machery, Nagel & Co.) Silica Gel G (Merck), and aluminum oxide G (Merck), plates were prepared according to Stahl (3). The plates were kept in a desiccator using calcium chloride and reactivated at 100° for 5 min. before use.

Methods

Separation of Amines.—Two per cent solutions of the amines were prepared using a 5% acetic acid solution in water. One-tenth microliter quantities were applied 1.5 cm. from the bottom edge of the plates. A distance of 10 cm. from the origin was marked off, and ascending chromatograms were run to this mark (at room temperature).

Quantitative Determination of Adrenaline.—*Weight/Area Relationship.*—A solution of adrenaline was prepared by dissolving 300 mg. of pure compound in 10 ml. of a 2% solution of acetic acid in water, in a 10-ml. volumetric flask. Using a micropipet, quantities of 1 to 5 μl. were applied to the plates 1.5 cm. from the bottom edges. Ascending chromatograms were run (at room temperature).

When the solvent front reached the 10 cm. mark (60–70 min.), the plates were removed and air dried, sprayed, and the spot areas were measured by means of a photovolt Densicord (model 52-C); the graph plotted by the instrument was rendered quantitative by an electronic integrator (Integraph model-49) adapted to the Densicord, and which calculated the spot area which then was expressed in the equivalent square centimeters by the use of a Gelman spot calculator. The area of the spots representing the various concentrations was taken and plotted, using the appropriate relationship.

Elution of the Spots.—A solution of adrenaline was prepared by dissolving 100 mg. of the amine in 3 ml. of a 2% solution of acetic acid in water (master solution). Five different concentrations

of the amine were prepared by diluting quantities of 1 to 5 μl. of the master solution into 10-ml. volumetric flasks and bringing up to the volume using the same solvent. The ultraviolet absorption of each solution was taken at 280 mμ, and from the values obtained, a standard curve was plotted.

Quantities of 1 to 5 μl. of the adrenaline solution (master solution) were applied to thin-layer plates of either silica gel or alumina, and the chromatograms were run in the usual manner.

After drying the plates, the spots were located using an ultraviolet lamp and were marked off. Each spot was then scraped off into small conical flasks. The adrenaline was eluted from the coating material by adding about 7 ml. of the solvent. Each flask was shaken well for about 10 min., centrifuged, and filtered into 10-ml. volumetric flasks. Solvent was added to volume.

No adrenaline residues were found on the filter, testing with the detection reagent (25).

A similar elution process was followed for a blank sample of the coating material alone.

Ultraviolet absorption of each solution was taken at the same wavelength as above (280 mμ), and the values obtained after reducing the absorption due to the blank were recorded next to the standard curve and compared.

To all solvent systems used, small quantities of sodium metabisulfite were added as antioxidant. All chromatograms were carried out in chromatographic chambers enriched in nitrogen atmosphere.

RESULTS AND DISCUSSION

All experiments were performed in duplicate.

The results for the separation of the amines, using alumina, cellulose, or silica gel plates and the various solvent systems, indicate that although there was a considerable movement of the amine spots on alumina plates, when No. *1* and *2* solvent systems were used, the mixture of the three amines could not be separated completely, since the observed R_f values were very close together. A complete separation, however, between dopamine and noradrenaline can be achieved, when the No. *1* solvent system is used.

Similarly on silica gel plates, separation between two amines can be observed, but not complete separation of the three amines.

On cellulose thin layer, solvents No. *1* and *2* showed a partial separation between the amine spots, but when phenol–water (solvent No. *5*) was used, the separation between the three amines was complete (Fig. 1). This indicates that among the layers studied, only that of cellulose, along with the phenol–water solvent system, can give a complete separation when even large quantities of the amines are used.

However, for very small loads (1–30 mcg.) combinations of the above-mentioned solvent systems and alumina, cellulose, or silica gel plates would give complete separation of the amines with the exception of alumina plate and the No. *3* solvent system. Furthermore, in no case was separation between adrenaline and noradrenaline observed when alumina and the No. *4* solvent system or silica gel and the No. *3* or *4* solvent systems were used, but a complete separation of these amines from dopamine always occurred.

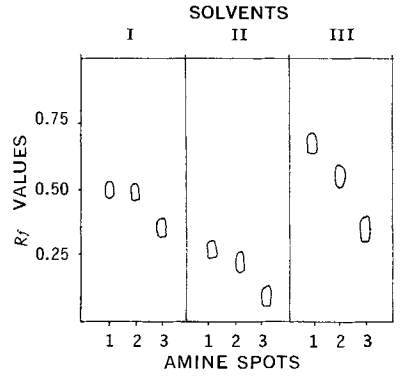

Fig. 1.—Thin-layer chromatography of adrenaline [1], dopamine [2], and noradrenaline [3] on cellulose layers using various solvent systems. Solvents: I, *n*-butanol–acetic acid–water, 4:1:5 v/v; II, *n*-amyl alcohol–acetic acid–water, 4:1:5 v/v; III, phenol–water, 8:2 w/w.

The results obtained for the quantitative estimation of adrenaline by applying the weight/area relationship method when *n*-butanol–acetic acid–water or phenol–water solvent systems were used are shown in Figs. 2 and 3, respectively. In Fig. 4 results obtained from the elution method are depicted.

For the weight/area method, a linear relationship between the spot area and the weight of the compound has been found when silica gel or alumina plates and either solvent systems No. *1* or *5* were used. This relationship shows the dependence of the area of the spot upon the amount of the sample used, and although the size of the spot varied with the thickness of the layers, the temperature of the experiment and the purity of the solvent used, the observed results were reproducible within ±5%. However, it is advisable ro run on the same plate both the standards and the sample.

Although the best separation of adrenaline from the other amines was achieved on cellulose plates,

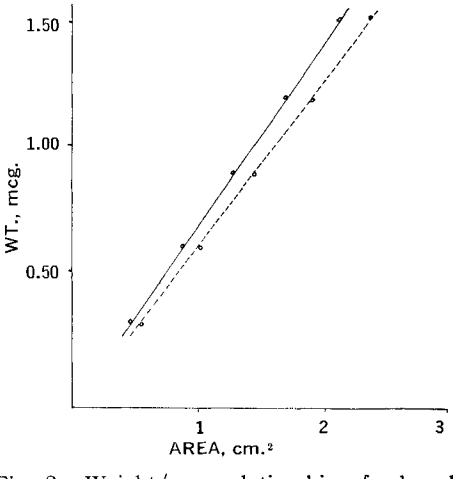

Fig. 2.—Weight/area relationship of adrenaline spots when *n*-butanol–acetic acid–water, 4:1:5 v/v solvent system, was used. Key: - - - - - -, silica gel plates; ——, alumina plates.

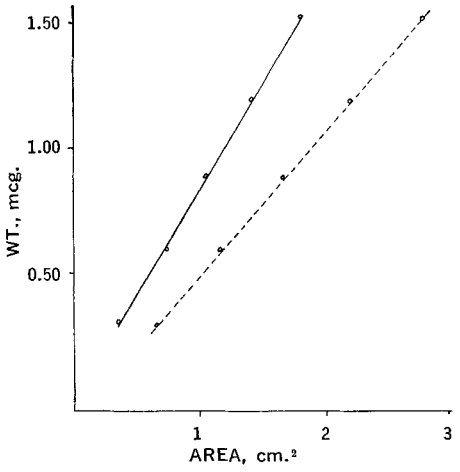

Fig. 3.—Weight/area relationship of adrenaline spots when phenol–water, 8:2 w/w solvent system, was used. Key: - - - - - -, silica gel plates; ——, alumina plates.

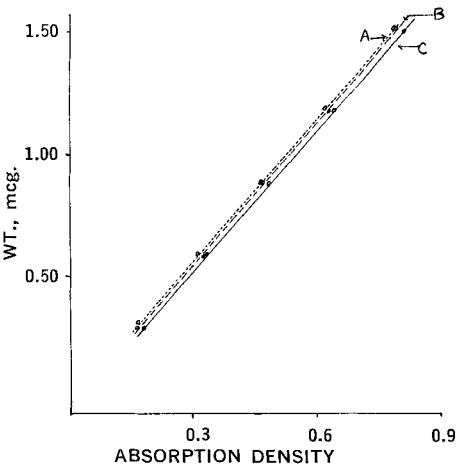

Fig. 4.—Adrenaline recovery after elution of the spots from thin-layer chromatograms of alumina (A) and silica gel (B). The straight line (C) indicates the blank. *n*-Butanol–acetic acid–water, 4:1:5 v/v solvent system, was used.

using the phenol–water solvent system, it was found that for the quantitative estimation of the separated amine, alumina or silica gel plates and the No. *1* solvent system gave the most accurately reproducible results (Fig. 4).

The recovery of the compounds, after the elution of the spots, was 93 and 97%, respectively, for thin layers of alumina or silica gel. The lower recovery on the alumina thin layer can be attributed to the strong adsorption forces by which the substance is held on the alumina (11). A recovery of below 85% was observed when cellulose layers were used. Here the adsorption and partition forces influence the recovery (11).

However, for quantitative determination of adrenaline, the above-mentioned methods may be regarded as simple and accurate, though the second method has been found more reliable, since factors which might have influenced the spot area in the first method are involved only to a certain extent in the elution method.

The use of these methods can also be extended for quantitative determination of other sympathomimetic amines and other compounds.

REFERENCES

(1) McGreer, E. G., and Clark, W. G., *J. Chromatog.*, **14**, 107(1964).
(2) Mattok, G. L., *ibid.*, **16**, 254(1964).
(3) Stahl, E., *Pharmazie*, **11**, 633(1956).
(4) Stahl, E., *Chemiker-Ztg.*, **82**, 323(1958).
(5) Stahl, E., *Pharmazeutische Rundschau I*, Spec. Edit. No. 2, 1959.
(6) Shepherd, D. M., and West, G. B., *Nature*, **169**, 797 (1952).
(7) West, G. B., *J. Pharm. Pharmacol.*, **11**, 595(1959).
(8) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *Nature*, **186**, 775(1960).
(9) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *J. Pharm. Pharmacol., Suppl.*, **12**, 203(1960).
(10) Beckett, A. H., and Choulis, N. H., *ibid.*, **15**, 236 (1963).
(11) Beckett, A. H., and Choulis, N. H., *XIII Intern. Kongr. Pharm. Wiss.*, Münster, Germany, 1963.
(12) Roberts, J., *J. Pharm. Pharmacol.*, **15**, 532(1963).
(13) Teicher, K., Mutschler, E., and Rochelmeyer, H., *Deut. Apotheker-Ztg.*, **100**, 283(1960).
(14) Waldi, D., *Arch. Pharm.*, **295**, 125(1962).
(15) Seher, A., *Mikrochim. Acta*, **1961**, 308.
(16) Purdy, S., and Truter, E. R., *Chem. Ind.*, **1962**, 506.
(17) Stahl, E., *Z. Anal. Chem.*, **181**, 303(1961).
(18) Breuner, M., and Niederwieser, A., *Experientia*, **16**, 378(1960).
(19) Pelka, J. R., and Metcalf, L. D., *Anal. Chem.*, **37**, 603(1965).
(20) Morrison, J. C., and Orr, J. M., *J. Pharm. Sci.*, **55**, 936(1966).
(21) Ganshirt, H., Koss, F. W., and Morianz, K., *Arzneimittel-Forsch.*, **10**, 943(1960).
(22) Zollner, N., Wolfram, G., and Amin, G., *Klin. Wochschr.*, **40**, 273(1962).
(23) Millett, M. A., Moore, W. E., and Seaman, J. F., *Anal. Chem.*, **36**, 491(1964).
(24) Eble, J. N., and Brooker, R. M., *Experientia*, **18**, 524 (1962).
(25) James, G., *Nature*, **161**, 551(1948).

Polymorphism and Drug Availability II

Dissolution Rate Behavior of the Polymorphic Forms of Sulfathiazole and Methylprednisolone

By W. I. HIGUCHI, P. D. BERNARDO, and S. C. MEHTA

The dissolution rate behavior of the two polymorphic forms of sulfathiazole and methylprednisolone has been studied. A correlation between the rates of reversion of the metastable forms to the stable forms during dissolution and the crystal growth rates of the stable form was found. Data obtained in a number of solvents showed that when the crystal growth rate of the stable phase was slow, there was little tendency for reversion during dissolution. These findings support the idea that the rate-determining step in the solvent mediated reversion of these polymorphs is the crystal growth of the stable phase. The recently presented mixture theory for dissolution rate was extended to explain the dissolution behavior of polymorphic mixtures. Experimental data on the effect of the agitation rate upon the dissolution rates of polymorphic mixtures agreed well with theory. It is proposed that this theory provides the explanation for the enigma of "the loss of sensitivity in distinguishing the difference in dissolution rates of polymorphs at high agitation rates."

FOR THERMODYNAMIC reasons (1) a drug exhibiting crystal polymorphism should possess different thermodynamic activities (or fugacities) depending upon its crystalline modification. Therefore, the drug release rate from any solid dosage form, *in vivo* or *in vitro*, may be strongly dependent upon this factor when the rate process is diffusion controlled. From the practical pharmaceutical standpoint, the existence of polymorphism may lead to good or bad consequences. The successful utilization of a polymorph of significantly greater thermodynamic activity than that of the stable modification may provide, in some instances, therapeutic blood levels for an otherwise inactive drug formulation. On the other hand, when the existence of multiple crystalline modifications goes unrecognized in a particular formulation, this may possibly result in too wide a range of dose-to-dose drug availability for the patient.

The purpose of this paper is to present some of the authors' studies on the dissolution rates of higher energy polymorphs of sulfathiazole and methylprednisolone. The data, when viewed in the light of the authors' crystal growth studies (2), provide a clear picture of what factors could be important in determining the physical kinetic stability of drug polymorphs. Furthermore, these studies appear to explain the mystery of the anomalous effect of agitation on the dissolution rate behavior of polymorphs (3–5).

Received April 28, 1966, from the College of Pharmacy, University of Michigan, Ann Arbor.

Accepted for publication September 20, 1966.

This investigation was supported by a grant from The Upjohn Co., Kalamazoo, Mich.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

EXPERIMENTAL

General Considerations.—The compounds selected for this study were sulfathiazole and methylprednisolone.[1] These are both known to exhibit dimorphism under usual conditions. The designations I and II will be used for the thermodynamically stable and the unstable forms, respectively.

Various aqueous and organic media were considered for this work. This provided a wide range of solubilities and a variation in the intermolecular interactions at the crystal–solution interface.

It was decided to employ methods involving dissolution from a plane surface. Thus, the techniques as described by Milosovich (6) for fast dissolution rates and by Higuchi *et al.* (7) for slow rates were used in this work. These arrangements with controlled agitation were expected to provide situations and yield results that could be handled conveniently by mathematics.

Preparation of Stable and Metastable Forms.—Sulfathiazole crystal forms are I, which melts at 174°–175°, and II which melts at 200°–201°. The recrystallization of sulfathiazole in warm ethyl alcohol gave form I which was hexagonal plates. Form II was obtained by using the method of Grove and Keenan (8), *i.e.*, by supersaturating a normal propanol solution, filtering, and allowing it to recrystallize over a water bath at approximately 80°. Elongated rods resulted. It was found that form II could also be obtained by recrystallizing from *sec*-butyl alcohol at room temperature. A third method for obtaining form II was also used so that verification of the appropriate form could be made. This was accomplished by melting sulfathiazole on a hot bar and then allowing it to crystallize at approximately 90°.

The form I of methylprednisolone was obtained by recrystallization from acetone, while form II was prepared by recrystallization from *tert*-butyl alcohol as was noted by Shell (9).

[1] The authors thank Dr. L. C. Schroeter, The Upjohn Co., for supplying us with samples of this material.

Characterization of Respective Forms.—Several methods were used in determining the appropriate crystal forms.

The powder method using the nickel-filtered copper radiation of a Siemens Crystalloflex IV counter-tube X-ray diffractometer provided one means of characterization. This was done for both sulfathiazole and methylprednisolone. Note recordings in Figs. 1–3.

For sulfathiazole, melting point determinations were also conducted. The microscope method using a Zeiss microscope equipped with a Kofler hot stage was used and results agreed with those in the literature (8). The characterization of the methylprednisolone could not be accomplished by this method since decomposition occurred.

Infrared spectra were also obtained to help characterize methylprednisolone forms I and II. The model 137, Perkin-Elmer spectrophotometer was used and data agreed with those in the literature (1).

TABLE I.—SOLUBILITIES AND ABSORBANCE MAXIMA

Solvent	Solubility at 30°, Gm./100 Gm.	$\lambda_{max.}$, mμ
Sulfathiazole		
Water	0.114	282
n-Propanol	0.277	291
sec-Butyl alcohol	0.149	291
95% Ethyl alcohol	1.02	288
75% (v/v) *n*-Propanol + 25% (v/v) ethyl alcohol	0.418	
40% (v/v) *sec*-Butyl alcohol + 60% (v/v) ethyl alcohol	0.545	
Methylprednisolone		
Water	0.0072	249
95% Ethyl alcohol	2.26	246
Decyl alcohol	0.269	243
75% (v/v) Water + 25% (v/v) ethyl alcohol	0.061	

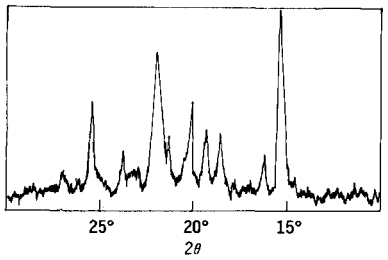

Fig. 1.—X-Ray recording of sulfathiazole form I.

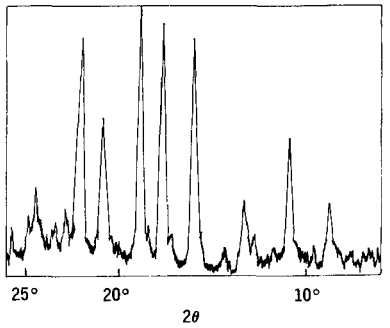

Fig. 2.—X-Ray recording of sulfathiazole form II.

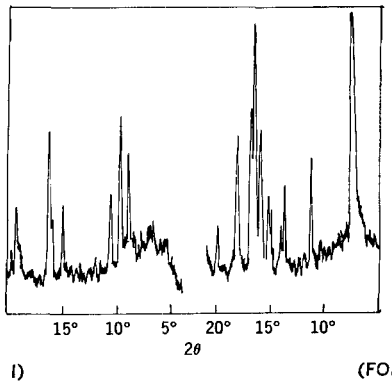

Fig. 3.—X-Ray recordings of methylprednisolone.

Solubility Determinations.—Excess sample was placed in the appropriate solvent and agitated in a water bath at 30.0° ± 0.1°. Samples were taken in duplicate at 12–24-hr. intervals. A preheated 10-ml. glass syringe was used to withdraw the samples which were filtered through a 0.45 μ membrane filter (Millipore HAWP 01300) by means of the syringe-Swinny-adapter assembly. The samples were then weighed and diluted for spectrophotometric assay. Reproducible data were obtained upon equilibration of the sample (Table I).

Apparatus and Procedure.—The apparatus and the procedure used were essentially those described by Milosovich (6). The sample was compressed by placing the powder in the die between the hardened steel block and a $^3/_8$ in. punch and compressing using a Carver press at approximately 2000 lb. pressure. The die had been previously ground to eliminate the tapered shoulder. This, therefore, ensured a constant surface area. The die containing the sample was mounted in the holder and placed into the dissolution solvent at zero time. The amount dissolved as a function of time was measured automatically by a Varian model G-10 recorder attached to a Beckman DU spectrophotometer through which the solution from the dissolution rate beaker was continuously circulated.

For the study of the dissolution rates of methylprednisolone in water, a second apparatus was necessary as the low water solubility of this compound demanded time intervals of 2 to 8 hr. The apparatus used was that described by Higuchi, Mir, and Desai (7). The tablet was made in the same manner as before and then the open end of the die was stoppered with a cork. This was placed in a Plexiglas holder which was mounted on the beaker cover. This was then placed into a 600-ml. water-jacketed beaker containing 200 ml. of water at 37°. A stainless steel stirrer, attached to a Bodine synchronous motor and mounted on the beaker cover, provided the necessary agitation. The speeds utilized were 10, 50, and 150 r.p.m. At time zero the entire unit was introduced into the solvent. Samples were pipeted out at specified times and assayed spectrophotometrically.

RESULTS AND DISCUSSION

Solvent Effects with Sulfathiazole.—Some typical dissolution rate results of the pure forms of sulfathiazole are given in Fig. 4. In all solvents the form I data were always linear as expected over the entire time period of the runs. With the form II experiments the results varied. Only in the two solvents, *n*-propanol and *sec*-butyl alcohol, were the rates for form II constant during the entire run. In many of the solvents initial constant rates were observed, but these decreased after some time and approached those for form I (see, *e.g.*, the II-in-ethanol and the II-in-50% ethanol–hexane curves in Fig. 4). In pure water and in water–ethanol mixtures containing up to 25% (v/v) ethanol, the form II rates and the form I rates could not be distinguished, even for initial rates. Conversion of II to I must be extremely rapid in water.

In all cases where the initial greater form II rates were observed, the ratios of the initial form II rate to that for form I in the same solvent were found to be essentially the same in all solvents. This ratio = 1.70 is the same as that reported by Milosovich for the sulfathiazole polymorphs in ethanol. According to the Noyes-Whitney relation, this ratio should correspond to the ratio of the solubilities of the two forms.

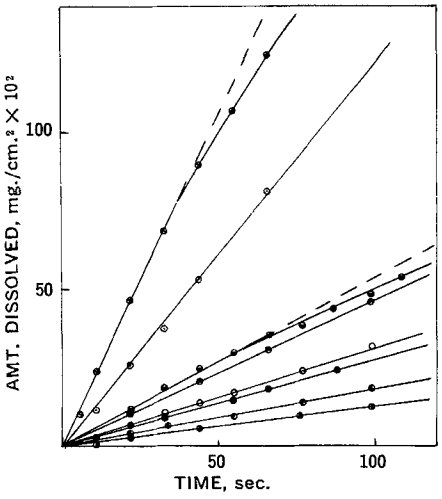

Fig. 4.—Dissolution rate of sulfathiazole (form I and form II) in various solvents. Key: ◑, I in *sec*-butyl alcohol, ◐, II in *sec*-butyl alcohol; ●, I in *n*-propanol; ◓, II in *n*-propanol; ○, I in 50% (v/v) mixture of ethyl alcohol and hexane; ◒, II in 50% (v/v) mixture of ethyl alcohol and hexane; ⊙, I in 95% ethyl alcohol; ⊖, II in 95% ethyl alcohol.

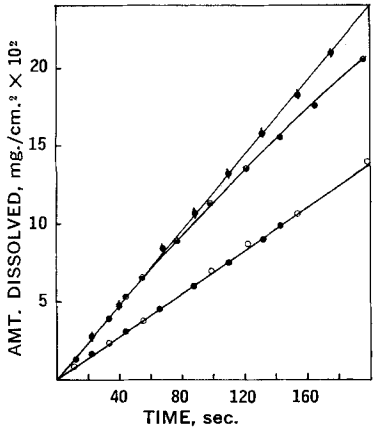

Fig. 5.—Effect of 0.1% gelatin on dissolution of sulfathiazole, form II in 40% (v/v) water + 60% (v/v) ethyl alcohol. Key: ●, form I in solvent; ○, form I in solvent + 0.1% gelatin; ◒, form II in solvent; ◓, form II in solvent + 0.1% gelatin.

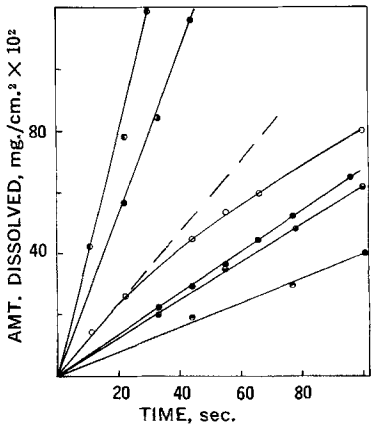

Fig. 6.—Dissolution rate of methylprednisolone (I and II) in various solvents. Key: ◓, I in decyl alcohol; ◒, II in decyl alcohol; ●, I in 50% (v/v) water–ethyl alcohol; ○, II in 50% (v/v) water–ethyl alcohol; ◑, I in ethyl alcohol; ◐, II in ethyl alcohol.

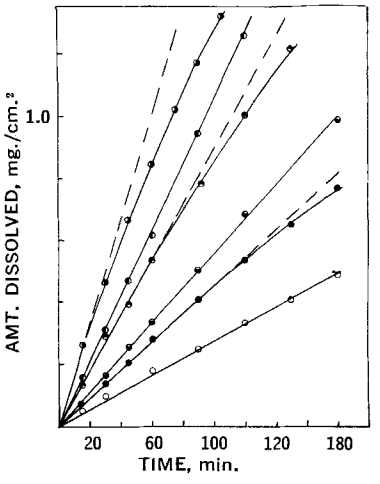

Fig. 7.—Dissolution rates of methylprednisolone (form I and form II) in water at 37° at agitation speeds of 10 r.p.m., 50 r.p.m., and 150 r.p.m. Key: ○, I at 10 r.p.m.; ●, II at 10 r.p.m.; ◒, I at 50 r.p.m.; ◓, II at 50 r.p.m.; ◐, I at 150 r.p.m.; ◑, II at 150 r.p.m.

These findings correlate with the authors' studies (2) on the crystal growth behavior of sulfathiazole form I. No measurable crystal growth of form I was found in *n*-propyl alcohol and in *sec*-butyl alcohol up to supersaturation ratios of 2.2 and 5.5, respectively. These supersaturations are far above the maximum supersaturation ratios (~1.7) available during the dissolution of form II in the present experiments. On the other hand, appreciable form I crystal growth rates were observed at supersaturation ratios below or near 1.7 in those solvents that have exhibited reversion in the present studies. Water and ethanol exhibited relatively rapid crystal growth rates at low supersaturations, and in the present studies these same solvents provided the most rapid reversion of II to I. Thus, one finds that reversion appears to occur rapidly in those solvents that allow relatively rapid crystal growth of the stable form and slowly in those solvents that require a relatively high supersaturation for the growth of the stable form.

In Fig. 5 the effect of 0.10% gelatin in 60% ethanol–water on the dissolution behavior of sulfathiazole can be seen. There appeared to be a retardation of the reversion of II to I. As the dissolution rate of I and the initial dissolution rate of II were not affected by the presence of gelatin, one can conclude that solution diffusional processes were not affected, and therefore gelatin must interfere at the crystal intreface. More studies with additives are planned for the future.

Solvent Effects with Methylprednisolone.—Some of the results in various solvents are presented in Figs. 6 and 7. The data given in Fig. 6 were obtained with the automatic recording apparatus. In the time ranges encountered here, the methylprednisolone form II data obtained in decyl alcohol did not show noticeable curvature with time. With 50% water–ethanol as the solvent, appreciable reversion of II to I was observed in less than 1 min. With ethanol as the solvent, the results were not conclusive as the dissolution was extremely rapid and disintegration of the tablet face was observed in some of the runs.

Because of the low solubility of methylprednisolone in water, the data given in Fig. 7 were obtained by the manual sampling procedure. The experiments were carried out at three agitation rates and much longer times were involved. It can be seen that, in water at 37°, reversion of II to I also occurred, although more slowly as reflected in these data.

If the results obtained in ethanol are excluded from consideration, these findings also correlate with the crystal growth data for form I in the same solvents (2). Thus, as was found with the sulfathiazole system, reversion was observed in those solvents (*e.g.*, water, water–ethanol mixtures) in which the crystal growth rates of form I were relatively rapid. In those solvents (*e.g.*, decyl alcohol) where the crystal growth rates of form I were extremely slow even at high supersaturation ratios, reversion was not observed in the experiments.

From these limited studies it appears that polymorphic reversion of those crystalline phases that are generally regarded as kinetically stable in the absence of solvent contact may be frequently controlled by the solvent-mediated crystal growth kinetics of the more stable phase and *not* by the dissolution rate of the less thermodynamically stable polymorph. This is perhaps not surprising if the crystal growth rates are controlled by a cooperative process such as two-dimensional nucleation. In these instances the reverse process, dissolution, likely would not be the reciprocal of growth but instead follow an energetically more comfortable route such as "peeling off" of molecular layers initiated at the corners and edges of the crystals.

Dissolution Rate from a Mixture of Two Polymorphs (Theory).—In order to be able to describe the results of the experiments with mixtures of polymorphs and of the authors' studies on the unusual effects of agitation on dissolution rates of polymorphs, a discussion of a quantitative theory is presented. This theory is an extension of that discussed recently (7) for the dissolution behavior of general polyphase mixtures.

Figure 8 schematically shows the three stages (from left to right) during the dissolution process. It is assumed that the mixture is initially uniform and reversion does not occur during dissolution. As dissolution of the drug begins by diffusion across the effective liquid diffusion layer of thickness h, only form II dissolves initially because C', the solution concentration at the tablet boundary–solution interface, is still greater than, C_s^{I} the solubility of form I. Thus, at time zero, $C' = C_s^{II}$, the solubility of form II. At later times

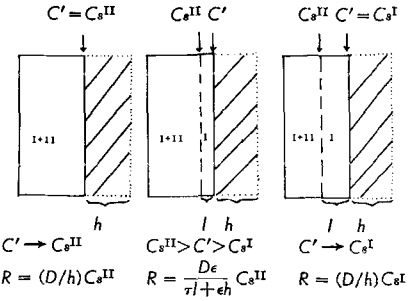

Fig. 8.—Mixture theory model.

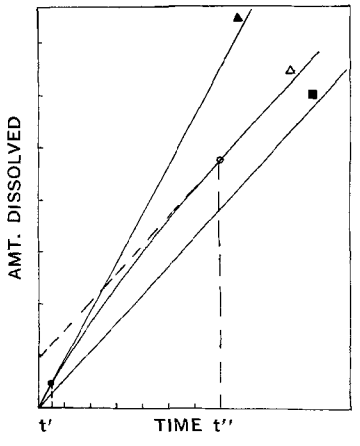

Fig. 9.—Comparison of dissolution rate of the two polymorphs and a mixture according to theory. Key: ▲, II; △, mixture; ■, I.

$C_s^{II} > C' > C_s^{I}$. Finally, in the steady-state, $C' \simeq C_s^{I}$, and both forms I and II dissolve at rates proportional to their percentages in the original mixture.

Mathematically, one may write for the dissolution rate per unit tablet area, R,

$$R = \frac{dw}{dt} = (D/h)C' = \frac{D\epsilon}{\tau l}(C_s^{II} - C') \quad \text{(Eq. 1)}$$

where

C' = concentration at the tablet–solution interface,
C_s^{II} = solubility of the form II,
ϵ = porosity of the form I residue layer,
τ = tortuosity of the form I layer,
h = diffusion layer thickness,
l = layer thickness of the form I layer,
w = amount dissolved per unit area of tablet at time, t,
D = diffusion coefficient.

Solving for C', one obtains

$$C' = \frac{C_s^{II}}{\left(1 + \frac{\tau l}{\epsilon h}\right)} \quad \text{(Eq. 2)}$$

whence

$$R = \frac{D\epsilon}{\tau l + \epsilon h} C_s^{II} \quad \text{(Eq. 3)}$$

R is also related to l and t by

$$R = H_{II}\frac{dl}{dt} \quad \text{(Eq. 4)}$$

where H_{II} is the amount of form II per unit volume in the original mixture.

Both Eqs. 3 and 4 will be examined in the time period up to the time, t'', when form I begins to dissolve. After $t = t''$ when a steady-state is reached, neither Eq. 3 nor Eq. 4 will correctly give the total dissolution rate. Instead one must write

$$R = \frac{D}{h} C_s^{I} \text{ for } t > t'' \quad \text{(Eq. 5)}$$

Therefore, when $0 < t < t''$,

$$H_{II}\left(\frac{dl}{dt}\right) = \frac{D\epsilon}{\tau l + \epsilon h} C_s^{II}$$

Integrating,

$$H_{II}\int_0^l dl(\tau l + \epsilon h) = \int_0^t C_s^{II} D\epsilon \, dt$$

or

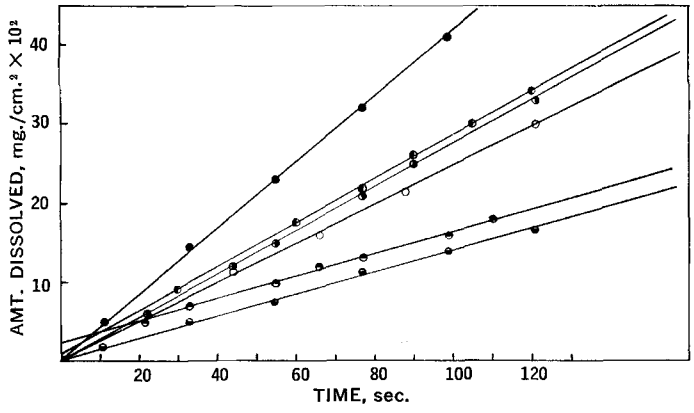

Fig. 10.—Dissolution rates of sulfathiazole (form I and form II and mixture of 80% I and 20% II) at two agitation speeds in *n*-propyl alcohol. Key: ◒, form I (*A*); ◓, mixture (*A*); ○, form II (*A*); ◑, form I (*B*); ◐, mixture (*B*); ●, form II (*B*); *A*, diffusion layer thickness = 5.62 × 10^{-3} cm.; *B*, diffusion layer thickness = 2.94 × 10^{-3} cm.

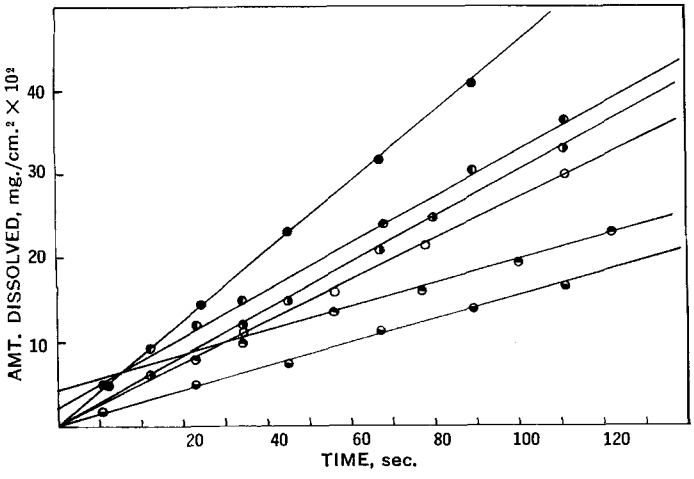

Fig. 11.—Dissolution rates of sulfathiazole (form I, form II, and mixture of 50% I and 50% II) at two agitation speeds in *n*-propyl alcohol. Key: ◒, form I (*A*); ◓, mixture (*A*); ○, form II (*A*), ◑, from I (*B*); ◐, mixture (*B*); ●, form II (*B*); *A*, diffusion layer thickness = 5.62 × 10^{-3} cm.; *B*, diffusion layer thickness = 2.94 × 10^{-3} cm.

TABLE II.—COMPARISON OF DIFFUSION LAYER THICKNESS TO INTERCEPT. SULFATHIAZOLE IN *n*-PROPYL ALCOHOL

Mixture Compn.	Diffusion Layer Thickness h, cm.	Ratio of h	Intercept	Intercept Ratio
50% II	5.62×10^{-3}	1.91	4.3	1.87
	2.94×10^{-3}	1	2.3	1
20% II	5.62×10^{-3}	1.91	1.7	1.85
	2.94×10^{-3}	1	.91	1

$$l^2 + \frac{2\epsilon h l}{\tau} = \frac{2C_s^{II} D\epsilon}{H_{II}\tau} t \qquad \text{(Eq. 6)}$$

If now one considers the situation when $C' \simeq C_s^{II}$ and defining t' as the maximum time up to which the dissolution will be essentially that from pure form II, one obtains from Eq. 6,

$$l = \frac{C_s^{II} D}{H_{II} h} t'$$

since

$$\frac{2\epsilon h l}{\tau} \gg l^2$$

Arbitrarily t' may be defined as the time up to which the dissolution rate is still within 99% of the pure form II rate. In this case

$$t' = \frac{0.01\ \epsilon h^2 H_{II}}{\tau\ C_s^{II}\ D} \qquad \text{(Eq. 7)}$$

The results of these theoretical considerations are graphically illustrated in Fig. 9. Here t' is shown as the point where deviation of the mixture curve from the pure form II curve begins to become important, and t'' is the point at which the rate of dissolution of the mixture becomes equal to the rate for pure form I as given by Eq. 5.

Finally, let us now obtain an equation for the intercept of the extrapolation of the linear portion of the mixture curve on the ordinate. If w″ is the amount dissolved at t'', one has then

$$\int_0^{w''} dw = \int_0^{l''} H_{II} dl \qquad \text{(Eq. 8)}$$

where

$$l'' = \frac{\epsilon h}{\tau}\left(\frac{C_s^{II} - C_s^{I}}{C_s^{I}}\right) \qquad \text{(Eq. 9)}$$

Integration of Eq. 8 gives

$$w'' = \frac{H_{II}\epsilon h}{\tau}\left(\frac{C_s^{II} - C_s^{I}}{C_s^{I}}\right)$$

Therefore, the intercept I will be given by

$$\frac{D}{h} C_s^{I}\, t'' + \mathrm{I} = w'' \qquad \text{(Eq. 10)}$$

where t'' is given by Eqs. 6 and 9. One has then

$$\mathrm{I} = \frac{H_{II}\epsilon h\,(C_s^{II} - C_s^{I})^2}{2\tau\ C_s^{I} C_s^{II}} \qquad \text{(Eq. 11)}$$

for the intercept.

Dissolution Rates from Mixtures, Effect of Agitation.—The effect of the agitation rate upon the dissolution rate, as predicted by the above theory, may be examined through the use of Eq. 11. Let us first note that Eq. 11 may be written

$$\mathrm{I} = Kh \qquad \text{(Eq. 12)}$$

where K is a constant for a given two-phase mixture of constant composition. The values of the diffusion layer thickness, h, at different agitation rates may be determined from the dissolution rate experiments with either of the pure polymorphs employing the Noyes-Whitney relation. If Eq. 12 is correct, the ratio of two h values should be the same as the ratio of corresponding intercepts.

Figures 10 and 11 present the experimental dissolution rate data in *n*-propanol at 30° for two mixtures of the sulfathiazole polymorphs obtained at two agitation rates corresponding to $h = 2.94 \times 10^{-3}$ cm. and $h = 5.62 \times 10^{-3}$ cm.

The intercepts were estimated as shown from the extrapolation of the linear portions of the data. These are given in column 4 of Table II. The ratio of the intercepts given in column 5 may be compared with the corresponding ratio of the h values given in column 3. The agreement between the h ratios and the intercept ratios is very satisfactory supporting the correctness of the theory.

Figure 12 presents the dissolution rate data obtained for the 50–50 mixture of methylprednisolone polymorphs at three agitation rates. Also presented, for reference purposes, are the pure form I

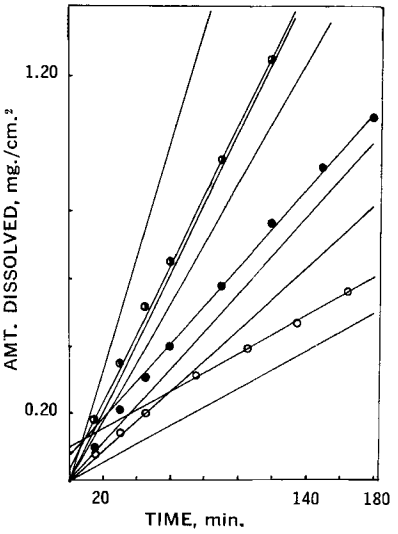

Fig. 12.—Dissolution of 50% I and 50% II mixture of methylprednisolone in water at agitation speeds of 10, 50, 150 r.p.m. Key: ○, mixture at 10 r.p.m.; ●, mixture at 50 r.p.m.; ◑, mixture at 150 r.p.m. Form II lines are those predicted if no reversion occurred.

TABLE III.—50% I AND II METHYLPREDNISOLONE MIXTURE IN WATER AT 37°

Agitation Speed, r.p.m.	Diffusion Layer Thickness h, cm.	Ratio h/h 150	Intercept	Ratio Intercept: Intercept 150
10	9.09×10^{-3}	3.68	.51	3.40
50	4.43×10^{-3}	1.79	.28	1.87
150	2.47×10^{-3}	1.00	.15	1.00

and form II data (taken from Fig. 9). Table III compares the ratio of the h values to the intercept ratios from the mixture data. Again one finds that the agreement of theory and data is excellent.

So far we have only examined the data with theory through the use of Eq. 11. Another look at the data, this time through Eq. 7, is helpful. Equation 7 predicts that the place where the dissolution data begins to deviate from the pure polymorph II line is proportional to the square of h. Therefore, for a given mixture, at low agitation rates, the data should follow the pure form II curve for a much longer time than at high agitation rates. An examination of the data given in Figs. 10–12 shows that the dependence of t' upon h^2 is essentially quantitatively followed in all instances.

The assumption that appreciable concomitant reversion did not occur in the runs with these mixtures should be a good one. Pure sulfathiazole form II in *n*-propanol showed no tendency to revert (Fig. 4). Pure methylprednisolone form II in water did show (Fig. 7) some reversion during dissolution. However, the mixture effect itself with the methylprednisolone 50–50 mixture appeared to be much larger during the times under consideration than the little reversion that did occur with pure form II. Therefore, the agreement of both the sulfathiazole and the methylprednisolone data with theory in the many aspects leaves little doubt that the theory is quantitatively sound.

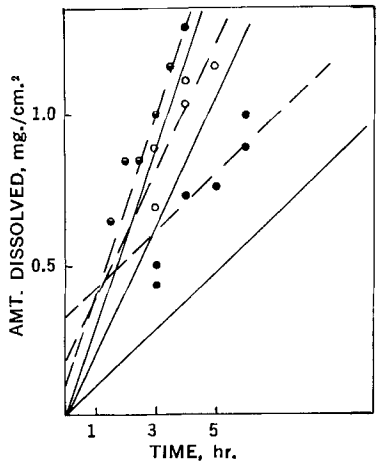

Fig. 13.—Interpretation of the dissolution rate data of Hamlin *et al.* Key: ●, form II at 0 r.p.m.; ○, form II at 6 r.p.m.; ◒, form II at 12 r.p.m.

Discussion of Some Previously Published Studies.—Hamlin, Nelson, Ballard, and Wagner first reported (3) the unusual effect of agitation upon the dissolution rate of a metastable polymorph. The material they investigated was methylprednisolone form II. These investigators found that upon increasing the agitation rate, it became increasingly difficult to distinguish the difference in the dissolution rates of form I and form II. Subsequently, Levy (4) and then Taylor and Wurster (5) observed similar effects with metastable methylprednisolone and prednisolone, respectively.

We propose that the answer to this mystery may be provided by the results of the authors' present studies. The following is the suggested description of the "anomalous" dissolution rate behavior.

First, it is necessary to have the reversion tendency of II to I neither very great (*e.g.*, sulfathiazole II in water at 30°) nor very small (*e.g.*, methylprednisolone in decyl alcohol at 30°) in the time range of interest. Methylprednisolone tablets in water at 37° (Fig. 9) appear to be in this "moderate" tendency category when the dissolution rate experiment is carried out for several hours under usual agitation conditions.

Then when the dissolution rate experiment is started, the initial rate will be dominated by the form II solubility, C_s^{II}. After some time, however, because of the reversion of II to I a layer of I will form if the rate of reversion is fast enough compared to the leaching-out rate of II. If the rate of reversion is not fast enough, the little form I that crystallizes out will probably flake out into the medium.

Assuming that a substantial deposit of I does form, some of the remaining II would leach out into the solution, and some would contribute to the deposit by further reversion. The net effect after a lag time should then be the same as having dissolution from a mixture of the two forms. That this situation is plausible may be seen by comparing the later time data of Fig. 7 with the early time data of Fig. 12.

At this point one may refer to Eq. 7. At high agitation rates h will be small. Therefore, the data in this case will, very quickly, become very like the data for pure form I. On the other hand at very low agitation rates, even though comparable reversion may have occurred, the dissolution rate

TABLE IV.—INTERCEPT AND h RATIOS CALCULATED BY THEORY FOR DATA OF HAMLIN *et al.*

Agitation, r.p.m.	Redrawn Slope of Form I	Slope from Lit.	D/h cm. hr.$^{-1}$	h (cm.)	Ratio $h:h_{12}$	Intercept	Ratio Intercept: Intercept$_{12}$
0	.096	.091 ± .021	.818	.022	3.06	.33	3.00
6	.208	.203 ± .027	1.77	.012	1.67	.18	1.64
12	.294	.276 ± .011	2.50	.0072	1.00	.11	1.00

may follow the expected pure form II behavior for much longer times.

The methylprednisolone form II data of Hamlin *et al.* (3) have been replotted in Fig. 13 and compared with theory (dotted lines) assuming a constant K value (Eq. 12). The corresponding h ratios and the intercept ratios are given in columns 6 and 8 of Table IV.

Because the use of a constant K value is subject to question here, the agreement of the theory with data must be regarded as only qualitatively satisfactory. However, the authors believe that the present arguments are the most satisfactory ones for explaining this phenomenon.

A quantitative model that can account for simultaneous reversion and dissolution is presently being investigated. Results of these studies will be reported at a later date.

REFERENCES

(1) Higuchi, W. I., Lau, P. K., Higuchi, T., and Shell, J. W., *J. Pharm. Sci.*, **52**, 150(1963).
(2) Bernardo, P. D., and Higuchi, W. I., unpublished data.
(3) Hamlin, W. E., Nelson, E., Ballard, B. E., and Wagner, J. G., *ibid.*, **51**, 432(1962).
(4) Levy, G., and Procknal, J. A., *ibid.*, **53**, 656(1964).
(5) Wurster, D. E., and Taylor, P. W., *ibid.*, **54**, 670(1965).
(6) Milosovich, G., *ibid.*, **53**, 484(1964).
(7) Higuchi, W. I., Mir, N. A., and Desai, S. J., *ibid.*, **54**, 1405(1965).
(8) Grove, D. C., and Keenan, G. L., *J. Am. Chem. Soc.*, **63**, 97(1941).
(9) Shell, J. W., private communication.

Enzyme Inhibitors XVI

Mode of Binding of Some 9-(2-Hydroxyalkyl)-6-(substituted)-purines to Adenosine Deaminase

By HOWARD J. SCHAEFFER and CHARLES F. SCHWENDER

The syntheses of a variety of 6-substituted-9-(2-hydroxybutyl)-, 9-(2-hydroxyheptyl)-, and 9-(2-hydroxyoctyl)purines have been completed. Those compounds with a 6-amino or a 6-methylamino group were inhibitors of adenosine deaminase, the compounds with a 6-amino group being more active than those compounds with a 6-methylamino group. For a series of 9-substituted adenines, the decreasing order of binding was: 9-(2-hydroxypropyl) > 9-(2-hydroxyethyl) ≅ 9-(2-hydroxybutyl) >9-(2-hydroxyheptyl) > 9-(2-hydroxyoctyl). From the data, it is concluded that there is a specific binding site for the terminal methyl group in 9-(2-hydroxypropyl)adenine and that the hydroxyl binding site and the main hydrophobic site of adenosine deaminase cannot be bridged by adenine derivatives which are substituted at the 9-position by straight-chain alkyl group bearing a hydroxyl group on carbon 2.

RECENTLY EVIDENCE was presented that there exists on the enzyme, adenosine deaminase, a nonpolar area which is involved in binding, by means of hydrophobic interactions, the alkyl group of some 9-alkyladenines (1). In addition, evidence was presented that if a hydroxyl group is attached to the alkyl group of a 9-alkyladenine, the hydroxyl group may either increase or decrease the binding of the inhibitor to the enzyme depending on the position of the hydroxyl group on the alkyl chain (1, 2). On the basis of these studies, it was suggested that in the reversible complex between a 9-(hydroxyalkyl)adenine and adenosine deaminase there is only one hydroxyl binding site on adenosine deaminase, and that this site on the enzyme is an area two to three carbon atoms removed from the site on the enzyme which binds the 9-position of the purine nucleus of the inhibitor (2). In order to study further the binding sites of this enzyme, it is apparent that it should be possible to prepare compounds which would occupy the hydrophobic binding region as well as the hydroxyl binding site of adenosine deaminase; such compounds should be bound more tightly to the enzyme. Compounds which may be able to occupy both binding sites on this enzyme are the 9-(2-hydroxyalkyl)-6-substituted purines. The present paper describes the synthesis and enzymatic evaluation of these compounds as inhibitors of adenosine deaminase.

Received September 14, 1966, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo 14214.
Accepted for publication October 18, 1966.
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CHEMISTRY

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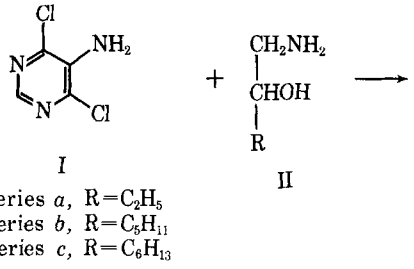
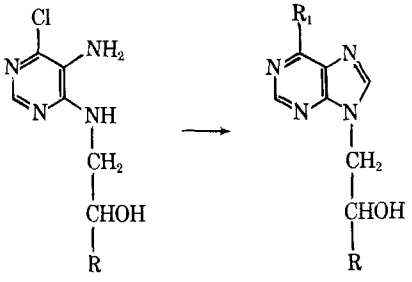
The compounds which were selected for synthesis are the 6-substituted purines which are substituted at the 9-position by either a 2-hydroxybutyl, a 2-hydroxyheptyl, or a 2-hydroxyoctyl group. The general method of synthesis is a modification of the

procedure previously utilized to prepare 9-alkyl-6-chloropurines (3, 4) and is outlined in Scheme I. Condensation of 5-amino-4,6-dichloropyrimidine (I) with the appropriate amino alcohol; *i.e.*, 1-amino-2-butanol (II*a*), 1-amino-2-heptanol (II*b*), or 1-amino-2-octanol (II*c*) gave the corresponding 5-amino-6-chloro-4-(2-hydroxyalkylamino)pyrimidine which on treatment with ethyl orthoformate and hydrochloric or ethanesulfonic acid produced the desired 6-chloro-9-(2-hydroxyalkyl)purine. When 6-chloro-9-(2-hydroxybutyl)- and 6-chloro-9-(2-hydroxyheptyl)purines were allowed to react with ammonia, methylamine, dimethylamine, 1 *N* hydrochloric acid, or thiourea, the desired 6-substituted derivatives were obtained. In the case of 6-chloro-9-(2-hydroxyoctyl)purine, only the 6-amino and the 6-mercapto derivatives (V*c*, IX*c*) were prepared.



I II

series *a*, R=C_2H_5
series *b*, R=C_5H_{11}
series *c*, R=C_6H_{13}



III

IV, R_1=Cl VII, R_1=NMe_2
V, R_1=NH_2 VIII, R_1=OH
VI, R_1=NHMe IX, R_1=SH

Scheme I

EXPERIMENTAL[1]

5 - Amino - 4 - chloro - 6 - (2 - hydroxybutylamino)pyrimidine (IIIa).—A mixture of 1.08 Gm. (6.62 mmoles) of I, 650 mg. (7.29 mmoles) of 1-amino-2-butanol (5), and 739 mg. (7.30 mmoles) of triethylamine in 50 ml. of 1-butanol was heated at reflux temperature for 22 hr. After the reaction mixture was evaporated *in vacuo* to a syrup, the crude product was crystallized and recrystallized from water and gave the crystalline product, 743 mg. (51.6%), m.p. 160°. ν in cm.$^{-1}$ (KBr): 3425 (OH); 3275 (NH); 1670 (NH); 1585 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 305 (1.22); H_2O, 265 (0.830) and 292 (0.848); 0.1 *N* NaOH, 265 (0.839) and 292 (0.882).

Anal.[2]—Calcd. for $C_8H_{13}ClN_4O$: C, 44.34; H, 6.05; N, 25.86. Found: C, 44.40; H, 6.15; N, 26.14.

6-Chloro-9-(2-hydroxybutyl)purine (IVa).—A mixture of 5.66 Gm. (25.1 mmoles) of III*a* and 2.09 ml. (25.1 mmoles) of concentrated hydrochloric acid in 100 ml. of triethyl orthoformate was stirred at room temperature for 2.5 hr. After the reaction mixture was evaporated *in vacuo* to a crude liquid product, crystallization of the syrup from chloroform–hexane and recrystallization yielded 3.09 Gm. (54.3%) of solid product, m.p. 125°. ν in cm.$^{-1}$ (KBr): 3275 (OH); 1590 and 1555 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 267 (0.832); H_2O, 267 (0.816); 0.1 *N* NaOH, 266 (0.860).

Anal.—Calcd. for $C_9H_{11}ClN_4O$: C, 47.68; H, 4.89; N, 24.71. Found: C, 47.88; H, 5.01; N, 24.76.

6-Amino-9-(2-hydroxybutyl)purine (Va).—In a stainless steel bomb a mixture of 305 mg. (1.34 mmoles) of IV*a* in 20 ml. of liquid ammonia was heated at 50° for 21 hr. After the ammonia had evaporated, the residual solid was recrystallized from 5% methanol in chloroform and hexane to give the white solid product; yield, 184 mg. (66.4%), m.p. 184°. ν in cm.$^{-1}$ (KBr): 3350 (OH); 3200 and 1710 (NH); 1630 and 1570 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 259 (1.08); H_2O, 261 (1.21); 0.1 *N* NaOH, 261 (1.17).

Anal.—Calcd. for $C_9H_{13}N_5O$: C, 52.16; H, 6.32; N, 33.80. Found: C, 51.96; H, 6.20; N, 33.53.

6-Methylamino-9-(2-hydroxybutyl)purine (VIa).—A mixture of 472 mg. (2.08 mmoles) of IV*a* and 20 ml. of aqueous methylamine (40%) was heated at 85° in a steel bomb for 68 hr. After the reaction mixture had been evaporated *in vacuo*, the residual material was extracted with hot ethyl acetate. Addition of hexane to the extract yielded the white crystalline product, 221 mg. (50.4%), m.p. 39°. The product was recrystallized from ethyl acetate–hexane and gave the analytical sample; yield, 164 mg. (37.5%), m.p. 39°. ν in cm.$^{-1}$ (KBr): 3350 (OH); 3250 and 1620 (NH); 1580 and 1540, sh (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl–10% EtOH, 262 (1.48); H_2O–10% EtOH, 265 (1.40); 0.1 *N* NaOH–10% EtOH, 265 (1.40).

Anal.—Calcd. for $C_{10}H_{15}N_5O$: C, 54.27; H, 6.83; N, 31.66. Found: C, 54.19; H, 7.10; N, 31.45.

6-Dimethylamino-9-(2-hydroxybutyl)purine Hydrochloride (VIIa).—A mixture of 499 mg. (2.20 mmoles) of IV*a* and 5 ml. of ethanol and 25 ml. of 25% aqueous dimethylamine solution was heated at reflux for 4 hr. After the reaction mixture was evaporated *in vacuo*, the residual liquid was extracted with chloroform. The extract was cooled and hydrogen chloride gas was passed through the solution. The crude hydrochloride salt of the product which precipitated was collected by filtration; yield, 533 mg. (89.6%), m.p. 188–190.5°. Recrystallization of the crude product from isopropyl alcohol gave the pure material; yield, 316 mg. (53.0%), m.p. 189–192°. ν in cm.$^{-1}$ (KBr): 3300 (OH); 2700 (acidic hydrogen); 1650 (NH^+); 1590 and 1540 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 268 (1.63); H_2O, 275 (1.65); 0.1 *N* NaOH, 275 (1.71).

Anal.—Calcd. for $C_{11}H_{18}ClN_5O$: C, 48.60; H, 6.68; Cl, 13.04; N, 25.77. Found: C, 48.88; H, 6.75; Cl, 13.23; N, 25.50.

6-Hydroxy-9-(2-hydroxybutyl)purine Hydrochlo-

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 202 spectrophotometer; the enzyme studies were done on a Gilford instrument model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

ride (VIIIa).—A solution of 399 mg. (1.76 mmoles) of IV*a* in 10 ml. of 1 *N* hydrochloric acid and 5 ml. of water was heated at reflux for 30 min. Evaporation of the reaction mixture *in vacuo* gave a syrupy material which was recrystallized from ethanol–ether; yield, 339 mg. (78.7%), m.p. 188.5–193°. ν in cm.$^{-1}$ (KBr): 3450 (OH); 2800–2300 (acidic hydrogen); 1720 (C=O, enol); 1680 (C=N^{+}—H); 1560 and 1540 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 250 (1.11); H_2O, 250 (1.14); 0.1 *N* NaOH, 256 (1.22).

Anal.—Calcd. for $C_9H_{13}ClN_4O_2$: C, 44.17; H, 5.36; Cl, 14.49; N, 22.90. Found: C, 44.17; H, 5.35; Cl, 14.54; N, 23.04.

6-Mercapto-9-(2-hydroxybutyl)purine (IXa).—A mixture of 402 mg. (1.77 mmoles) of IV*a* and 135 mg. (1.77 mmoles) of thiourea in 10 ml. of 1-propanol was heated at reflux for 15 min. Upon cooling the reaction mixture, the crystalline product precipitated and was collected by filtration, 281 mg. (70.8%), m.p. 277–280° Recrystallization of the product from methanol–propanol gave the analytical material, m.p. 276–280°. ν in cm.$^{-1}$ (KBr): 3450 (OH); 2700 (acidic hydrogen); 1590 and 1545 sh (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 10% EtOH–0.1 *N* HCl, 326 (1.90); 10% EtOH–H_2O, 323 (2.24); 10% EtOH–0.1 *N* NaOH, 313 (2.12).

Anal.—Calcd. for $C_9H_{12}N_4OS$: C, 48.19; H, 5.39; N, 24.98; S, 14.30. Found: C, 48.33; H, 5.38; N, 25.08; S, 14.29.

5 - Amino - 4 - chloro - 6 - (2 - hydroxyheptylamino)pyrimidine (IIIb).—A mixture of 283 mg. (1.72 mmoles) of I, 249 mg. (1.90 mmoles) of 1-amino-2-heptanol (6), and 253 mg. (2.50 mmoles) of triethylamine in 25 ml. of 1-butanol was refluxed for 17.5 hr. under a nitrogen atmosphere. Evaporation of the reaction mixture *in vacuo* gave a liquid which was crystallized from ethanol–water to give 328 mg. (73.5%), m.p. 120°. Further recrystallization from ethanol–water yielded 267 mg. (59.9%) of the analytical sample, m.p. 121°. ν in cm.$^{-1}$ (KBr): 3350 (OH); 3250 (NH); 1640 (NH); 1575 (C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl, 305 (1.14); H_2O, 265 (0.821) and 293 (0.877); 0.1 *N* NaOH, 265 (0.809) and 293 (0.842).

Anal.—Calcd. for $C_{11}H_{19}ClN_4O$: C, 51.04; H, 7.36; Cl, 13.74; N, 21.65. Found: C, 51.14; H, 7.50; Cl, 13.91; N, 21.47.

6-Chloro-9-(2-hydroxyheptyl)purine (IVb).—A mixture of 638 mg. (2.46 mmoles) of III*b* and 0.4 ml. of concentrated hydrochloric acid (4.78 mmoles) was stirred at room temperature in 40 ml. of triethyl orthoformate for 9.5 hr. The reaction mixture was evaporated *in vacuo* to a syrupy residue which was extracted with hot hexane. Cooling the extract yielded 342 mg. (51.8%) of pure white solid product, m.p. 96°. Recrystallization of the material from chloroform–hexane yielded the analytical sample, m.p. 96°. ν in cm.$^{-1}$ (KBr): 3325 (OH); 1590 and 1560 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 10% EtOH–0.1 *N* HCl, 266 (0.912); 10% EtOH–H_2O, 266 (0.929); 10% EtOH–0.1 *N* NaOH, 267 (0.956).

Anal.—Calcd. for $C_{12}H_{17}ClN_4O$: C, 53.63; H, 6.38; Cl, 13.19; N, 20.85. Found: C, 53.53; H, 6.30; Cl, 13.48; N, 20.63.

6-Amino-9-(2-hydroxyheptyl)purine (Vb).—A mixture of 402 mg. (1.49 mmoles) of IV*b* in 25 ml. of methanolic ammonia (20%) was heated at 80–84° for 20.5 hr. in a steel bomb. The reaction mixture was evaporated *in vacuo* to a residual solid, which was recrystallized from ethanol–water and gave 190 mg. (51.2%) of pure white crystalline solid, m.p. 190–193°. ν in cm.$^{-1}$ (KBr): 3400 (OH); 3350 and 1710 (NH); 1620 and 1570 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 10% EtOH–0.1 *N* HCl, 260 (1.39); 10% EtOH–H_2O, 261 (1.41); 10% EtOH–0.1 *N* NaOH, 261 (1.39).

Anal.—Calcd. for $C_{12}H_{19}N_5O$: C, 57.81; H, 7.68; N, 28.09. Found: C, 57.78; H, 7.64; N, 27.90.

6 - Methylamino - 9 - (2 - hydroxyheptyl)purine (VIb).—A mixture of 498 mg. (1.85 mmoles) of IV*b* in 5 ml. of ethanol and 20 ml. of 40% aqueous methyl amine solution was heated at reflux for 3 hr. The reaction mixture was evaporated *in vacuo* to a residual glass, which was recrystallized from chloroform–hexane, giving 239 mg. (49.1%) of white crystalline product, m.p. 112–113°. ν in cm.$^{-1}$ (KBr): 3400 (OH); 3175 (NH); 1625 (NH); 1570 and 1530 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 10% EtOH–0.1 *N* HCl, 261 (1.83); 10% EtOH–H_2O, 266 (1.58); 10% EtOH–0.1 *N* NaOH, 265 (1.78).

Anal.—Calcd. for $C_{13}H_{21}N_5O$: C, 59.30; H, 8.04; N, 26.60. Found: C, 59.35; H, 7.88; N, 26.41.

6 - Dimethylamino - 9 - (2 - hydroxyheptyl)purine (VIIb).—In a steel bomb was heated a mixture of 536 mg. (1.94 mmoles) of IV*b* in 5 ml. of methanol and 20 ml. of aqueous dimethylamine (25%) at 86° for 18.5 hr. After the reaction had been evaporated *in vacuo* to a liquid residue, the crude product was extracted from the residue with hot hexane. Cooling of the hexane extract yielded 356 mg. (62.2%) of a glassy material which upon recrystallization from hexane gave the white crystalline solid; yield, 280 mg. (49.0%), m.p. 55–59°. ν in cm.$^{-1}$ (KBr): 3475 (OH); 1610 and 1570 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 5% EtOH–0.1 *N* HCl, 268 (1.57); 5% EtOH–H_2O, 274 (1.66); 5% EtOH–0.1 *N* NaOH 274 (1.66).

Anal.—Calcd. for $C_{14}H_{23}N_5O$: C, 60.61; H, 8.72; N, 25.25. Found: C, 60.90; H, 8.44; N, 25.33.

6-Hydroxy-9-(2-hydroxyheptyl)purine Hydrochloride (VIIIb).—A mixture of 317 mg. (1.18 mmoles) of IV*b*, 7.1 ml. of 1 *N* HCl, and 5 ml. of water was heated at reflux for 30 min. The reaction mixture was evaporated *in vacuo* and gave white solid material. The crude product was recrystallized from ethanol saturated with hydrogen chloride giving the pure product; yield, 185 mg. (54.7%), m.p. 197–201°. ν in cm.$^{-1}$ (KBr): 3475 (OH); 2700–2300 (acidic hydrogen); 1725 (C=O, enol); 1680 (C=N^{+}—H); 1570 and 1540 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 10% EtOH–0.1 *N* HCl, 248 (1.14); 10% EtOH–H_2O, 248 (1.18); 10% EtOH–0.1 *N* NaOH, 253 (1.28).

Anal.—Calcd. for $C_{12}H_{19}ClN_4O_2$: C, 50.22; H, 6.68; Cl, 12.36; N, 19.54. Found: C, 50.46; H, 6.66; Cl, 12.31; N, 19.54.

6-Mercapto-9-(2-hydroxyheptyl)purine (IXb).—A mixture of 468 mg. (1.80 mmoles) of IV*b* and 137 mg. (1.80 mmoles) of thiourea in 10 ml. of 1-propanol was refluxed for 15 min. Upon cooling the reaction mixture, the crystalline material which precipitated was collected by filtration, 325 mg. (67.6%), m.p. 263–267°. The product was purified by recrystallization from ethanol; yield, 280 mg. (58.4%), m.p. 267–271°. ν in cm.$^{-1}$ (KBr): 3375 (OH); 2700

(acidic hydrogen); 1595 and 1560 (C=C, C=N). $\lambda_{max.}$ in mμ (ε × 10^{-4}): 10% EtOH–0.1 *N* HCl, 325 (1.93); 10% EtOH–H_2O, 322 (2.10); 10% EtOH–0.1 *N* NaOH, 313 (1.87).

Anal.—Calcd. for $C_{12}H_{18}N_4OS$: C, 54.10; H, 6.81; N, 21.03; S, 12.04. Found: C, 54.07; H, 6.63; N, 21.08; S, 12.09.

5 - Amino - 4 - chloro - 6 - (2 - hydroxyoctylamino)pyrimidine (IIIc).—A mixture of 482 mg. (2.94 mmoles) of I, 466 mg. (3.21 mmoles) of 1-amino-2-octanol (7), and 404 mg. (4.00 mmoles) of triethylamine in 25 ml. of 1-butanol was heated at reflux temperature for 24.5 hr. The reaction mixture was evaporated *in vacuo* to a liquid residue which after crystallization from methanol–water gave 273 mg. (76.3%), m.p. 111–114°. Further recrystallizations of the solid from ethanol–water gave the analytical product, m.p. 115–116.5°, 109 mg. (30.5%). ν in cm.$^{-1}$ (KBr): 3450 (OH); 3300, 3150, 1660 (NH); 1580 (C=N). $\lambda_{max.}$ in mμ (ε × 10^{-4}): 0.1 *N* HCl, 305 (1.15); H_2O, 265 (0.873) and 293 (0.891); 0.1 *N* NaOH, 265 (0.842) and 293 (0.865).

Anal.—Calcd. for $C_{12}H_{21}ClN_4O$: C, 52.83; H, 7.76; Cl, 13.00; N, 20.54. Found: C, 52.77; H, 7.74; Cl, 13.15; N, 20.64.

6-Chloro-9-(2-hydroxyoctyl)purine (IVc).—A mixture of 2.72 Gm. (9.96 mmoles) of IIIc and 667 mg. (6.01 mmoles) of ethanesulfonic acid in 120 ml. of triethyl orthoformate was stirred at room temperature for 30 min. After the reaction mixture was cooled, 1.62 Gm., m.p. 125°, of solid material was collected by filtration. The filtrate was evaporated *in vacuo* and gave a syrup which was extracted with hot hexane. The hexane extract was cooled and gave 1.18 Gm. of additional solid, m.p. 91°. The crude solid fractions were combined, 2.80 Gm. (99.2%), and recrystallized from chloroform–hexane giving the pure white crystalline product; yield, 1.36 Gm. (48.3%), m.p. 71–73°. ν in cm.$^{-1}$ (KBr): 3350 (OH); 1590 and 1560 (C=C, C=N). $\lambda_{max.}$ in mμ (ε × 10^{-4}): 10% EtOH–0.1 *N* HCl, 262 (0.735); 10% EtOH–H_2O, 263 (0.738); 10% EtOH–0.1 *N* NaOH, 263 (0.747).

Anal.—Calcd. for $C_{13}H_{19}ClN_4O$: C, 55.21; H, 6.77; Cl, 12.54; N, 19.81. Found: C, 55.27; H, 6.91; Cl, 12.82; N, 19.41.

6-Amino-9-(2-hydroxyoctyl)purine (Vc).—A mixture of 281 mg. (0.992 mmoles) of IVc in 20 ml. of methanolic ammonia was heated at 80° for 66 hr. in a steel bomb. The reaction mixture was evaporated to a residual solid which was recrystallized from ethanol–water giving 202 mg. (77.6%), m.p. 186–189°. Further recrystallizations from ethanol–water gave the analytical sample; yield, 148 mg. (56.7%), m.p. 186–189°. ν in cm.$^{-1}$ (KBr): 3300 (OH); 3250 (NH); 1700 (NH); 1610 and 1560 (C=C, C=N). $\lambda_{max.}$ in mμ (ε × 10^{-4}): 10% EtOH–0.1 *N* HCl, 258 (1.18); 10% EtOH–H_2O, 260 (1.26); 10% EtOH–0.1 *N* NaOH, 260 (1.26).

Anal.—Calcd. for $C_{13}H_{21}N_5O$: C, 59.30; H, 8.04; N, 26.60. Found: C, 59.08; H, 8.17; N, 26.29.

6-Mercapto-9-(2-hydroxyoctyl)purine (IXc).—A mixture of 279 mg. (0.985 mmole) of IVc and 83.7 mg. (1.10 mmoles) of thiourea in 15 ml. of 1-propanol was heated at reflux temperature for 15 min. Upon cooling the reaction mixture, a precipitate formed which was collected by filtration, 242 mg. (87.9%), m.p. 263–267°. The crude product was recrystallized from 1-propanol; yield, 98.4 mg. (35.7%), m.p. 264–267°. ν in cm.$^{-1}$ (KBr): 3450 (OH); 2650 (acidic hydrogen); 1600 and 1570 sh, (C=C, C=N). $\lambda_{max.}$ in mμ (ε × 10^{-4}): 10% EtOH–0.1 *N* HCl, 323 (1.78); 10% EtOH–H_2O, 322 (1.64); 10% EtOH–0.1 *N* NaOH, 310 (1.90).

Anal.—Calcd. for $C_{13}H_{20}N_4OS \cdot 1/2C_3H_8O$: C, 56.10; H, 7.80; N, 18.05; S, 10.32. Found: C, 56.00; H, 7.57; N, 17.90; S, 9.93.

The sample was dried at 100° *in vacuo* for 30 hr. and reanalyzed.

Anal.—Calcd. for $C_{13}H_{20}N_4OS$: C, 55.68; H, 7.22; N, 19.98; S, 11.44. Found: C, 55.83; H, 7.20; N, 19.62; S, 11.20.

Reagents and Assay Procedure.—Adenosine and adenosine deaminase (type I, calf intestinal mucosa) were purchased from the Sigma Chemical Co. The assay procedure for reversible inhibitors has previously been described (1, 2, 8) and is a modification of the general procedure described by Kaplan (9). The measurements of the initial rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6. The stock solutions of the enzyme, substrate, and inhibitors were prepared in 0.05 *M* phosphate buffer at pH 7.6. Those inhibitors which were only slightly soluble in phosphate buffer were dissolved in phosphate buffer containing 10% dimethylsulfoxide and evaluated as previously described (10). In order to determine the concentration of inhibitor required for 50% inhibition, a plot of V_0/V *versus* I was made where V_0 = initial velocity of the uninhibited enzymic reaction, V = initial velocity of the inhibited reaction at various concentrations of inhibitor, and I = the various inhibitor concentrations (8). Each plot was made with five different concentrations of inhibitor; each point for each plot is an average of at least three assays, and each reported value is an average of at least two different determinations of the concentration of inhibitor required for 50% inhibition of the enzyme. The concentration of adenosine was 0.066 m*M* in all experiments. The ratio of the m*M* concentration of the inhibitor for 50% inhibition to the m*M* concentration of the substrate $[I/S]_{0.5}$; *i.e.*, the inhibition index, was used to compare the inhibitory properties of the various compounds.

RESULTS AND DISCUSSION

As reported earlier, the authors believe that in the formation of a reversible enzyme–inhibitor complex with adenosine deaminase, the enzyme has a polar and a nonpolar region in the area where the 9-substituent of a 6-substituted purine is bound (1, 2). If the polar area (hydroxyl binding region) and the nonpolar area (hydrophobic region) of the enzyme are spatially closely related, it should be possible to synthesize inhibitors which take advantage of both the polar and nonpolar regions of adenosine deaminase. One way in which these two areas on the enzyme may be related to each other is in a "linear" manner; *i.e.*, in which the carbon chain at the 9-position of the purine nucleus is unbranched and has a hydroxyl group at the 2-position of the alkyl chain.

In agreement with previous findings, those 9-substituted compounds with a 6-chloro, a 6-dimethylamino, a 6-hydroxy, or a 6-mercapto group were either noninhibitory or only weakly inhibitory com-

TABLE I.—INHIBITION INDEX AND PARTIAL INHIBITION OF ADENOSINE DEAMINASE BY SOME 9-(HYDROXYALKYL)ADENINES

Compd.[a]	No.	mM Concn. for 50% Inhibition[b]	$[I/S]_{0.5}$[b]
Ad CH_2—CH—C_2H_5 OH	V*a*	0.078 ± 0.003[c]	1.2 ± 0.04[c]
Ad CH_2—CH—C_5H_{11} OH	V*b*	0.187 ± 0.001	2.8 ± 0.02
Ad CH_2—CH—C_8H_{13} OH	V*c*	0.209 ± 0.001	3.2 ± 0.02
Ad CH_2—CH_2 OH	XII	0.070 ± 0.004	1.1 ± 0.05[d]
Ad CH_2—CH—CH_3 OH	XIII	0.016 ± 0.002	0.25 ± 0.02[d]

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 m*M*. In those cases where a higher concentration is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/V *vs. I*. [c] Average deviation. [d] Data taken from *Reference 2*.

pared to the corresponding 6-amino derivative when evaluated against adenosine deaminase (2). However, an examination of Table I reveals some interesting features about the binding of some 9-substituted adenines to adenosine deaminase. Thus, as the 9-substituent of the adenine derivative is increased from a 2-hydroxybutyl group (V*a*) to a 2-hydroxyheptyl group (V*b*) to a 2-hydroxyoctyl group (V*c*), the compounds become progressively weaker as inhibitors. For comparison the $[I/S]_{0.5}$ of 9-(2-hydroxyethyl)adenine (XII) is 1.1, and the $[I/S]_{0.5}$ of 9-(2-hydroxypropyl)adenine (XIII) is 0.25. Similar results were obtained with the 6-methylamino derivatives (VI*a* and VI*b*) whose $[I/S]_{0.5}$ are 2.7 and 5.0, respectively.

From these results the conclusion is that the hydroxyl binding region and the principal hydrophobic binding region on the enzyme cannot be bridged by a straight chain compound with a 2-hydroxy group. However, there appears to be a specific binding region for a methyl group as noted by the increase in binding by XIII compared to XII. This methyl binding region has limited bulk tolerance[3] since the binding was decreased when the chain was lengthened from V*a* to V*b* to V*c*. Studies are continuing in an attempt to prepare 9-substituted adenines which can bind to both the hydroxyl and hydrophobic binding region of adenosine deaminase. Preliminary results with some hydroxylated branched chain compounds of the general structure of 9-(1-hydroxy-2-alkyl)-6-substituted purines indicated that these compounds are binding to both regions and will be the subject of a future paper.

REFERENCES

(1) Schaeffer, H. J., and Vogel, D., *J. Med. Chem.*, **8**, 507 (1965).
(2) Schaeffer, H. J., Vogel, D., and Vince, R., *ibid.*, **8**, 502(1965).
(3) Montgomery, J. A., and Temple, C., Jr., *J. Am. Chem. Soc.*, **79**, 5238(1957).
(4) Temple, C., Jr., Kussner, C. L., and Montgomery, J. A., *J. Med. Pharm. Chem.*, **5**, 866(1962).
(5) Lucas, H. J., and Ghirardelli, R., *J. Am. Chem. Soc.*, **79**, 734(1957).
(6) Freon, P., and Ser, S., *Compt. Rend.*, **222**, 447(1946).
(7) Gakenheimer, W. C., and Hartung, W. H., *J. Org. Chem.*, **9**, 858(1944).
(8) Baker, B. R., and Sachdev, H. S., *J. Pharm. Sci.*, **52**, 933(1963).
(9) Kaplan, N. O., in "Methods of Enzymology," vol. II, Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1955, p. 473.
(10) Schaeffer, H. J., and Odin, E., *J. Med. Chem.*, **9**, 576 (1966).

[3] It is also possible that the area on the enzyme beyond the methyl binding region is quite polar and thereby repels the longer chain compounds (V*a*, V*b*, and V*c*).

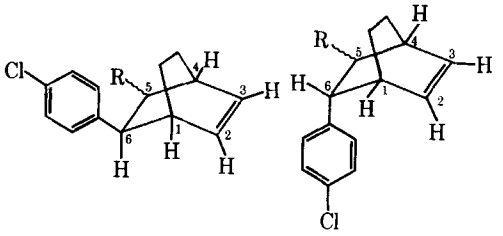
NMR Spin–Spin Decoupling Studies of Some 5,6-Disubstituted Bicyclo[2.2.2]oct-2-enes

Assignment of Vinylic Protons and Demonstration of Allylic Coupling

By DAVID B. ROLL*, BERNARD J. NIST, and ALAIN C. HUITRIC

Assignment of the NMR signals of the vinylic protons has been accomplished by spin–spin decoupling in endo-5-nitro-exo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene (I), endo-5-amino-exo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene hydrobromide (IV), endo-5-cyano-exo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene (V), endo-5-cyano-endo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene (VII), all of which show significant differences in the chemical shifts of the two vinylic protons. A long-range coupling of 1.5 c.p.s. is demonstrated between H-1 and H-3 and between H-4 and H-2 in these systems.

In two recent publications (1, 2) the authors have reported partial analyses of the NMR spectra of some 5,6-disubstituted bicyclo[2.2.2]-oct-2-enes. The analyses have now been expanded by spin–spin decoupling through frequency sweep double resonance technique.



I, R = NO_2, endo
III, R = NH_2, endo
IV, R = NH_3^+, endo[1]
V, R = CN, endo
VIII, R = CN, exo
II, R = NO_2, exo
VI, R = CN, exo
VIII, R = CN, endo

DISCUSSION

In the initial analysis of the spectra of compounds I and II (1) it was possible to assign the signals of H-1, H-4, H-5, and H-6, but unambigous differentiation of the signals of H-2 and H-3 was not possible, even though these signals are separated by about 23 c.p.s. in the spectrum of compound I measured in carbon tetrachloride at 60 Mc. In the isomeric compound II, in which the nitro group is exo and the aromatic group endo to the double bond, the difference in the chemical shift of the vinylic protons is much smaller (1). The differences in chemical shifts of H-2 and H-3 are attributed to the long-range shielding effects resulting from the magnetic anisotropy of the aromatic ring (3) and the nitro group (4).

Assignment of the signals of H-2 and H-3 in the spectrum of I has now been accomplished by spin–spin decoupling. Pertinent portions of the spectrum and decoupled spectra of I are given in Fig. 1.

Only that portion of the spectrum including the signals of H-1 through H-6 is given in each case. In spectrum F, prior to decoupling, the signals of the vinylic protons appear essentially as triplets centered at τ 3.46 and 3.85. Components of the triplet at τ 3.46 are further split by about 1.5 c.p.s., and those of the upper field triplet are broadened, indicating the presence of long-range coupling. Irradiation at the resonance of H-1, spectrum E, caused the triplet at τ 3.46 to collapse into a doublet, J_{23} = 8.5 c.p.s., while the triplet at τ 3.85 now appears as a quartet, J_{32} = 8.5 and J_{34} = 6.0 c.p.s. These results establish the signal at τ 3.46 as that of H-2. The sharpening of the signal at τ 3.85 into a well-defined quartet by irradiating at the signal of H-1 shows that H-3 is coupled slightly with the allylic H-1 in spectrum F.

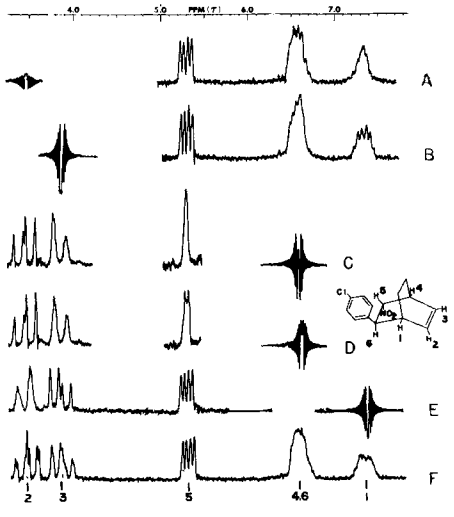


Fig. 1.—Portions of NMR spectrum and decoupled spectra of endo-5-nitro-exo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene; 60 Mc., about 1 *M* in carbon tetrachloride with TMS internal reference.

Received August 22, 1966, from the College of Pharmacy, University of Washington, Seattle 98105.
Accepted for publication October 5, 1966.
* Public Health Service Predoctoral Fellow 5-F1-GM-18,795. Present address: Pesticides Research Laboratories, Department of Health, Education, and Welfare, Perrine, Fla.
[1] As the hydrobromide salt.

Irradiation at the position of overlapping signals of H-4 and H-6 (spectra C and D) caused the triplet at τ 3.85 to collapse into a doublet, $J_{23} = J_{32} =$ 8.5 c.p.s., and simultaneously produced a sharp quartet, $J_{23} = 8.5$ and $J_{21} = 6.5$ c.p.s., for the signal of H-2. This confirms the assignment of H-2 and H-3 and also shows an allylic coupling of 1.5 c.p.s. between H-2 and H-4 in spectrum F. The sharp singlet of H-5 in spectrum C shows that H-5 is simultaneously decoupled from H-4 and H-6, while in spectrum D H-4 is not completely decoupled from H-5.

Additional evidence for long-range allylic coupling between H-1 and H-3 is found in spectrum B. Irradiation at the position of the signal of H-3 produced a much better resolved signal for H-1. Irradiation at H-3 also caused a narrowing of the combined signals of H-4 and H-6 as expected due to decoupling of H-3 from H-4. Spectrum A shows the expected narrowing of the signal of H-1 by irradiation at the signal of H-2.

Compound III was obtained by the reduction of I with iron in acetic acid by the method of Kornblum and co-workers (5). The spectrum of the free amine (not shown) was measured in tetrachloroethylene with TMS as internal reference. The vinylic protons give two triplets, centered at τ 3.57 and 3.89, which are quite analogous in shape to those of the corresponding nitro compound I. The spectrum has a signal at τ 7.10 which integrates to 1 proton, a signal at τ 7.60 which integrates to 2 protons, and a signal at 7.83 which accounts for 1 proton. Signals of the remaining hydrogens, including the amino protons, are overlapping at higher field. The spectrum of the hydrobromide salt of III, determined in D_2O with sodium 3-(trimethylsilyl)-1-propane sulfonate as internal reference, has the following pattern: the vinylic protons give 2 triplets centered at τ 3.03 and 3.39; there are unresolved singlets each integrating to 1 proton, at τ 5.82, 6.66, 6.90, and 7.25. The signals of the remaining ring protons are overlapped over a broad region at higher field and the exchangeable protons gave a signal at τ 4.89. The signal at τ 5.82 has experienced the largest downfield shift upon protonation and is assigned to H-5. Decoupling studies of the spectrum of this amine salt in D_2O made it possible to assign the signals of H-1 through H-6. Irradiation of the signal at τ 6.66 caused the signal of H-5 at τ 5.82 to appear as a poorly resolved doublet with separation of about 4.5 c.p.s., and caused the signal of the vinylic proton at τ 3.39 to collapse to a doublet with separation of about 7.5 c.p.s. These results show that the signal at τ 6.66 is due to H-4 and that the signal at τ 3.39 is that of the vinylic H-3. Irradiation at τ 7.25 caused the triplet at τ 3.03 to collapse to a doublet with separation of about 7.5 c.p.s. and a narrowing of the signal at τ 6.90. Irradiation at τ 6.90 caused a narrowing of the signal at τ 5.82 but did not cause any change in the signal of the vinylic protons. These results show that the signal at τ 7.25, 3.03, and 6.90 are due to H-1, H-2, and H-6, respectively.

The spectra of compounds V, VI, and VII have been published previously (2). The spectra of V and VII show a greater difference in chemical shifts of the vinylic protons than the spectrum of VI. The assignment of the signals of the vinylic protons was not done in the original publication. This has now been done by double resonance for compounds V and VII. A large solvent effect on chemical shift in going from deuterochloroform to pyridine was observed for H-5 of VII, causing the overlapped signals of H-5 and H-6 in chloroform to appear as discrete peaks in pyridine (2), but the pattern of the vinylic protons was almost identical in the two solvents.

Pertinent portions of the spectrum and decoupled spectra of VII measured in pyridine are given in Fig. 2. Spectrum C shows the signals of H-1 through H-6 without decoupling. The assignment of H-5 at τ 6.64 and H-6 at τ 6.82 was accomplished by deuteration at C-5 (2). The vinylic protons give partially overlapped sextets (doublets of triplets with minor splitting of 1.5 c.p.s.) centered at τ 3.48 and 3.67. The signals of the bridgehead protons appear at τ 7.14 and 7.39. Spectrum B shows the effects of irradiating at τ 7.14. The signal of H-5 has collapsed to a doublet, $J_{56} = 10$ c.p.s., showing that the signal at τ 7.14 is due to H-4 and that H-4 is now decoupled from H-5. In addition, the signal at τ 3.67 has collapsed to a broad doublet with separation of about 8.5 c.p.s., and the minor splitting of 1.5 c.p.s., due to allylic long-range coupling has disappeared in the signal at τ 3.48. This permits assignment of the signal at τ 3.67 to H-3 and that at τ 3.48 to H-2. These assignments are confirmed by irradiation of the signal of the other bridgehead proton (H-1) at τ 7.39, spectrum A, which causes no change in the signal of H-5 but causes the signal at τ 3.48 to collapse to a broad doublet with separation of about 8.5 c.p.s. and causes the signal at τ 3.67 to become a triplet with separation of 8.5 c.p.s., $J_{12} \simeq J_{23} = 8.5$ c.p.s.

The decoupled spectra of V are not shown. The original spectrum measured in deuterochloroform without decoupling (2) shows two sextets (essentially triplets with minor splitting of 1.5 c.p.s.) for the vinylic protons centered at τ 3.42 and 3.68; a sharp quartet for H-5 at τ 7.16; a signal accounting for two hydrogens at τ 6.97 which shows the poorly re-

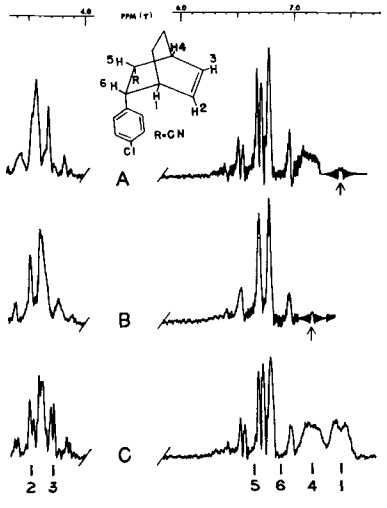


Fig. 2.—Portions of the NMR spectrum and decoupled spectra of endo-5-cyano-endo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene; 60 Mc. about 1 *M* in pyridine with TMS as internal reference.

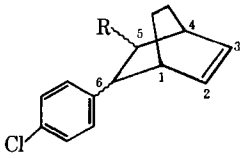
solved quartet of H-6 overlapped by the signal of one of the bridgehead protons; and the signal of the other bridgehead proton at τ 7.41. Decoupling was done in deuterochloroform. Irradiation at τ 7.41 did not cause any change in the quartet of H-5 but caused the poorly resolved signal of H-6 to appear as a sharper doublet and also caused the sextet at τ 3.42 to collapse to a doublet with separation of 8.5 c.p.s., with simultaneous change of the sextet at τ 3.68 to a quartet of coupling constants of 8.5 and 6.0 c.p.s. These changes in the pattern of the vinylic protons are analogous to those shown in spectrum E of Fig. 1, compound I. The results allow the assignment of the signal of H-1 at τ 7.41; H-2 at 3.42; H-3 at 3.68; and $J_{23} = J_{32} =$ 8.5 and $J_{21} = J_{34} =$ 6.0 c.p.s. The long-range allylic coupling of 1.5 c.p.s. is again demonstrated. These assignments are confirmed by irradiation in the vicinity of τ 6.97 where the other bridgehead proton (H-4) overlaps with the signal of H-6. This caused a collapse of the signal at τ 3.68 to a doublet with separation of 8.5 c.p.s. and a simultaneous change of the signal at τ 3.42 to a quartet, $J_{23} = 8.5$ and $J_{21} = 6.0$ c.p.s., changes similar to those seen in spectra C and D of Fig. 1.

The spectrum of exo-5-cyano-exo-6-(*p*-chlorophenyl)-bicyclo[2.2.2]oct-2-ene (VIII) was determined in deuterochloroform and in pyridine. The spectra are not shown. In deuterochloroform the signals of H-5 and H-6 overlap to give a single peak at τ 7.02. The signals of the bridgehead protons are centered at about τ 7.11 and 7.40. The signals of the vinylic protons at τ 3.50 and 3.76 have a pattern of complex triplets analogous to those of H-2 and H-3 in spectrum F of Fig. 1. In the spectrum measured in pyridine the signals of the vinylic protons appear at τ 3.62 and 3.89 and there is no significant change in the pattern of the complex triplets. The signals of the bridgehead protons are centered at about τ 7.27 and 7.51. The most significant difference in the spectra from the two solvents is a separation of the signals of H-5 and H-6 in pyridine which appear at τ 6.88 and 7.08 and give a pattern analogous to that of H-5 and H-6 of VII in pyridine, spectrum C, Fig. 2. The signal of one of these two hydrogens is shifted downfield, while the other one experiences very little change in pyridine compared to deuterochloroform. This effect has been observed in other isomers of VIII (2) where the assignment of H-5 and H-6 had been done through deuteration and in each case it was the signal of the hydrogen on the cyano-bearing carbon which experienced the downfield shift. Decoupling was done on VIII in pyridine by irradiation of the positions of the signals of the bridgehead protons. Irradiation at τ 7.51 caused the vinylic signal at τ 3.62 to collapse to a doublet and did not bring any change in the signal at 6.88, while irradiation at τ 7.27 caused a collapse of the signal of the vinylic proton at τ 3.89 to a doublet and caused the quartet at 6.88 to appear as a doublet with separation of 10.5 c.p.s. This shows that the bridgehead proton giving the signal at 7.51 is located between the vinylic proton responsible for the signal at τ 3.62 and the proton responsible for the signal at τ 7.08 while the bridgehead proton giving the signal at τ 7.27 is located between the other vinylic proton, τ 3.89, and the proton giving the signal at τ 6.88. If, as observed in other isomers (2), it is H-5 which experiences the deshielding effect in pyridine then the following chemical shifts would hold: H-1, 7.51; H-2, 3.62; H-3, 3.89; H-4, 7.27; H-5, 6.88; H-6, 7.08.

A summary of chemical shifts is given in Table I.

In all bicyclo[2.2.2]oct-2-enes with a nitro or cyano group at C-5 and a *p*-chlorophenyl group at C-6 in which the signals of the vinylic protons could be differentiated by spin–spin decoupling the signal of H-2 was found to be further downfield. Since the spatial orientation of the functional groups varies in these compounds and since the long-range shielding effects due to the magnetic anisotropy of the aromatic and nitro groups are affected by con-

TABLE I.—SUMMARY OF CHEMICAL SHIFTS IN τ UNITS



		Solvent	Vinylic H-2	Vinylic H-3	Bridgehead H-1	Bridgehead H-4	H-5	H-6
I,	Ar exo R = NO_2, endo	CCl_4	3.46	3.85	7.35	~6.59	5.32	~6.59
II,	Ar endo R = NO_2, exo	CCl_4	[a]	[a]	7.27	6.68	5.67	6.35
IV,	Ar exo R = NH_3^+ endo	D_2O[b]	3.03	3.39	7.25	6.66	5.82	6.90
V,	Ar exo R = CN, endo	$CDCl_3$	3.42	3.68	7.41	~6.97	7.16	~6.97
VI,	Ar endo R = CN, exo	$CDCl_3$	[c]	[b]	[d]	[d]	7.55	6.90
VII,	Ar endo R = CN, endo	Pyridine	3.48	3.67	7.39	7.14	6.64	6.82
VIII,	Ar exo R = CN, exo	$CDCl_3$ Pyridine	[e]	[e]	[e]	[e]	[e]	[e]

[a] The signals were not differentiated. The partially overlapped signals are centered at τ 3.58. See *Reference 1* for spectrum. [b] Sodium 3-(trimethylsilyl)-1-propane sulfonate was used as internal reference. [c] The signals are partially overlapped at τ 3.64 and were not differentiated. See *Reference 2* for spectrum. [d] Signals of the bridgehead protons, centered at about τ 7.17 and 7.31 are partially overlapped. See *Reference 2* for spectrum. [e] See text for discussion.

formational changes, it is dangerous to make any generalization regarding relative shielding effects of these functional groups on the vinylic protons in these systems. This could best be accomplished on mono-substituted compounds.

EXPERIMENTAL

The NMR spectra were determined with a Varian A-60 and/or HR-60 spectrometer. Decoupling was achieved by frequency sweep, double resonance procedure with a Varian DA-60IL spectrometer.

Compounds I and II have been reported in a previous paper (1) as were compounds V–VIII (2).

endo - 5 - Amino - exo - 6 - (p - chlorophenyl)-bicyclo[2.2.2]oct-2-ene Hydrobromide.—The amine was obtained in 48% yield by the reduction of the corresponding nitro compound I with iron in acetic acid by the method of Kornblum and co-workers (5) and as previously described for analogous compounds (6). The hydrobromide salt was formed by bubbling HBr gas into a ligroin solution of the amine. The salt was recrystallized from a mixture of benzene and ethanol, m.p. 247–250° (Kofler micro hot stage).

Anal.—Calcd. for $C_{14}H_{17}BrClN$: C, 53.44; H, 5.45. Found: C, 53.03; H, 5.38.

REFERENCES

(1) Roll, D. B., Nist, B. J., and Huitric, A. C., *Tetrahedron*, **20**, 2851(1964).
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Synthesis of Tropine-Labeled Atropine I

Micro Methods for the Synthesis of Tropine and for Its Esterification with Tropic Acid

By GILBERT C. SCHMIDT, THOMAS E. ELING*, and JOHN C. DRACH†

Yields for each step in the esterification of tropine and tropic acid have been determined with micro and semimicro quantities of reactants. In a similar way, the Robinson condensation has been studied. Data are compared with the findings of other investigators. In the esterification of micro quantities of tropine and tropic acid, 70–75 per cent of theoretical atropine yields are obtained routinely. Based on any intermediate in the Robinson condensation, micro quantities of reactants routinely produce 67–72 per cent of theoretical tropine yields. Predicted yields of 47–54 per cent atropine from Robinson intermediates were confirmed by synthesis of atropine from each of the Robinson reactants. The procedures are designed for the synthesis of labeled tropine and tropine-labeled atropine from labeled arabinose.

THE ESTABLISHMENT of metabolic pathways in tropine metabolism awaits the development of synthetic methods for selectively labeling the tropine ring. The fate of this heterocycle, free or as a structural component of atropine and related compounds, is essentially unknown because suitably ring-labeled compounds are not available in amounts adequate for animal studies.

Tritium-labeled atropine (1) and randomly-^{14}C-labeled atropine (2) are of limited usefulness because the labeling is not specific. Biosynthetic atropines (3, 4) are suitably labeled, and could be used to determine the metabolic fate of the tropine moiety, but complexities of preparation have limited their availability and prevented their use in animal studies. Fodor *et al.* (5) and Werner *et al.* (6) prepared *N*-methyl-^{14}C-tropine, then esterified it with unlabeled tropic acid to obtain tropine-labeled atropine. Subsequent metabolic studies (7), although not extensive, clearly indicate the need to label tropine and atropine in other positions. During studies of atropine toxicity, this need became very apparent to the authors.

The extreme toxicity of atropine and related compounds is an added complication, because low doses must be used for *in vivo* studies, and minor metabolites are correspondingly difficult to detect. Compounds of reasonably high specific

Received September 16, 1966, from the Children's Hospital, the Division of Pharmacology, Department of Pediatrics, and the Department of Biological Chemistry, Medical College of Alabama, University of Alabama, Birmingham. 35233.
Accepted for publication October 19, 1966.
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This investigation was supported by research grant GM-13844 (formerly grant GM-08447) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and research grant AFOSR 87-65,66 from the U. S. Department of the Air Force, Washington, D. C.
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activity are required, the use of unlabeled carriers in synthetic procedures is not practical, and a requirement for microsynthetic methods is imposed. For selective labeling of tropine, versatile microsynthetic methods are not available. Furthermore, micro procedures for the esterification of tropine and tropic acid have been used infrequently, by very few investigators (5, 6, 8), and many details essential for good reproducibility have not been published.

The authors concluded that labeling atropine in the tropine moiety would depend on increased reproducibility of published esterification procedures (5–9) and on adaptation of the Robinson condensation for use with micro quantities of reactants. A semimicro, prototype method evolved from quantitated studies of these two reaction sequences. From similar studies of the prototype, there resulted a reproducible microsynthesis of tropine and atropine. Detailed microsyntheses of atropine and tropine, employing 100–200 mg. of reactants, are reported. Results of both the micro and semimicro methods are compared with observations of other investigators. The feasibility of this approach to tropine and atropine labeling is established. Establishment of these pathways, and the availability of a microsynthesis of succindialdehyde from arabinose (10), provide the first feasible approach to labeling tropine in other than the *N*-methyl position.

EXPERIMENTAL

Preparation of O-Acetyltropic Acid Chloride—The method of Wolffenstein and Mamlock (9) was modified for use with micro quantities of reactants. For the synthesis of *O*-acetyltropic acid, 120 mg. tropic acid and 0.12 ml. acetyl chloride[1] were introduced into a 10-ml. round-bottom flask,[2] the flask was stoppered, and the reaction mixture allowed to stand for 12–15 hr. at room temperature. The resulting mass was heated at 100° for 7 min., and the excess acetyl chloride removed at 50°, *in vacuo*, using a flash evaporator.[3] Crystallization of the oily product, which otherwise required several days, was initiated by seeding with a crystal of pure *O*-acetyltropic acid.[4] When stored for 2–4 hr., in a vacuum desiccator, over calcium chloride, the product crystallized as a solid mass which was immediately converted to the acid chloride. To the *O*-acetyltropic acid, contained in the original reaction vessel and usually weighing 148 mg.,[1] was added 0.66 ml. thionyl chloride. The reaction vessel was fitted immediately with a calcium chloride drying tube and the reaction mixture heated, on an oil bath, at 50°, for 1 hr., during which period the mass liquefied and gas evolved. The drying tube was removed, the flask was fitted with a short-path vacuum distillation apparatus, and the excess thionyl chloride removed, at 70°, *in vacuo*.[5] The last traces of thionyl chloride were removed by the addition and *in vacuo* distillation of six 0.5-ml. portions of anhydrous benzene.[6] The product, a lemon-yellow oil having a strong and disagreeable odor, was reacted immediately with tropine HCl.[7]

Preparation of Tropine HCl—To 100 mg. dry tropine,[8] contained in the 10-ml. flask in which it had been prepared, was added approximately 5 ml. anhydrous ether. Tropine HCl[9] precipitated instantaneously when anhydrous HCl was passed carefully into the ether solution. The flow of gas was interrupted immediately, ether was removed quickly, *in vacuo*, and the crystalline product stored immediately,[10] within the reaction vessel, in a vacuum desiccator, over fresh NaOH pellets, until used for esterification.[11]

Esterification of O-Acetyltropic Acid Chloride and Tropine Hydrochloride—Thoroughly dried tropine HCl, 124 mg., was added to 160 mg. freshly prepared *O*-acetyltropic acid chloride, still contained in the original reaction vessel.[9–11] While stirring frequently, the reaction mixture was heated for 20–25 min., under anhydrous condition, in an oil bath that had been adjusted previously to 100° The resulting *O*-acetyltropine HCl was cooled to room temperature, then treated *in situ*[12] with 0.66 ml. water and maintained at 50° for 24 hr. The reaction mixture was cooled quickly to 0° and the atropine precipitated by careful, dropwise addition of 6–8 drops of saturated, aqueous NaOH. While maintaining the temperature at 0°, the aqueous

[1] In some micro experiments, 395 mg. tropic acid and 0.36 ml. acetyl chloride were used. After recrystallization, 148 mg. *O*-acetyltropic acid was removed, transferred to a thoroughly dried 10-ml. flask, then converted to the acid chloride as described here.

[2] Micro-Ware, Vineland, N. J.

[3] Rinco Instrument Co., Greenville, Ill.

[4] If the reaction mixture is not seeded, crystallization is delayed and atropine is contaminated with an unidentified, low-melting impurity. This difficulty is avoided by rapid crystallization and immediate conversion of the product to the acid chloride.

[5] Anhydrous conditions are essential and a dry ice–acetone trap must be placed between the distillation apparatus and the vacuum source.

[6] Unless all traces of thionyl chloride are removed, yields in the subsequent esterification are prohibitively low. The benzene must be anhydrous and the total time to effect all additions and removals should not exceed 15 min.

[7] The product is extremely sensitive to moisture and must be reacted immediately with tropine HCl. Prior to beginning the synthesis of *O*-acetyltropic acid chloride, tropine was prepared, converted to the HCl, and stored in a vacuum desiccator for 24 hr.

[8] Tropine was prepared within a day or two of use and was stored over NaOH pellets until converted to the HCl. Longer period of storage results in significant decomposition of tropine, which can be purified by vacuum distillation.

[9] Formation of the HCl by dissolving tropine in methanolic HCl and removing the solvent *in vacuo* was satisfactory at the semimicro level but gave inadequate yields at the micro level.

[10] Because tropine HCl is extremely hygroscopic, very rigid precautions to exclude moisture must be observed if a crystalline product is to be obtained.

[11] Tropine HCl and *O*-acetyltropic acid were always esterified in the reaction vessel that contained the acyl halide. Prior to esterification, this vessel was fitted with a standard taper T-tube, the side arm of which carried a calcium chloride tube, and the vertical arm of which contained a stirring rod secured in place with rubber tubing. This arrangement assured exclusion of moisture and allowed for manual stirring during the esterification.

[12] Experience has shown that, under the conditions stated, esterification is complete. During heating, the originally plastic mass liquefies, then becomes extremely viscous when cooled. When treated with water and stirred, it gradually dissolves to form a tan solution. If the esterification mixture is heated longer or more vigorously than stated, a brown to dark brown solution results when the product is treated with water. Under the latter conditions, yields are reduced from 10–30%.

phase was removed by careful decantation, the product washed with three 1-ml. portions of ice water, and the washed product seeded with a crystal of pure atropine.[13]

The crystalline, atropine free base was dried immediately in a vacuum desiccator over $CaCl_2$. When dry, the product was dissolved in 75 ml. anhydrous petroleum ether,[14] the solution was concentrated to approximately 10 ml., and purified atropine precipitated by cooling in the deep-freezer. Petroleum ether was carefully decanted from the crystals and the product dried for 12 hr. in a desiccator over $CaCl_2$ before converting to atropine sulfate. The dried, purified atropine base, usually 70% of theoretical yields, was dissolved in acetone and the calculated amount of 2 *N* H_2SO_4 in acetone was added. Atropine sulfate precipitated immediately. When cooled in a deep-freezer to insure complete precipitation, then washed with cold acetone and dried, the collected crystals represented 95–98% conversion to the sulfate.

Preparation of Acetone Dicarboxylic Acid—Acetone dicarboxylic acid was prepared from citric acid by modification of the general scheme outlined by Adams *et al.* (14).

For microsynthesis, 2.2 ml. fuming H_2SO_4 (20% excess SO_3), contained in a 100-ml. beaker, was cooled to −8° in a salt and ice bath. Using a thermometer as a stirring rod, 1.0 Gm. of citric acid was added, with constant stirring during approximately 15 min., at a rate slow enough to maintain the temperature at −5° or lower throughout the addition. The beaker and contents were removed from the cooling bath and stirred on a magnetic stirrer, at room temperature, for about 1 hr., until the evolution of carbon monoxide ceased. The reaction mixture was cooled again to −8°, and 4 Gm. chipped ice was added, carefully and in small portions, so the temperature never exceeded 0°. After the addition of all the ice, the reaction was cooled again to −8°, the product was collected on a sintered-glass filter, and was used at once.[15]

Preparation of Succindialdehyde—Succindialdehyde was prepared *in situ* by hydrolysis of 2,5-diethoxytetrahydrofuran,[16] by a simplified modification of the procedure of Fakstorp (15). To 2 ml. water and 5 μl. concentrated HCl, contained in a 50-ml. beaker, was added 0.17 ml. diethoxytetrahydrofuran.[17] The mixture was stirred at room temperature for 10 min., then used immediately for the synthesis of tropanone.[18]

Synthesis of Tropanone—After preliminary experiments the following "standard" procedure for the synthesis of micro quantities of tropanone was adopted. When molecular ratios were altered, the weight of methylamine hydrochloride was the reference point because this reactant can be weighed easily. Correspondingly more succindialdehyde was made available by simple proportionate changes during its preparation, and the amount of acetone dicarboxylic acid was altered by the use of a greater proportion of the moist material.

To the solution of succindialdehyde, prepared as described above and representing 1 mmole dialdehyde, was added 1 mmole acetone dicarboxylic acid, freshly prepared and still in the moist state.[19] Saturated aqueous Na_2HPO_4 was added to adjust the pH to 4.5–5.0, the solution was transferred to a 250-ml. side arm conical flask, and the system purged with nitrogen for 10 min. After the addition and dissolution of 1 mmole solid methylamine HCl, the system was quickly evacuated to remove most of the gas phase, then closed and allowed to remain under partial vacuum, at room temperature, for 12 hr. The slight evacuation was then repeated and the reaction mixture allowed to remain at room temperature for an additional 12 hr.

After 24 hr., the system was opened and the solution saturated with K_2CO_3, added in small portions, while stirring constantly and cooling the reaction mixture in a salt–ice bath. The alkaline solution was cooled to −5°, extracted with 100 ml. petroleum ether (boiling range 30–80°),[20] and the organic layer removed and reserved. The cooling, saturation, and extraction of the aqueous phase were repeated 6 times, the reaction vessel and separator were washed with petroleum ether, and the combined extracts dried over anhydrous Na_2SO_4. Portions were transferred to a sublimation flask,[21] solvent was removed *in vacuo* at 40° on the flash evaporator, and the Na_2SO_4 finally washed with anhydrous petroleum ether. After the *in vacuo* removal of this final solvent aliquot, crude oily brown tropanone remained in the sublimation flask. The product, contained in the sublimation flask was dried overnight in a desiccator over NaOH and $CaCl_2$.[22]

After drying, the product was purified by sublimation. A cold finger filled with dry ice and acetone was inserted into the sublimation flask containing the crude product. Tropanone was sublimed at 40°

[13] Atropine free base precipitated as a brownish, very viscous oil that adhered to the sides of the reaction vessel. The aqueous phase and washings were easily removed by careful decantation. The product quickly crystallized when maintained at 0° and seeded with pure atropine.

[14] Boiling range 30–60°.

[15] In semimicro experiments it was found that a very carefully washed product may be stored in a desiccator for 4 or 5 days, but that washing and recrystallization are accompanied by significant losses of product. Losses may be minimized by using ice-cold anhydrous ethyl acetate. If traces of sulfuric acid remain, there is significant decomposition in 2–3 hr. For calculation of yields the product was washed with ice-cold, anhydrous ethyl acetate and dried in a desiccator for 2 days. A yield of 95% and a product melting at 135° were obtained. When recrystallized from ethyl acetate, acetone dicarboxylic acid melted at 138°. To prevent losses during the synthesis of tropine, acetone dicarboxylic acid was neither washed nor recrystallized, but was used immediately in the Robinson condensation.

[16] Beacon Chemical Industries, Inc., Cambridge, Mass.

[17] In semimicro experiments, 1 vol. diethoxytetrahydrofuran, 16 vol. water, and 0.02 vol. concentrated HCl was mixed, then stirred until the translucent mixture became crystal clear

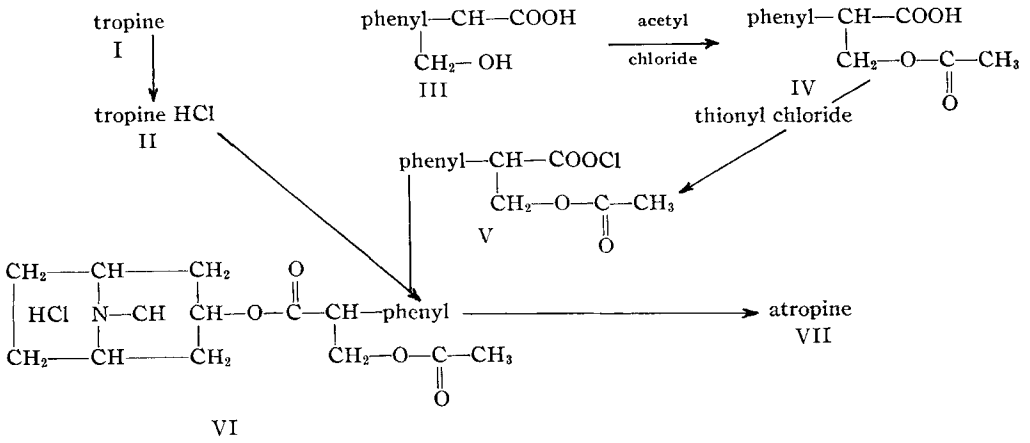
[18] To determine the extent of hydrolysis as a function of time, aliquots were treated with 2,4-dinitrophenylhydrazine, as described by Shriner *et al.* (16). A quantitative yield of the bis-dinitrophenylhydrazone, m.p. 270–275°, without recrystallization, was obtained after 5 min. in micro experiments. At the semimicro level, yields of 95–100% were always obtained when the opaque reaction mixture had become crystal clear.

[19] Twenty per cent of the moist weight of the product resulting from 1 Gm. citric acid corresponds to 1 mmole of acetone dicarboxylic acid.

[20] Allied Chemical Corp. The solvent must have a boiling range of 30–80 or extraction is incomplete and there are appreciable decreases in tropanone yield. Diethyl ether and a continuous extractor were used in semimicro experiments, but difficulties were encountered at the micro level with this solvent. For this reason, petroleum ether and manual extraction were used in micro experiments.

[21] This flask had a 250-ml. capacity and was of the design described by Werner (6).

[22] Care must be exercised in removing the solvent, and the use of a vacuum desiccator is inadvisable, because the low vapor pressure of tropanone results in losses on undue exposure to vacuum.



Scheme I

at 1 × 10^{-4} mm. mercury,[23] for 10 min. The vacuum was disconnected, dry ice and acetone were removed, the cold finger was removed from the flask, and the accumulated tropanone was washed into a 150-ml. beaker using small portions of absolute ethanol. The operation was repeated 2 times, using 10 and 15-min. sublimation periods. The combined ethanol solutions of sublimed tropanone were evaporated to dryness, on the micro flash evaporator, in a previously weighed 25-ml. flask. The product crystallized quickly on cooling, and was dried in a desiccator overnight.[22,24]

Reduction of Tropanone to Tropine—To the tropanone (approximately 100 mg. from 67.5 mg. methylamine HCl), contained in the 25-ml. flask in which it had been dried, was added 5 ml. absolute ethanol, a magnetic stirring bar, and 0.5 ml. of a suspension of Raney nickel in absolute ethanol.[25] The flask was placed in a water bath, attached to a gas buret, and the reaction mixture continuously stirred while flushing the system seven times by alternate admission and expulsion of hydrogen. Finally, the system was filled with hydrogen, closed, and continuously stirred until reduction was complete.[26]

To remove Raney nickel, the ethanol solution was filtered through a sintered-glass filter into a 25-ml. round-bottom flask, the original flask and nickel were washed repeatedly with small portions of absolute ethanol, and most of the solvent removed on the flash evaporator. When the volume of ethanol had been reduced to approximately 10 ml., the solution was transferred to a tared 10-ml. flat-bottom flask, evaporated to dryness, and both flask and contents dried in a vacuum desiccator[27] before conversion to the hydrochloride.

[23] Two stage vacuum pump, Welch Scientific Co., Chicago, Ill.

[24] Tropanone yield was determined from the weight of this product, m.p. 42°.

[25] To prepare Raney nickel, 2.5 Gm. of alloy (Raney Nickel Co., Chattanooga, Tenn.) was heated at 50° for 15 min. with a solution of 3.2 Gm. NaOH in 12.5 ml. water. The product was washed with water, ethanol, and absolute ethanol, then suspended in absolute ethanol. The final suspension represented 35 mg./ml. of Raney nickel.

[26] When reduction was complete, a 1-μl. aliquot gave a negative test when spotted on filter paper and treated with 2,4-dinitrophenylhydrazine solution. It is sometimes necessary to add additional aliquots of Raney nickel to effect complete reduction.

[27] Conversion to the hydrochloride is carried on in this vessel. The product is white, crystalline, m.p. 63–65°, and adequately pure for conversion to the HCl without recrystallization. Picrate darkens at 275° and melts at 295°.

RESULTS AND DISCUSSION

For the synthesis of tropine-labeled atropine, the two key steps are synthesis of labeled tropine and esterification of the labeled heterocycle with unlabeled tropic acid. Good reproducible yields with micro quantities of reactants must be obtainable with each of these steps for the synthesis of tropine-labeled atropine to be feasible.

Microesterifications of tropine and tropic acid have been done by very few investigators (5, 6, 8). All reported procedures are modifications of the Wolffenstein and Mamlock method (9) and involve conversion of tropine (I) to the hydrochloride (II), conversion of tropic acid (III) to *O*-acetyltropic acid chloride (V) *via* the intermediate acetyl compound (IV), esterification of II and V to form *O*-acetylatropine HCl (VI), and mild, selective hydrolysis to the free alkaloid (VII). (Scheme I.)

Although 63–70% yields have been reported for the microesterification of tropine and tropic acid, there have been no detailed studies of the method. Lack of reproducibility was disclosed in semimicro studies and was re-encountered at the micro level. Werner's data (6) indicate a similar difficulty. He obtained 70% yields of tropine-labeled atropine after a single esterification, but isolation of unreacted tropic acid and two esterifications were required to obtain a 63% yield of tropic acid-labeled atropine. Because of these inconsistencies, the authors quantitatively studied each step in the reaction sequence. Results of these studies are compared in Table I with summarized observations of other investigators.

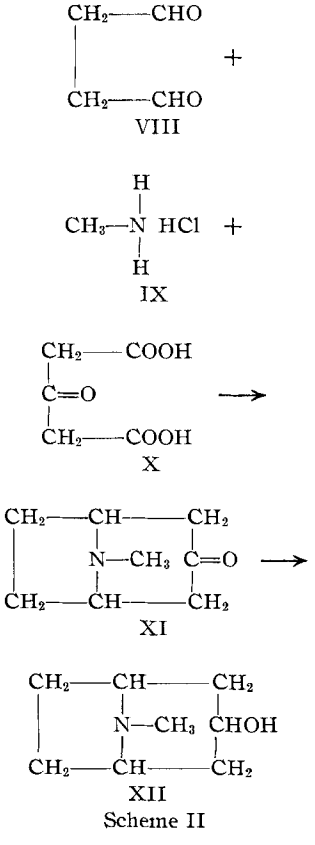
From the studies summarized in Table I, there evolved a reproducible microesterification of tropine and tropic acid. In contrast to others, equimolar quantities of tropine and tropic acid are used, and the method is equally adaptable for labeling atropine in either of these component moieties. The problem of synthesizing tropine-labeled atropine is thereby reduced to that of labeling the heterocycle. The need is for a microsynthesis of tropine that is adaptable for the introduction of appropriately labeled intermediates.

The classical approach to tropine synthesis is the method of Robinson (11), as modified by Schopf and Lehman (12). Succindialdehyde (VIII), methylamine HCl (IX), and acetone dicarboxylic acid (X) are condensed under essentially physiological condi-

TABLE I—COMPARISON OF OBSERVED AND REPORTED DATA FOR THE ESTERIFICATION OF TROPINE AND TROPIC ACID

	Micro[a]	Semimicro[b]	Werner[c]	Werner[d]	Fodor[e]	Mamlock[f]
Wt. of tropic acid used	120 mg.	1.5 Gm.	395 mg.	...	...	3.0 Gm.
Yield of *O*-acetyltropic acid	148 mg. (100%)	1.81 Gm. (100%)	495 mg. (100%)	...	...	...
M.p.[g] *O*-acetyltropic acid	87–88°	89–90°	...	...	...	...
Wt. *O*-acetyltropic acid used	148 mg.	1.81 Gm.	495 mg.	130 mg.	...	...
Yield *O*-acetyltropic acid chloride	160 mg. (99%)	1.95 Gm. (99%)	540 mg. (100%)	...	...	...
Wt. tropine used[h]	100 mg.	1.27 Gm.	...	98.8 mg.	620 mg.	...
Yield tropine HCl	124 mg. (99%)	1.56 Gm. (98%)	...	124.3 mg. (101%)	260 mg. (84.4%)	...
M.p. tropine HCl	280°	280°	...	200	...	...
Wt. tropine HCl used	124 mg.	1.56 Gm.	505 mg.	124.3 mg.	263 mg.	3.2 Gm.
Wt. *O*-acetyltropic acid chloride used	160 mg.	1.95 Gm.	540 mg.	141 mg.	320 mg.	...
Yield of atropine[i] free base	144 mg. (70%)	1.95 Gm. (75%)	430.9 mg. (63%)	142 mg. (70%)	120 mg. (63%)	(80%)
M.p.[j] atropine free base	114–116°	116–117°	...	115°	108°	115°
Mixed m.p. atropine free base	114–116°	114–116°	...	...	...	...
M.p. atropine picrate	174–175°	174–175°	...	...	...	...
M.p.[k] atropine sulfate	191°	191°	189°	189°	...	...

[a] Micro procedure described in this communication. [b] Semimicro procedure described in this communication. [c] Micro method of Werner *et al.* (6) for labeling tropic acid moiety. [d] Micro method of Werner *et al.* for labeling the tropine moiety (6). [e] Fodor *et al.* (5). [f] Wolffenstein and Mamlock (9). [g] All melting points determined with Fisher-Johns melting point apparatus. [h] Fodor used tropine picrate and converted to the HCl with an ion exchange resin. [i] Yields based on tropine except for Werner's tropic acid labeling, which was based on radioactive tropic acid and represents the sum of two esterifications. [j] Melting point reported by Fodor is for hyoscyamine. [k] "Merck Index," 7th ed., Merck & Co., Inc., Rahway, N. J., p. 111, reports melting points for the sulfate from 190–194°.



Scheme II

tions to yield tropanone (XI), which then is reduced to tropine (XII). (Scheme II.)

Although the scheme is inherently versatile and therefore potentially suited for radiosynthesis, there have been surprisingly few studies of the reaction (11–13) and no reference to its use at the micro level.[28] In Table II the results of semimicro and microsyntheses, developed during the studies described in this communication, are compared with the findings of other investigators.

To determine the effect of hydrogen-ion concentration and potential losses from decreasing weights of reactants, concentration of all reactants was fixed at 0.20 *M*, while decreasing the weight of methylamine HCl from 33.0 Gm. to 1.69 Gm. at each of four hydrogen-ion levels within the range pH 4.0–pH 10.5. Neither decreasing weights nor varying hydrogen-ion level had significant effects on tropanone yields. The weight of methylamine HCl was then fixed at 1.69 Gm., equimolar concentrations of reactants were used, and concentration was varied from 0.01–0.20 *M*, at each of the four hydrogen-ion levels previously employed. There were no significant differences in tropanone yields, thereby validating arbitrary choices of both concentration and hydrogen-ion level, within the ranges studied. In comparison, Keagle and Hartung (13) reported no significant differences in tropanone yields within the concentration range 0.04–0.16 *M* and the pH range pH 5.0–pH 11.0. Agreement with Keagle and Hartung's findings is apparent.

There is general agreement with Schopf and

[28] Werner's microsynthesis of *N*-methyl-labeled tropine and atropine were published just prior to completion of this investigation.

TABLE II—COMPARISON OF OBSERVED AND REPORTED YIELDS FOR THE SYNTHESIS OF TROPANONE BY THE MODIFIED ROBINSON CONDENSATION

Method	Wt. of Amine HCl	Molarity Amine HCl	Molecular Ratios of Amine–Aldehyde–Acid	Initial[a] pH	Tropanone,[b] Yield, %	Identification[c]
						Distils 113–115°, 25 mm.
	1.69 Gm.	0.01 *M*	1:1:1	4.0	71	Distils 72–74°, 10 mm.
Semimicro[d]	to	to	1:1:1	4.5	75	M.p. free base 41–42°
	33.8 Gm.	0.20 *M*	1:1:1	7.0	73	M.p. picrate, 220°
			1:1:1	10.5	69	M.p. oxime, 111°
						M.p. dipiperonylidene derivative, 214°
	67.5 mg.	0.01 *M*	1:1:1	5.0	70	
	67.5 mg.	0.02 *M*	1:1:1	5.0	70	
	67.5 mg.	0.01 *M*	1:2:2	5.0	65	M.p. free base, 42°
Micro[d]	67.5 mg.	0.02 *M*	1:2:2	5.0	71	M.p. picrate, 220°
	135 mg.	0.02 *M*	2:1:2	5.0	68	M.p. oxime, 111°
	135 mg.	0.04 *M*	2:1:2	5.0	72	M.p. dipiperonylidene
	135 mg.	0.02 *M*	2:2:1	5.0	67	derivative, 214°
	135 mg.	0.04 *M*	2:2:1	5.0	69	I.R. analysis
Werner (6)	67.5 mg.	0.008 *M*	1:2:3	5.6	70	M.p. free base, 42°
			4:2:5	3.0	47	
			4:2:5	5.0	54	
			4:2:5	7.0	65	
			4:2:5	9.0	66	
Schopf and Lehman (12)	...	0.04 *M*	4:2:5	11.0	86	M.p. picrate, 220°
			4:2:5	13.0	3	
			2:1:1	3.0	68	
			2:1:1	5.0	83	
			2:1:1	7.0	78	
			2:1:1	9.0	61	
			2:1:1	11.0	64	
			2:1:1	13.0	5	
	2.7 Gm.	0.04 *M*	2:1:2	5.2	58	
	2.7 Gm.	0.04 *M*	2:1:2	7.1	62.4	
Keagle and Hartung (13)	2.7 Gm.	0.04 *M*	2:1:2	7.1	62.4	M.p. picrate, 220°
	2.7 Gm.	0.04 *M*	2:1:2	11.02	60.3	M.p. dipiperonylidene
	2.7 Gm.	0.08 *M*	2:1:2	11.02	60.3	derivative, 214°
	21.6 Gm.	0.16 *M*	2:1:2	...	60–65	

[a] Hydrogen-ion concentration measured with Corning model 7 pH meter. [b] Yields for micro and semimicro procedures and for Werner's method are as the free base, after purification by sublimation. Other yields are as the picrate. [c] Dipiperonylidene derivative and picrates prepared as described by Keagle and Hartung (13). Melting points reported in this communication were determined with a Fisher-Johns melting point apparatus. For infrared analysis, samples were compared with authentic samples of tropine and tropinone as picrates using a Perkin-Elmer model 221 I.R. spectrophotometer. [d] Semimicro and micro procedures described in this communication.

Lehman (12), but their data indicate a possible interdependence of pH optima and ratios of reactants. When both amine and keto acid were in excess, tropanone yields were appreciably higher at pH 11.0, but when only the amine was in excess, yields were appreciably higher at pH 5.0. Because of the narrow pH range studied, our data do not completely exclude an interdependence. Results obtained in semimicro experiments, with varying ratios of reactants at pH 5.0 and pH 7.0, showed no dependence of tropanone yield on molecular ratios. These findings were confirmed in microsyntheses at pH 5.0, as shown in Table II. All data reported here were obtained when diethoxytetrahydrofuran was used as the source of succindialdehyde, while both Schopf and Lehman and Keagle and Hartung prepared the aldehyde from pyrrole *via* the intermediary succinaldoxime. The furan derivative is a far superior aldehyde source. Based on experience with both pyrrole and diethoxytetrahydrofuran, the authors concluded that Schopf and Lehman's data probably reflect a lack of reproducibility arising from the use of pyrrole.

During adaptation and modification of the prototype procedure, previously described experiments were repeated on a more limited scale. The data, which are not presented here, showed that flexibility of pH was no different when microquantities of reactants were used. An initial pH of 5.0 was chosen arbitrarily because carbon dioxide evolution is readily apparent and is a reliable, visual indication that the condensation is progressing properly. Because carbon dioxide is totally or partially bound, this guide is not available at hydrogen-ion levels near and above neutrality.

Changes in buffer composition were considered for possible effects on tropanone yields. The micro procedure described here was compared in parallel experiments with Werner's method (6). Identical 70% yields of pure tropanone were obtained, thereby confirming Werner's work and indicating that acetate offered no advantage over phosphate. Phosphate was then compared with citrate at different hydrogen-ion levels and different reaction times. Maximum yields of 50% pure tropanone were isolated when citrate buffers were used. The authors

could not confirm the claims of Hungarian workers (17) that the use of citrate buffers results in tropanone yields of 90% or more and a decrease in reaction time to 6 hr. The intent was to isolate pure tropanone in maximum yields, and the variables introduced by their use of the reineckate as a criterion for completeness of condensation was not considered. For our purposes, phosphate and acetate were equally effective and both were superior to citrate. Phosphate was chosen on the basis of more extensive experience with this system.

Further studies with the microsynthetic method, shown in Table II, were limited on the basis of probable conditions in radiosyntheses. Variations of methylamine weight from 67.5–135 mg., concentrations from 0.01–0.04 *M*, and molecular ratios within the probable experimental range had no significant effect on tropanone yields. Similarly, the use of an excess of one or more reactants had no effect. These observations confirm, for the micro modification, the results obtained with the semimicro prototype; reproducibility is good, there is a flexibility in actual quantities and molecular ratios of reactants, and there is no requirement for an excess of any particular intermediate.

The micro condensation was developed as an instrument for tropine labeling and is well suited for this purpose. It is flexible and reproducible. Equimolar ratios, or any desired proportion of reactants, can be used without affecting yields. There is no requirement for added carriers and specific activity of the product is limited only by the specific activities of the intermediates.

CONCLUSIONS

The esterification of tropine and tropic acid has been studied in detail, using both semimicro and microquantities of reactants. Yields for each step in the sequence have been determined and have been compared with the findings of other investigators. From these studies there evolved a micro procedure for esterifying tropine and tropic acid. Equimolar quantities of reactants are employed and the method is equally suited for labeling either of the component moieties of atropine. With proper attention to detail, esterification is unusually reproducible and yields of 70–75% atropine free base are obtained routinely.

In a similar way, the Robinson condensation has been studied. There has evolved a reproducible micro method for condensing equimolar quantities of methylamine HCl, acetone dicarboxylic acid, and succinic dialdehyde. There exists an ideal flexibility of molecular ratios, concentrations of reactants, and hydrogen-ion levels. The method is ideally suited for labeling tropine through any or all intermediates of the Robinson condensation, and yields of 67–72% tropine are obtained routinely.

Based on studies of the esterification and the condensation, yields of 47–54% atropine free base should result from any precursor of the Robinson condensation. This prediction has been confirmed by synthesis of atropine from each of the three compounds. This study, and Werner's work, clearly establish the feasibility of using the Robinson condensation to label tropine. The feasibility of esterifying labeled tropine with unlabeled tropic acid has been established. Results of this study, and a preliminary report describing a prototype synthesis of succindialdehyde from arabinose (10), make available for the first time a synthetic method for labeling the carbon skeleton of the tropine moiety. Details of the synthesis will be published in the second paper of this series.

REFERENCES

(1) Schmidt, H. L., and Werner, G., *Ann.*, **656**, 149 (1962).
(2) Geiling, K. M. K., *Med. Ann. District Columbia*, **201**, 197(1951).
(3) Leete, E., Marion, L., and Spenser, I. D., *Can. J. Chem.*, **32**, 1116(1964), Leete, E., Marion, L., and Spenser, I. D., *Nature*, **174**, 650(1954).
(4) Kaczkowski, J., Schutte, H. R., and Mothes, K., *Naturwiss.*, **47**, 304(1960).
(5) Fodor, G., and Janszo, G., Otvos, L., and Benfi, D., *Ber.*, **93**, 2681(1960).
(6) Werner, G., Schmidt, H. L., and Kassner, E., *Ann.*, **644**, 109(1961).
(7) Werner, G., and Schmidt, H. L., *Naturwiss.*, **46**, 626(1959).
(8) Murray, A., and Williams, D. L., "Organic Synthesis with Isotopes," part I, Interscience Publishers, Inc., New York, N. Y., 1958, p. 550.
(9) Wolffenstein, R., and Mamlock, L., *Ber.*, **41**, 723 (1908).
(10) Schmidt, G. C., Drach, J., and Eling, T. E., Abstracts from the American Association for the Advancement of Science, Montreal, Quebec, Canada, December 30, 1964.
(11) Robinson, R., *J. Chem. Soc.*, **111**, 762(1917).
(12) Schopf, A., and Lehman, G., *Ann.*, **518**, 5(1935).
(13) Keagle, A. C., and Hartung, W. H., *J. Am. Chem. Soc.*, **68**, 1608(1946).
(14) Adams, R., Chiles, M. M., and Rasswoller, G. F., "Organic Synthesis," coll. vol. I, John Wiley & Sons, Inc., New York, N. Y., 1946, p. 10.
(15) Fakstrop, J., Raleigh, D., and Schnupp, L., *J. Am. Chem. Soc.*, **72**, 869(1950).
(16) Shriner, R. L., Fuson, R. L., and Curton, D. Y., "The Systemic Identification of Organic Compounds," 5th ed., John Wiley & Sons, Inc., New York, N. Y., 1964, p. 253.
(17) Simonyi, G., and Takar, O., *Acta Chim. Acad. Sci. Hung.*, **6**, 365(1955).

Microbiological Methods for Quality Control of Membrane Filters

By FRANCES W. BOWMAN, MIRIAM P. CALHOUN, and MACK WHITE

Microbiological methods are presented for quality control of membrane filters of 0.45 and 0.22 μ porosity.

ALTHOUGH MEMBRANE filters have been produced for more than 100 years, they were not widely used in the United States until after World War II. According to a report by Goetz (1), the Germans made dramatic use of them during the war for preventing epidemics that could have been caused by contaminated water supplies. Using membrane filters, they developed a rapid test to determine the safety of public water supplies after bombings.

The technology for producing membrane filters was later brought to the U. S. where, under the sponsorship of the U. S. Department of the Army, extensive research was conducted on their production. This research resulted in practical procedures for commercial production. The filters were produced from cellulose esters in sheets approximately 150 μ thick. The pore sizes were predetermined and controlled in the manufacturing process.

At the present time, 12 distinct pore sizes or classes ranging from 8 μ (class 1) downward in pore size to 10 mμ (class 12) are commercially available. According to the reports by the manufacturers, some of the membranes are composed of pure cellulose acetate, whereas others contain a mixture of cellulose acetate and cellulose nitrate. Due to the demand for these membranes, an Interim Federal Specification (2) was published for the purpose of securing bids for the Defense Supply Agency. This federal specification includes methods for evaluating the quality of each class of membrane.

In the past decade, scientists have found innumerable applications for the membrane filters as an analytical tool. The filters have been widely used in the sampling and measurement of airborne particulates, microbiological analysis of water and foods, clinical diagnosis, sterile filtration, beer manufacturing, and sterility testing.

Holdowsky (3) first suggested the use of bacterial retentive membranes in sterility testing of antibiotics to provide a means of removing the antibiotic from the test medium. Using the membrane filtration technique, Bowman (4) developed procedures for soluble antibiotics, and the Antibiotic Regulations were subsequently amended to include the membrane filtration sterility test. For this test, the Antibiotic Regulations state that the bacterial membrane filter should have a nominal porosity of 0.45 $\pm$ 0.02 μ, a diameter of approximately 47 mm., and a flow rate of 55 to 75 ml. of distilled water passing each sq. cm. of filter area per min., with a differential pressure of 70 cm. of mercury at 25°. Bacterial retentive membranes that fulfill these specifications are available from a number of suppliers. Most manufacturers conduct laboratory quality control tests. In addition to these tests, it is desirable that laboratories conducting sterility tests assess the quality of the membranes to be used. Therefore, a reliable standard method for evaluating the bacterial retentive membranes was sought.

Among the methods currently used for determining pore size are Sauer's pore-size bubble test (5), Erbe's pore-size distribution determination (6), Ritter's mercury intrusion measurement of pore radius (7), and particle passage methods (8). The latter are the most commonly used, and are the basis of the current pore-size rating system. These tests tell whether the filter will retain particles larger than the nominal pore size. For large pores, colored or radioactive particles are used; for microbiological filters, bacteria are used.

The particle passage method provides a practical method for determining the quality of class 7 (0.45-μ) membranes used for sterility testing. Special equipment such as that used for the mercury intrusion and other methods is not needed. Most of the equipment and materials required for this test are found in any laboratory performing membrane filtration sterility tests. For this reason, a passage test using *Serratia marcescens* ATCC 14756 has been developed for accepting the membranes used for the sterility testing of antibiotics under the certification program. The acceptance test employs a general inspection level I, double sampling plan, MIL-STD-105, at a 1% acceptable quality level. For example, if a lot (batch) of 1201 to 3200 membrane filters is to be sampled for tests, 32 filters

Received October 27, 1966, from the Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education and Welfare, Washington, D. C. 20204.
Accepted for publication November 10, 1966.

will be selected randomly from the lot and tested for retention of *S. marcescens*. If none is found defective, the batch is acceptable. If one is defective, a second test is performed on an additional 32 filters. If two or more are found defective in either the first or the first and the second test, the lot is rejected. The double sampling plan allows acceptance of acceptable quality filters with minimum testing of one sample; the second sample allows for the probability occurrence of one defective membrane in a sample of 64 filters having an average quality level of 1% defective.

ACCEPTANCE TEST PROCEDURE FOR CLASS 7 MEMBRANES

Bacterial Culture.—Prepare a suspension of *S. marcescens* ATCC 14756 as follows. Transfer the organism to a Roux bottle containing 300 ml. of U.S.P. peptone–casein agar, incubate at 25° for 24 hr., and harvest the growth with 50 ml. of sterile 0.1% (w/v) peptone diluting fluid and sterile glass beads. Perform a viable cell count on the suspension and determine the dilution that will yield between 50,000 and 100,000 cells/ml.

Apparatus.—Assemble 32 filtration units as shown in Fig. 1. Each unit consists of a 1-L. side-arm Pyrex suction flask (*1*) fitted with a No. 8 rubber stopper which has a hole in the center for the support of a 300-ml. Pyrex filter funnel. The fritted-glass base which supports the membrane is held to the 300-ml. funnel with a spring action aluminum clamp. One end of a 2-in. long piece of vacuum tubing ($^1/_4$-in. bore and $^3/_{16}$-in. wall) is attached to the side-arm of the 1-L. flask. At the other end (*2*) a female adapter, Luer-Lok, is inserted to hold a Swinny hypodermic adapter (*3*) containing a 13-mm. diameter microfiber glass air filter. A male adapter, Luer-Lok (*4*), attached to the Swinny adapter is inserted in additional tubing which is connected to the vacuum source. The top of the side-arm flask and the base of the funnel are covered

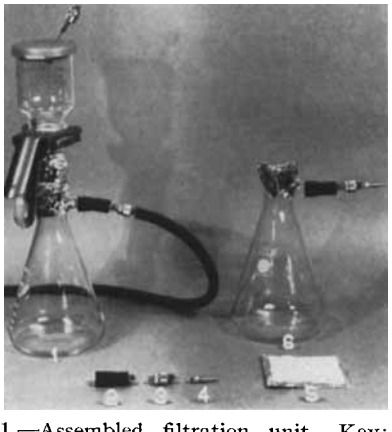


Fig. 1.—Assembled filtration unit. Key: *1*, Pyrex suction flask for filtration; *2*, female adapter; *3*, Swinny adapter with air filter; *4*, male adapter; *5*, gauze square; *6*. Pyrex suction flask for incubation.

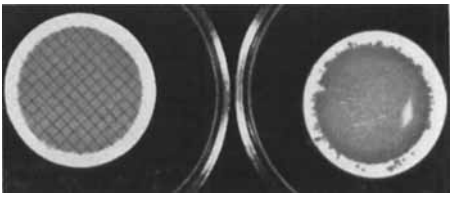


Fig. 2.—Membrane with satisfactory growth of *S. marcescens* (left) and unsatisfactory growth (right) after incubation in acceptance test.

with aluminum foil. The top of the funnel is closed by a rubber stopper pierced with an 18-gauge hypodermic needle attached to a Swinny adapter as described above. After the entire unit is autoclaved for 1 hr. at 121°, a sterile class 7 membrane is aseptically placed on the fritted-glass base of the funnel with flat bladed forceps to avoid damaging the membrane.

Test Procedure.—Lift the stopper of each of the 32 filter funnels prepared as described above and carefully pour in 200 ml. of U.S.P. peptone broth that has been seeded with 1 ml. of the *S. marcescens* suspension appropriately diluted as previously described. Replace the stopper firmly on the funnel and apply negative pressure to start the filtration. When the filtration has been completed, stop the vacuum and allow air to be drawn rapidly into the flask through the air filter inserted in the rubber tubing. With the funnel still attached, remove the rubber stopper from the suction flask and cover the top of the flask containing the filtrate with a presterilized gauze square approximately 11.5 × 11.5 cm. (*5*), saturated with 5% phenol. Remove each membrane aseptically and place it on the surface of peptone–casein agar in a sterile Petri dish (20 × 100 mm.). Incubate the 32 agar Petri dishes containing the membranes and the 32 side-arm flasks (*6*) at 32°. Observe the growth on the membranes after 24 and 48 hr., and examine the filtrates in the flask after 5 days' incubation. No growth of organisms in the filtrates proves complete retention of the organisms by the membranes and indicates their suitability. As shown by the membrane on the left in Fig. 2, growth on the plates should be confluent and contained on that part of the membrane inside the filtering area. Growth of the organisms on the edge of the membrane, as shown on the right in Fig. 2, indicates faulty technique and may produce growth in the filtrate. Side leakage can occur if the membrane is not perfectly sealed in the unit by the clamp. Therefore, extreme care should be taken when assembling the units to avoid leakage.

Evaluation of Results.—If no growth is detected in any of the 32 filtrates after 5 days and the growth of *S. marcescens* on the membranes is confluent, the batch of membranes is acceptable for sterility testing. If two or more filtrates show growth, the batch is rejected. However, if there is growth at the edge of a filter and the related broth exhibits growth, the test is judged invalid, and it is repeated in full. The results of tests judged invalid are not considered a part of the supplier's quality performance record.

If one filtrate shows growth, a repeat test is performed on 32 additional membranes. If no growth

TABLE I.—RESULTS OF ACCEPTANCE TEST ON CLASS 7 MEMBRANES[a]

Mfgr.	Batch	Filtrates—First Test	Filtrates—Second Test	Membranes—First Test	Membranes—Second Test
A	1	0	NA	Satisfac.	NA
	2	0	NA	Satisfac.	NA
	3	0	NA	Satisfac.	NA
B	1	0	NA	Satisfac.	NA
	2	1/32	0	Satisfac.	Satisfac.
	3	0	NA	Satisfac.	NA
C	1	0	NA	Satisfac.	NA
	2	0	NA	Satisfac.	NA
	3	0	NA	Satisfac.	NA

[a] 0, no growth; NA, not applicable; 1/32, one filtrate with growth out of 32 tested; satisfactory, confluent growth confined to the filtering area.

is detected in any of the additional 32 filtrates after 5 days at 32°, the batch of membranes is acceptable for sterility testing. If growth is detected in one or more of the filtrates, the batch is rejected.

Experimental.—In order to evaluate some of the commercially-produced class 7 membrane filters, membranes were obtained from three different manufacturers. An acceptance test, as described previously, was performed on three batches from each manufacturer. The manufacturers were identified as *A*, *B*, and *C* for the evaluation studies. It can be concluded from the results shown in Table I that all membranes tested were acceptable for sterility testing. A retest was necessary in only one out of nine acceptance tests.

DISCUSSION

Although class 7 (0.45-μ) and class 9 (0.22-μ) membranes are both used to sterilize liquids by filtration, the latter are always used with solutions containing sera, plasma, trypsin, or other enzymes where species of *Pseudomonas* of small size are known to occur. The class 7 filter, having larger pores, yields flow rates approximately 3 times those of the class 9 filters, but can only be used for sterile filtration where prior experience indicates that organisms smaller than 0.45 μ are not present in the solution to be filtered. For example, in the production of penicillinase (9) from *Bacillus cereus* ATCC 13061, it was found in this laboratory that the class 7 membrane was not adequate for sterilizing the enzyme. This membrane removed all of the *B. cereus* from the broth in which the enzyme was produced, but on several occasions the broth was also heavily contaminated with a small Gram-negative bacillus which the membrane did not filter out completely. However, in every case the class 9 membrane was able to retain all cells of this organism and yield a sterile penicillinase. The organism was identified as a species of *Pseudomonas* and was later used to develop an acceptance test for 0.22-μ filters. Its ability to pass through a class 7 is dependent on its small size (approximately 0.3 × 1.0 μ). It has been successfully used in the quality control of the class 9 membranes for over 1 year and has been transferred approximately 200 times with no increase in its size. Since it proved to be so useful in testing class 9 membranes, the organism was deposited with the American Type Culture Collection where it was given the accession number ATCC 19146.

The fact that class 7 membranes (0.45 μ) will not retain all microorganisms does not preclude their use in sterility testing. Investigational studies proved that the passage of these small *Pseudomonas* cells occurs only when the population size is relatively high. Therefore, the passage of a few cells is not significant, since the majority of the cells are retained on the membrane and their recovery in the sterility test is assured. For this reason, class 7 membranes, which provide a better flow rate than class 9 membranes, were selected for the antibiotic sterility tests.

As previously mentioned, class 9 membranes are used in this laboratory to sterilize penicillinase for use in testing insoluble penicillins, such as benzathine penicillin G. The sterile enzyme is aseptically added to sterile thioglycollate medium for use in the direct method sterility test. The class 9 membranes purchased for sterile filtration are tested by using the same sampling plan and the same equipment as that used in the acceptance test for class 7, except for changes in the organism and the procedure as described below.

ACCEPTANCE TEST PROCEDURE FOR CLASS 9 MEMBRANES

Maintain *Pseudomonas* sp. ATCC 19146 on U.S.P. peptone–casein agar. Transfer the organism to a conical flask containing 50 ml. of trypticase 2% (w/v) which contains 0.6% sodium citrate, and incubate the flask at 25° until the viable cell count reaches approximately 1,000,000 cells/ml. Filter 50 ml. of the broth suspension through a class 7 membrane into a sterile flask. Transfer the membrane to the surface of a sterile peptone–casein agar in a 20 × 100-mm. Petri dish. Incubate the Petri dish at 32° and observe the growth after 24 and 48 hr. Transfer three 1-ml. aliquots of the filtrate that has passed through the 0.45-μ membrane to each of three tubes containing 10 ml. of thioglycollate or any other suitable liquid nutrient medium. Filter the remaining portion of the filtrate through a class 9 membrane into a second sterile receiving flask. Incubate the flask for 5 days at 32°. Transfer the class 9 membrane to the surface of sterile agar in a Petri dish (20 × 100 mm). After 24–48 hr., the class 7 membrane should show confluent growth and after 48–72 hr., the class 9 membrane should show individual colonies of the test organism. The three tubes of thioglycollate medium should show growth. The filtrate that passed through the class 9 membrane should not show growth after 5 days' incubation at 32°. The evaluation of the results are the same as for the class 7 membranes except that the test organism must pass through the class 7 membrane and be retained by the class 9 membrane in each of the 32 tests performed.

SUMMARY

A microbiological method has been developed for performing an acceptance test for class 7 (0.45-μ) membrane filters, used in sterility testing, and class 9 (0.22-μ) membrane filters, used in sterile filtration. *S. marcescens* is used as the test organism for the

former and *Pseudomonas* sp. for the latter. The sampling plan of MIL-STD-105-D is used for the statistical evaluation of the membranes for acceptance or rejection.

REFERENCES

(1) Goetz, A., Fiat Final Report 1312, Joint Intelligence Objective Agency, U. S. Department of Commerce, Washington, D. C., 1947, p. 8.
(2) Interim Federal Specification, Disk Filter, Membrane, Bacteriological Particulate, Defense Medical Supply Center, Brooklyn, N. Y.
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Mannich Bases from 2-Phenylindolizines I

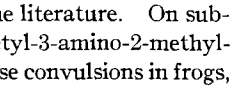
3-Alkyl-1-dialkylaminomethyl Derivatives

By WILLIAM B. HARRELL* and ROBERT F. DOERGE

In a search for new therapeutic agents, a series of Mannich derivatives of 3-methyl-2-phenylindolizine and 3-ethyl-2-phenylindolizine has been prepared. The mechanism by which 2,3-disubstituted indolizines participate in the Mannich reaction has been examined and alternate S_N1 and S_N2 routes have been proposed. 1-Diethylaminomethyl-3-methyl-2-phenylindolizine has been tested and found to have central nervous system depressant activity.

MANY PHYSIOLOGICALLY active compounds have the indole nucleus in their structure. The similarity between the indole and indolizine nuclei has prompted speculation that indolizine analogs of biologically important indoles could conceivably have potent physiological activity (1–3). It is this concept, coupled with the observation that only a few such investigations had been reported in this area, that encouraged the consideration of the indolizine nucleus for the present investigation.

Reports on the biological activity of indolizines are indeed very scarce in the literature. On subcutaneous injection 1-acetyl-3-amino-2-methylindolizine was shown to cause convulsions in frogs, mice, and rabbits (4). β-(1-Indolizinyl)-α-aminopropionic acid, the indolizine analog of the amino acid tryptophane, has been prepared (2) and shown to inhibit indole formation by an indole-accumulating mutant of *Salmonella typhimurium* (5). A series of arylindolizines was synthesized by Venturella for pharmacological testing (3, 6, 7), but to date no report on the activity of these compounds has appeared in the literature.

Having decided upon the indolizine structure, attention was focused on the various possibilities of chemical modification which might logically be expected to produce physiologically active compounds. Among the biologically active indoles, many are aminoalkyl derivatives such as serotonin, bufotenine, psilocin, reserpine, and lysergic acid diethylamide, all of which are known to have pronounced activity on the central nervous system. It was noted that in all of these compounds, the indole nitrogen and the extra-indole nitrogen are separated by four carbon atoms  , a spatial arrangement which might be a contributing factor in determining their activities. It was therefore reasoned that certain aminoalkylindolizines with the same carbon spread between the nitrogens might also possess activity on the central nervous system. After considering several possible chemical modifications, the Mannich reaction was chosen because the resulting dialkylaminomethyl substituent, when introduced at the C-1 position, would give the desired intramolecular spread. A comparison between bufotenine (I) and the Mannich bases (II) obtained by the reaction of 2,3-disubstituted indolizines with formaldehyde and dimethylamine illustrates this similarity.

Received July 20, 1966, from the School of Pharmacy, Oregon State University, Corvallis. 97331.
Accepted for publication October 11, 1966.
Abstracted from a thesis submitted by William B. Harrell to the Graduate School, Oregon State University, in partial fulfillment of Doctor of Philosophy degree requirements.
* Fellow of the American Foundation for Pharmaceutical Education, 1963–1964 and 1965–1966. National Science Foundation Science Faculty Fellow, 1964–1965. Present address: Department of Pharmaceutical Chemistry, School of Pharmacy, Texas Southern University, Houston.

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3-Alkyl-1-dialkylaminomethyl Derivatives

By WILLIAM B. HARRELL* and ROBERT F. DOERGE

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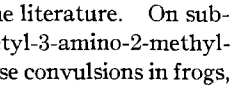
Having decided upon the indolizine structure, attention was focused on the various possibilities of chemical modification which might logically be expected to produce physiologically active compounds. Among the biologically active indoles, many are aminoalkyl derivatives such as serotonin, bufotenine, psilocin, reserpine, and lysergic acid diethylamide, all of which are known to have pronounced activity on the central nervous system. It was noted that in all of these compounds, the indole nitrogen and the extra-indole nitrogen are separated by four carbon atoms  , a spatial arrangement which might be a contributing factor in determining their activities. It was therefore reasoned that certain aminoalkylindolizines with the same carbon spread between the nitrogens might also possess activity on the central nervous system. After considering several possible chemical modifications, the Mannich reaction was chosen because the resulting dialkylaminomethyl substituent, when introduced at the C-1 position, would give the desired intramolecular spread. A comparison between bufotenine (I) and the Mannich bases (II) obtained by the reaction of 2,3-disubstituted indolizines with formaldehyde and dimethylamine illustrates this similarity.

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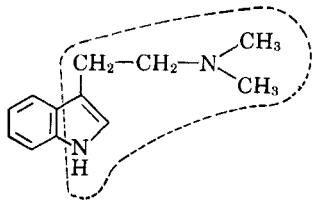
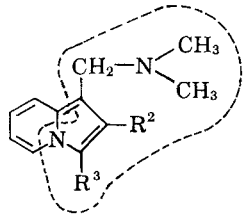


I



II

The purpose of this investigation was to synthesize several 1-substituted Mannich bases derived from selected 2-phenylindolizines to be evaluated for possible activity on the central nervous system. If interesting activity was found, a study of the relationship of chemical structure with activity would be conducted.

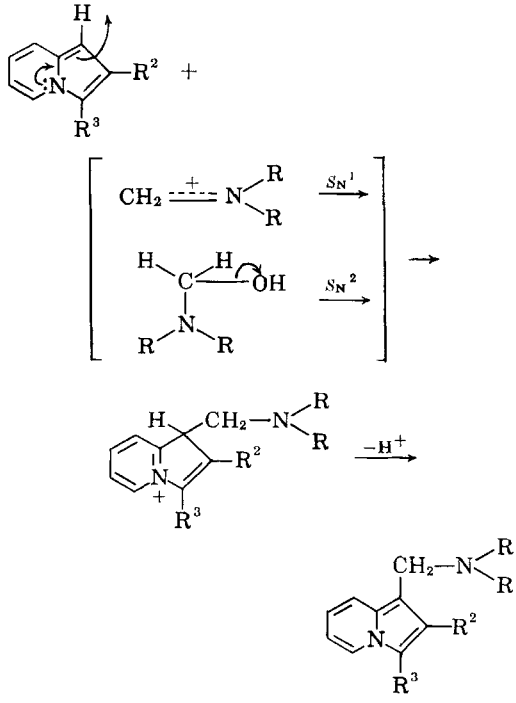
Preliminary pharmacological data were obtained for 1-diethylaminomethyl-3-ethyl-2-phenylindolizine (IV*b*). When injected in mice as a suspension in gum acacia, the compound exerted a depressant action on the central nervous system. The approximate LD_{50} was determined and found to be in the range of 70–100 mg./Kg. These compounds will be investigated more extensively and the results reported elsewhere.

DISCUSSION

The indolizines employed in this investigation were 3-methyl-2-phenylindolizine (III) and 3-ethyl-2-phenylindolizine (IV). These compounds were prepared by the Chichibabin synthesis (8) which involves condensing α-picoline with the appropriate phenacyl bromide and heating the resulting picolinium bromide in aqueous sodium bicarbonate.

There have been only two reports in the literature on the synthesis of Mannich bases from indolizines. Rossiter and Saxton obtained 2,3-dimethyl-1-dimethylaminomethylindolizine as an unstable oil using glacial acetic acid as the solvent (9). Carbon and Brehm (2), employing the same reaction conditions, prepared 3-acetyl-1-dimethylaminomethylindolizine. The present investigators, however, found that the acid conditions employed by these two groups gave undesirable results when applied to 2-phenylindolizines. Before proceeding further, it was considered advisable to examine more closely the mechanics of the Mannich reaction (10) with the hope that a set of reaction conditions could be devised that would be more favorable than those previously described in the literature.

The mechanism by which indolizines participate in the Mannich reaction has not been previously discussed in the literature, although several mechanisms have been proposed for the participation of ketones and other active hydrogen compounds (11–18). While most of the more prominent theories propose that the reaction results from a nucleophilic attack on a species of the type $(R_2N{=}CH_2)^+$ or $R_2N{-}CH_2OH$, the nature of the attacking nucleophile has not been clearly defined. According to Cummings and Shelton (14), two possibilities exist with ketones: attack by a carbanion of the type $R{-}\overset{O}{\overset{\|}{C}}{-}CH_2^-$, enhanced by running the reaction in basic medium, and attack by the enol form $R{-}\overset{:OH}{\overset{|}{C}}{=}CH_2$, which seems to be favored in acid medium. The present authors observed that ionization of indolizines in the presence of base is not likely to occur at the C-1 or C-3 position, the positions which are involved in the Mannich reaction, because of the high electron density at these positions (19, 20). It was also observed that, if such a carbanion should exist, it would be highly unstable due to the lack of resonance-stabilization. Since the enamine nature of indolizines has been observed (21) and because the similarity of enamines to enols is well established, the authors propose that indolizines participate in the Mannich reaction either by an S_N1 or S_N2 mechanism. (Scheme I.)



Scheme I

An important side reaction which can cause interference if conditions are not carefully controlled is the condensation of the indolizine with formaldehyde to form bis(indolizinyl)methanes. As a further means of control, the authors prepared bis(3-ethyl-2-phenyl-1-indolizinyl)methane (VI) and bis(3-methyl-2-phenyl-1-indolizinyl)methane (V) by reacting the corresponding indolizine with formalde-

hyde in the absence of a secondary amine. These are new compounds and the methods of synthesis are reported under *Experimental*. The compounds and their pertinent analytical data are given in Table I.

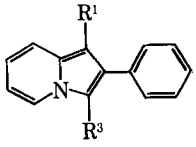
EXPERIMENTAL

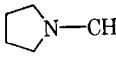
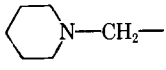
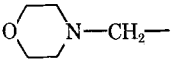
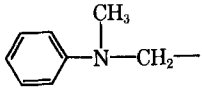
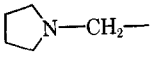
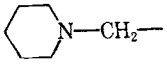
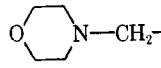
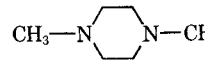
All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were provided by Weiler and Strauss Microanalytical Laboratory, Oxford, England. Preliminary pharmacological screening was performed by Dr. Robert Brummett, Department of Pharmacology, University of Oregon Medical School, Portland, Oreg.

Materials.—The preparation of 3 - methyl - 2 - phenylindolizine and 3-ethyl-2-phenylindolizine has been previously reported in the literature (22, 23).

Preparation of Mannich Bases.—The following general procedure was employed in the synthesis of these compounds. The formaldehyde (0.05 mole) and the secondary amine (0.05 mole) were combined and allowed to stand at room temperature for 15 min. The mixture was then added to a solution containing the indolizine (0.01 mole) dissolved in 30

TABLE I.—MANNICH BASES PREPARED



Compd.	Empirical Formula	R¹	R³	M.p.	Yield, %	Recrystn. Solvent	Anal., % Calcd.	Found
III*a*	$C_{18}H_{20}N_2$	$(CH_3)_2NCH_2$-	CH_3-	66–67°	86	Acetone–water	C, 81.78 H, 7.63 N, 10.60	C, 81.72 H, 7.71 N, 10.65
III*b*	$C_{20}H_{24}N_2$	$(C_2H_5)_2NCH_2$-	CH_3-	81–82°	89	Dioxane–water	C, 82.15 H, 8.27 N, 9.58	C, 82.36 H, 8.42 N, 9.34
III*c*	$C_{20}H_{22}N_2$		CH_3-	102–104°	89	Dioxane–water	C, 82.72 H, 7.64 N, 9.65	C, 82.45 H, 7.49 N, 9.40
III*d*	$C_{21}H_{24}N_2$		CH_3-	104.5–106.5°	90.5	Dioxane–water	C, 82.85 H, 7.95 N, 9.20	C, 82.79 H, 8.08 N, 9.05
III*e*	$C_{20}H_{22}N_2O$		CH_3-	133–134°	78	Dioxane–water	C, 78.40 H, 7.24 N, 9.14	C, 78.14 H, 7.31 N, 8.97
III*f*	$C_{23}H_{22}N_2$		CH_3-	131–132°	82.8	Acetone	C, 84.63 H, 6.79 N, 8.58	C, 84.60 H, 6.90 N, 8.47
IV*a*	$C_{19}H_{22}N_2$	$(CH_3)_2NCH_2$-	C_2H_5-	65.5–66.0°	87	Acetone–water	C, 81.97 H, 7.97 N, 10.06	C, 82.02 H 8.02 N, 10.15
IV*b*	$C_{21}H_{26}N_2$	$(C_2H_5)_2NCH_2$-	C_2H_5-	56–57°	80	Ethanol	C, 82.31 H, 8.55 N, 9.14	C, 82.16 H, 8.65 N, 9.21
IV*c*	$C_{21}H_{24}N_2$		C_2H_5-	78–79°	76	Ethanol	C, 82.85 H, 7.95 N, 9.20	C, 82.89 H, 8.04 N, 9.13
IV*d*	$C_{22}H_{26}N_2$		C_2H_5-	101–102°	79	Ethanol	C, 82.97 H, 8.23 N, 8.80	C, 83.07 H, 8.38 N, 8.80
IV*e*	$C_{21}H_{24}N_2O$		C_2H_5-	78–79°	75	Ethanol	C, 78.72 H, 7.55 N, 8.74	C, 78.88 H, 7.55 N, 8.81
IV*f*	$C_{22}H_{27}N_3$		C_2H_5-	117–118°	76	Acetone–water	C, 79.24 H, 8.16 N, 12.60	C, 79.05 H, 8.24 N, 11.96

ml. dioxane. The reaction flask was stoppered and allowed to stand at room temperature for 24 hr. In most instances the product crystallized on evaporation of the solvent under a stream of cold air accompanied by vigorous scratching with a glass rod. In cases where no crystallization occurred on evaporation, the residual oil was taken up in a minimum quantity of alcohol or acetone and placed in the freezing compartment of the refrigerator. The product crystallized after remaining in the refrigerator for periods ranging from 5 days to 3 weeks. The preparation of 3-ethyl-2-phenyl-1-pyrrolidinomethylindolizine is described in the following section and is typical of the experimental procedures employed.

3-Ethyl-2-phenyl-1-pyrrolidinomethylindolizine (IVc).—To 30 ml. *p*-dioxane was added 5.34 Gm. pyrrolidine (0.075 mole) and 6 ml. of 37% aqueous formaldehyde (0.075) mole and the resulting solution allowed to stand at room temperature for 15 min. To this mixture 3.3 Gm. 3-ethyl-2-phenylindolizine (0.015 mole) was added, and the flask was swirled until a clear solution was obtained. The reaction mixture was then set aside at room temperature for 24 hr. The clear solution containing the product was poured into an evaporating dish and cold air was blown across the surface to remove the solvent. Crystallization of the product occurred upon scratching the walls of the container with a glass rod during the evaporation process. The product was removed by filtration, recrystallized from ethanol, and yielded 3.5 Gm. (76%), m.p. 78–79°.

Bis(3 - methyl - 2 - phenyl - 1 - indolizinyl)methane (V).—Ten milliliters of 37% aqueous formaldehyde (0.125 mole) and 3 Gm. 3-methyl-2-phenylindolizine (0.015 mole) were dissolved in 30 ml. dioxane and the mixture was allowed to stand at room temperature for 48 hr. Crystallization of the product commenced after the first 24 hr. At the end of the 48-hr. period, 20 ml. of water was added in 5-ml. portions over a 2-hr. interval to complete crystallization. Rapid addition of the water tended to cause precipitation of an oil. The flask was allowed to stand an additional 24 hr. in the refrigerator. The crystalline product was then filtered and washed with 50% ethanol. The yield was 2.7 Gm. (87%), m.p. 162–163° dec. The product was recrystallized from hot acetone–water.

Anal.—Calcd. for $C_{31}H_{26}N_2$: C, 87.29; H, 6.14; N, 6.57. Found: C, 86.84; H, 6.14; N, 6.65.

Bis(3-ethyl-2-phenyl-1-indolizinyl)methane (VI).—Three milliliters of 37% aqueous formaldehyde (0.037 mole) and 1 Gm. 3-ethyl-2-phenylindolizine (0.0045 mole) were dissolved in 10 ml. of glacial acetic acid and the mixture was allowed to stand at room temperature for 48 hr. An equal volume of water was then added and the solution was made slightly basic with ammonium hydroxide. The gritty precipitate was filtered, washed with distilled water, and air dried. The product was dissolved in a minimum amount of hot ethyl acetate and, after cooling, was placed in the freezer compartment of the refrigerator. Crystallization occurred after 2 days in the refrigerator. The yield was 0.71 Gm. (70%), m.p. 106–107°.

Anal.—Calcd. for $C_{33}H_{30}N_2$: C, 87.19; H, 6.65; N, 6.16. Found: C, 87.29; H, 6.70; N, 6.20.

SUMMARY

Twelve new Mannich bases and two new bis-(indolizinyl)methanes derived from two indolizines have been synthesized. One of the compounds, 1 - diethylaminomethyl-3-methyl-2-phenylindolizine, has been tested and found to have CNS depressant activity.

REFERENCES

(1) Buu-Hoï, N. P., Jacquignon, P., Xuong, N. D., and Lavit, D., *J. Org. Chem.*, **19**, 1370(1954).
(2) Carbon, J. A., and Brehm, S., *ibid.*, **26**, 3376(1961).
(3) Venturella, V. S., *J. Pharm. Sci.*, **52**, 868(1963).
(4) Hirosawa, T., *Proc. Japan Pharmacol. Soc.*, **12**, 218 (1938).
(5) Lingens, F., *Z. Physiol. Chem.*, **331**, 56(1963).
(6) Venturella, V. S., *J. Pharm. Sci.*, **53**, 107(1964).
(7) *Ibid.*, **53**, 1166(1964).
(8) Chichibabin, A. E., *Chem. Ber.*, **60**, 1607(1927).
(9) Rossiter, E. D., and Saxton, J. E., *J. Chem. Soc.*, **1953**, 3654.
(10) Mannich, C., and Krösche, W., *Arch. Pharm.*, **250**, 647(1912).
(11) Alexander, E. R., and Underhill, E. J., *J. Am. Chem. Soc.*, **71**, 4014(1949).
(12) Bodendorf, K., and Koralewski, G., *Arch. Pharm.*, **271**, 101(1933).
(13) Cromwell, N. H., *J. Am. Chem. Soc.*, **68**, 2634(1949).
(14) Cummings, T. F., and Shelton, J. R., *J. Org. Chem.*, **25**, 419(1960).
(15) Feldman, J. R., and Wagner, E. C., *ibid.*, **7**, 31(1942).
(16) Lieberman, S. V., and Wagner, E. C., *ibid.*, **14**, 1001(1949).
(17) Roth, H. J., *Arch. Pharm.*, **294**, 623(1961).
(18) VanMarle, C. M., and Tollens, B., *Chem. Ber.*, **36**, 1351(1903).
(19) Fukui, K., Yonezawa, T., Nagata, C., and Shingu, H., *J. Chem. Phys.*, **22**, 1433(1954).
(20) Longuet-Higgins, H. E., and Coulson, C. A., *Trans. Faraday Soc.*, **43**, 87(1947).
(21) Colonna, M., Bruni, P., and Monti, A., *Gazz. Chim. Ital.*, **94**, 509(1964).
(22) Bragg, D. R., and Wibberley, D. G., *J. Chem. Soc.*, **1962**, 2627.
(23) Burrows, E. T., Holland, D. O., and Kenyon, J., *ibid.*, **1946**, 1083.

Mechanism of Crystallization of Hydrocortisone by Ultrasonic Irradiation

By STANLEY L. HEM*, DONALD M. SKAUEN, and HAROLD M. BEAL

Reproducible, reduced crystal size distributions resulted from insonating a supersaturated solution, whose background particle count is controlled, at a fixed temperature. The crystallization temperature of the insonated and uninsonated samples was dependent on the number of heterogeneous nuclei present. The ultrasonic energy used in this study was in excess of the amount required to reduce the particle size. Ultrasound acted in the initial phase of crystallization. Ultrasound is believed to act by eroding, shattering, or in some way disrupting nuclei that form in a normal manner.

Ultrasound has been effective in reducing the particle size of a large number of substances. It is surprising to note the many theories which have been suggested to explain the action of ultrasound in producing small, uniform crystals. The simplest suggestion, that the reduction in particle size is due to agitation, has been rejected by several authors (1, 2).

Experimentation on supercooled Wood's metal (3, 4) led to the conclusion that friction between the solid and the melt is responsible for the reduction in grain size. It has been generalized that the increased crystallization rate of metals is due to an increased number of crystallization centers as a result of ultrasonic scattering of the microcrystals forming in the melt (5). Ultrasound may increase the number of crystal nuclei by shattering or eroding the nuclei that occur in a normal manner (6, 7). A number of reports have concluded that ultrasound accelerates the process of spontaneous crystallization and plays an essential role in the formation of nuclei (8). Brown (9) and Mazhul (10, 11) have stated that cavitation bubbles themselves act as nuclei for the crystals to initially form around.

The increased crystallization velocity of supercooled benzophenone was attributed to the localized removal of heat at the phase boundaries (12).

Evaporation from the surface of the cavitation bubble during cavity growth may cause sufficient cooling to initiate nucleation (13).

The large pressures which occur after the collapse of the cavitation cavity may cause a sufficient change in melting point to cause nucleation (14).

The effect of ultrasound was hypothesized to be analogous to an increase in the supersaturation of the solution (15).

Thus, it has been established that ultrasonic treatment during a phase change can improve the product in terms of an increased rate of crystallization and a smaller, more uniform size distribution. At present no clear understanding of the mechanism of ultrasonic crystallization is available.

EXPERIMENTAL

Cohn's (16) investigation of the factors involved in the ultrasonic crystallization of hydrocortisone was chosen as a foundation for this study. The conditions in terms of solvent, degree of supersaturation, and treatment temperature that were reported to yield the smallest particle size were used. Thus, a saturated solution of hydrocortisone in ethanol was prepared at 65°.

The solubility curve for hydrocortisone in ethanol has been reported by Cohn (17). The pertinent values for this study are given in Table I.

Table I.—Solubility of Hydrocortisone in Ethanol

Temp., °C.	Concn., mg./ml.
65	63.1
55	42.3
50	36.2
45	31.5
40	25.4
35	21.5
30	17.7
25	15.4

The solution was filtered while at 65° through either a 0.45-μ membrane filter or a Whatman No. 42 filter paper which was also maintained at 65°. A 10-ml. sample of the saturated solution was transferred by a warm pipet to the treatment vessel and covered. The solution was permitted to cool from 65° to the bath temperature.

The Sonifier, model S-125,[1] provided the ultrasonic energy. It is a high-intensity piezoelectric type source with a frequency of 20 Kc./sec.

The geometry of the treatment vessel and the Sonifier tip was the same as reported by Cohn (16). The Sonifier tip was positioned 2.3 mm. from

Received July 28, 1966, from the Pharmacy Research Institute, University of Connecticut, Storrs.
Accepted for publication October 25, 1966.
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[1] Branson Sonic Power Co., Danbury, Conn.

the bottom of the 20-ml. beaker. The Sonifier, model S-125, has a fully transistorized power supply; no warm-up time was required as power was available immediately. This enabled treatment times of 5 sec. to be employed. Weissler's (18) method for measuring relative cavitation intensity confirmed that this geometry provided maximum ultrasonic effects.

Weissler's (18) method for measuring relative cavitation intensity was used to calibrate the Sonifier to permit operation at several intensities. The method is based on the sonochemical liberation of chlorine from carbon tetrachloride dissolved in water. *o*-Tolidine was reacted with the liberated chlorine and yielded a yellow color suitable for colorimetry. The absorbance at 436 mμ wavelength is linear with respect to the chlorine concentration. Various ultrasonic intensities were defined by plotting the absorbance against time. The slope of the straight line was used to designate a particular intensity. Calibration curves for the 3 cavitation levels used in this study are given in Fig. 1.

Weissler's (18) method requires measurement in an aqueous system. The addition of small amounts of such chemicals as petroleum ether, ethyl ether, and ethanol completely block the sonochemical decomposition of carbon tetrachloride. It has been suggested that these substances have a protective action on the solution. They have high vapor pressures and are thought to penetrate the cavitation bubble and interfere with the electronic effects associated with cavitation (19). It has also been suggested that the presence of organic liquids reduces the thermal conductivity of the gas-filled bubbles. This may reduce the thermal reactions occurring in the cavity. The possibility exists that the presence of these compounds causes a change in the decomposition products of carbon tetrachloride (20).

The crystallizations in this study were carried out in ethanol. Cavitation depends upon the surface tension, viscosity, density, vapor pressure, cohesive strength, and heat transfer of the liquid (21). At a fixed setting the amount of cavitation in an aqueous solution will differ from the amount in an alcoholic solution. The linear nature of the absorbance *versus* time plot would indicate a fairly simple, direct relationship among these solvent variables. Thus, it can be assumed that the relative cavitation levels in an aqueous solution will be directly related to the relative cavitation levels in an alcoholic solution.

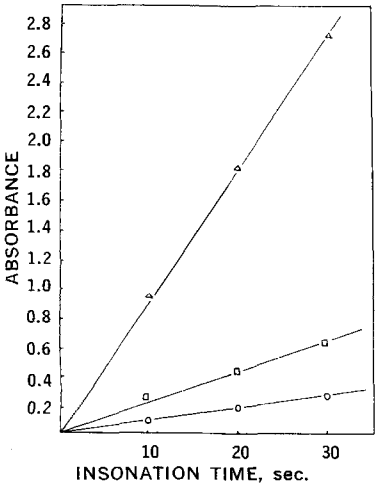

Fig. 1.—Calibration curves for measuring relative cavitation intensity. Key: △, 120 v., setting 8; □, 120 v., setting 2; ○, 100 v., setting 2.

The measurement of cavitation has been one of the major problems in ultrasonic research. There is no other satisfactory method available for measuring cavitation. Previous to Weissler's method it was impossible to accurately calibrate an instrument or monitor its day to day operation. In view of these conditions it was felt that this assumption was justified.

At a line voltage of 120 v. and a power level setting of 8 (maximum for the Sonifier), Weissler's (18) method gave a slope of 0.090. A line voltage of 120 v. and a power level setting of 2 gave a slope of 0.021. A slope of 0.009 was obtained at a line voltage of 100 v. and a power level setting of 2. These calibration curves are shown in Fig. 1.

These intensity levels are in the approximate ratio of 10:2:1. Although the cavitation levels were different in ethanol, it was assumed that these settings would also give ultrasonic intensities in ethanolic solutions in the ratio of 10:2:1.

The collection of a sample of crystals for evaluation was performed by vacuum filtration using a 0.20-μ membrane filter. Only a small sample of crystals was required for size analysis by the Coulter counter.[2] It was possible to obtain the necessary sample by quickly removing the mother liquor from 1 ml. or less of the crystallization suspension.

Particle size distributions were determined using a Coulter counter with a 70-μ aperture tube. It was found that an aqueous electrolyte of 0.9% sodium chloride saturated with hydrocortisone was suitable. The crystal sample was dispersed with 1 drop of 2% methylcellulose 400[3] prior to analysis. Enough sample was added to the counting vessel to give between 10,000 and 20,000 counts per 500 μl.

The particle size distributions were found to follow the log-normal distribution law. The geometric mean, $\overline{M}g$, the most appropriate measure of central tendency for log-normal systems, and the geometric standard deviation, σg, were used to characterize the results of the crystallizations. As these values are based on the measurement of 10,000 to 20,000 individual crystals, they are the best estimates available of the population mean and the population standard deviation for each case (22–24).

Two series of experiments were performed. In the first a saturated solution was prepared at 65°. The solution was filtered through a 0.45-μ membrane filter or Whatman No. 42 filter paper and transferred to the treatment vessel. A series was also performed on the unfiltered supernatant. Preparation of the solution, then filtering a portion through a membrane filter or filter paper was done to vary the number of heterogeneous particles present in the saturated solution. This was confirmed by the Coulter counter data in Table III.

[2] Coulter Electronics, Hialeah, Fla.
[3] Marketed as Methocel 400 by the Dow Chemical Co., Midland, Mich.

The 65° saturated solution was allowed to stand undisturbed for 2 hr., and then 10 ml. of supernatant was transferred by pipet to the treatment vessel. The Sonifier was positioned, and the solution was covered. A number of treatment bath temperatures were used varying from 55 to 30° in 5° intervals. The solution was examined 60 min. after reaching thermal equilibrium. A visual observation for the presence or absence of crystals was performed. This procedure was repeated with the application of ultrasound to the clear solution after thermal equilibrium in the treatment bath had been reached. Ultrasonic energy was applied for 1 min. at relative cavitation levels of 10 and 1. Each solution was visually examined after 1 hr. for the presence of crystals. These results are contained in Table II.

In order to evaluate the number of particles in the saturated solution before treatment, a particle count was run with the Coulter counter. Because the Coulter counter requires a conductive solution it was impossible to perform a background count on the alcoholic solutions. An aqueous 0.9% sodium chloride solution was saturated with hydrocortisone at room temperature. The clear supernatant was either taken as the sample or the solution was filtered through 0.45-μ membrane filter or Whatman No. 42 filter paper. The particle size distribution of each of these solutions was then determined by Coulter counter analysis. They are listed in Table III. The results were taken as approximations of the number of potential heterogeneous nuclei present in the crystallization solution.

TABLE II.—OCCURRENCE OF CRYSTALLIZATION IN FILTERED SOLUTIONS

Treatment Temp., °C.	Unfiltered Soln.			Whatman No. 42 Filter Paper			0.45-μ Membrane Filter		
	No US[a]	1[b]	10[b]	No US[a]	1[b]	10[b]	No US[a]	1[b]	10[b]
55	+	+	+	−	−	−	−	−	−
50	+	+	+	−	−	−	−	−	−
45	+	+	+	−	−	−	−	−	−
40	+	+	+	+	+	+	−	−	−
35	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+

[a] No ultrasound applied. [b] Relative cavitation intensity.

TABLE III.—COULTER COUNTER ANALYSIS OF SATURATED SOLUTIONS AFTER FILTRATION

D(diam.), μ	No. of Particles Larger than D in 0.5 ml. of Saturated Soln. After Indicated Treatment: Unfiltered Supernatant	Whatman No. 42 Filter Paper	0.45-μ Membrane Filter
1.25	78,000	31,000	250
2.00	28,000	7,500	62
3.00	12,000	2,500	18
5.00	3,700	700	5
6.00	2,000	350	—
8.00	800	150	—
10.00	300	100	—
12.00	140	35	—
14.00	100	30	—
16.00	40	22	—
18.00	21	14	—
20.00	8	4	—

TABLE IV.—PARTICLE SIZE DISTRIBUTIONS OBTAINED AT 35°

Insonation Time, sec.	Relative Cavitation Intensity 1: $\bar{M}g$	σg	2: $\bar{M}g$	σg	10: $\bar{M}g$	σg
60	4.7 μ	2.5	4.7 μ	2.5	4.9 μ	2.3
15	4.7 μ	2.4	4.9 μ	2.2	5.1 μ	2.3
5	4.8 μ	2.4	4.7 μ	2.6	4.9 μ	3.1

Control: $\bar{M}g$ 9.5 μ, σg 2.8

TABLE V.—PARTICLE SIZE DISTRIBUTIONS OBTAINED AT 25°

Insonation Time, sec.	Relative Cavitation Intensity 1: $\bar{M}g$	σg	2: $\bar{M}g$	σg	10: $\bar{M}g$	σg
60	4.7 μ	2.5	4.6 μ	2.3	4.6 μ	2.3
15	4.4 μ	2.4	4.5 μ	2.4	4.3 μ	2.4
5	4.6 μ	2.3	4.6 μ	2.3	4.3 μ	2.4

Control: $\bar{M}g$ 9.2 μ, σg 2.7

The second experiment examined the effect of ultrasonic intensity on the crystal size distributions. A saturated solution was prepared at 65°, filtered through a 0.45-μ membrane filter, and brought to thermal equilibrium in either a 25 or 35° water bath. Thirty-five degrees was chosen as one treatment temperature because this was the highest temperature at which crystals developed after filtration through a 0.45-μ membrane filter. The solution was clear to the eye at this point. It was then treated with ultrasound at a relative cavitation intensity of either 10, 2, or 1 for 60, 15, or 5 sec. The crystals were collected and their size distributions determined with the Coulter counter. The crystal size distributions are shown in Tables IV and V.

RESULTS AND DISCUSSION

The results of the crystallizations from filtered solutions are given in Table II. Table III lists the Coulter counter analysis of these solutions. The number of particles in the solution prior to treatment represents contamination by foreign material contained in either the hydrocortisone or the ethanol, or originating from the glassware. Undissolved hydrocortisone may also contribute to the total particle count.

It was observed that the crystallization temperature of the untreated samples decreased as the number of particles in the saturated solution was reduced. This behavior is in agreement with the heterogeneous nucleation theory.

Heterogeneous nucleation refers to nucleation which occurs on particles which are present in the solution. Filtration would remove some of these potential nucleation sites and cause a reduction in the temperature at which crystals appear. A similar observation has been reported by Melia and Moffitt (25).

Heterogeneous nucleation can be better understood by comparing it with homogeneous nucleation. In supersaturated solutions there is continual formation and dissolution of molecular clusters of

solute (26). The Becker-Doring theory (26, 27) and the Christiansen-Nielsen theory (27) view this as a series of bimolecular collisions. These clusters may continue to grow by collision or may shrink due to the loss of individual molecules. When the cluster reaches a point where the tendency to dissociate is balanced by the tendency to grow (in order to decrease surface free energy) it is termed a critical nucleus. The size of the critical cluster is unknown but it is thought to be several hundred molecules for most solids (27, 28). The tendency to grow is dominant once a cluster is larger than the critical nucleus. Nucleation followed by crystal growth then proceeds.

The energy barrier to nucleation may be reduced by the catalytic effect of foreign particles in the solution. The presence of these substrates for the critical nucleus permit nucleation to occur more readily (26, 29).

The activation energy of heterogeneous nucleation, $\Delta G_{het.}$, can be related to the activation energy of homogeneous nucleation, $\Delta G_{hom.}$ An equation (26) has been derived for the nucleation of vapor drops on surfaces in terms of the contact angle, θ.

$$\Delta G_{het.} = \frac{\Delta G_{hom.}\,(2 + \cos\theta)(1 - \cos\theta)^2}{4}$$

It is seen from the equation that $\Delta G_{het.}$ will always be smaller than $\Delta G_{hom.}$ If heterogeneous nucleation is possible it will cause crystallization to occur more readily than homogeneous nucleation. In most cases crystallization will be complete before homogeneous mechanisms have begun.

It was observed that ultrasound did not cause crystallization to occur at any temperature above the crystallization temperature of the untreated samples. This temperature was dependent on the number of particles present in the solution prior to insonation. The behavior of the insonated samples is in agreement with the heterogeneous nucleation theory. There is no indication that ultrasound initiates crystallization. It only affected solutions at or below their normal crystallization temperatures.

The particle size distributions obtained at 35° with varying cavitation intensities and treatment times are given in Table IV. The results of similar experiments at 25° are given in Table V. These results were obtained by Coulter counter analysis. This instrument proved very suitable for characterizing particle size distributions. A number of problems were encountered in calibrating the instrument, finding a suitable electrolyte, obtaining complete dispersion of the crystal cake, and developing an adequate technique. However, the advantages in terms of number of particles counted, reproducibility, and speed would recommend this technique for future particle size studies.

The particle size distributions show virtually no difference for treatment times ranging from 5 to 60 sec. Cohn (17) reported that treatment times of 1 to 15 min. showed no effect on particle size. This indicates that ultrasound acts at an early stage in the crystallization process, and that continued insonation has no effect.

Little change was noted in the particle size distribution when varying relative cavitation intensities were used. An excess of energy was available for this application.

Based on this study the following mechanism of ultrasonic crystallization is visualized. A cavitation bubble collapses with great force in the proximity of a nucleus or a growing crystallite. The high pressure generated is sufficient to erode, shatter, or in some way disrupt the small particle or crystallite. This results in the formation of new nuclei. These nuclei may grow into crystals or may be further dispersed by cavitation. The net effect is a great multiplication in the number of nuclei in the supersaturated solution. This results in reduced crystal size and an increased rate of crystallization. Ultrasound does not initiate nuclei but acts on nuclei which normally appear in the solution.

SUMMARY AND CONCLUSIONS

1. Crystal size was reduced by the application of ultrasound to a supersaturated solution of hydrocortisone in ethanol.

2. Reproducible distributions resulted from insonating a supersaturated solution, whose background particle count is controlled, at a fixed temperature.

3. The crystallization temperature of the supersaturated solution was observed to depend on the number of heterogeneous nuclei present.

4. Ultrasound did not initiate crystallization above the crystallization temperature of the untreated supersaturated solution.

5. Ultrasound acts in the initial phase of crystallization. The size distributions obtained after 5-sec. insonation compared favorably with those obtained after 60-sec. insonation.

6. The ultrasonic energy used in this study was in excess of the amount required to reduce the particle size.

7. Ultrasound is believed to act by eroding, shattering, or in some way disrupting nuclei that form in a normal manner. This effect increased the number of crystal nuclei and resulted in reduced particle size.

8. The Coulter counter was adapted for measuring the hydrocortisone crystals. The advantages of this technique in terms of number of particles counted, reproducibility, and speed would recommend it for future particle size studies.

REFERENCES

(1) Abramov, O. V., Neimark, V. E., and Teumin, I. I., *Fiz. Metal. i Metalloved.*, **13**, 875(1962); through *Chem. Abstr.*, **57**, 14832*f*(1962).

(2) Straughn, J. L., and Tarpley, W. B., *U. S. At. Energy Comm.*, NYO-7935(1959); through *Chem. Abstr.*, **54**, 19025*f* (1960).

(3) Schmid, G., and Roll, A., *Z. Elektrochem.*, **45**, 769 (1939); through Kapustin, A. P., "The Effects of Ultrasound on the Kinetics of Crystallization," Consultants Bureau, New York, N. Y., 1963, p. 16.

(4) Schmid, G., and Roll, A., *Z. Elektrochem.*, **46**, 653 (1940); through Kapustin, A. P., "The Effects of Ultrasound on the Kinetics of Crystallization," Consultants Bureau, New York, N. Y., 1963, p. 16.

(5) Kudryavtsev, B. B., in "Ultrasound in Industrial Processing and Control," Nozdreva, V. F., ed., Consultants Bureau, New York, N. Y., 1964, p. 34.

(6) Kapustin, A. P., *Zh. Tekhn. Fiz.*, **22**, 765(1952); through *Chem. Abstr.*, **50**, 6123*c*(1956).

(7) Polotskii, I. G., and Levin, G. I., *Kristallografiya*, **7**, 645(1962); through *Chem. Abstr.*, **57**, 14505*d*(1962).

(8) Chormonov, T. K., *Sb. Nauchn. Tr. Kazakhsk. Politekhn. Inst.*, **21**, 271(1960); through *Chem. Abstr.*, **60**, 12727*b*(1964).

(9) Brown, B., and Goodman, I. E., "High Intensity Ultrasonics," D. Van Nostrand Co., Princeton, N. J., 1965, p. 209.

(10) Mazhul, M. M., "Cavitation Phenomena and the Formation of Crystal Nuclei," Dissertation, 1954; through

Kapustin, A. P., "The Effects of Ultrasound on the Kinetics of Crystallization," Consultants Bureau, New York, N. Y., 1963, p. 14.
(11) Mazhul, M. M., *Uchenye Zapiski Beloruss. Univ., No. 32*, **1957**, 211; *Referat. Zhur., Met.*, Abstr. No. 24786 (1957); through *Chem. Abstr.*, **53**, 17619*c*(1959).
(12) Gunther, P., Zeil, W., Goring, W., and Gornhardt, J. F., *Z. Anorg. Allgem. Chem.*, **285**, 191(1956); through *Chem Abstr.*, **50**, 15157*c*(1956).
(13) Chalmers, B., G. M. Research Laboratory Symposium, 1963; through Hunt, J. D., and Jackson, K. A., "Nucleation of Solid in an Undercooled Liquid by Cavitation," presented to the International Symposium on Nucleation Phenomena, Case Institute of Technology, Cleveland, Ohio, April 1965.
(14) Hunt, J. D., and Jackson, K. A., "Nucleation of Solid in an Undercooled Liquid by Cavitation," presented to the International Symposium on Nucleation Phenomena, Case Institute of Technology, Cleveland, Ohio, April 1965.
(15) Glavatskii, D. E., and Kortnev, A. V., *Nauchn. Zap. Odessk. Politekhn. Inst.*, **41**, 22(1962); through *Chem. Abstr.*, **59**, 2232*g*(1963).
(16) Cohn, R. M., Ph. D. Dissertation, The Graduate School, University of Connecticut, Storrs, Conn., 1963.
(17) Cohn, R. M., and Skauen, D. M., *J. Pharm. Sci.*, **53**, 1040(1964).
(18) Weissler, A., *IRE Intern. Conv. Record, Part 6*, **1962**, 24.
(19) El'piner, I. E., "Ultrasound Physical, Chemical, and Biological Effects," Consultants Bureau, New York, N. Y., 1964, pp. 26, 27.
(20) Fitzgerald, M. E., Griffing, V., and Sullivan, J., *J. Chem. Phys.*, **25**, 926(1956).
(21) Frederick, J. R., "Ultrasonic Engineering," John Wiley & Sons, Inc., New York, N. Y., 1965, p. 118.
(22) Senders, V. L., "Measurement and Statistics," Oxford University Press, New York, N. Y., 1958, p. 316.
(23) Irani, R. R., and Callis, C. F., "Particle Size: Measurement, Interpretation, and Application," John Wiley & Sons, Inc., New York, N. Y., 1963, p. 41.
(24) Austin, J. B., *Ind. Eng. Chem., Anal. Ed.*, **11**, 338 (1939).
(25) Melia, T. P., and Moffitt, W. P., *J. Colloid Sci.*, **19**, 433(1964).
(26) Walton, A. G., *Science*, **148**, 601(1965).
(27) Laitinen, H. A., "Chemical Analysis," McGraw-Hill Book Co., New York, N. Y., 1960, pp. 127–133.
(28) Van Hook, A., "Crystallization Theory and Practice," Reinhold Publishing Corp., New York, N. Y., 1961, p. 113.
(29) *Ibid.*, pp. 127, 128.

Variability of Uniformity of Weight Test as an Indicator of the Amount of Active Ingredient in Tablets

By J. M. AIRTH, D. F. BRAY, and C. RADECKA

Fifty-four samples, embracing five drugs sold as compressed tablets, were obtained. Individual tablet weights were determined and individual chemical assays were carried out on 10 tablets from each sample. There was little or no relationship between the amount of active ingredient and tablet weight for promazine tablets in which the active ingredient formed a small proportion (15–23 per cent) of tablet weight, but this relationship was high for tolbutamide tablets in which the active ingredient formed a large proportion (73–90 per cent) of tablet weight. This observation suggests that the uniformity of weight test may sometimes be usefully employed instead of individual tablet chemical assays when the proportion of active ingredient in the tablets is high and that the emphasis in developing direct measures of content uniformity should be placed on preparations containing small proportions of active ingredient. The present data do not confirm the observation of Moskalyk *et al.* (1961) that "Greater deviations 'in active ingredient' were found to occur in lighter weight tablets" within preparations.

THE UNIFORMITY of weight test has been included in the "British Pharmacopoeia," the "United States Pharmacopeia," and the "National Formulary" for many years. Its purpose has been to provide an indication of the amount of active ingredient in each tablet. The validity of such a test depends, of course, on the assumption that the amount of active ingredient is directly proportional to the weight of the tablet. While various aspects of the uniformity of weight test have been studied previously (see for example *References 1–3*), general recognition of its inadequacy in certain situations has only recently been recognized. The 17th revision of the U.S.P. and the 12th edition of the N.F. included for the first time a content uniformity test for certain tablets. While determinations of the amount of active ingredient in individual tablets will be necessary for some preparations, there seems little point in taking these more costly observations in cases where the uniformity of weight test gives comparable results. It is the purpose of this paper to present some data which suggest that the uniformity of weight test may be satisfactory for some tablet preparations, but inadequate for others.

Received July 18, 1966, from the Research Laboratories, Food and Drug Directorate, National Health and Welfare Department, Ottawa, Ontario, Canada.
Accepted for publication November 7, 1966.
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MATERIALS AND METHODS

Samples of five drugs sold in compressed tablet form were procured from Canadian retail outlets in bottles of 100 tablets. A total of 54 samples from 36 different companies were obtained. In general, not more than one sample of each drug was obtained from each company, and in fact 53 of the possible 180 company-compound combinations are represented.

Kapustin, A. P., "The Effects of Ultrasound on the Kinetics of Crystallization," Consultants Bureau, New York, N. Y., 1963, p. 14.
(11) Mazhul, M. M., *Uchenye Zapiski Beloruss. Univ., No. 32*, **1957**, 211; *Referat. Zhur., Met.*, Abstr. No. 24786 (1957); through *Chem. Abstr.*, **53**, 17619*c*(1959).
(12) Gunther, P., Zeil, W., Goring, W., and Gornhardt, J. F., *Z. Anorg. Allgem. Chem.*, **285**, 191(1956); through *Chem Abstr.*, **50**, 15157*c*(1956).
(13) Chalmers, B., G. M. Research Laboratory Symposium, 1963; through Hunt, J. D., and Jackson, K. A., "Nucleation of Solid in an Undercooled Liquid by Cavitation," presented to the International Symposium on Nucleation Phenomena, Case Institute of Technology, Cleveland, Ohio, April 1965.
(14) Hunt, J. D., and Jackson, K. A., "Nucleation of Solid in an Undercooled Liquid by Cavitation," presented to the International Symposium on Nucleation Phenomena, Case Institute of Technology, Cleveland, Ohio, April 1965.
(15) Glavatskii, D. E., and Kortnev, A. V., *Nauchn. Zap. Odessk. Politekhn. Inst.*, **41**, 22(1962); through *Chem. Abstr.*, **59**, 2232*g*(1963).
(16) Cohn, R. M., Ph. D. Dissertation, The Graduate School, University of Connecticut, Storrs, Conn., 1963.
(17) Cohn, R. M., and Skauen, D. M., *J. Pharm. Sci.*, **53**, 1040(1964).
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(19) El'piner, I. E., "Ultrasound Physical, Chemical, and Biological Effects," Consultants Bureau, New York, N. Y., 1964, pp. 26, 27.
(20) Fitzgerald, M. E., Griffing, V., and Sullivan, J., *J. Chem. Phys.*, **25**, 926(1956).
(21) Frederick, J. R., "Ultrasonic Engineering," John Wiley & Sons, Inc., New York, N. Y., 1965, p. 118.
(22) Senders, V. L., "Measurement and Statistics," Oxford University Press, New York, N. Y., 1958, p. 316.
(23) Irani, R. R., and Callis, C. F., "Particle Size: Measurement, Interpretation, and Application," John Wiley & Sons, Inc., New York, N. Y., 1963, p. 41.
(24) Austin, J. B., *Ind. Eng. Chem., Anal. Ed.*, **11**, 338 (1939).
(25) Melia, T. P., and Moffitt, W. P., *J. Colloid Sci.*, **19**, 433(1964).
(26) Walton, A. G., *Science*, **148**, 601(1965).
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(28) Van Hook, A., "Crystallization Theory and Practice," Reinhold Publishing Corp., New York, N. Y., 1961, p. 113.
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TABLE I.—METHODS OF ASSAY

Drug	Samples, No.	S.D., % Recovery	Analytical Method
Promazine hydrochloride	5	0.37	Milne and Chatten (4)
Isoniazid	7	0.60	Scott (5)
Phenylbutazone	13	0.60	B.P. (6) with refinement to permit assay of 100 mg. of drug
Phenobarbital	7	0.60	Chatten (7) and/o Mattson (8)
Tolbutamide	22	0.32	U.S.P. (9)

Ten tablets were taken from each bottle for chemical assay. Two methods of selecting the tablets for assay were used. In 18 samples, 10 tablets were chosen in an essentially random manner. In the remaining 36 samples, 100 tablets were weighed and the 3 lightest, the 4 medium, and the 3 heaviest were selected for analysis.

The 10 selected tablets from each sample were individually weighed to the nearest 0.1 mg. and individually chemically assayed by the appropriate method indicated in Table I.

A single determination was carried out on each tablet by one of the authors (Radecka). Experience in this laboratory has shown that the assay error is uniformly small for all methods reported here. Values are given in Table I as standard deviations of per cent recovery.

RESULTS AND DISCUSSION

Linear regression techniques were used to study the relationship between amount of active ingredient and tablet weight. The slope (b) of the least squares regression line fitted to the data from each sample was compared with the one which indicated no relationship between active ingredient and tablet weight (β_0), and with the one which assumed the amount of active ingredient to be directly proportional to tablet weight (β_1). The former theoretical line passes through the bivariate mean of the sample data and has slope (β_0) equal zero, while the latter passes through the origin, and has slope $(\beta_1) = \frac{\Sigma xy}{\Sigma x^2}$ which in these data is extremely close to $\bar{y}/\bar{x}$, where y is the amount of active ingredient in a tablet in mg., and x is the tablet weight in mg.

A better estimate of β_1 could be obtained if the tablets had been procured directly from the manufacturer along with data indicating the actual amounts of active ingredient and other materials used. The authors have assumed that such data would give estimates of β_1 which would be essentially similar to those reported here.

It follows, therefore, that any given sample can be placed into one of four categories on the basis of the calculated value of the regression (b) of active ingredient on tablet weight ($P = .05$):

I, not significantly different from either β_0 or β_1.
II, significantly different from β_1, but not β_0.
III, significantly different from both β_1 and β_0.
IV, significantly different from β_0, but not β_1.

Both category I and category II imply the absence of a slope, and hence indicate no useful relationship between active ingredient and tablet weight. In contrast, both categories III and IV indicate the presence of a slope, in the latter case not demonstrably different from β_1, and hence suggest a situation in which the uniformity of weight test may possibly be used as an indicator of content uniformity. A typical example of a sample falling into each category is illustrated in Fig. 1.

Table II gives the number of samples in each category by drug and range of per cent active ingredient. The samples have been divided into a low group and a high group on the basis of per cent active ingredient, with the division occurring at 33%. This classification is an arbitrary one and is simply that suggested by the observed frequencies. Table II suggests that a relationship exists between the proportion of active ingredient and the category of response. Promazine tablets, having the smallest proportion of active ingredient, gave only category I and II responses, suggesting that the uniformity of weight test would be unsatisfactory as an indicator of content uniformity. Tolbutamide tablets, having the highest proportion of active ingredient, gave only category III and IV responses, suggesting that the uniformity of weight test may be usefully employed in such situations.

Since the drugs which gave the extreme responses were also those at the extremes in per cent active ingredient, it is impossible to state whether the observed relationship with category is a property of per cent active ingredient or a property of the drug examined. Only two drugs, phenylbutazone and

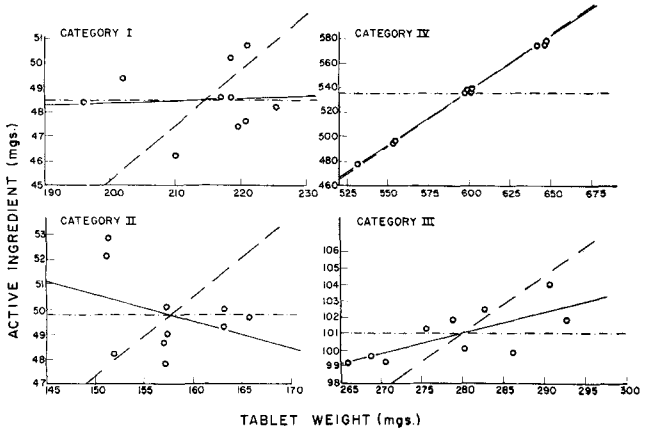


Fig. 1.—Examples of regression relationships. Key: category I, promazine, 23% active ingredient; category II, isoniazid, 32% active ingredient; category III, phenylbutazone, 36% active ingredient; category IV, tolbutamide, 90% active ingredient; · · · · · · · ·, β_0; — — —, β_1; ————, b.

TABLE II.—NUMBER OF SAMPLES IN EACH CATEGORY[a] INDICATED BY SIGNIFICANCE TESTS OF REGRESSION COEFFICIENTS

Drug	Range of % Active Ingredient		Category							
			I		II		III		IV	
	Low	High	Low	High	Low	High	Low	High	Low	High
Promazine	15–23		2		3		0		0	
Isoniazid	20–32		1		3		0		3	
Phenylbutazone	23–33	36–43	2		1		0		2	
				3		3		1		1
Phenobarbital	26–31	33–60	1		0		0		3	
				0		0		2		1
Tolbutamide		73–90								
				0		0		4		18

[a] See text for description of categories.

TABLE III.—NUMBER OF SAMPLES BY PER CENT ACTIVE INGREDIENT AND CATEGORY

Active Ingredient, %	Cagetory	
	I and II	III and IV
Low	13	8
High	6	27

phenobarbital, gave sufficiently varied responses and were represented over a sufficiently wide range of per cent active ingredient to give an indication of the effect of per cent active ingredient free from the effect of drug. Table II illustrates that the results of this examination are inconclusive.

The summary given in Table III illustrates the apparent relationship between per cent active ingredient and category. While some caution is needed in interpreting this table due to the partial confounding of drug with per cent active ingredient described in the preceding paragraph, it may be helpful to note that the interaction chi-square is significant at $P = 0.01$.

The data presented here were examined in the light of the observation of Moskalyk *et al.* (1) that, "Greater deviations," in active ingredient, "were found to occur in lighter weight tablets," within preparations. The variance about the line with slope β_1 was computed for the three largest and the three smallest tablets for all 54 samples. The ratio

$$\frac{\text{variance about the line with slope } \beta_1 \text{ of 3 largest tablets}}{\text{variance about the line with slope } \beta_1 \text{ of 3 smallest tablets}}$$

was formed for each sample. Values of the ratio less than 1 support Moskalyk's statement while those greater than 1 do not. In fact, 28 ratios were less than 1, and 26 were greater. This result is not different from the 1:1 ratio which is expected on the basis of no difference between the two variances. At one point in the paper of Moskalyk *et al.* the observation was restricted to "less uniformly mixed brands," these being the ones with large over-all variation about the line having slope β_1. If this hypothesis were true, the ratio would be expected to be small when the variance about the line for all 10 tablets is large, and tend toward 1 when the variance is small. No relationship between variance and ratio is apparent in these data (Fig. 2).

Since the preceding discussion is based on a model assuming that all points lie about the line passing through the origin and since this is not the best fitting line for all 54 samples, the same techniques

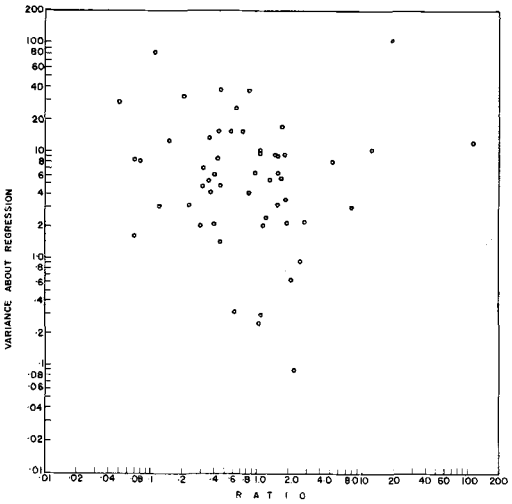


Fig. 2.—Lack of a relationship between variance about regression and ratio:

$$\frac{\text{variance about regression of larger tablets}}{\text{variance about regression of smaller tablets}}$$

were applied to the variation about the least squares line without restriction, *i.e.*, having slope b. A 31:23 ratio of ratios was obtained and the plot was similar to Fig. 2. Not all samples gave exactly similar results, but the conclusions are not different.

The present study fails to confirm the observation of Moskalyk *et al.* that "Greater deviations," in active ingredient," were found to occur in lighter weight tablets." A reason for the apparent contradiction may lie in the fact that no statistical tests were applied to the data bearing on this point in their paper.

REFERENCES

(1) Moskalyk, R. E., Chatten, L. G., Cox, C. E., and Pernarowski, M., *J. Pharm. Sci.*, **50**, 651(1961).
(2) Arambulo, A. S., Fu, H. S., and Deardorff, D. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 692(1953).
(3) Train, D., *ibid.*, **49**, 265(1960).
(4) Milne, J. B., and Chatten, L. G., *J. Pharm. Pharmacol.*, **9**, 686(1957).
(5) Scott, P. G. W., *ibid.*, **4**, 681(1952).
(6) "British Pharmacopoeia," the General Medical Council, Pharmaceutical Press, London, England, 1963, p. 603.
(7) Chatten, L. G., *J. Pharm. Pharmacol.*, **8**, 504(1956).
(8) Mattson, L. N., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 22(1954).
(9) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 765.

Antimalarial Agents IX

3-Alkylquinolones as Potential Repository Drugs

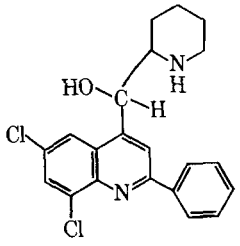
By J. H. BURCKHALTER and DOUGLAS G. MIKOLASEK

As a possible answer to the need for antimalarials with more prolonged activity as well as for drug resistant malaria, several compounds related structurally to endochin (II) have been synthesized. They failed to pass preliminary pharmacological requirements.

ALTHOUGH the 4-aminoquinoline antimalarial agents, chloroquine and amodiaquine, are generally effective suppressive agents in areas of endemic malaria, they must be readministered every few weeks to maintain therapeutic effectiveness (1). An objective of the present study is the synthesis of certain substituted quinolines which might show prolonged antimalarial activity. Should such agents be found, it is hoped that prolonged activity might also contribute effectiveness against the recent serious threat posed by resistant falciparum malaria (2).

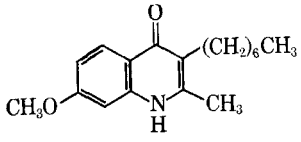
DISCUSSION

Compound I (SN-10,275) (3) once gave promise of possessing prolonged activity because 1 to 5% of the drug was still present in the plasma as long as 3 months following the last dose (4). However, photosensitivity manifest by severe itching prohibits administration to humans.



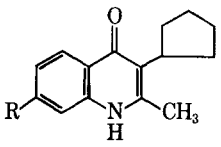
I

SN-10,275



II

SN-13,421 (Endochin)

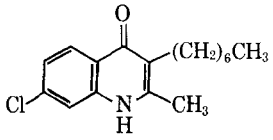


R

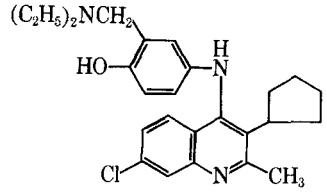
a, Cl

b, CH_3O

III



IV



V

Endochin (II) (SN-13,421) has shown prophylactic activity against *Plasmodium cathemerium* in the canary, *P. gallinaceium* in the chick, and *P. lophurae* in the turkey (5). In view of the lipophilic alkyl grouping in position 3 of the quinoline nucleus, it was believed that endochin or its variants might be held in the tissues and gradually released to contribute prolonged antimalarial activity (6). Such structures as III*a* and IV were proposed since they contain structural features of both endochin and I. Particularly, a substituent at the 2 position would not allow the deactivating 2-hydroxylation which occurs upon administration of quinine (7). Since quinoline antimalarials containing a basic side chain at position 4 generally possess greater potency than the parent compound, structure V was desired for comparative biological evaluation with types III and IV.

Compounds of type III were synthesized by the Conrad-Limpach method (8) as outlined in Scheme I.

Ethyl α-cyclopentylacetoacetate (VI) was prepared from bromocyclopentane and ethyl acetoacetate according to the method of Rydon (9). Two methods were employed for the formation of the anilinocrotonates (VII). It was desirable to use conditions that were as mild as possible to avoid any formation of an anilide (IX) which could result at higher temperatures through a Knorr-type condensation (10). In method *A*, a *m*-substituted aniline and ethyl α-cyclopentylacetoacetate (VI)

Received August 6, 1964, from the College of Pharmacy, University of Michigan, Ann Arbor.

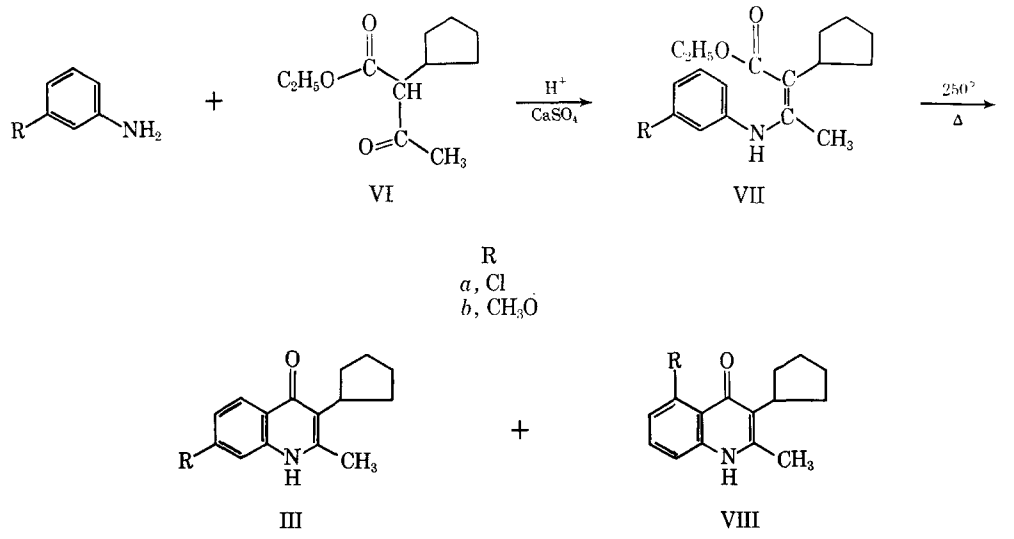
Accepted for publication October 5, 1966.

Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.

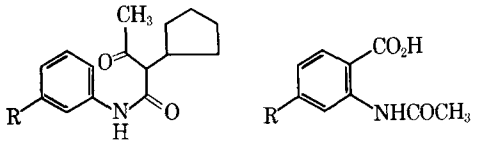
Abstracted from a thesis submitted by Douglas G. Mikolasek to the University of Michigan, Ann Arbor, in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported by fellowship funds from Parke, Davis and Co.

Previous paper: Nobles, W. L., Tietz, R. F., Koh, Y. S., and Burckhalter, J. H., *J. Pharm. Sci.*, **52**, 600(1963).



Scheme I



IX X

were heated in ethanol with an excess of anhydrous calcium sulfate and a catalytic amount of the appropriate aniline hydrochloride, according to an early procedure of Hauser and Reynolds (11). Anil formation has been shown to be acid catalyzed (12).

The second method (method *B*) involved the removal of water by azeotropic distillation and gave an increase in yield without apparent adverse reactions (13).

With the exception of ethyl β-(*m*-chloroanilino)-α-cyclopentylcrotonate, the anils were cyclized directly after removal of excess starting materials when it was found that ring closure occurred during attempted distillation. Cyclizations were carried out by addition of the anilinocrotonates (VII) to a refluxing mixture of diphenyl ether and biphenyl, according to the procedure of Hauser and Reynolds (14).

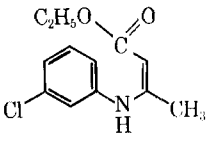
Infrared spectra of the products determined in potassium bromide disks show that the 7-substituted-3-cyclopentyl-2-methyl-4-quinolinols exist in the quinolone (III) form. Absorption peaks appear at 3330 cm.$^{-1}$ characteristic of NH stretching and at 1637 cm.$^{-1}$ characteristic of the lactam carbonyl stretching frequency (15).

A major difficulty with the Conrad-Limpach synthesis, as well as with the Skraup synthesis, is the formation of mixtures of 5- and 7-substituted quinolines when *m*-substituted anilines are used (16). Fortunately, only 3-cyclopentyl-7-methoxy-2-methyl-4-quinolone (III*b*) was isolated when ethyl α - cyclopentyl - β - *m* - methoxyanilinocrotonate (VII*b*) was cyclized. However, in the case of the reaction as applied to *m*-chloroaniline a small amount of solid, m.p. 274–276°, was isolated from the filtrate after removal of 7-chloro-3-cyclopentyl-2-methyl-4-quinolone (III*a*); it is undoubtedly the 5-chloro isomer (VIII). A comparison of its infrared spectrum with that of the 7-chloro isomer (III*a*) showed the two spectra to be very similar except in the region characteristic of aromatic substitution. The spectrum of the lower melting 5-chloro isomer (VIII) contained a broad double peak at 790–810 cm.$^{-1}$ characteristic of three adjacent free hydrogens, while this peak was missing from the spectrum of the 7-chloro isomer (17).

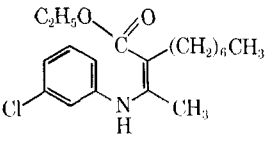
The structures of the 7-substituted quinolones (III*a* and *b*) were proved by oxidation to the appropriate *N*-acetyl-4-substituted anthranilic acids (X*a* and *b*) with alkaline potassium permanganate in the manner of Elderfield and Wright (18). 3-Cyclopentyl-2-methyl-7-methoxy-4-quinolone (III*b*) gave on oxidation *N*-acetyl-4-methoxyanthranilic acid (X*b*), m.p. 197–199°, corresponding to that reported by Friedlander, Bruckner, and Deutsch (19). The melting point of the isomeric *N*-acetyl-6-methoxyanthranilic acid is 176° (19).

Inasmuch as the melting points of the *N*-acetyl derivatives of both 4-chloroanthranilic acid and 6-chloroanthranilic acid are nearly identical (214 and 215°), it was necessary to hydrolyze the amide (X*a*) to the free base. The melting point of the 4-chloroanthranilic acid thus obtained (m.p. 235–238° dec.) corresponded to that reported by Cohn (20). He also reported the melting point of 6-chloroanthranilic acid to be 146–147° dec. (21).

The 7-chloro analog of endochin, 7-chloro-3-heptyl-2-methyl-4-quinolone (IV), was synthesized according to the general procedure of Leonard, Herbrandson, and Van Heynigen (22). The condensation of *m*-chloroaniline and ethyl acetoacetate proceeded smoothly to give ethyl β-*m*-chloro-anilinocrotonate (XI) in 81% yield. XI was alkylated with iodoheptane using sodium hydride in xylene



XI



XII

as the condensing agent. After removal of the solvent and excess starting materials under vacuum, the crude anil (XII) was added directly to a refluxing mixture of diphenyl ether and biphenyl. The yield of 7-chloro-3-heptyl-2-methyl-4-quinolone (IV) was disappointing; only 9% yield based on *m*-anisidine and ethyl acetoacetate was obtained. I.R. suggested a 7-chloro rather than a 5-chloro substituent.

In an attempt to study the effect of a basic side chain upon the antimalarial activity of compounds of the endochin type, it was decided to attach the 4-amino-α-diethylamino-*o*-cresol side chain to 7-chloro - 3 - cyclopentyl - 2 - methyl - 4 - quinolone (III*a*). Treatment of III*a* in methylene chloride with phosphoryl chloride gave 3-cyclopentyl-4,7-dichloro-2-methylquinoline in high yield. This intermediate was condensed with 4-amino-α-diethylamino-*o*-cresol dihydrochloride in the manner of Burckhalter and co-workers (23). 4-(7-Chloro-3-cyclopentyl - 2 - methyl - 4 - quinolylamino) - α-diethylamino-*o*-cresol dihydrochloride (V) was obtained in 54% yield.

PHARMACOLOGICAL RESULTS

Compounds III*a*, III*b*, IV, and V were evaluated as antimalarial agents by Dr. Paul E. Thompson, Research Laboratories, Parke, Davis and Co., Ann Arbor, Mich. III*a* and III*b* were examined for suppressive activity against *P. lophurae* in chicks, according to the method of Thompson (24). Both substances, fed in diet concentrations of 0.05 and 0.2%, were found to be inactive.

IV and V dihydrochloride were tested by Thompson against *P. berghei* in mice by subcutaneous injection. The former in a single subcutaneous dose of 400 mg./Kg. and the latter in twice daily doses of 5 mg./Kg. for 4 days failed to prevent the development of malarial infections. Since quinine is active at 25 mg./Kg., V is less than 5 times as active as quinine.

None of the compounds appear to offer promise as repository agents.

EXPERIMENTAL

Ethyl β - m - Chloroanilino - α - cyclopentylcrotonate (VII*a*).—*Method A.*—A modification of the general procedure of Hauser and Reynolds was used (11). To a mixture of 12.8 Gm. (0.1 mole) of *m*-chloroaniline and 18.2 Gm. (0.1 mole) of ethyl α - cyclopentylacetoacetate (9) in 20 ml. of absolute ethanol, 27 Gm. of anhydrous calcium sulfate and a catalytic amount (12) (0.01 Gm.) of *m*-chloroaniline hydrochloride were added. The mixture was stirred and heated for 4 hr. at 70–80°. The calcium sulfate was removed by filtration and the filtrate was concentrated and distilled under vacuum, giving 15 Gm. (55% yield) of the anilinocrotonate (VII*a*), b.p. 137° (0.2 mm.).

Anal.—Calcd. for $C_{17}H_{22}ClNO_2$: C, 66.33; H, 7.20. Found: C, 66.11; H, 7.29.

Method B.—The improved method of Hauser and Reynolds was employed (13). A mixture of 0.1 mole of *m*-chloroaniline, and 0.1 mole of ethyl α-cyclopentylacetoacetate (9) and 50 ml. of benzene was placed in a flask fitted with a Dean-Stark water separator and condenser. After addition of 0.5 ml. of acetic acid, the mixture was heated to reflux until water no longer separated. After 72 hr., 1.2 ml. of water had collected. The mixture was concentrated and excess *m*-chloroaniline and ester were distilled under vacuum, giving 22 Gm. (72% yield) of crude product. The VII*a* was pure enough to use in the next step.

7 - Chloro - 3 - cyclopentyl - 2 - methyl - 4 - quinolone (III*a*).—A solution of 15 Gm. (0.049 mole of ethyl β - *m* - chloroanilino - α - cyclopentylcrotonate (VII*a*) in 30 ml. of diphenyl ether was added dropwise to 150 ml. of a refluxing mixture of diphenyl ether and biphenyl, according to the procedure of Hauser and Reynolds (14). After the mixture was cooled, the gray solid which separated was collected on a filter, washed with petroleum ether (60–70°), and dried to yield 7 Gm. of crude III*a*, m.p. 334–336°. Recrystallization from dilute alcohol gave 5 Gm. (39% yield) of III*a*, m.p. 362–365° dec. $\nu_{max.}^{KBr}$ in cm.$^{-1}$ 3300 (NH), 1639 (lactam C=O), 780 (2-adj. free H's).

Anal.—Calcd. for $C_{15}H_{16}ClNO$: C, 68.83; H, 6.16. Found: C, 68.79; H, 6.28.

The filtrate from the reaction just described was diluted with 300 ml. of petroleum ether (60–70°) and the solid which separated was collected on a filter. After recrystallization from dilute alcohol, 1.3 Gm. of a white solid (VIII) was obtained, m.p. 276–278° corrected. Infrared: $\nu_{max.}^{KBr}$ in cm.$^{-1}$, 3300 (NH), 1637 (lactam C=O), 788, 812 (3-adj. free H's).

Anal.—Calcd. for $C_{15}H_{16}ClNO$: C, 68.83; H, 6.16. Found: C, 69.03; H, 6.21.

Proof of Structure III*a*: Its Oxidation to N-Acetyl-4-chloroanthranilic Acid (X*a*).—A suspension of 6 Gm. (0.023 mole) of III*a* in 1500 ml. of water made alkaline with 2.5 Gm. of potassium hydroxide was oxidized with 40 Gm. of potassium permanganate according to the procedure of Elderfield and Wright (18). After acidification of the concentrate with concentrated hydrochloric acid, 2 Gm. of *N*-acetyl-4-chloroanthranilic acid (X*a*) was obtained, m.p. 213.5–215.5° corrected. [Lit. (20) m.p. 215°.]

Hydrolysis of the amide (X*a*) in 10% hydrochloric acid, followed by neutralization with sodium bicarbonate, gave 0.2 Gm. of 4-chloroanthranilic acid, m.p. 234.5–237.5° corrected. [Lit. (20) m.p 235°.]

3 - Cyclopentyl - 2 - methyl - 7 - methoxy - 4-quinolone (III*b*).—A mixture of 12.3 Gm. (0.1 mole) of *m*-anisidine and 18.2 Gm. (0.1 mole) of ethyl α-cyclopentylacetoacetate (9) was condensed according to the method of Hauser and Reynolds (11). After distillation under vacuum, 14.5 Gm. (47% yield) of ethyl α-cyclopentyl-β-*m*-methoxyanilinocrotonate (VII*b*) was obtained, b.p. 150° (0.4 mm.), n_D^{27} 1.5252.

A solution of 14 Gm. (0.046 mole) of the foregoing anilinocrotonate (VII*b*) in 30 ml. of diphenyl ether was added dropwise to 250 ml. of a refluxing

mixture of diphenyl ether and biphenyl. The mixture was heated for an additional 30 min., cooled, and the light tan product collected on a filter, washed with petroleum ether (60–70°), and dried giving 5 Gm. (42% yield) of *IIIb*, m.p. 310–312° dec. Recrystallized from 75% ethanol, it melted at 312–313° dec.

Anal.—Calcd. for $C_{16}H_{19}NO_2$: C, 74.68; H, 7.44. Found: C, 74.64; H, 7.49.

Proof of Structure IIIb: Its Oxidation to N-Acetyl-4-methoxyanthranilic Acid (Xb).—A suspension of 6 Gm. (0.023 mole) of 3-cyclopentyl-2-methyl-7-methoxy-4-quinolone (*IIIb*) in 1500 ml. of water containing 2.5 Gm. of potassium hydroxide was oxidized with potassium permanganate according to the procedure of Elderfield and Wright (18). The mixture was filtered free of manganese dioxide, concentrated to one-third of its volume, and acidified with concentrated hydrochloric acid, giving 1.5 Gm. of crude *N*-acetyl-4-methoxyanthranilic acid (*Xb*). Recrystallization first from water and then from methanol gave 1 Gm. of white needles, m.p. 196.5–198.5° corrected. [Lit. (19) m.p. 197–199°.]

Ethyl β - m - Chloroanilinocrotonate (XI).—A mixture of 25.6 Gm. (0.2 mole) of *m*-chloroaniline and 26 Gm. (0.2 mole) of ethyl acetoacetate was condensed according to the general procedure of Hauser and Reynolds (11). Vacuum distillation gave 39 Gm. (81% yield) of XI, b.p. 139–141° (1.2 mm.). [Lit. (12) b.p. 145–148° (1.5 mm.).]

7 - Chloro - 3 - heptyl - 2 - methyl - 4 - quinolone (IV).—A solution of 39 Gm. (0.16 mole) of ethyl β - *m* - chloroanilinocrotonate (11) (XI) in 100 ml. of dry xylene was alkylated with 37 Gm. (0.113 mole) of 1-iodoheptane according to the procedure of Leonard, Herbrandson, and Van Heynigen (22). After filtration, and removal of solvent, the crude oily residue (40 Gm.) was cyclized directly.

To a refluxing solution of 500 ml. of diphenyl ether–biphenyl mixture, 40 Gm. of crude ethyl β - *m* - chloroanilino - α - heptylcrotonate (XII) was added dropwise. After the addition was complete, heating was continued for 20 min. and the mixture chilled. White plates were collected on a filter and washed with petroleum ether (60–70°). Recrystallization from 95% ethanol gave 4.3 Gm. (9% yield) of IV, m.p. 260–262°. $\nu_{max.}^{KBr}$ in cm.$^{-1}$ 3300 (NH), 1637 (lactam C=O), 775 (2 adj. free H's).

Anal.—Calcd. for $C_{17}H_{22}ClNO$: C, 69.97; H, 7.60. Found: C, 69.94; H, 7.44.

3 - Cyclopentyl - 4,7 - dichloro - 2 - methylquinoline.—A mixture of 25 Gm. (0.095 mole) of 7 - chloro - 3 - cyclopentyl - 2 - methyl - 4 - quinolone (*IIIa*) and 75 Gm. (0.5 mole) of phosphoryl chloride was heated on a steam bath for 2 hr. Excess phosphoryl chloride was removed *in vacuo*, and the residue taken up into 200 ml. of methylene chloride. This solution was allowed to flow slowly into a vigorously stirred mixture of ice and water. The two-phase solution was neutralized with concentrated ammonium hydroxide and the resulting mixture extracted with 100 ml. of methylene chloride. The extracts were washed with water and dried over anhydrous potassium carbonate. The dried solution was filtered through a layer of charcoal and diatomaceous earth,[1] and concentrated to dryness. Recrystallization of the residue from methanol gave 26 Gm. (98% yield) of product, m.p. 86–88°.

Anal.—Calcd. for $C_{15}H_{15}Cl_2N$: C, 64.30; H, 5.40. Found: C, 64.06; H, 5.44.

4 - (7 - Chloro - 3 - cyclopentyl - 2 - methyl - 4-quinolylamino) - α - diethylamino - o - cresol (V) Dihydrochloride.—A mixture of 5.2 Gm. (0.019 mole) of 3-cyclopentyl-4,7-dichloro-2-methylquinoline, 4.8 Gm. (0.019 mole) of 4-amino-α-diethylamino-*o*-cresol dihydrochloride (23), and 30 ml. of ethanol was refluxed for 24 hr. on a steam bath. The mixture was cooled, and the solid was collected on a filter giving 2.4 Gm. of orange crystals, m.p. 257–259° corrected. The filtrate was refluxed for an additional 24 hr., cooled, and the product again collected on a filter. After repetition of this procedure a second time, a total of 7.7 Gm. of crude product was obtained. Recrystallization from methanol–acetone gave 5.1 Gm. (54% yield) of V dihydrochloride as orange crystals, m.p. 252–253°. A sample for analysis was recrystallized twice from absolute alcohol, m.p. 252–254° dec.

Anal.—Calcd. for $C_{26}H_{32}ClN_3$ 0.2 HCl: C, 61.12; H, 6.31. Found: C, 60.93; H, 6.39.

REFERENCES

(1) *World Health Organ. Tech. Rept. Ser.*, **226,** 66(1961).
(2) Moore, D. V., and Lanier, J. E., *Am. J. Trop. Med. Hyg.*, **10,** 5(1961); Eyles, D. E., *et al.*, *ibid.*, **12,** 840(1963).
(3) Buchman, E. R., Sargent, H., Meyers, T. C., and Howton, D. R., *J. Am. Chem. Soc.*, **68,** 2710(1946).
(4) "A Survey of Antimalarial Drugs," Wiselogle, F. Y., ed., J. W. Edwards, Ann Arbor, Mich., 1946.
(5) *Ibid.*, p. 90.
(6) *Cf.* Elslager, E. F., and Thompson, P. E., "Abstracts of the Ninth National Medicinal Chemistry Symposium of the American Chemical Society," Minneapolis, Minn., June 1964, p. 6j.
(7) Brodie, B. B., Baer, J. E., and Craig, L. C., *J. Biol. Chem.*, **188,** 567(1951).
(8) Conrad, M., and Limpach, L., *Chem. Ber.*, **20,** 944, 948(1887).
(9) Rydon, H., *J. Chem. Soc.*, **1939,** 1544.
(10) Knorr, L., *Ann. Chem.*, **236,** 69(1886); **245,** 357 (1888).
(11) Hauser, C., and Reynolds, G., *J. Am. Chem. Soc.*, **70,** 2402(1948).
(12) Coffey, S., Thompson, J., and Wilson, F., *J. Chem. Soc.*, **1936,** 856.
(13) Reynolds, G. A., and Hauser, C. R., "Organic Syntheses," coll. vol. III, Horning, E. C., ed., John Wiley & Sons, Inc., New York, N. Y., 1955, p. 374.
(14) *Ibid.*, p. 593.
(15) Bellamy, L. J., "The Infrared Spectra of Complex Molecules," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958, p. 214.
(16) Elderfield, R. C., "Heterocyclic Compounds," vol. 4, John Wiley & Sons, Inc., New York, N. Y., 1952, p. 33.
(17) Bellamy, L. J., *op. cit.*, p. 78.
(18) Elderfield, R. C., and Wright, J., *J. Am. Chem. Soc.*, **68,** 1276(1946).
(19) Friedlander, P., Bruckner, S., and Deutsch, G., *Ann. Chem.*, **388,** 46(1912).
(20) Cohn, P., *Monatsh.*, **22,** 485(1901).
(21) *Ibid.*, **22,** 473(1901).
(22) Leonard, N., Herbrandson, H., and Van Heynigen, E., *J. Am. Chem. Soc.*, **68,** 1279(1946).
(23) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Jones, P. A., Holcomb, W. F., and Rawlins, A. L., *ibid.*, **70,** 1363(1948).
(24) Thompson, P. E., Moore, A. M., Reinertson, J. W., and Bayles, A., *Antibiot. Chemotherapy*, **3,** 399(1953).

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

Hepatotoxicity of a Series of Organotin Esters

By DAVID J. CALLEY, W. L. GUESS, and J. AUTIAN

Four organotin esters having use or potential use in the formulation of medically used plastics were evaluated for their toxic effects on the liver following oral administration. The liver damage is apparently the cumulative type, requiring several days to manifest damage in the form of elevated SGPT values and prolonged hexobarbital narcosis. The toxic potential was in general solubility related.

CERTAIN ORGANO-METALLIC compounds are currently being used as stabilizers in plastic formulations, curing agents for rubber formulations, antifungal agents, and many other industrial applications. The bulk of the toxicity investigations to date on organotin compounds have been conducted with the alkyl–tin salts of the general formula R—Sn—X, R being an alkyl group, Sn as quadrivalent tin, and X representing a halide, sulfate, or other salt-forming ion. An excellent paper by Barnes and Stoner (1) reviews many of the toxic aspects of these compounds. Relatively little has been published concerning the toxicity of the alkyl–tin esters, R—Sn—O—R, particularly with a view toward delineating the relationship which may exist between varying the number of alkyl groups and the oral toxicities of these compounds. In addition, certain differences have been noted by Barnes and Stoner as to species variations when using the salts of alkyl–tin compounds.

In view of the greater interest being shown in certain of the alkyl–tin esters for various uses in devices for medical and pharmaceutical applications, it was decided that an investigation of the acute oral toxicity of a series of organotin compounds be undertaken. The esters chosen for this study were the acetate esters of the di- and tributyl compounds. In addition the tetrabutyl compound was selected which naturally has no point in the molecule available for esterification. A fourth compound, dibutyltin di-(2-ethylhexoate), was included as a check for the effect of the ester moiety on the resulting toxicity.

EXPERIMENTAL

Materials.—The materials used in this study were: (*a*) tetrabutyltin, lot 42921; (*b*) tributyltin acetate, lot GPI-1281; (*c*) dibutyltin diacetate, lot GP-1108; (*d*) dibutyltin di-(2-ethylhexoate), lot 1246.

Received August 26, 1966, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin 78756.

Accepted for publication October 25, 1966.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

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

The authors thank Carlisle Chemical Works, Reading, Ohio, for supplying the organotin compounds used in this study.

Methods

Oral LD_{50} Determinations.—Since published data were not available, it was necessary to establish LD_{50} values and time of onset of toxicity. These values were determined by a single oral intubation of each organotin, dissolved in sesame oil, into groups of 10 male Swiss-Webster albino mice of uniform weight and age, which had been deprived of food for 24 hr. Dosage levels of organotin were spaced in geometric progression increasing by a factor of two. The volume of each dose was 0.5 ml./20 Gm. body weight. Following intubation, observations for mortality were recorded daily for 1 week.

Liver Function Tests.—Previous reports in the literature have indicated that certain organotins exert an hepatotoxic effect (2), while others indicate toxicity to the bile duct (3). A series of experiments was undertaken to define more clearly the hepatotoxicity of the tin esters. Rabbits were used to obtain direct and indirect evidence of acute toxicity to liver tissue. They were intubated with a single oral dose of each organotin, and the following liver function tests were performed.

(*a*) Serum glutamic-pyruvic transaminase (SGPT), as an index of hepatocellular injury. (*b*) Prothrombin time, to indicate liver damage in the absence of jaundice. (*c*) Alkaline phosphatase, as an early indicator of hepatic obstruction which might lead to jaundice. (*d*) Thymol turbidity, to differentiate cellular from obstructive hepatic disease.

Four groups of rabbits, three rabbits per group, were each administered approximately one-half the LD_{50} dose of each organotin. A fifth group, administered only the sesame oil vehicle, served as controls. Values obtained from each rabbit before organotin dosing also served as controls for determining acute response after dosing. Five-milliliter blood samples were withdrawn from the median ear artery before dosing and again at intervals of 48 and 144 hr. (6 days) after dosing.

Cholestasis in Mice.—Plaa and Becker (4) recently reported a reliable method for the determination of intrahepatic cholestasis. This method employs an intravenous dose of fluorescein dye, which accumulates in the gall bladder and bile duct in the absence of intrahepatic bile stasis. The dye is visualized under ultraviolet light as a brilliant yellow-green color. If cholestasis does occur due to a toxic response or liver damage, fluorescence in the biliary tract should be absent, using all-or-none criteria. This method was used in this study. Groups of 10 mice were orally intubated with 50 mg./Kg. of organotin (except dibutyltin diacetate at 25 mg./Kg.). After an interval of 7 days the fluorescein dye was injected intravenously *via* the

tail vein, 0.1 ml./10 Gm., with a concentration of 20 mg./10 ml. in 0.17 *N* $NaHCO_3$. Fifteen minutes later the animals were sacrificed and examined under ultraviolet light for yellow-green fluorescence.

Determination of Blood Concentration of Tin.—To prove gastrointestinal absorption, hence blood passage through the liver, it was necessary to determine blood levels of tin at various time intervals following oral intubation. Five-milliliter samples of blood were widthdrawn by cardiac puncture from New Zealand albino rabbits prior to a single intubation with one-half the LD_{50} dose of organotin. These blood samples were then assayed for presence of tin according to the method described by Feigl (5). Additional 5-ml. samples were taken at intervals of 1, 3, 5, and 18 hr. following organotin intubation, and similarly analyzed.

Hexobarbital Narcosis.—This study was conducted to determine (*a*) a direct CNS effect after a relatively short time lapse following a single oral dose of organotin and (*b*) the presence of liver damage or change in function after a relatively long period of time following a single oral organotin administration. For part (*a*) sodium hexobarbital, 60 mg./Kg., was administered intraperitoneally 1 hr. after organotin dosing, and in part (*b*) the sodium hexobarbital was administered 7 days after organotin dosing. In both cases one-fourth the LD_{50} dose was given. Sleeping time of dosed mice in parts (*a*) and (*b*) were compared with that of concurrent controls. Sixteen albino male mice per test group were used.

Pathology.—Liver and kidney tissues from animals exposed on an acute and subacute basis to doses of organotins were obtained immediately after sacrifice and preserved in buffered formalin solution. These were subsequently stained with the standard hematoxylin-eosin stain and examined for changes which might be attributed to the organotin compounds.

Response to Acute and Subacute Administration.—In order to broaden the toxicity profile on the compounds under study for effects on the liver, and perhaps to more accurately correlate data, some general observations were made on animals dosed on an acute and subacute basis. Consequently, one group of animals was administered oil solutions in smaller doses, but repeated at intervals over a 1 to 2-week period. A third group of animals was administered saturated aqueous solutions by the intraperitoneal route for acute observations.

RESULTS AND DISCUSSION

LD_{50} Determination.—The oral administration of toxic concentrations of organotin, as oil solutions, resulted in deaths over a long period of time. The earliest deaths began to appear about 8 to 12 hr. after dosing, but continued over a period of days. For purposes of calculation of LD_{50} values, an arbitrary period of 7 days was set for counting deaths due to organotin poisoning. After the 7-day period, any deaths occurring did not enter the calculations, because it was difficult to determine whether death was directly attributable to the compound or whether it occurred through a secondary depressive starvation for food and water. The results of this study are listed in Table I, with confidence limits calculated by the method of Weil (6) and Thompson and Weil (7). It should be noted that the trialkyl ester (tributyltin acetate) had a slightly higher toxicity than the di-ester, though not significantly so. The tetrabutyltin exhibited a very low order of toxicity. Dibutyltin di-(2-ethylhexoate) had an LD_{50} value approximately twice that of the di- and tributyl acetate esters. These data seemed to indicate that the alkyl moiety had less effect in the determination of death than the ester moiety.

Liver Function Tests in Rabbits.—Following a single oral dose (one-half the LD_{50} dose) of the organotin compounds, only the SGPT values showed any significant change from the normal control values. No significant SGPT elevations at the 48-hr. time interval were noted (Fig. 1). At the 144-hr. interval, however, values of all organotin-dosed rabbits showed significant ($P = 0.05$) increases over control values, except with dibutyltin diacetate. The prothrombin time, alkaline phosphatase, and thymol turbidity showed no significant changes from control values at any of the time periods.

SGPT is a highly specific index of hepatocellular injury and is much more sensitive to minimal or moderate damage to the liver than are the other hepatic function tests. The transaminase activity in blood serum parallels the degree of liver cell injury. Elevation of the SGPT indicates that one of the first hepatic responses to organotin poisoning is

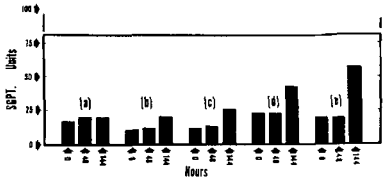


Fig. 1.—Serum glutamic-pyruvic transaminase (SGPT) values in rabbits receiving single oral doses of organotin (average of three rabbits), at 0, 48, and 144-hr. intervals. Key: (a) control rabbits; (b) rabbits receiving dibutyltin diacetate; (c) rabbits receiving dibutyltin di-(2-ethylhexoate); (d) rabbits receiving tributyltin acetate; (e) rabbits receiving tetrabutyltin.

TABLE I.—APPROXIMATE BLOOD LEVELS OF ORGANOTIN 3 hr. AFTER ORAL DOSAGE WITH LD_{50} VALUES

Compd.	Mol. Wt.	Single Oral Dose, mg./Kg.	Elemental Tin in Blood, p.p.m.	Oral LD_{50} Values, mg./Kg. ± S.E.
Dibutyltin diacetate	350.7	110	90	109.7 ± 24.2
Dibutyltin di-(2-ethylhexoate)	518.7	200	130	199.9 ± 43.2
Tributyltin acetate	348.7	300	140	99.1 ± 8.4
Tetrabutyltin	410.7	500	200	6000.0 ± 500.0

from parenchymal cells and involves the activity of one or more enzymes. In the case of the other liver function tests, none proved sensitive enough to detect subtle changes that may have occurred. Lack of any significant change in SGPT values from normal 48 hr. after oral dosing indicated that either the organotin does not reach the site of action in the liver cells in sufficient concentration to produce physiological disturbances of any great consequence within 48 hr., or that the changes produced there are produced slowly so that the accumulated response does not manifest itself until sometime after 48 hr. This also confirms other data indicating a delayed hepatotoxicity from the organotin compounds.

Intrahepatic Cholestasis and Jaundice in Mice.—In the course of the LD_{50} determination, it was noticed that three of the organotin compounds at the higher dose levels (200 mg./Kg.) caused visually evidenced jaundice in mice. Dibutyltin diacetate, tributyltin acetate, and dibutyltin di-(2-ethylhexoate) in large doses produced, in from 5 to 7 days, visually apparent discoloration of the skin, internal organs, and urine. Tetrabutyltin did not cause an apparent jaundiced discoloration in any of the test animals. Confirming evidence of intrahepatic cholestasis by the method of Plaa and Becker (5) is summarized in Table II. This table presents the total number of animals showing cholestasis at various dose levels, all exceeding 50 mg./Kg. These data indicated that all four compounds were at least capable of inducing cholestasis. Since none of the control animals showed cholestasis by the fluorescein dye method, this condition in the other animals must be attributed to the organotin.

Absorption of Organotin into Bloodstream.—Feigl spot tests for tin in the bloodstream of rabbits were negative prior to the oral administration of the four tin compounds. Five-milliliter blood samples taken 1, 3, 5, and 18 hr. after oral intubation of organotins were positive for the presence of tin in all cases. The test color intensity was compared with standards of known concentration. By this comparison it was found that increasing concentrations of tin appeared in the bloodstream, reaching a maximum at 3 hr. after intubation. Positive tests for tin at substantial levels were also obtained through 18 hr., at which time the study was terminated. The blood level of each organotin at the 3-hr. time period was calculated (Table I).

Results of the Feigl spot tests indicated that, with all four compounds, either the organotin itself or a metabolite was capable of being carried into the bloodstream in a reasonably short time in amounts large enough to create a pharmacological or toxicological response. In addition, the presence of relatively high levels of tin persistent in the blood stream after 18 hr. suggested the possibilities that either a large storage depot existed somewhere in the body from which amounts of organotin were steadily being released or that the organotin remained in circulation for a protracted length of time. Concurrent spot tests for tin in the bloodstream of rabbits which had been orally dosed 13 days prior to testing proved negative. This indicated that should a prolonged retention exist, it was depleted within the 13-day period.

TABLE II.—CHOLESTASIS 1 WEEK AFTER ORAL ORGANOTIN ADMINISTRATION

Compd.	Mice, No.	% Showing Cholestasis
Sesame oil controls	17	0.0
Dibutyltin diacetate	33	18.2
Tributyltin acetate	32	6.3
Tetrabutyltin	14	35.7
Dibutyltin di-(2-ethylhexoate)	36	22.2

TABLE III.—EFFECT OF ORGANOTINS ON HEXOBARBITAL NARCOSIS IN MICE

Compd.	Mice, No.	Oral Dose	Sleep Time ± S.E., min.	Significance, P
Control group (sesame oil)	14	0.5 ml./20 Gm.	29.0 ± 14	....
Dibutyltin diacetate	12	30 mg./Kg.	44.5 ± 11.9	0.01
Tributyltin acetate	13	50 mg./Kg.	43.1 ± 8.1	0.01
Dibutyltin di-(2-ethylhexoate)	8	40 mg./Kg.	37.7 ± 11.7	0.05

Hexobarbital Narcosis in Mice.—When sodium hexobarbital (60 mg./Kg.) was administered intraperitoneally 1 hr. after oral dosing with the organotins, there was no significant difference between the sleeping times of the test and the control groups of mice. This indicated that within the limitations of the technique, there was no significant influence on the CNS from these compounds within a 1-hr. time span. On the other hand, groups of mice dosed with sodium hexobarbital (60 mg./Kg.) intraperitoneally 1 week after a single dose of each organotin compound showed significant differences from control sleeping times (Table III). The mice received one-fourth LD_{50} doses. There was a significant prolongation of sleeping times among mice receiving dibutyltin diacetate and tributyltin acetate. This indicates that liver damage from these two compounds did occur to some extent by the end of 1 week. This was confirmed by pathologic manifestations in liver sections. The tetrabutyltin is apparently unstable as evidenced by an increasing toxicity with time. The oral LD_{50} value established on fresh material was approximately 6000 mg./Kg., but it was noted in sleeping-time studies that all mice dosed at one-fourth LD_{50} doses died almost immediately. Rechecking of data and materials indicated that the tetrabutyltin compound had decomposed. Therefore, the sleeping-time data for this compound was discarded. All other data reported in this paper were conducted on the fresh material and are therefore included.

Acute Pharmacological Response.—Response to single large oral doses of organotin dissolved in sesame oil produced no sudden or dramatic responses in mice, even in concentrations 5 to 8 times the LD_{50} value, suggesting slow gastric absorption. After 30 min. to 1 hr., a gradual depressive syndrome was noted, in which activity was decreased, respiration rate, and irritability reduced. Almost complete ptosis was observed after 1 to 2 hr. Death at higher dose levels occurred within 18 hr., but at the LD_{50} levels, death occurred over a period of several days.

Saturated aqueous solutions of each organotin compound were prepared in normal saline and injected, 0.5 ml./20 Gm., intraperitoneally in mice.

Two compounds, dibutyltin diacetate and tributyltin acetate, produced a rapid onset of symptoms, those caused by the former being most severe. Writhing and stretching were observed in both cases within 2 to 4 min. Those injected with dibutyltin diacetate showed severe depression, ataxia, and ptosis with death resulting within 30 to 35 min. due to apparent respiratory paralysis. Gasping with the mouth widely opened was a common response. Cyanosis preceded death. These symptoms were observed in mice receiving the tributyltin acetate, but onset was less rapid and severe, with death occurring between 30 to 60 min. Mice injected with tetrabutyltin showed stretching, but symptoms were very mild by comparison with those caused by the two previously mentioned (no deaths resulted). Solutions of dibutyltin di-(2-ethylhexoate) caused little response except depression, and no deaths occurred with this compound.

Subacute Responses.—Rats dosed repeatedly with one-half the LD_{50} dose at 3 to 5-day intervals generally showed little response either immediately after each dose or at the end of a 1-month time period, with the exception of those receiving dibutyltin diacetate. Slight temporary depression was noted with tributyltin acetate. In the case of the dibutyltin diacetate, diarrhea, ptosis, and general depression was noted, with individuals varying in their response. Onset of diarrhea was observed after the first dose, but the animals did not seem to become depressed to any extent until after receiving four to five doses. In these cases little was observed except depression. Death occurred in two of five rats after the fifth and sixth dose (total dose 250 to 300 mg./Kg.).

Rabbits dosed repeatedly with the same levels of dibutyltin diacetate tended to exhibit diarrhea to a greater extent than rats receiving the same dose. In one rabbit, following six repeated doses with dibutyltin diacetate at weekly intervals, complete paralysis of the hind legs resulted. Dosing was thereupon discontinued and no recovery was seen after 3 weeks. Other rabbits exhibited considerable malaise and some depression, but no paralysis was noted after seven doses.

Pathology.—The tissue sections submitted for pathological examination generally confirmed that the organotin compounds do exert a toxic effect on liver tissue. A significant finding is that liver damage is rather slowly manifested after a single oral dose. No pathology of liver tissue could be observed 3 days after a single dose of organotin, but definite pathology could be observed after 7 days. The primary change in liver tissue of rats and mice appears to be a moderate increase in reticuloendothelial cells and mild hepatocellular damage with cloudy swelling, which could cause retention of bile. (See Fig. 2.)

These data confirm prior indications of liver damage at the 7-day time interval as evidenced by the prolonged sleeping time from hexobarbital, by the elevated SGPT, by bile stasis indicated in the fluorescein test, and by jaundice from high doses in mice. Apparently there was no bile duct pathology during this time period as had been observed for salts of certain organotin compounds (1). This was somewhat surprising in view of the elimination of these compounds by way of the bile into the intestinal tract and finally through the feces.

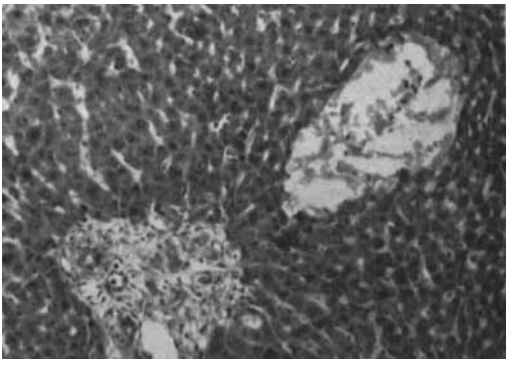


Fig. 2.—Liver of rat receiving dibutyltin diacetate, nine repeated oral doses of 50 mg./Kg., spaced over a 5-week period. The tissue shows cloudy swelling, most noticeable in the centrilobular location, with minimal fatty degeneration. A slight increase in fibrous connective tissue exists, and there are islands of acute necrosis, with minimal infiltration of polymorphonuclear leucocytes throughout the section.

SUMMARY

The results of the experiments on a series of organotin esters indicated that the compounds are absorbed from the gastrointestinal tract following oral administration in an oil solution. After absorption into the bloodstream, there is evidence that the compounds remain in the blood for periods in excess of 18 hr. and then are eliminated *via* the feces. In the passage of these compounds through the liver, there accumulates over a period of several days a uniform and general liver damage. This damage usually takes the form of cloudy swelling, mild hepatocellular involvement, and a moderate increase in reticuloendothelial cells following a single oral dose. There does not appear to be any significant difference between the hepatotoxic effects of the di- and tributyl esters, but the tetrabutyl compound exhibits a reduced toxicity. In view of the delayed effects of these compounds on liver tissue, it is suggested that long-term studies should be conducted on them.

REFERENCES

(1) Barnes, J. M., and Stoner, H. B., *Brit. J. Ind. Med.*, **15**, 15(1958).
(2) Cremer, J. E., *Biochem. J.*, **68**, 685(1958).
(3) Barnes, J. M., and Magee, P. N., *J. Pathol. Bacteriol.*, **75**, 267(1956).
(4) Plaa, G. L., and Becker, R. A., *J. Appl. Physiol.*, **20**, 534(1965).
(5) Feigl, F., *Inorg. Appl.*, **1**, 104(1954).
(6) Weil, C. S., *Biometrics*, **8**, 249(1952).
(7) Thompson, W. R., and Weil, C. S., *ibid.*, **8**, 51(1592).

Effects of β-Hydroxyethylhydrazine on Growth and Alkaloid Formation of *Datura stramonium*

By LEO A. SCIUCHETTI and GORDON K. NIELSEN*

The aerial parts of *Datura stramonium* were sprayed with aqueous solutions of β-hydroxyethylhydrazine (BOH) in strengths of 1, 0.01, and 0.0001 per cent. Treatment with 1 per cent of BOH reduced the height 54 per cent and the total dry weight 67 per cent. Malformations which were induced in the plants are described. The formation of flowers was inhibited. The concentration of leaf-tops alkaloid was decreased 45 per cent, while root alkaloid was increased 61 per cent. The total alkaloidal content per plant was only 26 per cent of controls. The habit of the groups treated with 0.01 and 0.0001 per cent of BOH resembled the controls. The concentration of alkaloids of the 0.01 per cent BOH group was decreased 17 per cent in the leaf-tops, but increased 161 per cent in the roots. The total alkaloidal content of the roots was 230 per cent of controls. In the 0.0001 per cent BOH group increases of 11, 103, and 209 per cent were found in the concentration of alkaloid of the leaf-tops, stems, and roots, respectively. The total alkaloidal content of this group was increased 31 per cent per plant, 99 per cent in the stems, and 219 per cent in the roots.

GOWING AND Leeper (1) in 1955 demonstrated that β-hydroxyethylhydrazine (BOH)[1] induced flowering in pineapples. In subsequent work these authors (2) tested 39 hydrazine derivatives for induction or inhibition of flowering and toxicity to the pineapple plant and found that BOH was one of six hydrazine derivatives which forced the pineapple to flower. The first effect of BOH was retardation of stem elongation which then was followed by floral initiation (3, 4). Reed (5) has indicated that BOH forces flowering in pineapple plants, possibly by reducing auxin concentration. This compound also was found to be a potent inhibitor *in vivo* of animal diamine oxidase (5).

B995, known chemically as *N*-dimethylaminosuccinamic acid, can also be considered to be a hydrazine derivative, *viz.*, the 1,1-dimethylhydrazide of succinic acid. Reed (6) has indicated that inhibition of shoot elongation in dwarf and tall peas by B995 was correlated with the inhibition of the oxidation of tryptamine-2-^{14}C to indoleacetaldehyde-2-^{14}C in homogenates prepared from epicotyls of young plants treated with B995. Previous investigations (7, 8) in this laboratory have shown that B995 caused significant reductions in height, growth, and the total alkaloid content of *Datura tatula* and *D. innoxia*. Plant maturity was notably delayed. It was also found that a 2% aqueous spray of B995 caused significant increases in the concentration of stem alkaloids.

B995 has induced interesting effects on the growth of various *Datura* species, and since the chemical structures of this compound and BOH are closely related, it was decided to determine the effects of various concentrations of BOH on the growth and alkaloid synthesis of *D. stramonium* L. Furthermore, BOH has not been reported to have been tested on medicinal plants.

EXPERIMENTAL

Growth of Plants.—*D. stramonium* plants employed in this investigation were germinated from seeds obtained from plants grown in the Oregon State University drug garden in 1961. The germination of the seeds, transplantation of the seedlings into individual 1-gal. metal containers, and soil composition are described in a previous publication (9). The growth studies were performed under greenhouse conditions at temperatures varying from 85° F. at night to 95° F. during the day. A humid condition was maintained in the greenhouse.

Treatment Plan.—A total of 72 five-week-old plants was selected on July 1, 1965 (zero time), for uniformity as to size and height from a pool of 100 plants grown in individual 1-gal. metal containers. The experimental period which started at this time lasted 4 weeks. Three harvests were made—at 0, 2, and 4 weeks. Each harvest consisted of 6 plants from each of the 4 groups, *viz.*, control plants (untreated plants), plants treated with 1% of BOH, those treated with 0.01% of BOH, and plants receiving treatment with 0.0001% of BOH. The first harvest, at zero time, however, consisted of 24 plants which were considered controls since none had been treated. At zero time, the plants were labeled according to treatment and randomized on a greenhouse bench.

The treatment solutions were prepared on a v/v basis from a stock preparation of β-hydroxyethylhydrazine.[2] At zero time, the aerial portions of the plants were sprayed to run-off with an atomizer with the previously designated solutions to assure an approximate equal dosage on a volume-to-unit area relationship. Specially prepared paper shields

Received August 15, 1966, from the Department of Pharmacognosy, School of Pharmacy, Oregon State University, Corvallis 97331.
Accepted for publication November 2, 1966.
* Participant in Undergraduate Science Education Program, National Science Foundation, grant GE 6467.
[1] Marketed as Omaflora by Industrial Chemicals Division of Olin Mathieson Corp., New York, N. Y.

[2] The BOH was generously supplied by Dr. S. I. Cohen, E. R. Squibb and Sons, Division of Olin Mathieson Chemical Corp., New York, N. Y.

prevented the solutions from entering the soil. Treatment according to group was conducted in a separate room to prevent cross-contamination. The plants received four weekly treatments. Height measurements were taken twice weekly, and the plants were observed periodically for any morphological changes.

The dosage regimens employed in this investigation were determined by a preliminary experiment performed 3 months earlier on *D. stramonium* and *D. meteloides*. Groups of 5 plants from each species received biweekly treatments by spraying to run-off for 5 weeks with the following strengths (v/v) of BOH: 1, 0.1, 0.01, 0.001, 0.0001%, and distilled water (controls). The 1% strength of BOH drastically inhibited plant growth and was phytotoxic which was manifested by the appearance of necrotic areas on the leaf blades. In *D. stramonium* the total fresh weight of the plants was only 8.7% of controls, and the average height of the treated plants at the end of the fifth week was only 6.6 cm. *versus* 27.3 cm. for controls. In *D. meteloides* growth was even more drastically inhibited, the total fresh weight being only 1.2% of controls. In this species the average height of the treated group at the end of the fifth week was 5.4 cm. *versus* 29.8 cm. for controls. The plants receiving the 0.1% strength of BOH were shorter than controls, and growth (fresh weight) was decreased 15 and 45% in *D. stramonium* and *D. meteloides*, respectively. The plants treated with 0.01 and 0.001% of BOH closely resembled the controls, whereas a slight increase was noted in height and growth in those receiving the weakest concentration (0.0001%) of BOH. Thus, it was decided to employ weekly treatments instead of biweekly treatments, and to use the previously designated concentrations of the chemical.

Measurements and Harvesting.—Height measurements were taken twice weekly from the soil line to the apex of the highest leaf. At harvest time each plant was immediately washed and cleaned, and the moisture was removed with towels. The plant then was divided into leaf-tops, stem, and root portions. The leaf-tops and stems were cut into small segments, and the fresh weight of each portion was taken immediately. Dry weights were taken after drying 48 hr. in a forced-air drier at 60° C. The plant parts were then ground to a No. 40 powder in a Wiley mill, pooled according to treatment and harvest, and stored in colored glass containers in a desiccator until subsequent analyses were performed.

Alkaloid Analysis.—The dried plant parts, employing pooled samples, were assayed for alkaloid concentration, expressed as scopolamine, according to the Brummett-Sciuchetti method (10). Two extractions were made per group, and each of the extracts was analyzed in duplicate. When duplicate determinations did not agree within a reading of 0.005 absorbance, two further extractions and analyses were made for each group. The total alkaloidal content per plant and per plant organ was obtained by multiplying the dry weight of the plant by the per cent of alkaloids obtained from the alkaloid analyses, and expressing the results in milligrams.

RESULTS

Growth Effects.—Plants treated with 1% of BOH were drastically inhibited in growth. Deformative effects were also noted. The height of this group was only 46% of the controls at the terminal harvest (Fig. 1). The newly formed leaves of this group appeared to be slightly chlorotic, whereas the older leaves were a darker green in color. The young leaves also became curled and wrinkled, and a characteristic yellow splotching was noted among the veins of this group as the leaves matured (Fig. 2). The newly formed leaves were smaller and narrower than similarly formed ones of the controls. Contrasted with this, the older leaves of the group treated with 1% of BOH were thicker than the controls. Numerous necrotic areas varying from 15 to 30 mm. in diameter were noted usually in the upper portion of the blade of these leaves. The width of the blades and petioles of the older leaves

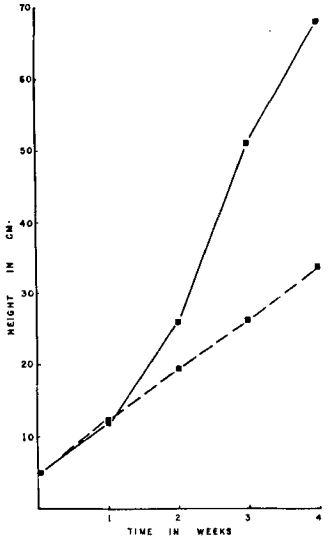


Fig. 1.—Effect of 1% BOH on height of *D. stramonium*. Key: ————, control; — — —, 1% BOH.

Fig. 2.—Habit of plants at final harvest. Key: X, untreated (control); A, 1% BOH.

TABLE I.—WEIGHT OF *D. stramonium* PLANT PARTS[a]

Treatment and Harvest Time, Wk.	Total Wt. Fresh, Gm.	Total Wt. Dry, Gm.	Total Wt. Control Dry Wt., %	Leaf-Tops Fresh, Gm.	Leaf-Tops Dry, Gm.	Leaf-Tops Control Dry Wt., %	Stems Fresh, Gm.	Stems Dry, Gm.	Stems Control Dry Wt., %	Roots Fresh, Gm.	Roots Dry, Gm.	Roots Control Dry Wt., %
Control, 0[b]	0.84	0.07	...	0.46	0.05	...	0.17	0.01	...	0.21	0.01	...
Control, 2	45.7	3.31	...	30.5	2.42	...	8.2	0.47	...	7.0	0.42	...
1.0% BOH,[c] 2	33.0	2.27	69	22.3	1.61	67	5.7	0.36	77	5.0	0.30	71
0.01% BOH, 2	38.0	2.45	74	24.2	1.74	72	7.3	0.37	79	6.5	0.34	81
0.0001% BOH, 2	43.6	2.93	89	28.8	2.09	87	9.8	0.51	109	5.0	0.33	79
Control, 4	171.5	18.81	...	68.0	7.54	...	74.8	8.80	...	28.7	2.47	...
1.0% BOH, 4	84.8	6.19	33	49.5	3.83	51	24.8	1.63	19	10.5	0.73	30
0.01% BOH, 4	155.7	16.91	90	61.3	6.57	87	69.2	8.16	93	25.2	2.18	88
0.0001% BOH, 4	157.1	18.19	97	63.3	7.01	93	67.3	8.62	98	26.5	2.56	104

[a] Mean weight per group of 6 plants. [b] Mean weight of all 24 plants which were harvested but not treated at zero time. [c] BOH is β-hydroxyethylhydrazine.

TABLE II.—CONCENTRATION OF ALKALOIDS OF STRAMONIUM PLANT PARTS[a]

Treatment and Harvest Time, Wk.	Leaf-Tops Alkaloids,[b] mg./Gm.	Leaf-Tops Control, %	Stems Alkaloids,[b] mg./Gm.	Stems Control, %	Roots Alkaloids,[b] mg./Gm.	Roots Control, %
Control, 0[c]	1.78	...	3.04	...	1.72	...
Control, 2	2.56	...	0.89	...	0.99	...
1% BOH, 2	2.38	93	1.11	125	1.80	182
0.01% BOH, 2	2.56	100	0.99	111	0.67	68
0.0001% BOH, 2	2.07	81	1.22	137	0.94	95
Control, 4	3.84	...	1.04	...	0.36	...
1% BOH, 4	2.12	55	1.09	105	0.58	161
0.01% BOH, 4	3.18	83	1.07	103	0.94	261
0.0001% BOH, 4	4.27	111	2.11	203	1.11	309

[a] Based on pooled samples of six plants per group. [b] Total alkaloids calculated as scopolamine. [c] Consisted of all four groups which were harvested but not treated at zero time.

TABLE III.—TOTAL ALKALOIDAL CONTENT[a] OF STRAMONIUM PER PLANT AND ORGAN

Treatment and Harvest Time, Wk.	Per Plant mg.	Per Plant Control, %	Leaf-Tops mg.	Leaf-Tops Control, %	Stems mg.	Stems Control, %	Roots mg.	Roots Control, %
Control, 0	0.14	...	0.08	...	0.04	...	0.02	...
Control, 2	7.04	...	6.20	...	0.42	...	0.42	...
1% BOH, 2	4.77	68	3.83	62	0.40	95	0.54	129
0.01% BOH, 2	5.05	72	4.45	72	0.37	88	0.23	55
0.0001% BOH, 2	5.26	75	4.33	70	0.62	148	0.31	74
Control, 4	38.99	...	28.95	...	9.15	...	0.89	...
1% BOH, 4	10.32	26	8.12	28	1.78	19	0.42	47
0.01% BOH, 4	31.67	81	20.89	72	8.73	95	2.05	230
0.0001% BOH, 4	50.96	131	29.93	103	18.19	199	2.84	319

[a] Calculated from dry weight and alkaloid analyses data; per plant = leaf-tops + stems + roots.

of this group was about 60% of the controls. The leaves of this group were extremely brittle. This brittleness was noted after the second treatment with 1% of BOH and became more pronounced with each subsequent treatment. Great care had to be taken in handling these plants since the leaves would break off from the plant simply by brushing against them. This characteristic was not noted in the other groups. The formation of floral buds and flowers did not occur in the 1% BOH group. On the other hand, the number of floral buds and flowers of the two groups receiving the weaker strengths of BOH was similar to the controls. The habit of the plants receiving treatments with 0.01 and 0.0001% of BOH in general resembled the controls.

Statistical analysis[3] of the height data indicated that the height of the plants treated with 1% of BOH was depressed significantly (at the 1% confidence level) as soon as 2 weeks following application of the chemical. The two weaker concentrations of BOH did not significantly alter the height of the plants.

The growth of the plants as indicated by dry weight data was markedly inhibited by treatment with 1% of BOH (Table I). Statistical analysis of the dry weight data indicated that with the lower concentrations (0.0001 and 0.01%) of BOH there was little difference in growth between treated and

[3] The statistical analyses of height and dry weight data were performed by Dr. R. G. Petersen, Oregon State University, Corvallis.

untreated plants. At a concentration of 1% of BOH, plant growth was depressed. This inhibition of growth became more pronounced with time. In considering the individual plant parts, the effect of 1% of BOH on the depression of growth was least pronounced in the roots and most pronounced on plant height. At the terminal harvest, total dry weight, leaf-top dry weight, and stem dry weight were significantly decreased at the 1% confidence level and root weight at the 5% confidence level.

Effect on Alkaloid Patterns.—At the terminal harvest the concentration of alkaloids of the group treated with 1% of BOH was reduced 45% in the leaf-tops, but increased 61% in the roots (Table II). Similar figures for the 0.01% BOH groups were a decrease of 17% and an increase of 161%. The concentration of stem alkaloids was not affected in either group. Significant increases in the concentration of alkaloids were found in the group treated with 0.0001% of BOH, *viz.*, twofold in the stems and threefold in the roots (Table II).

The total alkaloidal content of the plants receiving treatment with 1% of BOH was significantly decreased (Table III). At the terminal harvest, the total alkaloidal content per plant was only 26% of the controls. All of the plant organs indicated a significant reduction. This was due primarily to the inhibition of growth. Decreases of 19% per plant and 28% in the leaf-tops were noted in the total alkaloidal content of the group treated with 0.01% of BOH, while an increase of 130% was found in the roots. Significant increases of 31% in the leaf-tops and of 219% in the roots of the group receiving the 0.0001% treatment were noted.

DISCUSSION AND CONCLUSIONS

Treatment of *D. stramonium* with 1% of BOH definitely inhibited plant growth. Deformative effects were noted in the leaves. These were manifested by a curling and wrinkling of the newly formed leaves and the appearance of a characteristic yellow splotching among the veins of the leaves of the treated plants. The latter indicates a damaged root system which would limit growth. This suggests that the 1% strength of BOH was a growth inhibitor in *D. stramonium* rather than a growth retardant as has been indicated for B995 (3, 7, 8). Although BOH and B995 are closely related chemically, treatment of *Daturas* with approximately the same concentration of each chemical does not produce the same effect. The effects on the plants treated with 1% of BOH more closely resemble those noted when *Daturas* have been treated in this laboratory with maleic hydrazide or its ester. Other unusual effects noted in this group included the appearance of necrotic areas in the upper portion of the older leaves which had a reduction of about 40% in the width of their blades and petioles compared with leaves from untreated plants, an exceptional brittleness of the leaves, and inhibition of floral bud formation. Flowers did not appear in any of the plants of this group, while the formation of floral buds and flowers in the groups receiving 0.01 and 0.0001% of BOH approximated the controls.

The total alkaloidal content of the 1% BOH group was about one-fourth that of the controls at the final harvest. This was due primarily to the inhibition of growth, although the 45% reduction in the concentration of leaf-tops alkaloids was a contributing factor. The 61% increase in root concentration of alkaloids did not offset the latter since the roots represent slightly more than 10% of the total plant weight compared with 50% for the leaf-tops.

The habit of the plants treated with 0.01 and 0.0001% of BOH generally resembled the controls. Noteworthy was the effect that the treatments with the weaker strengths of BOH had on the concentrations of alkaloids in the plant organs. The concentrations of leaf alkaloids in the group treated with 0.01% of BOH was 83% of controls compared with 111% for the 0.0001% BOH group. This concentration was intermediate between the latter and the group treated with 1% of BOH. The concentration of root alkaloids was increased 161% in the 0.01% BOH group and 209% in the 0.0001% BOH group. This suggests that the synthesis and/or accumulation of alkaloids in this organ was significantly increased by the two weaker strengths of BOH. The only treatment which affected the concentration of stem alkaloids was that with 0.0001% of BOH which resulted in a twofold increase. Due to the phenomenal increase in the concentration of alkaloids in the stems and roots of this group, the total alkaloidal content per plant was increased 31%, that of the stems increased 99%, and that of the roots increased 219%. These increases were considered significant. No precise explanation is presently forthcoming as to why BOH in all three of the strengths employed significantly increased the concentration of root alkaloids.

REFERENCES

(1) Gowing, D. P., and Leeper, R. W., *Science*, **122**, 1267 (1955).
(2) Gowing, D. P., and Leeper, R. W., *Botan. Gaz.*, **123**, 34(1961).
(3) Cathey, H. M., *Ann. Rev. Plant Physiol.*, **15**, 271 (1964).
(4) Riggio-Bevilacqua, L., *Boll. Soc. Ital. Sper.*, **32**, 459, 1323(1956).
(5) Reed, D. J., *Science*, **148**, 1097(1965).
(6) *Ibid.*, **148**, 1469(1965).
(7) Sciuchetti, L. A., and Born, A. E., *J. Pharm. Sci.*, **54**, 285(1965).
(8) Sciuchetti, L. A., and Iturrian, R. C., *ibid.*, **54**, 1477 (1965).
(9) Sciuchetti, L. A., *ibid.*, **53**, 61(1964).
(10) Brummett, R. E., and Sciuchetti, L. A., *ibid.*, **49**, 274 (1960).

Kinetics of Aggregation of Denatured Proteins I

Methodology Based on the Multichannel Particle Size Analyzer

By NORMAN F. H. HO and WILLIAM I. HIGUCHI

The purpose of this study was to develop a method for studying the kinetics and the mechanisms of aggregation of denatured protein particles. It was found that a procedure based on the RIDL multichannel particle counter and size analyzer system was suitable. The precipitation behavior of urea denatured ovalbumin was studied near its isoelectric point. An evaluation of the data showed that the Smoluchowski theory for rapid aggregation of monodispersed particles and the self-preservation theory satisfactorily accounted for the results.

THE PROPENSITY of proteins to undergo gross physical and chemical changes by denaturation is realized in clinical biochemistry. This characteristic property is the basis for diagnostic procedures involving sedimentation, salt precipitation, and heat coagulation of proteins, such as the erythrocyte sedimentation reaction, the Weltman coagulation band technique, turbidity, and flocculation reactions (1, 2). More recently, medical researchers have developed the use of denatured protein particles complexed with radioisotopes to measure renal, liver, splenic, and pulmonary blood flow (3–5). One preparation, *i.e.*, the particle denatured human serum albumin —^{131}I complex, is used for the rapid diagnosis and location of pulmonary embolism by scintillation scanning on the basis of differential filtration of the tracer particles at the site of embolism (6). The particle size and stability of the suspension are critical problems of this preparation.

While the protein chemists have intensified their investigations on the denatured protein mainly in the direction to unravel the structure of the native protein and to study the kinetics, mechanisms, and energetics of the denaturation process itself, the kinetics of growth and aggregation of denatured protein particles has received little or no attention. Research on the particulate behavior of denatured proteins should contribute to the fundamental understanding of the techniques in clinical laboratory procedures involving sedimentation of denatured proteins and in the interpretation of relevant data. In the field of applied pharmaceutical chemistry, such studies should provide a basic approach to the preparation of protein particles tagged with radioactive substances for the rapid diagnosis of embolism. Through the application of controlled growth and aggregation, one should be able to "tailor-make" a stable denatured protein particle or aggregate of uniform and desired size.

The long range objective of the present research is to study this aspect of denaturation on a quantitative basis. The authors hope to examine the effects of protein species and concentration, the method of denaturation, pH, temperature, mono- and polyvalent electrolytes, solvents, and surface-active agents.

The purpose of this paper is to present a well-defined procedure for carrying out such studies. It involves the use of an electronic resistance counter with a multichannel particle size analyzer to follow changes in particle size distributions with time. For the model system studied here, the urea–pH induced denaturation of ovalbumin (7) was chosen taking advantage of the well-defined properties and the better controlled irreversible denaturation of ovalbumin.

EXPERIMENTAL

Materials and Method of Denaturation.—A single lot of twice crystallized, lyophilized ovalbumin[1] was used throughout the study without further recrystallization and stored in a tight container under refrigeration to maintain stability of the protein. Urea used was reagent grade. Buffer solutions were prepared from reagent grade salts and standardized with the Beckman zeromatic pH meter.

One-half and 1% ovalbumin solutions in 8 *M* urea–phosphate buffer were prepared in the following manner. Ovalbumin was dissolved in a small amount of 0.05 *M* phosphate buffer (pH 6.88 and previously filtered through 0.45 μ Millipore filters) without stirring. To this was added the freshly prepared phosphate buffered urea solution with gentle stirring. The final pH was 7.1. Denaturation of the protein was carried out at constant stirring of 20 r.p.m. with a synchronous motor and in a closed system thermostated at 30°. At the end of 5 days, the denatured protein solution was discarded to avoid the complications of cyanate formation from the alkaline hydrolysis of urea (8).

Procedure for Aggregation[2] Studies.—Samples of the urea-denatured ovalbumin were used for the

Received April 28, 1966, from the College of Pharmacy, University of Michigan, Ann Arbor.

Accepted for publication October 13, 1966.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported by fellowship 5-F1-GM-24,039 from the Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[1] Worthington Biochemical Corp., Freehold, N. J.

[2] Throughout this study "aggregation" means the formation of conglomerates of particles as distinguished from molecular aggregation.

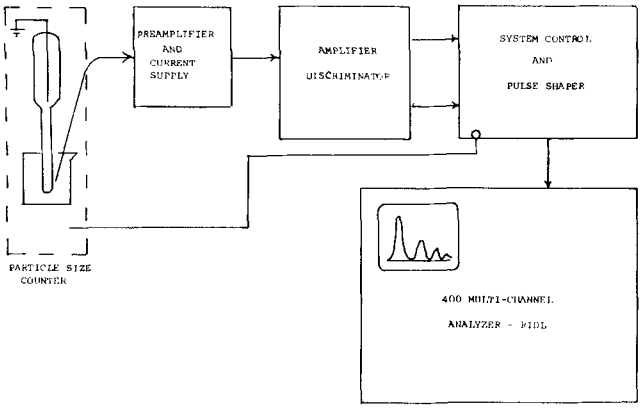


Fig. 1.—Particle counter and size analyzer system.

aggregation experiments after the viscosities of the solutions became constant. The viscosities were followed with an Ostwald viscometer. After denaturation periods of at least 18 hr., the protein solutions were diluted into 0.01 *M* acetate–normal saline buffer solutions, previously adjusted to pH 4.6 or 5.5, so that the final concentration was 1 mcg. denatured protein per ml. Also, normal saline adjusted to pH 8.7 with sodium hydroxide was used as a diluting solution. Initially, the solutions were mixed with gentle stirring and then allowed to stand without disturbance. Dilutions were made for several denaturation periods. The course of the aggregation with time was followed by the single channel Coulter counter, model A, and the RIDL multichannel particle analyzer. Both the 30-μ and 50-μ aperture tubes were used. For the counting runs, a further 1:11 dilution of the master suspension was necessary to minimize coincidence errors. The initial particle concentrations were in the neighborhood of 1×10^7 particles per ml. Since the aggregating system was somewhat sensitive to the method of dilution, a procedure was developed that did not influence the results. A 1-ml. transfer pipet, Kimax -51 No. 37034-A, blow-out type, was used to pipet and deliver an aliquot at the rate of 1 ml./15 sec. The delivery was performed with the tip of the pipet under the surface of the diluting medium and the remaining drops were gently blown out. The samples were then immediately counted.

Particle Counter and Size Analyzer System and Its Standardization.—To follow aggregation, changes in the particle size distribution as a function of time were followed by a new electronic resistance counter system shown schematically in Fig. 1. The system consists of a particle detector apparatus based on the Coulter counter[3] principle and a sophisticated transistorized RIDL 400 channel analyzer,[4] which was originally developed for γ-ray spectrometry. A constant current is supplied to the aperture tube of the detector apparatus. As a particle moves through the aperture, there is an abrupt change in the aperture current. The current signal is amplified and converted to a voltage pulse by the preamplifier. The pulse is then shaped and sent on to the pulse height analyzer where it is classified into one of the 400 channels and counted. Only one pulse can be analyzed at a time, but the analyzer performs this task in the order of milliseconds before accepting another pulse.

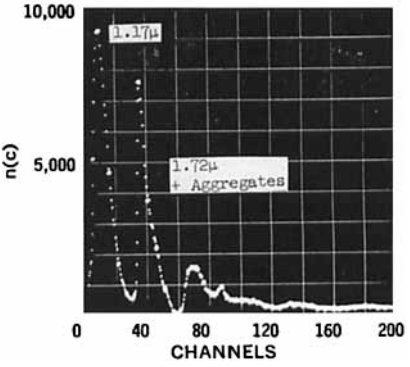


Fig. 2.—Differential distribution data for a mixture of a 1.17 and 1.72-μ diameter latices. Two-hundred channels used. n(c) is the concentration of particles in channel c.

The distribution of particle size densities can be oscillographically displayed or stored in memory. By utilizing only 100 channels at a time, it is possible to simultaneously hold in memory up to four particle size distributions which may be displayed independently or simultaneously on the oscilloscope screen. Corrections for background may also be programmed.

While in most of the present work the data were presented on the oscilloscope screen and photographed with a Polaroid camera, other convenient modes of data retrieval may be used. Numerical readout devices and conventional recorders are easily adapted to the instrument.

Standardization of the multichannel particle analyzer was accomplished with 1.17, 1.72, and 2.051-μ diameter polyvinyltoluene latices[5] suspended in 0.90% sodium chloride solutions. All of the standard latices were washed repeatedly with alcohol and water by centrifugation, and the latter two latices were aggregated to some degree with magnesium sulfate. Microscopic measurements and the Coulter counter, model A, served as a check. Figure 2 shows the differential distribution of a mixture of 1.17-μ and aggregated 1.72-μ diameter

[3] Coulter Electronics, Chicago, Ill.
[4] Radiation Instrument Development Laboratory, Inc., Melrose, Park, Ill.
[5] Dow Chemical Co., Midland, Mich.

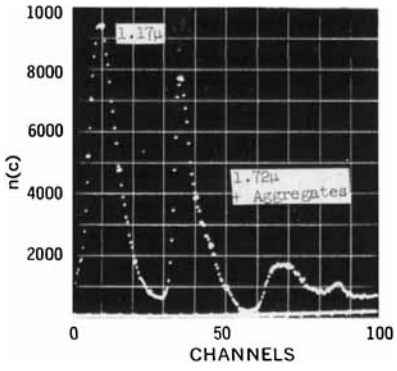


Fig. 3.—Same distribution as Fig. 2, but only the first 100 channels presented.

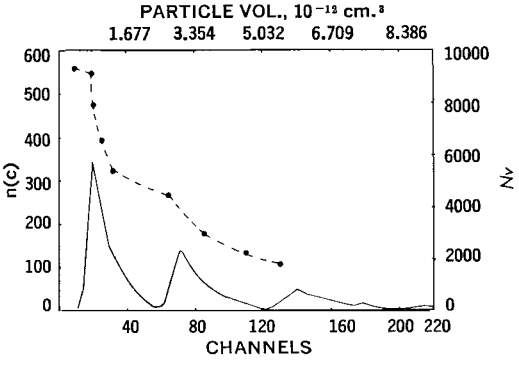


Fig. 4.—Analysis of a mixture of the 1.17-μ and an aggregated 1.72-μ diameter latices. Key: ———, differential distribution data obtained with multichannel system; – – – –, data obtained with Coulter counter, model A.

latices. It is the oscillographic spectrum actually displayed by the 400 multichannel analyzer. Here, however, only 200 channels have been used to describe the distribution. The channel number is proportional to particle volume and the x-axis is calibrated with at least two known standards. The total number of particles corresponding to a given channel is represented by a point so that there will be as many points on the smooth distribution curve as there are preselected number of channels. At the 10th channel in Fig. 2, there is essentially a unimodal distribution of the 1.17-μ particles. The sharp singlet peak for the 1.72-μ particles occurs at about the 35th channel, its doublet peak occurs at approximately the 70th channel, and the triplet aggregate at the 105th channel as expected. However, there is in addition an odd small peak at the 90th channel.[6] The somewhat broader distributions of the high order aggregates are probably due to the wider distributions of shapes (configurations).

[6] Tentatively, the authors have assigned both peaks at the 90th and 105th channels to triplet aggregates. Microscopically, the triplet aggregates appear in three general geometrical forms, *viz.*, the triangular, linear, and other angular arrangements. According to rough theoretical calculations on the effect of particle shape factors on the electronic particle detection system and qualitative experimental data on the rearrangement kinetics of the triplet aggregates, there would be approximately a spread of 10 to 20 channels between the more stable triangular triplet aggregate and the other triplet aggregates. This study is being pursued further.

The distribution of the 1.72-μ latices shows about 45% aggregation. When longer sampling times were taken, the statistics for the higher order aggregates improved and even 8-mers were detectable for the 1.72-μ aggregated system at approximately the 285th channel. There is relatively good linearity with this system. Figure 3 shows the same distribution as in Fig. 2 except that the first 100 channels have been expanded by means of the x-axis expansion control. Likewise, the second 100 channels may be expanded for examination of the higher order aggregates.

A quantitative analysis of a mixture of 1.17 and aggregated 1.72-μ latices was carried out with both the multichannel particle analyzer and the Coulter counter, model A, using a 30-μ aperture tube. As shown in Fig. 4, the multichannel analyzer gives directly a differential distribution, while the Coulter counter gives a cumulative distribution. Whereas the differential distribution data were obtained from one 50-μl. sample, the cumulative distribution was obtained with nine 50-μl. samples. The advantages of the multichannel system can be seen from Fig. 4. With the single channel instrument many sample readings must be taken to achieve the same degree of accuracy. For example, if the sixth point in the cumulative plot of Fig. 4 were not taken, it would have been impossible to decide whether the first two peaks were separate or possibly a single broad distribution. Also, the true particle distribution cannot be adequately resolved for rapid flocculating systems without laborious effort.

To compare the data from the multichannel analyzer with that from the Coulter counter, the differential distributions were integrated planimetrically. Therefore,

$$N_v = \int_v^\infty n(v)dv \qquad \text{(Eq. 1)}$$

where N_v is the integral number of particles larger than v, and $n(v)dv$ is the number of particles between the sizes v and $v + dv$. The results are tabulated (Table I) and compared with the Coulter counter data.

As the sophistication of the electronics of the multichannel analyzer allows one to detect rapid changes in particle size distributions, the direct display of the differential distribution and the efficient data recording techniques can be utilized with the aid of Eq. 1 to follow the course of aggregation of an initially monosized particle system as a function of time.

Recently, Samyn (9) reported that when suspension systems are highly polydispersed, the Coulter

Table I.—Comparison of Coulter Counter, Model A, and RIDL Multichannel Data on Mixed Latex Particles

Channels	Particle Vol. 10^{-12} cm.³	No. of Particles Beyond Stated Size RIDL Multichannel	Coulter, Model A
10	0.419	9256	9350
20	0.839	8462	8550
40	1.677	5421	5350
60	2.51	4760	4700
80	3.354	3239	3300
100	4.193	2380	2400
120	5.032	1851	1850

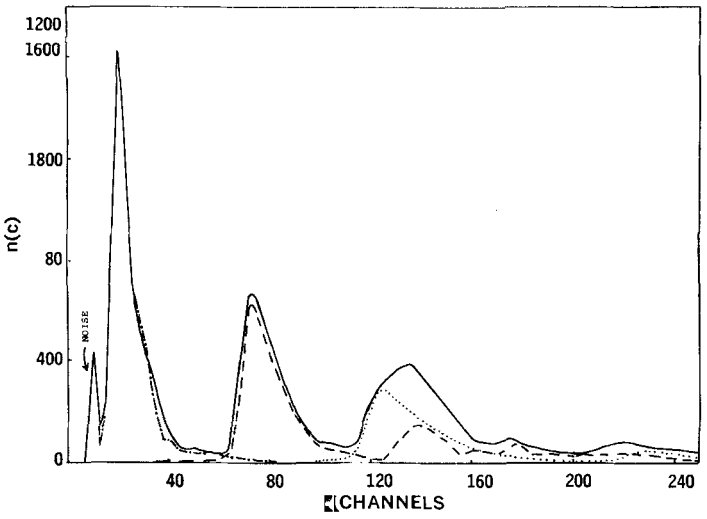


Fig. 5.—Distribution curves accounting for additivity in a polydispersed latex system by the RIDL multichannel analyzer. Key: ———, distributions of mixture of equal parts of 1.17, 1.72, and 2.05 μ; —·—·—, independent distribution of 1.17 μ; — — —, independent distribution of 1.72 μ; ....., independent distribution of 2.05 μ.

counter, model A, may show poor additive results. This prompted the investigation of the reliability of the multichannel analyzer in this aspect, at least in the range of sizes pertinent to our research. Figure 5 shows (broken curves) the independent distributions of 1.17-μ and aggregated 1.72 and 2.05-μ particles. The noise peak at the 10th channel serves as a reference from which the true particle distribution can be differentiated. Upon mixing equal parts of the latex suspensions, the resulting normalized distribution assuming additivity of the separate runs is shown by the smooth curve. It appears therefore that the multichannel analyzer system can give accurate results even when the polydispersity is of the order of thirtyfold in volume.

Because the latex particles are essentially monodisperse, the peak widths given in Figs. 2–5 may be used to estimate the instrument resolution. In the present size range this is found to be about ± 0.1 to 0.2 μ in the diameter. Except in unusual situations, most real suspension systems have broad enough particle size distributions so that the instrument resolution does not constitute a serious limitation.[7]

RESULTS AND DISCUSSION

Process of Denaturation.—The kinetics and mechanisms of denaturation of ovalbumin by urea are discussed in a number of references (7, 10–13). The initial stage is an intramolecular reaction involving the destruction of internal order. This may be interpreted as the unfolding of the helical structures of ovalbumin to a more randomly coiled arrangement accompanied by an increase in viscosity. Usually, this stage is measured by optical rotation. The second stage is characterized by an increase in viscosity of the solution without further change in optical rotation. In contrast to the relatively rapid increase in viscosity in the initial stage, the viscosity increases at a slower rate during this second stage and eventually reaches a plateau. It is believed that association of the denatured protein molecules primarily by exchange reaction with sulfhydryl and disulfide groups is an important factor in this second phase. In general, the concentrations of ovalbumin, urea and electrolytes, pH, and temperature all affect this over-all denaturation process.

With the ovalbumin–urea concentration ratios used in this study, the first stage of denaturation is completed within 3 hr. and the viscosity reached a plateau a few hours thereafter.

Aggregation Experiments.—Typical differential particle distributions at various time intervals, obtained directly from photographs of the oscillographic display of the multichannel analyzer, are shown in Fig. 6 for the aggregation of denatured protein. In this instance the 30-μ aperture tube was used. Since there was a noise contribution in channels 10 to 13, previously pointed out in Fig. 5, only that portion of the distribution from the 15th channel was used for further analysis.

Figure 7 gives the cumulative counts *versus* time curves showing aggregation of denatured ovalbumin at times greater than about 60 min. These results were planimetrically determined from data such as those given in Fig. 6. The pH is in the neighborhood of the isoelectric point for denatured ovalbumin. The two curves correspond to the 0.5% and the 1.0% protein denatured for 24 and 22 hr., respectively. For longer denaturation times the results were essentially the same.

Mass balance was approximately maintained after times greater than the time corresponding to the peak shown in Fig. 7. This was established by graphical integration,

$$M = \int_v^\infty \rho v n(v) dv \qquad \text{(Eq. 2)}$$

where M is the protein mass, ρ is the density, v is the volume of the particle, and $n(v)dv$ is the number of particles between the sizes v and $v + dv$.

The range of sizes observed in these experiments was 0.4 to 1.2-μ radius. The majority of the particles were between 0.4 to 0.8-μ radius. Under the microscope, $\times 970$ in immersion oil, aggregates were found to be nearly spherical in shape and were in the general magnitude of sizes detected by the multichannel analyzer.

[7] A detailed study of this question is underway and will be reported in a later communication.

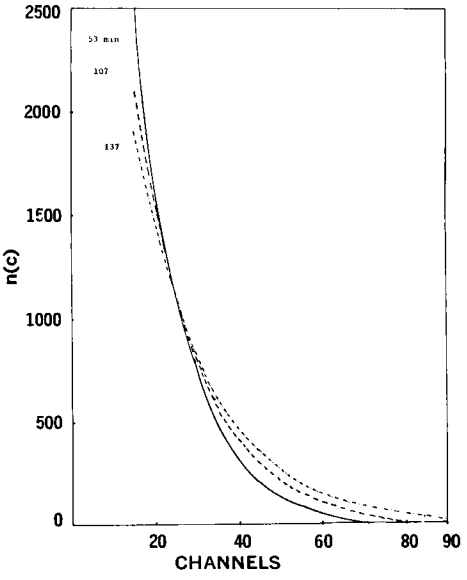


Fig. 6.—Differential distribution data showing aggregation of denatured ovalbumin particles with time in buffered saline, pH = 4.6.

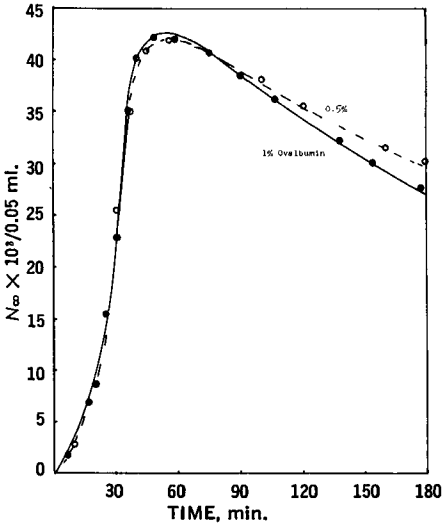


Fig. 7.—Changes in total denatured protein particulate concentration for sizes greater than 0.80-μ diameter as a function of time. Decreasing counts after 60 min. reflect aggregation of particles.

Comparison with Smoluchowski's Theory.—Smoluchowski worked out a basic theory of aggregation of spherical particles of a colloidal suspension (14). When the aggregation is controlled by Brownian diffusion in the absence of a force field, the aggregation of a relatively monodispersed system is given by the kinetic expression for bimolecular collision,

$$\frac{dN_\infty}{dt} = -KN_\infty^2 \qquad \text{(Eq. 3)}$$

Where N_∞ is the number of particles of all sizes, K is the rate constant, and t the time. Upon integration a linear relationship of $1/N_\infty$ *versus* t with slope K is predicted. For this case, the rate constant is:

$$K = \frac{4\pi DR}{W} \qquad \text{(Eq. 4)}$$

where D is the effective diffusion coefficient, R is the distance between centers of the particles, and W is the stability factor. By assuming the Stokes-Einstein relationship for translational diffusion, *i.e.*,

$$D = \frac{kT}{6\pi\mu a} \qquad \text{(Eq. 5)}$$

and the effective collision radius, $R = 2a$, and irreversible collision, $W = 1$, where k is the Boltzmann constant, μ the viscosity of the medium, T the absolute temperature, and a the particle radius, the theoretical maximum rate constant becomes

$$K = \frac{4kT}{3\mu} \qquad \text{(Eq. 6)}$$

At 30° the value of K is 5.6×10^{-12} cm.^{3} sec.$^{-1}$.

By using Eq. 1 the changes of the cumulative number of particles, N_∞, with time were determined by graphical integration with a planimeter of such differential particle distribution data as shown in Fig. 6. In the present experiments N_∞ corresponds to the total number of particles larger than the particle volume of 0.6289×10^{-12} cm.^{3} (15th channel), although the distribution extended to smaller sizes. The Coulter counter, model A, gave this quantity directly by using the appropriate threshold setting. In Fig. 8, the straight line relationship of the Smoluchowski-type plot is found. This is consistent with the theory of aggregation of essentially monodispersed sols. The experimental rate constants obtained with the 30 and 50-μ aperture tubes were not significantly different. The data much beyond 200 min. were believed to be unreliable because of the effects of sedimentation upon the aggregation rate. According to Müller (14), at room temperature, sedimentation may significantly affect the rate constant when the radius of some of the denatured ovalbumin particles are larger than 2 to 3 μ.

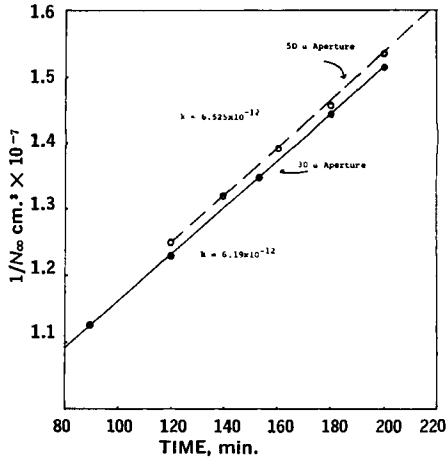


Fig. 8.—Linear plots showing agreement of the aggregation-time data with Smoluchowski theory.

In Table II the summary of some aggregation experiments of urea-denatured ovalbumin is given. The rate constant obtained from the multichannel analyzer and the Coulter counter compared well with each other and also with theory. Once the denatured protein solution reached a constant viscosity level, subsequent rate constants found did not differ from each other. There is a strong dependence of pH on the aggregation. Maximum aggregation was found near the isoelectric point. At pH 5.5 insignificant counts were detected with the 30-μ aperture tube. However, the Tyndall beam showed the presence of many submicron particles in a sol that remained stable for at least 4 weeks. At higher pH values, the Tyndall beam was weaker.

TABLE II.—SUMMARY OF DATA ON AGGREGATION OF UREA-DENATURED OVALBUMIN

Protein Concn., %	pH Buffered Normal Saline	Denaturation Period, hr.	$k \times 10^{-12}$ (cm.^{3}sec.$^{-1}$) Coulter, Model A	RIDL Multichannel
0.5	4.6	24	5.62	6.43
		64	5.90	6.19
	5.5	64	Insignificant counts,	
	8.7	64	but weak Tyndall beam observed.	
1.0	4.6	22	...	7.23
		49	7.1	7.67
	5.5	49	Insignificant counts,	
	8.7	49	but weak Tyndall beam observed.	

Comparison with the Self-Preserving Theory.—Friedlander and Swift (15) showed that certain forms of similarity or self-preserving functions can be applied to the solution of Smoluchowski's fundamental equation for aggregation. The relations depend upon the assumptions that the particle distribution in various sizes is continuous and that a particular form of the size spectrum is invariant with time. Hidy *et al.* (16, 17) have applied the theory to the case of discontinuous particle distributions.

In the theory of Friedlander and Swift the particle distribution is expressed as:

$$n(v, t) = \frac{N_\infty^2}{\phi} \psi_1(\eta) \qquad \text{(Eq. 7)}$$

where $\psi_1(\eta)$ is a dimensionless distribution function, the constant ϕ is the total volume fraction of particles:

$$\phi = \int_0^\infty vn(v)dv \qquad \text{(Eq. 8)}$$

and η is the dimensionless volume distribution:

$$\eta = \frac{vN_\infty}{\phi} \qquad \text{(Eq. 9)}$$

When $\eta = 1$, the average size of the particles with respect to volume is predicted. Furthermore,

$$N_v = N_\infty \int_\eta^\infty \psi_1(\eta)d\eta = N_\infty \psi_2(\eta) \qquad \text{(Eq. 10)}$$

where $\psi_2(\eta)$ is the dimensionless cumulative particle distribution. The terms $n(v)$, N_v, and N_∞, as defined before, are time-dependent; ψ_1 and ψ_2 are time-independent functions and, therefore, do not depend upon the initial size distributions.

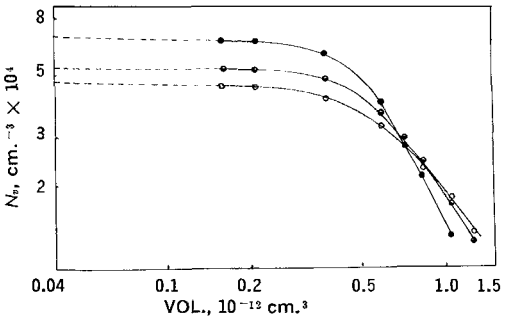


Fig. 9.—Cumulative size distributions for aggregating denatured ovalbumin particles. Key: ●, 53 min.; ◒, 107 min.; ○, 137 min.

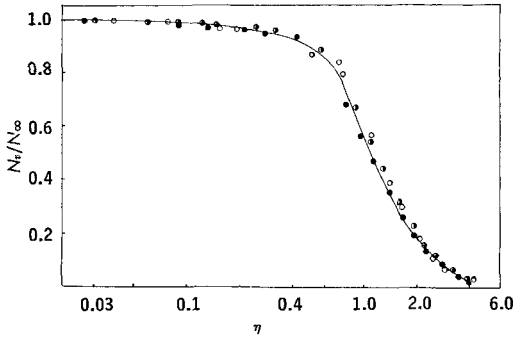


Fig. 10.—Test of self-preservation for aggregating denatured ovalbumin particles. In terms of dimensionless distribution functions, particle distributions taken at different times during the aggregation process fall approximately on a single curve. Data taken from Fig. 9.

The theory was applied to the denatured protein system for the data partially shown in Fig. 6. A log-log plot of N_v *versus* v is shown in Fig. 9 and values of N_∞ were determined by extrapolation of the curve to small values of v. When a Smoluchowski-type plot was made using the extrapolated values, the rate constant was 5.91×10^{-12} cm.^{3} sec.$^{-1}$, which is in good agreement with 6.19×10^{-12} cm.^{3} sec.$^{-1}$ previously found by using that part of the data at larger sizes. The volume fraction was 3.56×10^{-8} cm.^{3} for 0.05-ml. sample of the sol in contrast to the theoretical 3.58×10^{-8} cm.^{3} to indicate that after 53 min. practically all of the insoluble protein came into the detectable range of the RIDL analyzer system. In Fig. 10 the plots of N_v/N_∞ *versus* η at different time intervals shows the tendency of the distribution to preserve its form and gives ψ_2, the time-independent dimensionless cumulative distribution function, as predicted by Eq. 10.

According to Friedlander, if the $1/N_\infty$ *versus* t plot is linear, the rate of aggregation with the self-preserving size distribution can be described by:

$$\frac{dN_\infty}{dt} = \frac{-\alpha A_1 k T N_\infty^2}{3\mu} \qquad \text{(Eq. 11)}$$

where α is the collision efficiency having the same physical meaning as the factor W in Eq. 4, and

$$A_1 = \left[2 + 2 \int_0^\infty \eta^{1/3} d\psi_2 \int_0^\infty \eta^{-1/3} d\psi_2 \right] \quad \text{(Eq. 12)}$$

when $\alpha = 1$ and $A_1 = 4.0$, the rate constant of Eq. 11 reduces to that of Eq. 6 for the homogeneous case. Thus, A_1 is a significant parameter accounting for the effect of heterogeneity on the rate of aggregation. By comparing Eqs. 3 and 11,

$$\alpha = \frac{3K\mu}{A_1 kT} \quad \text{(Eq. 13)}$$

For the protein system, α was approximately 1, and A_1 was 4.148 ± 0.022. Then heterogeneity accounted for about 4% increase in the rate. From Eq. 11 the rate constant found was 5.81×10^{-12} cm.^{3} sec.$^{-1}$. The comparison of methods of determining the rate constant is summarized in Table III.

TABLE III.—COMPARISON OF METHODS OF DETERMINING RATE CONSTANTS OF AGGREGATION

Method of Treatment	Rate Constant, cm.^{3} sec.$^{-1}$
Smoluchowski's theory for monodispersed distribution taking N_∞ from	
(*a*) $\geq v = 0.6289 \times 10^{-12}$ cm.^{3}	6.19×10^{-12}
(*b*) $\geq v = 0.02 \times 10^{-12}$ cm.^{3}	5.91×10^{-12}
Self-preservation theory	5.81×10^{-12}

It should be noted that the approach of self-preservation theory offers a method of determining the aggregation rate constant independent of that approach which assumed a monodispersed system at initial periods of the aggregation process. Since the effective collision between particles was unity and the particle size distribution was essentially monodispersed, the rate constants determined by both methods were in good agreement.

Studies are continuing on the mechanism of the pH influence on the aggregation behavior. Experiments on the effects of electrolytes, temperature, and other additives are also planned.

REFERENCES

(1) Frankel, W., and Reitman, R., "Gradwohl's Clinical Laboratory Methods and Diagnosis," 6th ed., C. V. Mosby, St. Louis, Mo., 1964.
(2) Wuhrman, S., and Wunderly, R., "The Human Blood Proteins," Grune and Stratton, Inc., New York, N. Y., 1960.
(3) Biozzi, G., *et al.*, *J. Lab. Clin. Med.*, **51**, 230(1958).
(4) Torrance, H., and Glowenlock, A., *Clin. Sci.*, **22**, 413(1962).
(5) Taplin, G., Groswold, M., and Dore, E., *UCLA Report 481 (Biology and Medicine)*, 1961.
(6) Henkin, R., and Bishop, H., *J. Lab. Clin. Med.*, **60**, 709(1962).
(7) Simpson, R. B., and Kauzmann, W., *J. Am. Chem. Soc.*, **75**, 5139(1953).
(8) Stark, G., Stein, W., and Moore, S., *J. Biol. Chem.*, **235**, 3177(1960).
(9) Samyn, J., *J. Pharm. Sci.*, **54**, 1794(1965).
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(11) Steven, F., and Tristam, G., *Biochem. J.*, **73**, 86 (1959).
(12) Gagen, W., and Holme, J., *Phys. Chem.*, **68**, 723 (1964).
(13) Tanford, C., "Physical Chemistry of Macromolecules," John Wiley & Sons, Inc., New York, N. Y., 1961, p. 627.
(14) Kruyt, H. R., "Colloid Chemistry," vol. 1, Elsevier Publishing Co., Amsterdam, The Netherlands, 1952, Chap. 7.
(15) Swift, D. L., and Friedlander, S. K., *J. Colloid Sci.*, **19**, 621(1964).
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CHLOROQUINE has been shown to have an unexpectedly wide spectrum of therapeutic uses since its introduction into medicine as an antimalarial. The increased utilization of this drug at fairly high dose levels for extended periods has prompted reconsideration of its potential toxicity.

Visual disturbances, headache, bleaching of hair, electrocardiographic changes, and weight loss have been reported during short-term administration of chloroquine to healthy subjects (1). Electrocardiographic changes, consisting of lowered T wave, ST segment depression, and prolongation of QTc interval have occurred when

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REFERENCES

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(15) Swift, D. L., and Friedlander, S. K., *J. Colloid Sci.*, **19**, 621(1964).
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the drug was used in the treatment of conditions such as extraintestinal amebiasis (2). Similar changes have been noted during the course of acute, fatal, chloroquine poisoning (3). Muscle weakness and bilateral loss of knee and ankle reflexes have been demonstrated during extended chloroquine therapy in rheumatoid arthritis (4, 5).

Upon examination of several cases of acute chloroquine poisoning in children, a striking similarity to poisoning by quinidine was noted (6). It was suggested that the cardiac arrest which occurred might be due to a direct myocardial action, to anoxia from respiratory depression, or to both.

It has been reported that substantial differences exist in the toxicity of chloroquine for various laboratory animals, the compound being most toxic to the dog and least toxic to the mouse. The rat appeared to be more susceptible to the drug than the mouse. The study further indicated that chloroquine had a distinctly steeper dose-toxic response curve than other 4-aminoquinolines (7). This suggests that the drug has a narrow margin of safety, a factor which may be of great significance in therapeutic applications where high dose levels are necessary.

Long-term, chronic administration of chloroquine to rats indicated slight gross pathological changes (8). However, microscopic examination of tissues revealed a slowly developing necrosis of cardiac and skeletal muscle.

Because some of the cardiac effects of chloroquine might be due to the interference with metabolic processes of the myocardium, it appeared to be of interest to investigate the effect of the drug on some of the respiratory processes of heart homogenates.

METHODS

Conventional manometric techniques were used to study the effect of chloroquine on the oxidation of succinate, malate, and β-hydroxybutyrate by myocardial homogenates prepared from male Sprague-Dawley rats (9).

The animals were stunned by a blow to the head, exsanguinated, and the hearts removed immediately. Each heart was allowed to beat a few seconds in cold 0.1 *M* phosphate buffer, pH 7.35, after which the ventricular tissue was dissected away, cut open, washed in cold buffer, blotted dry on filter paper, and weighed on a torsion balance.

The homogenate was prepared by placing the tissue in a glass homogenizer containing sufficient cold buffer so that 0.5 ml. of homogenate contained 10 mg. of fresh tissue for the succinate studies or 40 mg. for the malate and β-hydroxybutyrate studies. Not more than 15 min. elapsed from the time the animals were sacrificed until the addition of the homogenate to the Warburg flasks. In some experiments the homogenates were aged before use by allowing them to remain in the refrigerator at 5° overnight followed by 5 hr. at room temperature.

The mitochondrial preparations utilized in the study of the effect of chloroquine on the oxidation of reduced nicotinamide adenine dinucleotide[1] were obtained by centrifuging homogenates, prepared in cold 0.25 *M* sucrose, at 700 × g for 12 min. at 2° in a Servall RC-2 refrigerated centrifuge equipped with a SS-34 rotor to remove cell fragments. The supernatant was then centrifuged at 10,000 × g for 12 min. The resulting pellet was dispersed in 0.25 *M* sucrose, and the suspension added to the flasks so that each contained mitochondria representing 100 mg. of ventricle. The suspension was found to oxidize NADH quite rapidly.

In all of the studies utilizing manometric techniques the center well of the Warburg flasks contained 0.2 ml. 10% KOH and a pleated strip of filter paper. All of these experiments employed air as the gas phase. A temperature of 30° was maintained during respiration determinations utilizing malate, β-hydroxybutyrate, and NADH as substrates. The effects of glutamate and chloroquine[2] on the oxidation of succinate were studied at 37°.

The buffer used for control of pH in the Warburg vessels during the succinate studies was 0.1 *M* potassium phosphate, pH 7.35, containing 0.01 *M* disodium ethylenediaminetetraacetate (EDTA) per liter as recommended by several investigators (10, 11). The main compartments of the flasks in which the oxidation of succinate by aged homogenates was studied contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-2} *M* chloroquine; 0.3 ml. of 5×10^{-4} *M* cytochrome c; 1.3 ml. of phosphate buffer. Distilled water was used to replace the chloroquine in the control flasks. The sidearm contained 0.4 ml. of 0.1 *M* succinate which was tipped into the main compartment at zero time. In some of the experiments with aged homogenates, 0.2 ml. of 10^{-3} *M* NAD, dissolved in phosphate buffer, replaced 0.2 ml. of the buffer.

When the effect of glutamate and chloroquine on the oxidation of succinate was studied, Warburg vessels with two sidearms were employed. One sidearm of each vessel contained 0.4 ml. of 0.1 *M* succinate and was tipped at zero time. The second sidearm contained 0.3 ml. of either distilled water or 5×10^{-3} *M* of chloroquine or 0.1 *M* glutamate and was tipped after 45 min. The main compartment contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-4} *M* cytochrome c; 1.0 ml. of phosphate buffer, and sufficient distilled water to give a final volume of 3.0 ml. In some experiments 0.3 ml. of 0.1 *M* glutamate or 0.3 ml. of 5×10^{-3} *M* chloroquine was placed in the main compartment instead of in the second sidearm.

In the study of the oxidation of malate and β-hydroxybutyrate, the main compartment of each flask contained 0.5 ml. of homogenate; 0.3 ml. of 5×10^{-3} *M* chloroquine or distilled water; 0.1 ml.

[1] Henceforth designated as NADH. The oxidized form will be designated as NAD.

[2] The chloroquine used in this study was graciously supplied by the Winthrop Laboratories, New York, N. Y., as Aralen Diphosphate.

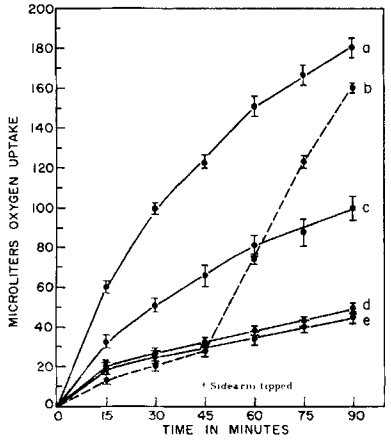

Fig. 1.—The effect of glutamate and chloroquine on succinate in heart homogenates. Key: curve a, glutamate in main compartment; curve b, glutamate tipped from sidearm; curve c, chloroquine in main compartment; curve d, control (no additions); curve e, chloroquine tipped from sidearm.

of 5×10^{-4} *M* cytochrome c; 0.1 ml. of 1.5×10^{-2} *M* $MgCl_2$; 0.1 ml. of 1.5×10^{-2} *M* nicotinamide; 0.1 ml. of 2×10^{-2} *M* adenosine triphosphate; 1.1 ml. of 0.1 *M* potassium phosphate buffer with no EDTA added, pH 7.35; and 0.4 ml. of 0.1 *M* substrate. When malate was employed as the substrate, 0.3 ml. of 1.5×10^{-2} *M* glutamate was included, and the 0.1 ml. of 1.5×10^{-2} *M* malate which was used in the β-hydroxybutyrate studies was excluded from the reaction mixture. Distilled water was added to make the final volume 3 ml.

A reaction mixture similar to that which has been used by other investigators (12) was employed to study the effect of chloroquine on oxidation of NADH by a suspension of swollen and/or fragmented mitochondria. The main compartment of each flask contained 0.75 ml. of a buffer, pH 7.4, consisting of 0.04 *M* potassium phosphate, 0.04 *M* tris (hydroxymethyl) aminomethane, and 0.4 *M* KCl; 0.15 ml. of 0.4 *M* KF; 0.15 ml. of 0.12 *M* $MgCl_2$; 0.5 ml. mitochondrial suspension, and 0.3 ml. 5×10^{-3} *M* chloroquine or distilled water. The sidearm, containing 0.4 ml. of 0.04 *M* NADH, was tipped at zero time. Distilled water was added to make the final volume 3 ml. Readings were taken every 5 min. during a 45-min. period.

The method described by Friedemann (13) was used for the determination of total α-keto acids present in the flasks after a 90-min. incubation of fresh homogenates with succinate as the substrate.

The effect of chloroquine on malic dehydrogenase was determined by following the rate of oxidation of NADH at 340 mμ according to the method of Ochoa (14). In these experiments a purified malic dehydrogenase, obtained from commercial sources,[3] was used, and the reaction was followed for 4 min. at 25° with a Beckman DU spectrophotometer equipped with a Guilford automatic recorder.

The possibility of complex formation occurring between chloroquine and NAD was studied spectrophotometrically using a Beckman DB and recording the spectra between 220 and 400 mμ. In these experiments 0.5 ml. of 5×10^{-5} *M* NAD, 0.5 ml. of 5×10^{-5} *M* chloroquine, 1.0 ml. of H_2O, and 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.35) were incubated for 10 min. at 25° prior to the determination. The reference cell contained the same reagents, except that NAD was replaced by buffer.

In order to study the chronic effects of chloroquine administration on the respiratory processes of the heart, young male rats weighing approximately 100 Gm. were separated into 2 groups of six animals each, further designated as groups A and B. Each rat in group A received daily intraperitoneal injections of 5 mg. of chloroquine phosphate in a volume of 0.5 ml. of distilled water for 7 days and 9 mg. of the drug in the same volume thereafter. Group B received 1.5 ml. of distilled water daily by the same method of administration. On the 14th day the animals were sacrificed, and heart homogenates prepared from each animal. The ability of heart homogenates prepared from animals in group A to utilize malate and β-hydroxybutyrate was compared to that of group B.

RESULTS AND DISCUSSION

Figure 1 shows that chloroquine enhanced the oxygen uptake of freshly prepared homogenates in the presence of added succinate. This effect was previously noted by Datta and Basu (15) with mouse liver homogenates, but not by Hess and Haugaard (16) who used rat heart slices and homog-

Table I.—Effect of Chloroquine on α-Keto Acid Accumulation

	Chloroquine Absent	Chloroquine Present
α-Keto acid/10 mg. tissue/90 min., mcg.	15.5	3.25
	15.0	5.0
	20.0	4.4
	19.0	5.5

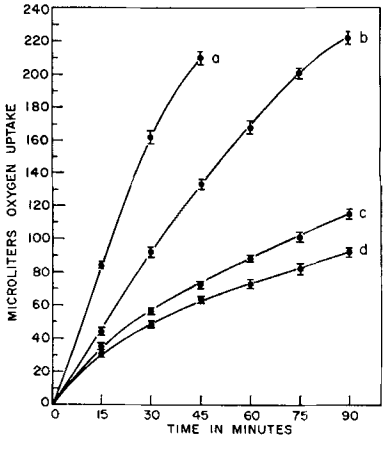

Fig. 2.—Oxidation of succinate by aged heart homogenates. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-3} *M*); curve c, NAD (final concentration 7×10^{-5} *M*); curve d, NAD (final concentration 7×10^{-5} *M*) and chloroquine (final concentration 5.4×10^{-3} *M*).

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

enates. The oxidation of succinate proceeds *via* the following pathway: succinate → fumarate → malate $\xrightarrow{NAD}$ oxaloacetate.

It has been reported that oxaloacetate is an inhibitor of succinate oxidation, *in vitro*, (10) and it follows that an accumulation of oxaloacetate in the Warburg vessels might cause a curtailment of oxygen consumption. On the other hand, chloroquine, by preventing the accumulation of oxaloacetate, could conceivably increase oxygen uptake. The data reported in Table I support this concept and show that after 90 min., the α-keto acid content of flasks to which chloroquine was added at the beginning of the experiment was much lower than that of the control flasks. Heim *et al.* (17) have reported that emetine prevents the accumulation of oxaloacetate and augments the oxygen consumption of heart homogenates respiring in the presence of added succinate. Wolff and Ball (10) showed that thyroxine evokes the same effect.

When chloroquine was added to the flasks 45 min. after the beginning of the experiments an enhancement of oxygen uptake was not observed, possibly because sufficient oxaloacetate had accumulated during the 45-min. period to exert an inhibitory effect. Heart tissue is rich in glutamate-oxaloacetate transaminase and, if the lowered oxygen consumption were due to an accumulation of oxaloacetate, the addition of glutamate would be expected to reverse the inhibitory effect of the oxaloacetate. As shown in Fig. 1, when glutamate was added to the flasks, either at the beginning of the experiment or after 45 min. had elapsed, the oxygen consumption was greatly increased. Therefore, it seems probable that glutamate, *via* transamination, removes the oxaloacetate while chloroquine, when added at the beginning of the experiment, appears to prevent the accumulation of the inhibitory compound. This suggests that the drug may inhibit at some point on the pathway between succinate and oxaloacetate.

With aged homogenates it was found that chloroquine evoked an appreciable inhibition of oxygen uptake in the presence of added succinate. When NAD was added at the beginning of the experiments, the oxygen consumption was markedly inhibited and further reduced, but only to a slight extent, by chloroquine. These results are shown in Fig. 2.

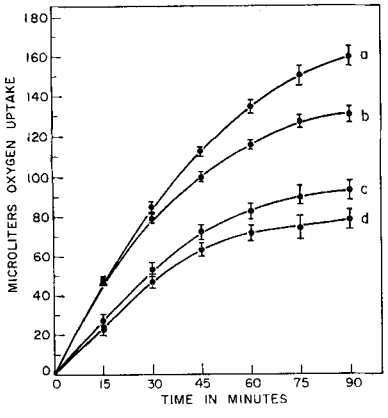

Fig. 3.—Respiration of rat heart homogenates in presence of malate. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*); curve c, endogenous respiration; curve d, endogenous respiration with added chloroquine.

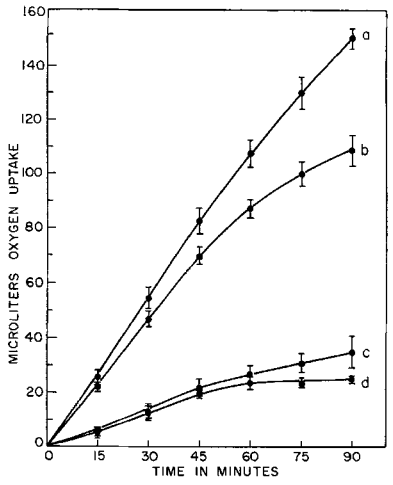

Fig. 4.—Oxidation of β-hydroxybutyrate by heart homogenates. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*); curve c, endogenous respiration; curve d, endogenous respiration with chloroquine added.

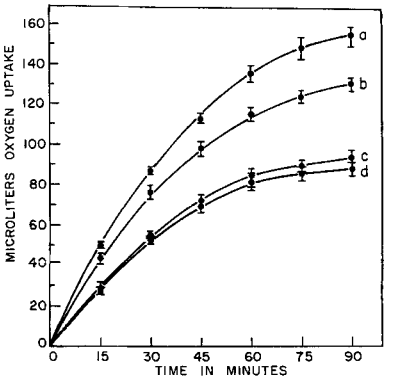

Fig. 5.—Oxidation of malate by heart homogenates from sham-injected and chloroquine-treated rats. Key: curve a, sham-injected animals; curve b, chloroquine-treated animals; curve c, endogenous respiration of chloroquine-treated animals; curve d, endogenous respiration of sham-injected animals.

The oxidation of malate, unlike that of succinate, is an NAD-linked process and contributes substantially to the production of high energy phosphate compounds. Figure 3 shows that chloroquine evokes an inhibitory effect on the oxidation of added malate by homogenates. Datta and Basu (15), utilizing considerably higher concentrations of chloroquine than those employed in this study, demonstrated a rather marked depression of malate oxidation by the drug with mouse liver homogenates.

The oxidation of β-hydroxybutyrate was inhibited by chloroquine, as shown in Fig. 4. It has

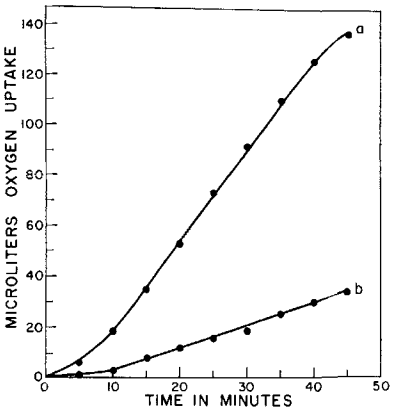

Fig. 6.—Effect of chloroquine on respiration of fragmented mitochondrial suspensions in presence of added NADH. Each point represents determination in duplicate from four animals. Key: curve a, control (no drug added); curve b, chloroquine (final concentration 5.4×10^{-4} *M*).

also been reported by Datta and Basu (15) that certain other NAD-linked reactions are inhibited by chloroquine.

In the animals chronically poisoned by chloroquine a significant loss of weight, similar to that reported by Fitzhugh *et al.* (1), was noted. The ability of heart homogenates, prepared from poisoned animals, to utilize β-hydroxybutyrate was not impaired, but the ability to oxidize malate was somewhat inhibited as illustrated in Fig. 5.

Studies of a preliminary nature were conducted to learn if a complex formation occurs between NAD and chloroquine. No alteration in the absorption spectra could be observed when solutions of the two compounds were mixed. The conclusions which may be drawn from such an observation are limited but present an indication that a complex formation does not occur.

Malic dehydrogenase activity was found to be not significantly affected by chloroquine, in confirmation of the work of Gerlack (18), who observed that the drug elicited no appreciable effect on the activity of either lactic or malic dehydrogenases.

Mushinski *et al.* (19) reported that chloroquine was a potent inhibitor of NADH-cytochrome c reductase in rat skeletal muscle preparations. If this mechanism is operative in heart tissue, it could explain the depression of malate oxidation without a direct effect on malic dehydrogenase. Malate is oxidized in the presence of malic dehydrogenase and NAD to oxaloacetate with the concomitant formation of NADH. NADH is then oxidized to NAD as hydrogen atoms are transferred to flavoproteins, a step which is followed by a series of oxidation–reduction reactions involving the cytochromes. It has been reported that the impermeability to extra-mitochondrial NADH is a characteristic of intact mitochondria (12). The oxidation of added NADH will occur, however, if the mitochondria are exposed to conditions, such as hypotonic media or rigorous isolation procedures, which cause the swelling and/or disruption of mitochondrial membranes. The mitochondrial preparation used in this study displayed a nearly linear oxidation of added NADH over a period of 30 min., and it was found that chloroquine, at a concentration of 5.4×10^{-4} *M*, elicited a marked inhibitory effect on the oxidative process, as shown in Fig. 6. This finding, together with the observed inhibitory effect of the drug on the oxidation of succinate by aged homogenates to which NAD had been added, and the insignificant effect evoked on a purified malic dehydrogenase, suggests that chloroquine may exert effects at or beyond the NAD–NADH step of terminal respiration.

REFERENCES

(1) Fitzhugh, O. G., Nelson, A. A., and Holland, O. L., *J. Pharmacol. Exptl. Therap.*, **93**, 147(1948).
(2) Sanghvi, L. M., and Mathur, B. B., *Circulation*, **32**, 281(1965).
(3) Mason, J. R., Khan, K., and Frewing, H. L., *J. Am. Med. Assoc.*, **188**, 187(1964).
(4) Cohen, A. S., and Calkins, E., *Arthritis Rheumat.*, **1**, 297(1958).
(5) Loftus, L. R., *Can. Med. Assoc. J.*, **89**, 917(1963).
(6) Cann, H. M., and Verhulst, H. L., *Pediatrics*, **27**, 95(1961).
(7) Wislogle, F. Y., "A Survey of Antimalarial Drugs, 1941–1945," J. W. Edwards, Inc., Ann Arbor, Mich., 1946, p. 94.
(8) Nelson, A. A., and Fitzhugh, O. G., *Arch. Pathol.*, **45**, 454(1948).
(9) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1957.
(10) Wolff, E. D., and Ball, E. G., *J. Biol. Chem.*, **224**, 1083(1957).
(11) Slater, E. C., and Cleland, K. W., *Biochem. J.*, **55**, 566(1953).
(12) Devlin, T. M., and Bedell, B. H., *J. Biol. Chem.*, **235**, 2134(1960).
(13) Friedemann, T. E., in "Methods in Enzymology," vol. III, Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1957, p. 414.
(14) Ochoa, S., *ibid.*, vol. I, p. 735.
(15) Datta, A. G., and Basu, U. P., *J. Sci. Ind. Res.*, **14C**, 61(1955).
(16) Hess, M. E., and Haugaard, N., *Circulation Res.*, **6**, 256(1958).
(17) Heim, H. C., Froede, H. C., and Erwin, V. G., *J. Pharmacol. Exptl. Therap.*, **137**, 107(1962).
(18) Gerlach, U., *Klin. Wochschr.*, **36**, 376(1958).
(19) Mushinski, J. F., Yielding, K. L., and Munday, J. S., *Arthritis Rheumat.*, **5**, 118(1962).

Effect of Ultraviolet Irradiation on Chlorpromazine II

Anaerobic Condition

By CHIAN L. HUANG* and FREEMAN L. SANDS

In this report, ultraviolet light-catalyzed reaction of chlorpromazine under anaerobic condition is presented. It appears that chlorpromazine behaves differently to ultraviolet energy under aerobic and anaerobic conditions. Under the former condition oxidation products prevail, and the sulfoxide and *N*-oxide are formed; however, under the latter condition, the polymerization process predominates and the polymer was found to be the major product. The biological activity of this compound was tested on human volunteers; this study indicated that this compound caused a negligible skin irritation but produced a bluish-purple color resembling that of the skin discoloration observed in patients. Hydrogen chloride liberated in the initial phase of the photochemical reaction may be responsible for the skin rash reported in patients receiving daily maintenance dose of chlorpromazine. Formation of a polymer coupled with melanin may be the cause of the skin discoloration observed in patients under prolonged and high dose of chlorpromazine treatment.

SINCE THE introduction of chlorpromazine in the clinical treatment of psychosis, a number of cases of photosensitivity or photoallergy, namely, abnormal skin reactions (1–16) and ocular changes (8–19), associated with this drug have been reported. Concurrently, these investigators pointed out that skin discoloration occurred on the exposed portions of the body of patients receiving a long-term medication of large doses (300–500 mg. three times a day) of phenothiazines, especially chlorpromazine. The rate of occurrence of the skin discoloration is about 0.1–0.2% of the patients treated with chlorpromazine. The incidences of the ocular changes seem to precede the skin pigmentation in reference to the reports that many patients had ocular changes before skin pigmentation took place (20–22), and the majority of patients with skin changes also had ocular complications (23). It appears that these bizarre phenomena are directly associated with photo energy of short wavelength; however, the nature of these abnormal reactions is not well understood.

It is the purpose of this study to investigate the *in vitro* effect of ultraviolet energy on an aqueous solution of chlorpromazine which might furnish clues toward the nature of the photochemical reactions taking place in patients under chlorpromazine medication.

In a previous communication (24), the effect of ultraviolet (U.V.) irradiation (25–29) on an aqueous solution of chlorpromazine under aerobic conditions was presented; two oxidation products, chlorpromazine sulfoxide and chlorpromazine *N*-oxide, were identified, and the physical properties of 10 other compounds were described. Apparently, oxygen (30) present in the medium played an important role in the formation of oxidation products of chlorpromazine in this photochemical reaction. The site of attack by the oxygen was found to take place first on the S function at position 5 followed by the terminal N.

In this report, the photo-induced reaction products of chlorpromazine in an aqueous solution under anaerobic condition are presented. The chromatographic pattern of the products formed under the anaerobic condition was quite different from that of the aerobic condition. A polymerization reaction was found to be a major process taking place under these conditions, and a polymer and a dimer were isolated from the reaction mixture. Evidently, the cleavage of the chlorine function (31) at position 2 resulted in the formation of a free radical which prompted these reactions. In a preliminary test, an aqueous suspension of the polymer injected intracutaneously to two volunteers exhibited a discoloration resembling that of the photosensitivity observed in patients receiving chlorpromazine medication. The formation of a polymer in the skin might be a cause of photosensitivity reported in patients under a long-term medication of large doses (300–500 mg. three times a day) of chlorpromazine. Promazine and 2-hydroxypromazine were also isolated and identified. Negative tests with ninhydrin and nitroprusside reagents on the reaction products isolated in this experiment indicated that there is no demethylation occurring under these conditions (32).

Received June 9, 1966, from the Medical Research Department, Spring Grove State Hospital, Baltimore, Md.
Accepted for publication October 25, 1966.
This project was supported in part by research grants MY-5785 and MY-7617 from the National Institute of Mental Health, U.S. Public Health Service, Bethesda, Md.
The authors thank Dr. Kaneo Hirano for his technical assistance in this study.
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METHOD[1]

Chlorpromazine hydrochloride (5 Gm.) was dissolved in freshly boiled water (500 ml.) and placed in a glass ampul (10 ml.), which was previously filled with nitrogen gas. The ampul was replenished with nitrogen gas and sealed under nitrogen atmosphere. Fifty ampuls thus prepared were placed in a box with aluminum foil linings on inside walls and subjected to ultraviolet irradiation from a Spectroline B-100 lamp (120 w.) at a distance of 20 cm. At every 24-hr. interval, one ampul was removed from the box and the content examined for the extent of the photochemical effect. It was found that all the starting material was consumed completely within 5 days. The time sequential chromatographic patterns and the ultraviolet absorption spectra of the irradiated media are shown in Fig. 1.

At the end of 5 days, U.V. irradiation was terminated. The ampuls were removed from the box, and the contents emptied into a container filled with nitrogen. It was noted that the colorless solution at the beginning had changed to deep-brown at the termination of the irradiation, and the pH had shifted from 4.5 to 1.9 (33). The results of alkalimetry revealed that approximately 0.8 mole of hydrogen chloride per mole of starting material was liberated in the medium. The solution was adjusted to pH 7 with 10% NaOH solution and the yellowish green precipitate which appeared was collected by centrifugation. The precipitate was washed 3 times with water, and the washings were added to the supernatant liquid.

The supernatant liquid was tested for the presence of dimethylamine according to the procedure described by Yamamoto and Fujisawa (32). Although the product recovered from the reaction vessel (containing dichlorodiphenyldisulfimide reagent) acquired a light brown color at the end of the experiment, it gave the same melting point as that of the starting material, m.p. 211–213°. Therefore, it was concluded that no dimethylamine was present in the irradiated solution of chlorpromazine and no demethylation took place under these conditions.

Thick-Layer Chromatography.—The precipitate collected above was extracted with a large quantity of cold methanol. The methanol extract was condensed to a small volume and subjected to thick-layer chromatography. Approximately, 1.5 ml. of an aliquot specimen was placed linearly in a 0.5-cm. band about 4 cm. from the bottom of a 15 × 48-cm. plate (coated with a 0.5-mm. layer of Silica Gel G and activated at 100° for 1.5 hr.). The chromatogram was developed in a nitrogen atmosphere in a chamber containing a solvent system, ethyl acetate–ethanol–water (8:3:3), for 7 hr. to a height of approximately 31 cm. After the chromatogram was removed from the solvent system, it was dried, examined under a U.V. lamp, and covered with a sheet of plastic wrap (Saran wrap); this left about a 2-cm. strip on one side of the plate uncovered. The exposed strip was sprayed with 50% sulfuric acid to produce color. Twelve fractions with R_f 0–0.78 and the color varying from pink to purple were observed. Using this sprayed strip as a guide, the chromatogram was fractionated, and each fraction was removed from the plate and extracted with the above solvent system. The extract of each fraction was rechromatographed twice for further purification. Only a trace of the starting material was recovered from the medium after 5 days of irradiation.

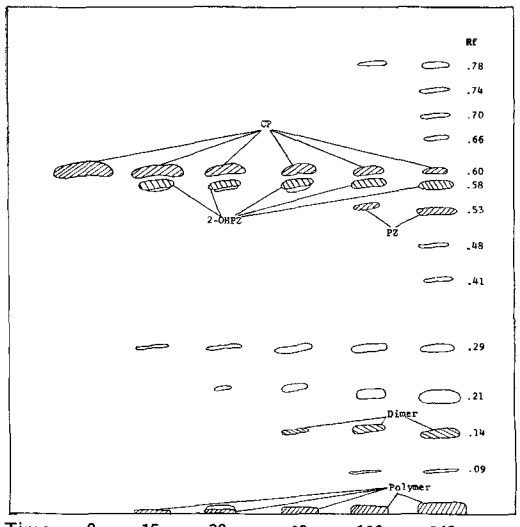


Fig. 1.—Sequential thin-layer chromatographic patterns of ultraviolet light-induced reaction products of chlorpromazine under anaerobic condition. Key: CP, chlorpromazine; 2PZ-OH, 2-hydroxypromazine; PZ, promazine.

Isolation of Promazine.—Fraction *6* was rechromatographed on a thick-layer plate several times for purification. After the final methanol eluate was evaporated to dryness, an oily residue was obtained. The physical properties of this compound—namely, R_f (0.46), color reaction with 50% sulfuric acid (orange), $\lambda_{max.}$ (208, 256, 300 mμ), and I.R. spectrum, were found to be identical with those of a reference promazine. The melting point of the picrate of this compound was found to be 143–144°, and the mixed melting point with an authentic promazine picrate did not show depression (142–144°).

Isolation of 2-Hydroxypromazine.—Fraction *4* was eluted with methanol from a thick-layer plate. The methanol extract was evaporated to dryness and the residue was rechromatographed three times for further purification. The purified fraction had an R_f value of 0.48 and a reddish-purple color reaction with 50% sulfuric acid. The melting point (132–133.5°), U.V. (212, 254 mμ), and I.R. spectra of this compound were found to be identical with those of a reference 2-hydroxypromazine. The color reaction of this compound compared with that of 2-hydroxypromazine (2-PZOH) is shown in Table I.

Isolation of the Dimer.—*Scheme I.*—Fraction *11* (R_f 0.09) was eluted with hot methanol. The methanol eluate was evaporated to dryness and the residue recrystallized from methyl ethyl ketone to a yellow

[1] Melting points were taken on a Fisher-Johns apparatus and are uncorrected. Molecular weights were determined by using a Mechrolab vapor pressure osmometer. Ultraviolet absorption spectra were obtained in a Bausch & Lomb spectronic 505 and infrared absorption spectra were taken in a Perkin-Elmer 237 spectrophotometer.

TABLE I.—COLOR REACTION OF FRACTION 4 AND 2-PZOH

	Ehrlich's Reagent		$FeCl_3$		2,6-Dichloro-
	Preheating	Postheating	Reagent	$NaIO_4$	quinone Chlorimide
2-PZOH	Reddish-purple	Deep green	Brown	Green	Greenish-gray
Fraction 4	Pinkish-purple	Green	Brown	Green	Greenish-gray

powder. This material had a purple color reaction with 50% sulfuric acid. The melting point of this compound was above 300° (loses color reaction with 50% sulfuric acid at 250°). Ultraviolet absorption peaks were found at 262 and 313 mμ. A quaternary ammonium salt of this compound was prepared by reacting an acetone solution of this compound (1 mole) with methyliodide (1 mole) at room temperature. The mixture was shaken for a few minutes, and ether was added to precipitate the quaternary ammonium salt (m.p. > 300°). The molecular weight of this compound was found to be 816; this is equivalent to the molecular weight of the quaternary ammonium salt of a dimer (31).

Anal.—Calcd. for $(C_{18}H_{21}N_2SI)_2$: mol. wt., 848.7; C, 50.94; H, 4.99; N, 6.61; S, 7.55. Found: C, 50.44; H, 5.13; N, 6.71; S, 7.43; mol. wt., 816.

Isolation of the Polymer.—The greenish-yellow residue left after the methanol extraction above was purified as follows. This material was extracted with chloroform and the chloroform extract was condensed to a small volume and precipitated with *n*-heptane. The yellowish-brown precipitate was filtered and dried. This material was slightly soluble in acetone, ethyl acetate, and ethanol. Both the sodium fusion and Beilstein's flame tests gave a negative reaction for chlorine. It had a bluish-purple color reaction with 50% sulfuric acid, and a green color with 1% ferric chloride reagent. The R_f value on a thin-layer chromatogram was 0–0.01. This compound was found to be extremely resistant to oxidation by hydrogen peroxide. Attempts to degradate this compound with acid or base at elevated temperatures and pressures failed to produce any product. This material is a greenish-brown powder, with m.p. > 300° (loses color reaction with 50% sulfuric acid at 235°). Its picrate has a m.p. 144–145°. The infrared absorption spectrum is shown in Fig. 2. This compound does not rotate polarized light. The proposed structure of this compound is illustrated in Scheme I.

Anal.—Calcd. for $(C_{17}H_{18}N_2S \cdot {}^1/_2H_2O)n$: C, 70.10; H, 6.24; N, 9.63; S, 11.00. Found: C, 70.47; H, 5.90; N, 9.22; S, 10.91; mol. wt., 1990.

A quaternary ammonium salt of the polymer was prepared by refluxing methanol solution of this compound (1 mole) with methyliodide (1 mole) for 15 min. After cooling, ether was added into the mixture to precipitate the quaternary ammonium salt. This compound is a yellowish-brown powder (m.p. 239–245°) with ultraviolet absorption peaks at 220 and 262 mμ.

Anal.—Calcd. for $(C_{18}H_{21}N_2SI)n$: C, 50.95; H, 4.99; N, 6.60; S, 7.56. Found: C, 50.87; H, 5.07; N, 6.36; S, 7.79.

A Preliminary Test on Skin.—Ten milligrams of the polymer was suspended in 10 ml. of normal saline solution, and 0.1 ml. each of the suspension was injected intracutaneously into the inner side of forearms of two volunteers. It produced a bluish-purple stain resembling that of the skin discoloration observed in patients (1–16). The irritating effects of this compound on the injected site was almost negligible. The color of the injected site faded and the skin returned to its normal color within 3 days.

CONCLUSION AND DISCUSSION

Liberation of hydrogen chloride and the subsequent formation of a polymer of chlorpromazine in the tissues has been suggested as a possible cause of the photosensitivity and skin discoloration of patients receiving chlorpromazine medication for a prolonged period.

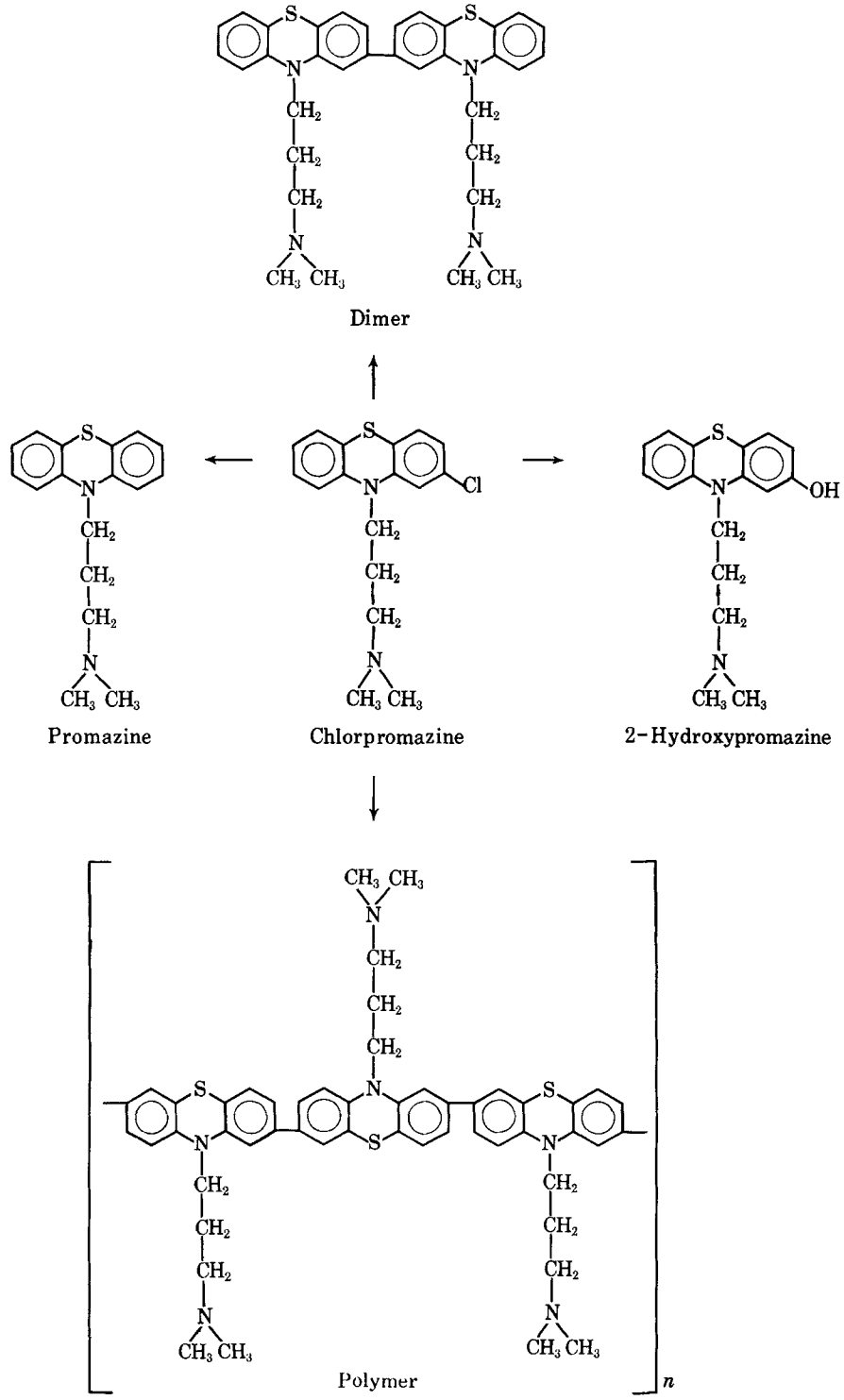
The hydrogen chloride in the initial phase of photochemical reaction could be responsible for the skin rash (7) observed in patients who were exposed to sunlight. The skin irritability of the patients subsided after they took cover from the sunlight; however, the inflammatory signs remained for hours. It could be due to the residual effects of the hydrogen chloride on the tissues. The chloride function at position 2 of the chlorpromazine molecule seems to be cleaved easily under these conditions; this is evidenced by a shift of the pH toward an acidic side (33) and an increase in the concentration of hydrogen chloride in the irradiated medium after 15 min. Similar findings, namely, cleavage of the $SO_2N(CH_3)_2$ group from thioproperazine to form sulfuric acid has been reported (32).

As the results of the cleavage of the chloride function (31), a carbonium ion is created at position 2. On the other hand, there exists in the chlorpromazine molecule a carbon at position 7 which acquires a relatively high electron density due to the greater *o,p*-directing force of the N at position 10. Thus, it has been speculated that a free radical attack on position 7 triggered by the carbonium ion at position 2 takes place, and a polymer with 2–7 linkage would be formed. Also the formation of a dimer from chlorpromazine with a possible 2–2 linkage has been found in this study (32, 34). Possible roles this compound might have in connection with the ocular and skin changes are currently under investigation.

The polymer isolated from the reaction medium is a greenish-brown powder, insoluble in almost all organic solvents except hot methanol, and resistant to acidic or basic hydrolysis at elevated pressures and temperatures. The intracutaneous injection of this polymer produced a purple color in the injected area of skin resembling that of the photosensitivity observed in patients. Therefore, the polymer formed in tissues as the results of the photo-induced polymerization reaction may be responsible for the skin discoloration.

This photochemical reaction takes place quite rapidly in reference to the observations in two patients (who were receiving 600 mg. three times a day of chlorpromazine medication); 15–30 min. of exposure to a bright sunlight was enough to produce a skin discoloration preceded by a condition

of sunburn. This indicated that photo-induced reaction of chlorpromazine took place at the site of exposure. It appears that chlorpromazine or its metabolites are in a bound state with melanin in tissues, since the drug has been demonstrated to be capable of forming complexes with melanin *in vitro*



Illustrated Structures of Ultraviolet Light-Induced Reaction Products of Chlorpromazine Under Anaerobic Condition

Scheme I

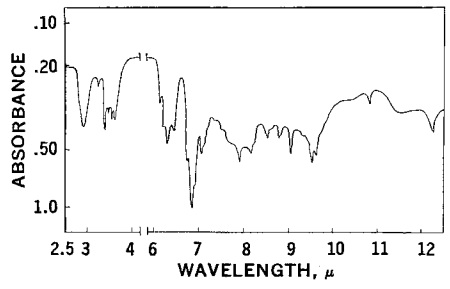


Fig. 2.—Infrared absorption spectrum of the polymer taken on a Perkin-Elmer model 237 spectrophotometer.

(35) and *in vivo* (36–39); however, skin discoloration would not occur without photo energy to trigger the polymerization reaction of chlorpromazine. Since chlorpromazine and its polymer react readily with silver, iron, and other metal ions to produce a color or a precipitate, the reagents currently applied to stain melanin (7) in tissues are expected to react with this drug or its derivatives to produce a melanin-like stain (7, 38).

The skin discoloration produced by the polymer injected intracutaneously to two volunteers faded, and the skin resumed its normal color within 3 days. These findings indicated that the polymer is transported from the site of administration or metabolized. Accordingly, a polymer of the same nature was isolated from a large amount of urine specimens from patients receiving a long-term medication of chlorpromazine (40). This finding suggested that the polymer formed in the skin by a photo-induced reaction is metabolized and excreted in urine. It is speculated that part of the polymer might be deposited or picked up by other organs during the course of transportation; this may be responsible for the appearance of a melanin-like material in the organs not being exposed to sunlight (41). Organs such as hairs and nails which are less affected by the normal process of metabolism would be expected to have a relatively high concentration of the drug. As the results of the accumulation of the drug, the hair might alter its shade because of a possible formation of the polymer, though the rate of formation would be lower than that occurring in the skin. It would be of interest to see further observations on this subject. The polymer of chlorpromazine may be bound to protein or deposited in fat-rich tissues; however, attempts were not made to clarify this matter in this study.

The polymers isolated from the U.V. irradiated medium showed a bluish-purple color reaction with 50% sulfuric acid; however, the reaction is rather sluggish—namely, the color reaction takes 30–60 sec. to develop, and the color density reaches its peak after 2–3 hr. The U.V. absorption peaks were found at 208 and 263 mμ; this indicated a shift toward the longer side of wavelength. There is no evidence of a 310-mμ peak when compared with chlorpromazine (207, 256, and 310 mμ).

The quaternary ammonium salt of this polymer is less soluble in organic solvents than the free base. In the U.V. irradiated medium under the anaerobic condition, 12 fractions (Table II) were detected on a thin-layer chromatogram; however, the sulfoxide could not be demonstrated. As the reaction proceeded and the starting material, chlorpromazine, was consumed, the concentration of the polymer increased in an almost parallel relationship to the irradiation time. The relationship of the appearance of various fractions *versus* irradiation time is illustrated in Fig. 1. The isolated polymer reacted readily with methyliodide to form a quaternary ammonium salt; this indicated the presence of an intact dimethylamino side chain.

Various organic solvents were used in the preparation of chlorpromazine solution for irradiation under anaerobic condition. Chloroform is the only sol-

TABLE II.—PHYSICAL PROPERTIES OF PHOTODEGRADATION PRODUCTS OF CHLORPROMAZINE UNDER ANAEROBIC CONDITION

Fraction	R_f (TLC[a])	Color Reactions: 50% H_2SO_4	Color Reactions: 1% $FeCl_3$	Nature	U.V. (QAS[b]) mμ	M.p., °C. (QAS[b])
1	0.78	Reddish-purple	Brownish-green	...	...	...
2	0.73	Brownish-purple	Brownish-green	...	...	...
3	0.69	Orange	Brown	...	...	...
4	0.66	Purple	Blue (changes to reddish-purple)	...	220, 265, 320	198–200°
5	0.48	Reddish-purple	Brownish-purple	2-PZOH[c]	212, 254	...
6	0.46	Orange	Orange	PZ[d]	208, 256, 300	190–194 (Picrate, 143–144)
7	0.30	Purple	Purple	...	219, 263, 316	195–199
8	0.28	Purple	Reddish-purple	...	219, 262, 312	192–198
9	0.26	Bluish-purple	Purple	...	218, 262, 313	193–198
10	0.20	Bluish-purple	Purple	...	218, 262, 313	190–197 (Picrate, 167–171)
11	0.14	Reddish-purple	Reddish-purple	CP-dimer[e]	262, 313	(Picrate, 136–142)
12	0	Bluish-purple	Green	Polymer	208, 263	235–239 (Picrate, 149–155)

[a] Thin-layer chromatography. [b] Quaternary ammonium salt. [c] 2-Hydroxypromazine. [d] Promazine. [e] Chlorpromazine dimer.

vent which prevented the formation of the polymer, and other solvents, such as benzene, ethylene dichloride, ethyl acetate, and carbon tetrachloride, failed to suppress the polymerization reaction. The chromatographic pattern of the ethylene dichloride solution of chlorpromazine resembles that of the deoxygenated aqueous solution. A deoxygenated chlorpromazine solution in phosphate buffer solution (pH 7.8), treated with U.V. energy under the same conditions, failed to prevent the polymer formation. It was of interest to observe that ascorbic acid added to the aqueous solution of chlorpromazine prevented the formation of the polymer, whereas sodium bisulfite did not inhibit the polymerization reaction under the same conditions. The aqueous medium containing maleic acid produced only a trace of the polymer. Free radical (24–27) formation elicited by the short wavelength photon is apparently the key step toward the formation of the polymer. However, these free radicals are detectable only in the solvent media, and merely a trace or none of them exists in a solid state.

It appears that the intact chlorpromazine molecules are deposited in the tissues or organs either in the free or bound form (41). When these tissues or organs are exposed to ultraviolet energy, photochemical reactions take place to cleave the chloride function on the chlorpromazine molecules to form hydrogen chloride and, on the other hand, creating free radicals at the site of irradiation. Immediately, a polymerization reaction of these free radicals proceeds to form a polymer which deposits at the site of irradiation along with melanin (7, 38) to cause the skin discoloration. Although hydrogen chloride liberation and the polymer formation are proposed as the main cause of the skin irritation and the abnormal skin discolorations, caution must be taken to differentiate between the photosensitivity of this nature and that of a photo allergy or drug allergy developed in individuals who are sensitive to even a low dose of phenothiazines.

REFERENCES

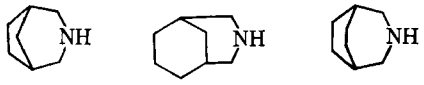
(1) Schwank, R., and Jirasek, L., *Cesk. Dermatol.*, **36**, 4(1961).
(2) Knox, J. M., *Ann. Allergy*, **16**, 749(1961).
(3) Hayes, G. B., Lyle, C. B., Jr., and Wheeler, C. E., Jr., *Arch. Dermatol.*, **90**, 471(1964).
(4) Zelickson, A. S., and Zeller, H. C , *J. Am. Med. Assoc.*, **188**, 394(1964).
(5) Ban, T. A., and Lehman, H. E., *Can. Psychiat. Assoc. J.*, **10**, 112(1965).
(6) Zelickson, A. S., *J. Am. Med. Assoc.*, **194**, 670(1965).
(7) Satanove, A., *ibid.*, **191**, 263(1965).
(8) Greiner, A. C., and Nicolson, G. A., *Can. Med. Assoc. J.*, **91**, 627(1964).
(9) Perry, T. C., Culling, C. F. A., Berry, K., and Hansen, S., *Science*, **146**, 81(1964).
(10) Feldman, P. E., and Frierman, B. D., *Am. J. Psychiat.*, **121**, 187(1964).
(11) Tridici, L. M., Schiele, B. C., and McClanahan, W. S., *Minn. Med.*, **48**, 569(1965).
(12) Brill, H., Schiele, H. G., and Delong, S. L., *Am. J. Psychiat.*, **122**, 326(1965).
(13) Cairns, R. J., Capoose, H. S., and Gregory, I. D. R., *Lancet*, **I**, 239(1965).
(14) Richards, F., *ibid.*, **II**, 437(1965).
(15) Nicolson, G. A., Greiner, A. C., McFarlane, W. J. G., and Baker, G. A., *ibid.*, **I**, 344(1965).
(16) Massey, L. W. C., *Can. Med. Assoc. J.*, **92**, 186(1965).
(17) Barsa, J. A., and Saunders, J. C., *Psychopharmacologia*, **7**, 138(1965)
(18) Rutschmann, J., Kalberer, F., Schalch, W., and Stähelin, H., *Psychopharm. Serv. Cent. Bull.*, **2**, 73(1962).
(19) Ellis, P. P., *Arch. Ophthamol.*, **74**, 96(1965).
(20) Mathalone, M. B. R., *Lancet*, **II** (7403), 111(1965).
(21) Delong, S. L., Poley, B. J., and McFarlane, J. R., Jr., *Arch. Ophthamol.*, **73**, 611(1965).
(22) Barsa, J. A., Newton, J. D., and Saunders, J. C., *J. Am. Med. Assoc.*, **193**, 10(1965).
(23) Siddall, J. R., *Arch. Ophthalmol.*, **74**, 460(1965).
(24) Huang, C. L., and Sands, F. L., *J. Chromatog.*, **13**, 246(1964).
(25) Forrest, I. S., Forrest, F. M., and Berger, M., *Biochim. Biophys. Acta*, **29**, 441(1958).
(26) Lagercrantz, C., *Acta Chem. Scand.*, **15**, 1545(1961).
(27) Lagercrantz, C., and Yhland, M., *ibid.*, **16**, 508(1962).
(28) Wollmann, M., and Keleti, T., *Arzneimittel-Forsch.*, **12**, 360(1962).
(29) Borg, D. C., and Cotzias, G. C., *Proc. Natl. Acad. Sci.*, **48**, 643(1962).
(30) Martin, H. F., Price, S., and Gudzinowicz, B. J., *Arch. Biochem. Biophys.*, **103**, 196(1963).
(31) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *Biochem. Pharmacol.*, **12**, 779(1963).
(32) Yamamoto, R., and Fujisawa, S., *Yakugaku Zasshi*, **82**, 1396(1962).
(33) Felmeister, A., and Discher, C. A., *J. Pharm. Sci.*, **53**, 756(1964).
(34) Jung, E. G., Schwarz-Speck, M., and Kormany, G., *Schweiz. Med. Wochschr.*, **93**, 249(1963).
(35) Forrest, I. S., Gutmann, F., and Keyzer, H., *Rev. Aggressol.*, **7**, 147(1966).
(36) Potts, A. M., *Invest. Ophthalmol.*, **3**, 405(1964).
(37) Blois, M. S., Jr., *J. Invest. Dermatol.*, **44**, 475(1965).
(38) Zelickson, A. S., *J. Am. Med. Assoc.*, **194**, 670(1965).
(39) Meier-Ruge, W., Kalberer, F., and Cerletti, A., *Experientia*, **22**, 153(1966).
(40) Huang, C. L., *Intern. J. Neuropharmacol.*, to be published.
(41) Forrest, F. M., Forrest, I. S., and Roizin, L., *Rev. Aggressol.*, **4**, 259(1963).

Esters of Aminoalcohols Derived from 3-Azabicyclo[3.2.2]nonane

By PRAMOD P. KARKHANIS* and W. LEWIS NOBLES

A series of esters of the type I and II was prepared utilizing 3-azabicyclo[3.2.2]nonane. The pharmacological testing of one of these esters is reported.

COMPOUNDS having interesting pharmacological activity have been prepared from one or more of the parent amines, 3-azabicyclo[3.2.1]-octane (I), 3-azabicyclo[3.3.1]nonane (II), and 3-azabicyclo[3.2.2]nonane (III) (1).



I II III

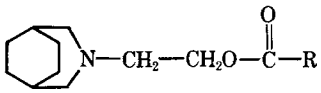
The use of 3-azabicyclo[3.2.2]nonane, extended in other areas of research, was initiated earlier at this institution by Blanton and Nobles (2, 3). These authors investigated the ketonic Mannich bases and their derivatives obtained from 3-azabicyclo[3.2.2]nonane. The ketonic Mannich bases have been shown to exhibit activity against *Staphylococcus aureus, Candida albicans, Trichophyton mentagrophytes,* and *Trichomonas foetus* (4).

Krieger (5) has demonstrated the usefulness of the guanidine derivative of 3-azabicyclo[3.2.2]-nonane as a possible hypotensive agent. Moreover, Martell and Soine (6) have reported that the esters of 3-hydroxy-1-azabicyclo[3.3.1]-nonane and 8-hydroxy-1-azabicyclo[4.3.0]nonane are potent anticholinergic agents.

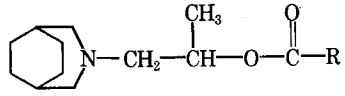
These observations prompted the investigations reported in this paper. The object of the present work was to prepare and examine some of the esters of the amino alcohols obtainable from 3-azabicyclo[3.2.2]nonane and to determine whether they possessed significant pharmacological action.

DISCUSSION

Types of compounds prepared from 3-azabicyclo[3.2.2]nonane are shown as types I and II.

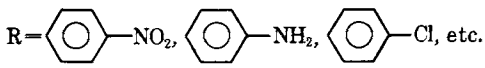


Type I



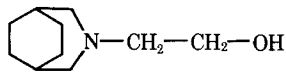
Type II

where



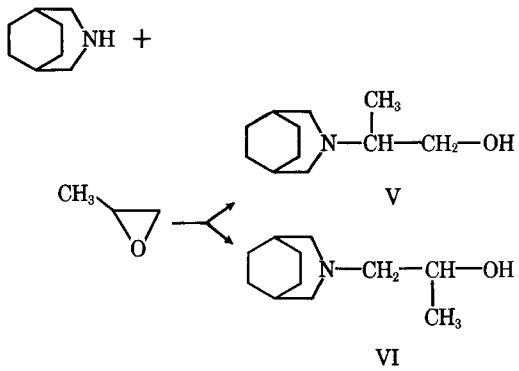
The preparation of 20 new esters of types I and II, starting with 3-azabicyclo[3.2.2]nonane,[1] was accomplished by well-known methods described in the literature.

A modification of the procedure described by Horne and Shriner (7), utilizing ethylene oxide at a lower temperature, was employed to obtain 3-azabicyclo[3.2.2]nonane-3-ethanol (IV) from 3-azabicyclo[3.2.2]nonane (8).



IV

When propylene oxide is treated with 3-azabicyclo[3.2.2]nonane using the same conditions as those used for ethylene oxide, there is a possibility of forming either 2-(3-azabicyclo[3.2.2]nonyl)-3-propanol (V) or 3-(3-azabicyclo[3.2.2]nonyl)-2-propanol (VI) or a mixture of both of these isomers, depending upon the point of ring opening (Scheme I).



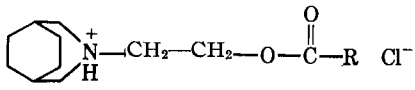
Scheme I

When the alcohol was chromatographed on plates coated with Silica Gel G in solvent systems such as ethanol, methanol, chloroform, benzene, and benzene–methanol (40:60), it demonstrated only one sample spot upon visualization with acidic potassium permanganate solution, indicating the formation of only one isomer.

Received April 25, 1966, from the School of Pharmacy, University of Mississippi, University 38677.
Accepted for publication November 3, 1966.
Abstracted in part from a thesis submitted by Pramod P. Karkhanis to the Graduate School, University of Mississippi, University, in partial fulfillment of Master of Science degree requirements.
* Present address: Miles Laboratories, Inc., Elkhart, Ind.

[1] 3-Azabicyclo[3.2.2]nonane was supplied through the courtesy of Eastman Chemical Products, Inc., Kingsport, Tenn.

TABLE I.—COMPOUNDS OF TYPE I



Compd.	R	Yield, %[a]	M.p., °C.[b]	Molecular Formula	Anal.[c] Calcd.	Found
1	Phenyl	61.5	230 dec.	$C_{17}H_{23}NO_2 \cdot HCl$	C, 65.91	65.71
					H, 7.81	7.84
					N, 4.52	4.45
2	*p*-Nitrophenyl	67.3	203 dec.	$C_{17}H_{22}N_2O_4 \cdot HCl$	C, 57.54	57.12
					H, 6.57	6.54
					N, 7.90	7.77
3	*p*-Aminophenyl	89.0	256 dec.	$C_{17}H_{24}N_2O_2 \cdot HCl$	C, 62.85	62.08
					H, 7.76	7.89
					N, 8.62	8.45
4	Benzilic	98.0	198 dec.	$C_{24}H_{29}NO_3 \cdot HCl$	C, 69.30	70.02
					H, 7.27	7.58
					N, 3.37	3.52
5	Mandelic	96.5	136 dec.	$C_{18}H_{25}NO_3 \cdot HCl$	C, 63.61	63.46
					H, 7.71	7.95
					N, 4.12	4.27
6	Cinnamyl	68.9	241 dec.	$C_{19}H_{26}NO_2 \cdot HCl$	C, 67.74	67.74
					H, 8.08	7.89
					N, 4.15	4.46
7	*m*-Bromophenyl	64.3	219–220 dec.	$C_{17}H_{22}BrNO_2 \cdot HCl$	C, 52.52	52.75
					H, 5.96	6.02
					N, 3.60	3.26
8	*p*-Chlorophenyl	64.7	252–253 dec.	$C_{17}H_{22}ClNO_2 \cdot HCl$	C, 59.29	59.37
					H, 6.73	6.65
					N, 4.07	4.20
9	*o*-Nitrophenyl	64.7	204–205 dec.	$C_{17}H_{22}N_2O_4 \cdot HCl$	C, 57.54	57.68
					H, 6.57	6.65
					N, 7.89	7.79
10	*o*-Aminophenyl	84.8	244 dec.	$C_{17}H_{24}N_2O_2 \cdot HCl$	C, 62.85	63.08
					H, 7.75	8.26
					N, 8.62	8.78

[a] All the compounds were recrystallized from ethanol–ethylacetate and ether mixture. [b] All the melting points are uncorrected. [c] Elemental analyses were done through the courtesy of Dr. P. N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

The NMR data[2] (see under *Experimental*) indicated the formation of 3-(3-azabicyclo[3.2.2]-nonyl)-2-propanol (VI). Contrary to expectations, this alcohol failed to undergo the haloform reaction. Fuson and Bull (9) have indicated that only certain secondary alcohols undergo a haloform reaction.

The alcohol, 3-(3-azabicyclo[3.3.2]nonyl)-2-propanol, on oxidation (10) with chromic acid and sulfuric acid below 20°, gave a compound which reacted with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone derivative. The oxidation product did not reduce either Tollen's reagent or Schiff's reagent. The infrared spectrum of the oxidation product showed a strong peak in the region of 1725 to 1705 cm.$^{-1}$. These qualitative tests strongly suggest that the compound thus obtained is a ketone; therefore, the alcohol formed by the reaction of propylene oxide and 3-azabicyclo[3.2.2]-nonane should be 3-(3-azabicyclo[3.2.2]nonyl)-2-propanol.

Two different methods were employed to obtain the esters of 10 aromatic acids. The acid chloride method, as described by Martell and Soine (6), was used for the preparation of the esters of all acids except benzilic and mandelic acids. In general, the reaction proceeded smoothly to yield the expected products. It may be noted, however, that the yields obtained were between 60 and 70%.

A second method, as described in the literature (8), was employed to obtain esters from benzilic and mandelic acids. The yields were consistently in the range of 95–98%.

The esters of *p*-amino and *o*-amino acids were prepared by reducing the nitro groups of *p*-nitro and *o*-nitro benzoates, as described by Wright and Freifelder (11).

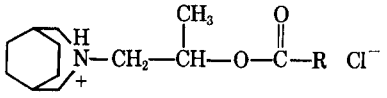
Pharmacological Results.—The compound 2-(3-azabicyclo[3.2.2]nonyl)-ethyl benzilate hydrochloride (Table I, compound *4*) was tested for biological activity. This compound was screened for potential pharmacodynamic properties. Intraperitoneal injection of 100 mg./Kg. of the compound in 12 mice resulted in weak central nervous system depression and in weak psychotrophic and anticonvulsant properties in 70% of the mice tested. The activity had its onset within 3 min. with an intraperitoneal injection of 500 mg./Kg., and the duration of the activity was observed to range from 24 to 72 hr.

The side effects of the compound with a dose of 500 mg./Kg. were characterized by marked ataxia, muscle weakness, and dyspnea.

When the same compound was tested for cardiovascular, somatic, and visceral effects in dogs anesthetized by pentobarbital, intravenous injections of

[2] The interpretation of the NMR data was done through the courtesy of Dr. R. L. Settine, Department of Chemistry, The University of Mississippi, University.

TABLE II.—COMPOUNDS OF TYPE II



Compd.	R	Yield, %[a]	M.p., °C.[b]	Molecular Formula	Anal.[c] Calcd.	Found
1	Phenyl	64.7	218 dec.	$C_{18}H_{25}NO_2 \cdot HCl$	C, 66.75	66.57
					H, 8.09	8.11
					N, 4.33	4.33
2	*p*-Nitrophenyl	63.8	247 dec.	$C_{18}H_{24}N_2O_4 \cdot HCl$	C, 58.60	58.35
					H, 6.83	6.93
					N, 7.60	7.63
3	*o*-Nitrophenyl	60.0	194 dec.	$C_{18}H_{24}N_2O_4 \cdot HCl$	C, 58.60	58.79
					H, 6.83	7.00
					N, 7.60	7.61
4	3,4,5-Trimethoxyphenyl	60.7	197–198 dec.	$C_{21}H_{31}NO_5 \cdot HCl$	C, 60.93	60.88
					H, 7.79	7.73
					N, 8.57	8.54
5	*p*-Aminophenyl	81.8	256 dec.	$C_{18}H_{26}N_2O_2 \cdot HCl$	C, 63.79	63.45
					H, 8.03	7.79
					N, 8.26	8.05
6	*o*-Aminophenyl	86.3	222 dec.	$C_{18}H_{26}N_2O_2 \cdot HCl$	C, 63.79	63.73
					H, 8.03	8.20
					N, 8.26	8.29
7	Benzilic	93.7	176 dec.	$C_{25}H_{31}NO_3 \cdot HCl$	C, 69.83	69.97
					H, 7.50	7.77
					N, 3.26	3.24
8	Mandelic	94.9	181 dec.	$C_{19}H_{27}NO_3 \cdot HCl$	C, 64.48	63.21
					H, 7.97	8.05
					N, 3.95	3.99
9	*m*-Bromophenyl	63.5	299 dec.	$C_{18}H_{24}BrNO_2 \cdot HCl$	C, 53.68	53.88
					H, 6.25	6.27
					N, 3.47	3.23
10	*p*-Chlorophenyl	66.6	246–247 dec.	$C_{18}H_{24}ClNO_2 \cdot HCl$	C, 60.40	60.33
					H, 7.08	6.99
					N, 3.91	3.89

[a] All the compounds were recrystallized from ethanol–ethylacetate and ether mixture. [b] All the melting points are uncorrected. [c] Elemental analyses were done through the courtesy of Dr. P. N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

1 and 10 mg./Kg. resulted in slight hypotensive effects, characterized by moderate inhibition of the linguo-mandibular reflex, apparently not related to the hypotensive effect.

EXPERIMENTAL

Compounds of Type I (Table I)

3-Azabicyclo[3.2.2]nonane-3-ethanol.—A solution of 125 Gm. (1.0 mole) of 3-azabicyclo[3.2.2]-nonane in 125 Gm. of methanol was treated over a 1-hr. period with 59.5 Gm. (1.35 mole) of ethylene oxide. The temperature of the reaction was maintained below 47° by keeping the reaction mixture in ice water. The reaction mixture was stirred for 1.75 hr. after the addition of ethylene oxide had been completed. Distillation of the product after removal of the methanol yielded 139 Gm. (82%) of 3-azabicyclo[3.2.2]nonane-3-ethanol, b.p. 94°/1.8 mm.

Anal.—Calcd. for $C_{10}H_{19}NO$: C, 70.96; H, 11.31; N, 8.28. Found: C, 70.93; H, 11.06; N, 8.29.

2-(3-Azabicyclo [3.2.2]nonyl)-ethyl Chloride Hydrochloride.—A solution of 33.8 Gm. (0.2 mole) of 3-azabicyclo[3.2.2]nonane-3-ethanol in 175 ml. of dry benzene was placed in a three-necked flask. The mixture was stirred as 32.1 Gm. (0.27 mole) of thionyl chloride in dry benzene was added dropwise. The reaction was slightly exothermic. After the addition of thionyl chloride had been completed, the mixture was refluxed for 3 hr. and then cooled. The solid product was collected by filtration and was washed with benzene yielding 42.0 Gm. (95%) of 2-(3-azabicyclo[3.2.2]nonyl)-ethyl chloride hydrochloride, m.p. 225° (sublimation).

2-(3-Azabicyclo [3.2.2]nonyl)-ethyl Benzilate Hydrochloride.—A 50-ml. ether suspension of 6.7 Gm. (0.03 mole) of 2-(3-azabicyclo[3.2.2]-nonyl)-ethyl chloride hydrochloride was treated with 1.5 Gm. of sodium hydroxide in 25 ml. of water. The aqueous layer was extracted with two 50-ml. portions of ether and discarded. The ether extracts and ether solution were combined, dried over anhydrous magnesium sulfate, and then poured into 6.6 Gm. (0.02 mole) of benzilic acid in isopropanol. The solvents were distilled at 80°. The reaction then was maintained under total reflux for 3 hr. The solid that recrystallized from the mixture was collected by filtration and recrystallized from isopropanol to yield 8.23 Gm. (98%) of 2-(3-azabicyclo[3.2.2]nonyl)-1-ethyl benzilate hydrochloride, m.p. 198° dec.

Anal.—Calcd. for $C_{18}H_{25}NO_3.HCl$: C, 63.61; H, 7.71; N, 4.12. Found: C, 63.43; H, 7.95; N, 4.27.

2-(3-Azabicyclo [3.2.2]nonyl)-ethyl Benzoate Hydrochloride.—A solution of 3.6 Gm. (0.021 mole) of 3-azabicyclo[3.2.2]nonane-3-ethanol was made in 150 ml. of dry benzene and 3 ml. of triethylamine.

Three grams (0.021 mole) of benzoylchloride in 75 ml. of dry benzene was added dropwise over a period of 30 min. with stirring. The mixture was heated at 70° with stirring for 2.5 hr. The mixture was then cooled, and the triethylamine hydrochloride was removed by filtration. The benzene solution of the free base was washed with four 60-ml. portions of water, dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue was dissolved in 75 ml. of dry ether; the hydrochloride was formed by passing anhydrous hydrogen chloride through the solution. The product was recrystallized from ethanol, ethyl acetate, and ether, yielding 4.0 Gm. (61%) of 2-(3-azabicyclo[3.2.2]-nonyl)-1-ethyl benzoate hydrochloride, m.p. 230° dec.

Anal.—Calcd. for $C_{17}H_{23}NO_2 \cdot HCl$: C, 65.91; H, 7.81; N, 4.52. Found: C, 65.71; H, 7.84; N, 4.45.

2-(3-Azabicyclo [3.2.2]nonyl)-ethyl-(p-amino) Benzoate Hydrochloride.—A solution of 2.1 Gm. (0.006 mole) of 2-(3-azabicyclo[3.2.2]nonyl)-ethyl-(*p*-nitro) benzoate hydrochloride in 25 ml. of water was placed in a Parr low-pressure hydrogenator. The solution then was hydrogenated in the presence of 0.2 Gm. of 5% palladium-on-activated charcoal under 5 lb. of pressure of hydrogen. The reduction was complete in 40 min. The solution absorbed 2 lb. of hydrogen. The solution was then filtered from the catalyst and was concentrated under reduced pressure to a syrup. About 15 ml. of absolute ethanol was added to it, and the mass was triturated. The product so obtained was recrystallized from absolute ethanol, yielding 1.7 Gm. (89%) of 2-(3-azabicyclo[3.2.2]nonyl)-1-ethyl-(*p*-amino) benzoate hydrochloride, m.p. 256° dec.

Anal.—Calcd. for $C_{17}H_{24}N_2O_2 \cdot HCl$: C, 62.85; H, 7.76; N, 8.62. Found: C, 62.80; H, 7.89; N, 8.45.

Compounds of Type II (Table II)

3-(3-Azabicyclo[3.2.2]nonyl)-2-propanol.—A solution of 100 Gm. (0.8 mole) of 3-azabicyclo[3.2.2]-nonane in methanol was treated over a 1-hr. period with 48 Gm. (0.83 mole) of propylene oxide. The temperature of the reaction was maintained below 47° by keeping the reaction mixture in ice water. The reaction mixture was stirred for 1.75 hr. after the addition of propylene oxide had been completed. Distillation of the product after removal of the methanol yielded 135 Gm. (93%) of 3-(3-azabicyclo-[3.2.2]nonyl)-3-propanol, b.p. 96°/1.7 mm.

Anal.—Calcd. for $C_{11}H_{21}NO$: C, 72.08; H, 11.55; N, 7.64. Found: C, 72.59; H, 11.69; N, 7.50.

The NMR spectrum in carbon tetrachloride was δ = 1.70 (d, probably CH_3—C, 3H), 1.70 (t, probably ring protons, 10H), 2.23 (d, probably N—CH_2, 2H), 2.65 (m, probably CH_2—N, 4H), 3.28 (s, probably C—OH, 1H), and 3.74 (m, probably —CH—O, 1H), p.p.m.

3-(3-Azabicyclo [3.2.2]nonyl)-propane-2-one.—To 13.7 Gm. (0.075 mole) of 3-(3-azabicyclo[3.2.2]-nonyl)-2-propanol in 35 ml. of acetone was added 7.35 Gm. of concentrated sulfuric acid in a few milliliters of water with cooling and stirring. Then 50 ml. of an aqueous solution containing 10 Gm. of CrO_3 and 16 Gm. of sulfuric acid was added dropwise below 20° by keeping the reaction mixture in an ice bath. The mixture was stirred for 3 hr., and then the solvent was evaporated. The residue was diluted with 250 ml. of water. The crystals formed were removed by filtration, washed several times with water, and recrystallized from water yielding 7.1 Gm. (51%) of 3-(3-azabicyclo[3.2.2]nonyl)-propane-2-one, m.p. 129–131° dec.

Anal.—Calcd. for $C_{11}H_{19}NO$: C, 72.81; H, 10.56; N, 7.72. Found: C, 72.56; H, 10.38; N, 7.61.

REFERENCES

(1) Krieger, H., *Suomen Kemistilehti*, **36A**, 2(1963); through *Chem. Abstr.*, **58**, 8849(1963).
(2) Blanton, C. D., Jr., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 878(1962).
(3) *Ibid.*, **52**, 46(1963).
(4) Smith Kline & French Laboratories, Philadelphia, Pa., unpublished data.
(5) Krieger, H., *Suomen Kemistilehti*, **B35**, 185, 218 (1962); through *Chem. Abstr.*, **59**, 573(1963); **59**, 1152(1963).
(6) Martell, M. J., Jr., and Soine, T. O., *J. Pharm. Sci.*, **52**, 331(1963).
(7) Horne, W. H., and Shriner, R. L., *J. Am. Chem. Soc.*, **54**, 2925(1932).
(8) "3-Azabicyclo [3.2.2] nonane Properties—Reactions," Chemical Division, Eastman Chemical Products, Inc., Kingsport, Tenn., 1962.
(9) Fuson, R. C., and Bull, B. A., *Chem. Rev.*, **15**, 275 (1934).
(10) Olomucki, M., and Marszak, I., *Compt. Rend.*, **246**, 1714(1958); through *Chem. Abstr.*, **52**, 17096*i*(1958).
(11) Wright, H. B., and Freifelder, M., *J. Am. Chem. Soc.*, **71**, 1513(1949).

Determination of Chlorobutanol in Pharmaceuticals by Gas Chromatography

By K. T. KOSHY*, R. C. CONWELL, and R. N. DUVALL

A gas chromatographic procedure has been developed for the determination of chlorobutanol in a variety of pharmaceutical preparations. Numerous columns were investigated and found useful. Chlorobutanol was separated from its degradation products on all of these columns. Analysis of synthetic mixtures and some commercial preparations containing chlorobutanol indicated that the procedure was applicable to such systems. Peak heights relative to a standard were used to calculate unknown concentrations. If desired, cyclohexanol, camphor, or menthol can be used as the internal standard.

CHLOROBUTANOL U.S.P. is widely used as a preservative and/or an antiseptic and local anesthetic in ophthalmic, otic, nasal, and parenteral preparations. There is considerable interest, therefore, in methods for analyzing these preparations.

Several analytical procedures have been reported for the determination of chlorobutanol. Most of these methods involve the decomposition of chlorobutanol by heating with alkali and subsequent determination of the liberated chloride ion by one of the conventional chloride procedures. The official assay in U.S.P. XVII is based on this principle, utilizing the Volhard method for the determination of the chloride ion. Sinton (1) employed a gravimetric method and Taub and Luckey (2) a turbidimetric procedure. Jensen and Jannke (3) have described a method based on the decomposition of chlorobutanol by a known amount of alkali and determination of the excess alkali. They also reported an assay involving hydrolysis of chlorobutanol to acetone, steam distillation, and iodometric determination of the acetone in the distillate. Vastagh (4) and Ray and Basu (5) distilled chlorobutanol from pharmaceutical formulations and determined chloride ion in the distillate, either argentometrically or iodometrically, following treatment with alkali. All these methods have the inherent disadvantage of not differentiating between free chloride ion present in the formulation or that formed on normal decomposition or alkaline hydrolysis of chlorobutanol. In addition, these procedures are not applicable if other components of the formulation produce halide ions when hydrolyzed with alkali.

Lach *et al.* (6) have described a sensitive amperometric titration for determination of the chloride ion liberated by alkaline hydrolysis of chlorobutanol. For those materials already containing chloride ions from other components in the sample or from normal decomposition of chlorobutanol, they determined the free chloride ion initially and then the total chloride ions after treatment with alkali. The difference represents the chloride contribution by chlorobutanol. This procedure is not directly applicable to samples containing organic compounds which liberate halide ions on treatment with alkali.

Birner (7) has described a procedure for the determination of chlorobutanol by polarography. While this procedure is very sensitive and specific for chlorobutanol, its application to pharmaceutical formulations involves prior separation by steam distillation.

Rehm and Mader (8) reported a procedure based on measurement of the colored ferric hydroxamate complex formed from ferric ion and the reaction product of hydroxylamine and chlorobutanol in alkaline solution. Ketones, aldehydes, esters, imides, acid chlorides, and acid anhydrides interfere since they also form hydroxamates; ferric ion complexing anions such as tartrate, citrate, phosphate, and phthalate also interfere unless a prior distillation step is used (9). Application of the Fujiwara alkali–pyridine reaction for chloroform to the colorimetric determination of chlorobutanol in commercial parenteral dosage forms was recently reported by Chafetz and Mahoney (10). Other polyhalogen compounds interfere as would also chlorobutanol degradation products containing the polyhalo moiety.

From the above discussion it is evident that there is a need for an improved method for the analysis of chlorobutanol preparations. Chlorobutanol is volatile and should lend itself to gas chromatographic analysis, but no such method has been reported in the literature. The purpose of this study was to develop a rapid, specific, gas–liquid chromatographic procedure for the determination of chlorobutanol. Such a method would not be subject to the limitations of the techniques discussed above and would be suitable for evaluating stability samples containing

Received September 19, 1966, from the Product Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, Ind.
Accepted for publication November 23, 1966.
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chlorobutanol, especially those involving new packaging materials. A number of chromatographic columns were investigated in order that chlorobutanol could be effectively separated from the wide variety of drugs and solvents with which it is commonly used.

EXPERIMENTAL

An F and M model 810 dual column gas chromatograph with a dual flame ionization detector and a Minneapolis Honeywell recorder was used.

Anhydrous chlorobutanol U.S.P. (S. B. Penick and Co., New York, N. Y.) was used without further purification as a standard and in the preparation of synthetic mixtures. The material was found to contain 98.4% chlorobutanol when assayed by the official procedure.

A number of chromatographic columns were investigated. Table I lists the different stationary phases and column conditions that were found satisfactory for chlorobutanol separation. Silanized Chromosorb W, 70–80 mesh (Johns-Manville, New York, N. Y.) packed in 0.25-in. aluminum tubing was used as column support. The gas flow rates were: helium, 60 ml./min.; hydrogen, 60 ml./min.; air, 300 ml./min.

Applicability of gas chromatography to the evaluation of stability samples containing chlorobutanol was investigated. A 0.125% solution was hydrolyzed in 1.0 *M* phosphate buffer, pH 7.9 at 70°, aliquots were withdrawn at periodic intervals, and these were analyzed with the polypropylene glycol column listed in Table I.

Synthetic mixtures containing known amounts of chlorobutanol were prepared with three solvents: water, light liquid petrolatum, and propylene glycol. Thiamine mononitrate and methapyrilene hydrochloride were used as active constituents in aqueous solutions, menthol and camphor in the light liquid petrolatum solutions, and benzocaine in the propylene glycol solutions. These solvents are representative of those usually present in formulations containing chlorobutanol. Compositions of these mixtures are shown in Table II. Diisodecyl phthalate was used as the stationary phase in the analysis of these solutions. Concentrations were calculated from peak heights, using a standard chlorobutanol solution chromatographed concurrently with the sample.

The utility of the gas chromatographic procedure for the analysis of commercial chlorobutanol preparations was also investigated. Various samples of unknown age, obtained from retail pharmacies, were analyzed for chlorobutanol content using the diisodecyl phthalate column.

Table I—Columns Found Satisfactory for Chlorobutanol Separation[a]

Stationary Phase	Column Length, ft.	Column Temp.[b]	Retention Time, min.
Diisodecyl phthalate,[c] 10%	6	140	4.95
Silicone 550,[c] 10%	8	130	3.15
Diethyleneglycol succinate,[c] 20%	5.5	150	3.45
Paraffin grease,[d] 10%	8	150	2.10
1,2,3-Tris(2-cyanoethoxy)-propane,[e] 10%	8	140	4.65
Polypropylene glycol,[f] 10%	6	150	4.20
Polyethylene glycol 4000,[g] 10%	6	160	2.70
Polyethylene glycol 1000,[g] 10%	8	150	5.10

[a] Silanized Chromosorb W, 70–80 mesh packed in 0.25-in. aluminum tubing as support. [b] Injection port and detector were maintained at 225°. [c] Wilkens Instrument and Research, Inc., Walnut Creek, Calif. [d] Available as Apiezon L from Applied Science Laboratories, Inc., State College, Pa. [e] Applied Science Laboratories, Inc., State College, Pa. [f] Marketed as Ucon 50 HB 5100 by Union Carbide Corp., New York, N. Y. [g] Marketed as Carbowax by Union Carbide Corp., New York, N. Y.

Table II—Analysis of Known Solutions of Chlorobutanol

	Solvent	Chlorobutanol, % Added	Found	Recovery
Thiamine mononitrate, 1.0%	Water	0.150	0.148	98.7
		0.300	0.300	100.0
		0.400	0.404	101.0
		0.500	0.497	99.4
Methapyrilene hydrochloride, 2.0%	Water	0.200	0.199	99.5
		0.300	0.299	99.7
		0.400	0.399	99.8
Camphor, 2.5%	Light liquid petrolatum	0.80	0.79	98.8
		1.00	0.99	99.0
Menthol, 1.8%		1.20	1.19	99.2
Benzocaine, 5.0%	Propylene glycol	0.50	0.50	100.0
		1.00	1.00	100.0
		1.50	1.54	102.7

RESULTS AND DISCUSSION

The various stationary phases that were found satisfactory for chlorobutanol separation along with column conditions and respective retention times obtained from a 0.5% aqueous solution of chlorobutanol are listed in Table I. Chlorobutanol was separated from its primary degradation products, chloroform and acetone, on all of these columns. The choice of such a variety of columns increases the applicability of the gas chromatographic procedure to the separation of chlorobutanol from the ingredients commonly used with this preservative. A typical chromatogram of a mixture of chlorobutanol, menthol, camphor, and eucalyptus and cinnamon oils obtained on the diisodecyl phthalate column is shown in Fig. 1. The good resolution of such a complex mixture indicates the versatility of this column in separating volatile constituents that are commonly present with chlorobutanol.

Nair and Lach (11) have shown that the degradation of chlorobutanol in buffered aqueous solution is first order with respect to chlorobutanol concentration. Figure 2, based on gas chromatographic analysis of degraded solutions, demonstrates the pseudo first-order kinetics for chlorobutanol decomposition and suggests the usefulness of this technique in stability evaluation of chlorobutanol formulations. The polypropylene glycol column, as described in Table I, was used in this experiment.

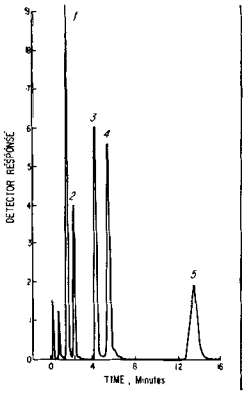

Fig. 1—Chromatogram of a mixture containing chlorobutanol on the diisodecyl phthalate column. Key: 1, eucalyptus oil; 2, chlorobutanol; 3, camphor; 4, menthol; 5, cinnamon oil.

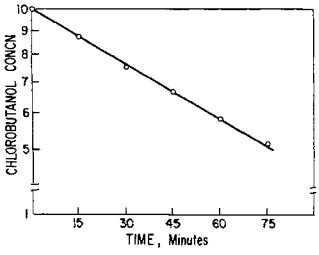

Fig. 2—Plot showing the pseudo first-order degradation of chlorobutanol in buffer pH 7.9 at 70° as determined by gas chromatographic analysis.

Results of the analysis of known mixtures containing chlorobutanol are given in Table II. The diisodecyl phthalate column was used as the stationary phase. The aqueous preparations were injected directly. Reproducibility was poor when the light liquid petrolatum preparations were injected directly but dilution with chloroform prior to injection eliminated this problem and the data presented were obtained in this manner. Tailing of the product solvent peak caused interference in the preparations containing propylene glycol. These were diluted with water and the chlorobutanol extracted with chloroform prior to analysis by comparison with a standard treated in a similar manner. The results indicate quantitative recovery of concentrations ranging from 0.15 to 1.50% in the three solvent systems, and per cent recoveries indicate no significant differences between these solvent systems.

Some commercial preparations containing chlorobutanol were analyzed, and the results are shown in Table III. Depending on the nature of the solvent, these formulations were treated as outlined above. The glycerin-containing product was too viscous to inject directly and was, therefore, analyzed in the same manner as the propylene glycol preparations.

TABLE III—ANALYSIS OF COMMERCIAL PREPARATIONS CONTAINING CHLOROBUTANOL

Prepn.	Solvent	Chlorobutanol, % Label Claim	Chlorobutanol, % Found
A_1	Water	0.15	0.05
A_2	Water	0.15	trace
B	Light liq. pet.	1.0	0.28
C	Water	0.3	0.41
D	Glycerin	3.0	2.98
E	Propylene glycol	0.4	0.39
F	Light liq. pet.	1.0	1.0
G	Water	0.5	0.51

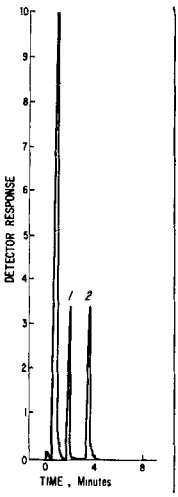

Fig. 3—Chromatogram of a chloroform solution of chlorobutanol and cyclohexanol on the diisodecyl phthalate column. Key: 1, chlorobutanol; 2, cyclohexanol.

Preparation *A* was an ophthalmic preparation packaged in a plastic container. Two different samples were obtained, and it is obvious that the container did not afford sufficient protection against chlorobutanol loss. Preparation *B* was a nasal decongestant packaged in a glass bottle with a rubber dropper assembly. The general appearance of the package indicated that this product was of considerable age, and analysis showed that the chlorobutanol content was considerably below the label claim, probably due to loss through the closure. Product *C*, packaged in a plastic container, was found to contain chlorobutanol in excess of the label claim, presumably the result of the excess needed to maintain the label claim through the product shelf life. Preparations *D* through *G* were found to conform to the label claim.

All chlorobutanol determinations reported in this paper were calculated from peak heights by comparison with a standard chlorobutanol solution. A standard was used for each analysis. The relationship between peak height and concentration was found to be linear in the concentration ranges investigated. However, internal standards are very useful and popular, and it appears that several compounds could be used for this purpose. The potentiality of camphor and menthol as internal standards is evident from Fig. 1, and Fig. 3 illustrates the possibility of using cyclohexanol as an internal standard.

REFERENCES

(1) Sinton, F. C., *J. Assoc. Offic. Agr. Chemists*, **21**, 557 (1938).
(2) Taub, A., and Luckey, W. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **32**, 28(1943).
(3) Jensen, H., and Jannke, P., *ibid.*, **37**, 37(1948).
(4) Vastagh, G., *Pharm. Zentralhalle*, **78**, 497(1937); through *Chem. Abstr.*, **31**, 7595[3] (1937).
(5) Ray, N., and Basu, U. P., *Indian J. Pharm.*, **12**, 6 (1950).
(6) Lach, J. L., Nair, D., and Blaug, S. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 46(1958).
(7) Birner, J., *Anal. Chem.*, **33**, 1955(1961).
(8) Rehm, C. R., and Mader, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 621(1957).
(9) Goddu, R. F., LeBlanc, N. F., and Wright, C. F., *Anal. Chem.*, **27**, 1251(1955).
(10) Chafetz, L., and Mahoney, R. W., *J. Pharm. Sci.*, **54**, 1805(1965).
(11) Nair, A. D., and Lach, J. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 390(1959).

Technique for the Preservation of Tetracycline Fluorescence in Ascites Tumor Cells

By ELIZABETH P. BARBOUR, JAMES R. THOMPSON, and CARL C. RIEDESEL

Fluorescence microscopy has a potential application in cellular pharmacology for the study of intra- and intercellular distribution of pharmacological agents. A method is reported for preparing permanent preparations of tetracycline-retaining ascite tumor cells. A combination of glutaraldehyde fixation followed by glycerol dehydration was found satisfactory.

During an investigation concerning tetracycline retention by ascites tumor cells using fluorescence microscopy, it became essential to make permanent preparations of the cells for more detailed observation. It became apparent immediately that application of traditional histologic techniques would not suffice because tetracycline fluorescence is easily quenched or displaced by otherwise suitable fixatives and dehydrating agents. Numerous agents were screened in an effort to develop a satisfactory method for preserving tetracycline fluorescence. Under the conditions reported in this paper, a combination of glutaraldehyde fixation followed by glycerol dehydration proved to be the most satisfactory technique. Fluorescence microscopy has a potential application in cellular pharmacology for the study of intra- and intercellular distribution of pharmacological agents. For this reason, the technique found useful in preserving tetracycline fluorescence is described.

MATERIALS AND METHODS

All microscopic examinations of the cell preparations were made using a Zeiss GFL fluorescence microscope with a mercury light source (HB200). Excitation filters BG-38 and BG-12 were used along with barrier filters 41 and 53. These filters permit the best observation of tetracycline fluorescence. At the same time they eliminate autofluorescence of the ascites cell which may be seen when wider ranges of the U.V. spectrum are employed. Photomicrographs were taken using a Zeiss Ikon camera attachment and 35-mm. Panatomic X film.

The Ehrlich ascites tumor cells are strain ES obtained from Cailleau (1). They are maintained in Swiss mice by weekly intraperitoneal transfer. All cells used in these investigations were removed from the host 6 to 10 days following passage to assure an optimal growth phase.

All chemicals and reagents employed were of C.P. grade or the equivalent. The following substances were selected for evaluation as fixatives: acetone, diethyl ether and alcohol (1:1), formalin, and glutaraldehyde. A wide range of concentrations of each agent was employed to find the most efficient concentration. The following substances were evaluated as dehydrating agents: acetone, diethyl ether, ethanol, and glycerol. Each of these reagents was similarly tested at a variety of concentrations. A variety of exposure times ranging from 2 to 30 min. in the fixatives and dehydrants was employed.

The general procedure was as follows. Several milliliters of ascites cell suspension were withdrawn from the host animal 15 min. following the intraperitoneal injection of 0.1 mg. of tetracycline. This particular dosage and time lapse were selected to assure adequate fluorescence in the tumor cells. The cell suspension was diluted with an equal volume of Hank's balanced saline, then fixed with one of the fixatives. In the case of control preparations, these were examined and photographed immediately following dilution in Hank's saline solution. Ascites cells which were subjected to fixation were exposed for the appropriate time interval, centrifuged lightly, then washed with several changes of Hank's saline solution. The cells were then either examined under the microscope or further treated by exposure to a dehydrating agent through a series of 3 to 5 min. changes in progressively higher concentrations of the dehydrant. At each step samples of the cells were photographed to determine what changes could be observed in the fluorescence pattern in the cells when compared to control specimens.

As this work progressed, it became obvious that fixation in glutaraldehyde dissolved in Sorensen's phosphate buffer was the most satisfactory fixative. The best preservation of fluorescence was obtained when glutaraldehyde fixation was followed by glycerol dehydration. For this reason, the protocol of the glutaraldehyde–glycerol technique is given in more specific detail: fixation is accomplished by diluting the ascites cell suspension with an equal volume of 4% glutaraldehyde (redistilled) in Sorensen's phosphate buffer, pH 7.4, and allowing the cells to remain in the solution for 5 min. Longer fixation seems to produce no deleterious effect, but offers no real advantage. Following fixation and light centrifugation (100 g) the cells are run through a series of 3 min. changes in progressively higher concentrations of glycerol.

RESULTS

As can be seen from the photomicrographs submitted, this technique does not appear to decrease intensity of fluorescence, or displace the tetracycline within the cell. On the other hand, all other com-

Received April 25, 1966, from the Department of Physiology and Pharmacology, School of Pharmacy, University of the Pacific, Stockton, Calif.

Accepted for publication August 5, 1966.

The authors acknowledge the financial support of this investigation by the Milheim Foundation for Cancer Research.

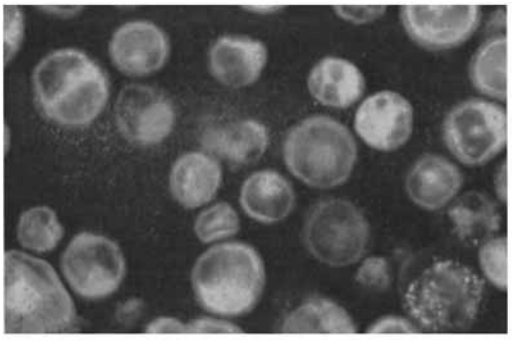

Fig. 1.—Ascites tumor cells freshly removed from the peritoneal cavity of a tetracycline treated mouse. X960.

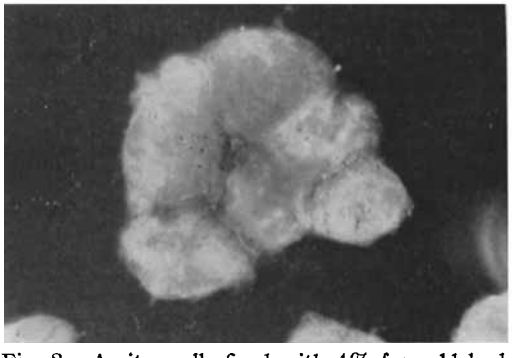

Fig. 2.—Ascites cells fixed with 4% formaldehyde for 2–3 min. X960.

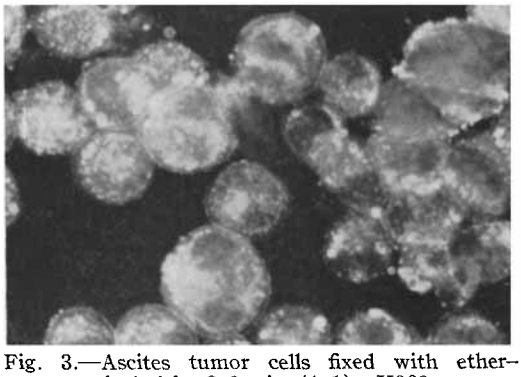

Fig. 3.—Ascites tumor cells fixed with ether–alcohol for 2–3 min. (1:1). X960.

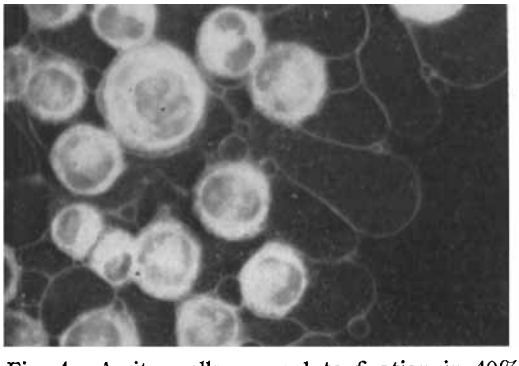

Fig. 4.—Ascites cells exposed to fixation in 40% acetone. X960.

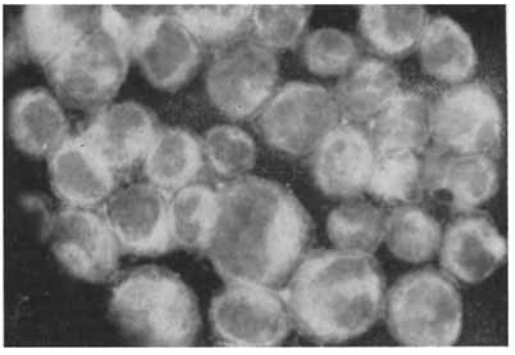

Fig. 5.—Ascites tumor cells fixed in 4% glutaraldehyde. X960.

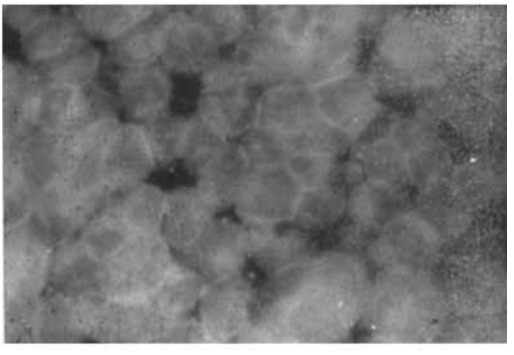

Fig. 6.—Effect of acetone dehydration after glutaraldehyde fixation. X960.

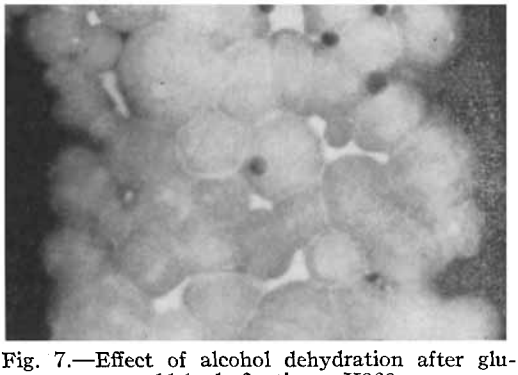

Fig. 7.—Effect of alcohol dehydration after glutaraldehyde fixation. X960.

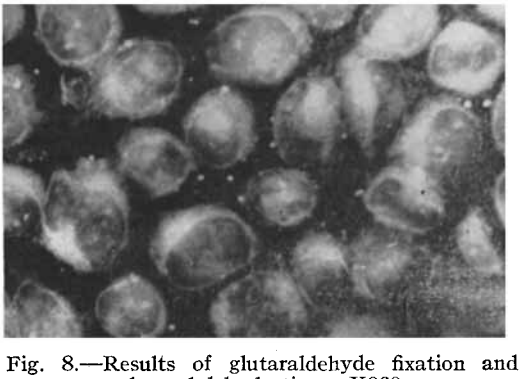

Fig. 8.—Results of glutaraldehyde fixation and glycerol dehydration. X960.

binations of fixatives and dehydrating agents appeared to introduce artifacts, some of these being visible in the photomicrographs.

No photomicrograph is submitted for ascites cells not exposed to tetracycline since at the particular wavelength employed there is no autofluorescence; hence, it is not possible to take a photomicrograph of the cells against the darkfield background. The first five photomicrographs were taken of cells before exposure to dehydrating agents. Figure 1 illustrates the appearance of tetracycline-containing tumor cells freshly removed from the host animal but having been subjected to no treatment other than their removal from the host. They may be considered as control preparations. In such cells the tetracycline seems to be located in the cytoplasmic portion of the cell, with concentration in the perinuclear zone. Figure 2 shows the effect of 4% formalin fixation for 2 min. There is an apparent loss of definition and diffusion of the tetracycline. Higher concentrations of formalin or longer fixation periods further exaggerated these observations. Figure 3 shows the effect of diethyl ether–alcohol fixation for 2 min. The tetracycline complex appears to be precipitated as judged by the altered pattern of fluorescence. The effect of acetone fixation is shown in Fig. 4. There is extensive blebbing with the suggestion that tetracycline has diffused into the karyoplasm. Figure 5 is a photomicrograph of cells fixed with glutaraldehyde. One sees an apparent intensification of the fluorescence. This was a consistent finding in all glutaraldehyde preparations.

The remaining photomicrographs demonstrate the effects of several dehydrating agents. Figure 6 illustrates the result of acetone dehydration following glutaraldehyde fixation. Even when the total dehydrating time is kept to 4 min. there appears to be a reduced fluorescence. The use of ethanol dehydration following glutaraldehyde fixation (Fig. 7) seems to reduce sharpness of detail of the preparation although there is no loss of fluorescence. Areas of bright fluorescence in some of the intercellular spaces suggests a possible leakage of fluorescent substance from the cells. The final photomicrograph (Fig. 8) shows the result of glutaraldehyde fixation followed by glycerol dehydration in accordance with the protocol described in this report. There is no loss of cellular detail, nor any apparent loss or distortion of fluoresence.

REFERENCE

(1) Cailleau, R., and Costa, F., *J. Natl. Cancer Inst.*, **26**, 271(1961).

Hydrolysis of Methylparaben

By N. N. RAVAL and E. L. PARROTT

The hydrolysis of methylparaben in aqueous solution at 70, 80, and 85° in pH range from 6 to 8 has been studied.

SINCE their introduction into the United States in the 1930's, the parabens have been widely used as preservatives in foods, drugs, and cosmetics (1–5). The lack of toxicity and the broad spectrum of preservative activity of methylparaben have been reported (6–8). The correlation of preservative activity with the binding of methylparaben to other substances has led to more effective and intelligent product formulation (9–11).

Methylparaben is stable in air. Aqueous solutions of methylparaben buffered at pH 3 and 6 showed no decomposition when heated for 2 hr. at 100° or for 30 min. at 120° (12). As the removal of the ester portion by hydrolysis yields *p*-hydroxybenzoic acid, which possesses insignificant preservative activity, this investigation was undertaken to study the extent of hydrolysis of methylparaben in a pH range utilized in pharmaceuticals.

EXPERIMENTAL

A 0.1-Gm. quantity of methylparaben[1] was accurately weighed into a 100-ml. volumetric flask and dissolved in a sufficient amount of buffer to bring to a volume of 100 ml. of solution. The solution was filled and sealed into 10-ml. ampuls. The ampuls were placed in appropriate constant-temperature baths, and after thermal equilibrium was attained, an ampul was removed and, by means of iced water, was cooled to 25°. This solution was analyzed for zero-time concentration of methylparaben.

Five milliliters of the solution was withdrawn, adjusted to pH 7, and sufficient distilled water was added to bring the volume to 100 ml. A 10-ml. aliquot was extracted with four 10-ml. portions of anhydrous ether. The ether was evaporated and the residue was dissolved in 100 ml. of distilled water. The absorbance of the solution was measured at 255 mμ using a Beckman DU spectrophotometer. By means of a standard absorption curve, the concentration of methylparaben was determined. Ampuls were removed at definite intervals of time and analyzed by this procedure, which is essentially that of Aalto *et al.* (1).

All chemicals were reagent grade. McIlvaine's buffer was used for pH 6 and 7; Palitzsch's buffer was used for pH 8 and 9 (13). All buffer solutions were adjusted at and the pH determined at the temperature of hydrolysis by means of Beckman Zeromatic pH meter.

RESULTS AND DISCUSSION

The rate of hydrolysis of methylparaben was followed at pH 6, 8, and 9 at 70, 80, and 85°. A plot of the logarithm of concentration against time produced a straight line. The observed velocity constant k can be evaluated by multiplying the slope of the line by 2.303. The half-life or time required

Received September 19, 1966, from the College of Pharmacy, University of Iowa, Iowa City, Ia. 52240.
Accepted for publication November 1, 1966.
Abstracted from a thesis submitted by Nayankumar N. Raval to the Graduate College, University of Iowa, in partial fulfillment of Master of Science degree requirements.

[1] The methylparaben used was Methyl Parasept, Heyden Newport Chemical Corp.

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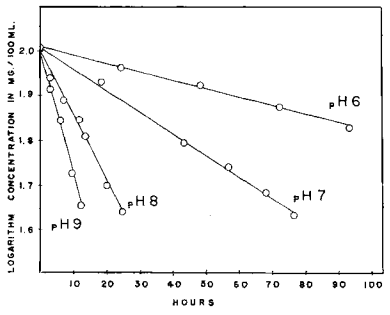

Fig. 1—Pseudo first-order hydrolysis of methylparaben at 85°.

for half of a given amount of methylparaben to decompose can be evaluated by dividing 0.693 by the velocity constant (14). Figure 1 is typical of the results obtained. The experimentally determined velocity constants and half-life periods are given in Table I.

As the hydrolysis appears to follow a first-order relationship, the energy of activation E_a was calculated using the logarithmic form of the Arrhenius equation between the limits of absolute temperature T_2 and T_1 (15).

$$\log \frac{k_2}{k_1} = \frac{E_a}{2.303\,R}\left(\frac{T_2 - T_1}{T_2 T_1}\right)$$

The apparent energy of activation is approximately 24 Kcal./mole. The value for the velocity constant for the reaction was calculated at 25 and 121°. The calculated velocity constants and half-life periods at 25° are listed in Table II.

As autoclaving may be required of certain pharmaceutical solutions, the per cent decomposition of a methylparaben solution in an autoclave was experimentally determined. After autoclaving for 30 min. at pH 6 and 9, there remained 94.5 and 58.0% of the initial concentration, respectively. Using $k_{121°} = 0.105$ hr.$^{-1}$ at pH 6 as calculated from the Arrhenius equation, the predicted decomposition was 5.13%; the experimental decomposition was 5.5%. Likewise, at pH 9 the predicted loss was 48.36%; the experimental decomposition was 42.0%.

TABLE I—HYDROLYSIS OF METHYLPARABEN AT VARIOUS TEMPERATURES AND pH'S

Temp.	pH		$k \times 10^2$	$t_{1/2}$
70°	6.0	±0.050	0.123 hr.$^{-1}$	565.2 hr.
	8.0		0.737	94.0
	9.0		1.842	37.6
80°	6.0		0.189	364.7
	8.0		2.303	30.0
	9.0		4.900	14.1
85°	6.0		0.485	142.9
	7.0		1.076	64.7
	8.0		3.290	21.0
	9.0		6.810	10.1

TABLE II—THEORETICAL VELOCITY CONSTANTS AND HALF-LIFE PERIODS FOR THE HYDROLYSIS OF METHYLPARABEN AT 25° AS CALCULATED FROM AN ARRHENIUS PLOT

pH	$k \times 10^5$	$t_{1/2}$
6.0	0.432 hr.$^{-1}$	6675 days
8.0	3.236	892
9.0	6.998	412

REFERENCES

(1) Aalto, T. R., Firman, C. C., and Rigler, N. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 449(1953).
(2) Neidig, C. P., and Burell, H., *Drug Cosmetic Ind.*, **54**, 408(1944).
(3) Gottfried, N. S., *Am. J. Hosp. Pharm.*, **19**, 310(1962).
(4) Sokol, H., *Drug Std.*, **20**, 89(1962).
(5) Wedderburn, D. L., "Advances in Pharmaceutical Sciences," Academic Press Inc., New York, N. Y., 1964, p. 195.
(6) Matthews, C., Davidson, J., Bauer, E., Morrison, J., and Richardson, A., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 260(1956).
(7) Jones, P. S., Thigpan, D., Morrison, J., and Richardson, A. P., *ibid.*, **45**, 268(1956).
(8) Lawrence, C. A., *ibid.*, **44**, 457(1955).
(9) Pisano, F. D., and Kostenbauder, H. B., *ibid.*, **48**, 310(1959).
(10) Blaug, S., and Ahsan, S., *J. Pharm. Sci.*, **50**, 441 (1961).
(11) Guttman, D., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 659(1956).
(12) Phares, C., Goldschmidt Chemical Corp., personal communication.
(13) Britton, H. T. S., "Hydrogen Ions," 4th ed., Chapman and Hall Ltd., London, England, 1955, vol. I, pp. 356, 361.
(14) Hamill, W. H., and Williams, R. R., Jr., "Principles of Physical Chemistry," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1959, Chap. XII.
(15) Daniels, F., and Alberty, R. A., "Physical Chemistry," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1961, p. 315.

Possible Ethanol-Induced Tolerance in Rats

By THEODORE H. EICKHOLT, LEO J. SCHILLACI*, and S. ALAN SEARCY*

A tilting-plane technique was employed to determine the effects of alcohol on performance of rats with specific attention to development of possible tolerance. Significant differences were obtained only on the first day of treatment with possible tolerance developing in only 1 day. No significant learning effects could be shown over the 3-week period.

A VARIETY of tests have been employed for determining performance in humans and animals while under alcohol administration (1–6). The many variables in response to pretreatment, age, sex, strain, and stress confounds the study of tolerance to alcohol using these tests (7–16). Behavioral tolerance has been investigated (17) and was reported in monkeys following once daily administration, 2 Gm./Kg. i.v., for only 4 consecutive

Received June 6, 1966, from the Department of Pharmacology, School of Pharmacy, Northeast Louisiana State College, Monroe.
Accepted for publication October 5, 1966.
* Special Problems Undergraduate Students in Pharmacology.

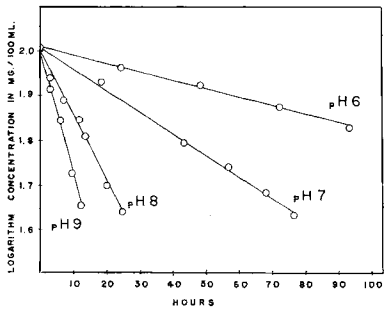

Fig. 1—Pseudo first-order hydrolysis of methylparaben at 85°.

for half of a given amount of methylparaben to decompose can be evaluated by dividing 0.693 by the velocity constant (14). Figure 1 is typical of the results obtained. The experimentally determined velocity constants and half-life periods are given in Table I.

As the hydrolysis appears to follow a first-order relationship, the energy of activation E_a was calculated using the logarithmic form of the Arrhenius equation between the limits of absolute temperature T_2 and T_1 (15).

$$\log \frac{k_2}{k_1} = \frac{E_a}{2.303\,R}\left(\frac{T_2 - T_1}{T_2 T_1}\right)$$

The apparent energy of activation is approximately 24 Kcal./mole. The value for the velocity constant for the reaction was calculated at 25 and 121°. The calculated velocity constants and half-life periods at 25° are listed in Table II.

As autoclaving may be required of certain pharmaceutical solutions, the per cent decomposition of a methylparaben solution in an autoclave was experimentally determined. After autoclaving for 30 min. at pH 6 and 9, there remained 94.5 and 58.0% of the initial concentration, respectively. Using $k_{121°} = 0.105$ hr.$^{-1}$ at pH 6 as calculated from the Arrhenius equation, the predicted decomposition was 5.13%; the experimental decomposition was 5.5%. Likewise, at pH 9 the predicted loss was 48.36%; the experimental decomposition was 42.0%.

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REFERENCES

(1) Aalto, T. R., Firman, C. C., and Rigler, N. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 449(1953).
(2) Neidig, C. P., and Burell, H., *Drug Cosmetic Ind.*, **54**, 408(1944).
(3) Gottfried, N. S., *Am. J. Hosp. Pharm.*, **19**, 310(1962).
(4) Sokol, H., *Drug Std.*, **20**, 89(1962).
(5) Wedderburn, D. L., "Advances in Pharmaceutical Sciences," Academic Press Inc., New York, N. Y., 1964, p. 195.
(6) Matthews, C., Davidson, J., Bauer, E., Morrison, J., and Richardson, A., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 260(1956).
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(9) Pisano, F. D., and Kostenbauder, H. B., *ibid.*, **48**, 310(1959).
(10) Blaug, S., and Ahsan, S., *J. Pharm. Sci.*, **50**, 441 (1961).
(11) Guttman, D., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 659(1956).
(12) Phares, C., Goldschmidt Chemical Corp., personal communication.
(13) Britton, H. T. S., "Hydrogen Ions," 4th ed., Chapman and Hall Ltd., London, England, 1955, vol. I, pp. 356, 361.
(14) Hamill, W. H., and Williams, R. R., Jr., "Principles of Physical Chemistry," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1959, Chap. XII.
(15) Daniels, F., and Alberty, R. A., "Physical Chemistry," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1961, p. 315.

Possible Ethanol-Induced Tolerance in Rats

By THEODORE H. EICKHOLT, LEO J. SCHILLACI*, and S. ALAN SEARCY*

A tilting-plane technique was employed to determine the effects of alcohol on performance of rats with specific attention to development of possible tolerance. Significant differences were obtained only on the first day of treatment with possible tolerance developing in only 1 day. No significant learning effects could be shown over the 3-week period.

A VARIETY of tests have been employed for determining performance in humans and animals while under alcohol administration (1–6). The many variables in response to pretreatment, age, sex, strain, and stress confounds the study of tolerance to alcohol using these tests (7–16). Behavioral tolerance has been investigated (17) and was reported in monkeys following once daily administration, 2 Gm./Kg. i.v., for only 4 consecutive

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days (18). Metabolic tolerance has been indicated by a lower blood alcohol level as early as the third day in self-maintained intoxicated rats receiving increased alcoholic intakes (19), and it has been postulated that metabolic tolerance may serve as a limiting factor in alcohol consumption (20). The rate of metabolism of ethanol in humans has been shown to be increased after a 3 to 14-day period of induced intoxication (21). The same has been shown in rats along with fine structural changes in the liver (22, 23).

It was the purpose of this study to demonstrate the effect of alcohol in rats through the use of a tilting-plane technique with specific attention to the development of any degree of tolerance observed over a 2-week administration period.

EXPERIMENTAL

The technique, the tilting-plane technique, proposed by Arvola, Sammalisto, and Wallgren appeared most satisfactory for quantitating equilibrium and motor coordination (24). The test is suitable for differentiating certain degrees of alcohol intoxication. The rat is placed on a board, the board is tipped up, and the angle at which the animal slides is determined. The surface of the plane is rough and the rat clings with its claws to the surface. A deeply intoxicated rat cannot cling to the plane and slides down the plane at a lower angle than an unintoxicated rat. The method has been used and found satisfactory by others (24–27).

Twenty Wistar male rats, 200 Gm. average, were used. Ten served as a control group and 10 were used in the test group.

The first week without any drug administration both groups were put on the tilting-plane and 10 readings of each animal on each of 4 consecutive days were made for control, learning, pretreatment averages. The second and third weeks, the test animals received 2 Gm./Kg. body weight of a 10% solution of ethanol i.p. This dose, 2 Gm./Kg., was chosen as that maximally tolerated and in best agreement with that used in the literature. At this dosage, the animals at first appeared nearly unconscious, but activity after 60 min. was satisfactory for testing on the tilting-plane.

The controls were injected with an equivalent volume of normal saline thus subjecting them to the same handling (stress) as the test group.

Both groups were again placed on the tilting-plane and 10 readings of each animal, on each of 4 consecutive days, for the second and third weeks were made. Readings were taken 60 min. after injections. The room temperature was maintained at 24°.

The tilting-plane was made from rough pine wood with the dimensions being 23.5 × 44 cm. All animals were placed on the plane and raised through an angle of 90° in 5 sec. and the angle at which the animals slid was recorded.

The averages of all readings were determined and the t test of significance performed.

RESULTS AND DISCUSSION

After the first week of alcohol injections, approximately 50% of the rats appeared to enjoy the injection, they were much easier to handle, and would remain quiet during the injection. The same was not true for the saline injected rats. From the averages of the readings taken, the comparisons with t tests and standard deviations were calculated and are shown in Tables I, II, and III.

In the tables, the animals are referred to as control and test groups. A further delineation as to treatment periods within groups is indicated by "Control Learn, Control Test" and "Test Learn, Test Test." The first day control learn indicates the first day the control group was placed on the tilting-plane during the first week learning period. Likewise, the first day test learn indicates the first day the test group was placed on the tilting-plane during the first week learning period.

The first day control test indicates the first day the control group, receiving comparable volume saline injections, was placed on the tilting-plane during the first week of the test period, actually the second week in the test program. Likewise, the first day test test indicates the first day the test group receiving alcohol injections was placed on the tilting-plane during the first week of the test period, again, actually the second week in the test program.

Furthermore, the sixth day control test would indicate the sixth day the control group, receiving the saline injections, was placed on the tilting-plane during the second week of the test period, actually the third week in the test program. Likewise, the sixth day test test indicates the sixth day the test group receiving alcohol injections was placed on the tilting-plane during the second week of the test period, again actually the third week in the test program.

These results show that in the test animals there was a significant difference between the first day trial on the tilting-plane and the first day of test (t = 5.79), and also the fourth day of trial and the first day of test (t = 3.41). Likewise, the first and fourth day control learn animals showed a significant difference from the first day test test animals (t = 4.03 and t = 5.22, respectively). The first day control test animals were not significantly different from the first day test test animals at P 0.05 but were at P 0.1 (t = 2.09), a t value greater than 2.101 being required at P 0.05.

However, in a comparison of the second day of trial on the plane and the second day of test, in all cases of comparison, there were no statistically significant differences. The animals were apparently not as intoxicated the very next day after receiving the same dosage of alcohol. Therefore, it would appear possible to induce alcohol tolerance in 1 day as determined by this method. Behavioral tolerance has been reported to occur in monkeys in 4 days and a metabolic tolerance has been reported in rats in 3 days (18, 19).

Alcohol metabolism may have been altered very rapidly in this study, although Wistar rats have been shown to select less alcohol than G-4 rats when given free choice (9). What this would imply regarding the respective strains' metabolism is questionable. The animals used in this study were males, and males also have been shown to drink more alcohol than females, another possible factor influencing tolerance (11). Operant performance has also been shown to vary between alcohol-preferring and nonpreferring animals (12).

Statistical evaluation of control animals showed no significant difference in these animals over the

whole 3-week period. Apparently with this method, learning played no significant part in this study. It has been reported, however, that alcohol-treated rats appear to learn more slowly (2).

All of the animals' weights were followed; however, no significant differences were determined between the control and test animals.

TABLE I.—STANDARD DEVIATIONS AND *t* VALUES OF VARIOUS CONTROL AND TEST GROUP ANIMALS

	S.D.	*t*
First week test test *vs.* second week test test	5.08	1.64
First week control test *vs.* first week test test	5.46	0.89
800 Control test *vs.* 800 test test	4.66	1.07
First day control test *vs.* first day test test	6.03	2.09[a]
Second day control test *vs.* second day test test	6.36	0.40
Third day control test *vs.* third day test test	6.06	1.44
Fourth day control test *vs.* fourth day test test	5.70	1.09
Fifth day control test *vs.* fifth day test test	4.35	0.99
Sixth day control test *vs.* sixth day test test	4.46	1.15
Seventh day control test *vs.* seventh day test test	4.88	1.71
Eighth day control test *vs.* eighth day test test	4.57	1.45

[a] Significant at *P* 0.1.

TABLE II.—STANDARD DEVIATIONS AND *t* VALUES OF VARIOUS CONTROL AND TEST GROUP ANIMALS

	S.D.	*t*
First day control learn *vs.* first day control test	5.63	0.62
Second day control learn *vs.* second day control test	5.02	0.66
Third day control learn *vs.* third day control test	4.64	0.87
Fourth day control learn *vs.* fourth day control test	4.11	0.54
First day test learn *vs.* first day test test	3.50	5.79[a]
Second day test learn *vs.* second day test test	6.78	1.51
Third day test learn *vs.* third day test test	6.53	1.00
Fourth day test learn *vs.* fourth day test test	6.74	0.55
Fourth day test learn *vs.* first day test test	5.59	3.41[a]
Fourth day control learn *vs.* first day test test	4.36	5.22[a]
First day control learn *vs.* fourth day control learn	3.79	1.73
First day control learn *vs.* fourth day control test	3.71	0.42
First day control learn *vs.* eighth day control test	3.79	1.58
First day test learn *vs.* fourth day test learn	4.80	0.26
First day test learn *vs.* fourth day test test	5.13	0.48
First day test learn *vs.* eighth day test test	3.63	1.35

[a] Significant at *P* 0.05.

TABLE III.—STANDARD DEVIATIONS AND *t* VALUES OF VARIOUS CONTROL AND TEST GROUP ANIMALS

	S.D.	*t*
First day control learn *vs.* first day test learn	2.76	1.49
First day control learn *vs.* fourth day test learn	5.16	0.53
First day control learn *vs.* first day test test	3.99	4.03[a]
First day control test *vs.* first day test test	6.03	2.09
First day control test *vs.* fourth day test test	7.11	1.42
First day control test *vs.* eighth day test test	6.27	0.41

[a] Significant at *P* 0.05.

SUMMARY AND CONCLUSIONS

Male Wistar rats were subjected to a tilting-plane technique to determine the effects of alcohol on their performance. Subjected first to a 1-week learning period, this was followed by a 2-week test period. Control rats received normal saline and test rats received 2 Gm./Kg. of alcohol. All animals were placed upon the tilting-plane and the angle at which they would slide off was recorded. Significant differences were obtained between first day control learn *versus* first day test test, first day control test *versus* first day test test, first day test learn *versus* first day test test, fourth day test learn *versus* first day test test, and fourth day control learn *versus* first day test test. As measured by this method, tolerance would seem to appear after only 1 day. No significant learning effect could be shown by this method in the control animals over the entire 3-week period.

REFERENCES

(1) Denenberg, V. H., Pawlowski, A. A., and Zarrow, M. X., *Quart. J. Studies Alc.*, **22**, 14(1961).
(2) Pawlowski, A. A., Denenberg, V. H., and Zarrow, M. X., *ibid.*, **22**, 232(1961).
(3) Boyd, E. S., Morken, D. A., and Hodge, H. C., *ibid.*, **23**, 34(1962).
(4) Harris, H. E., Piccolino, E. B., Roback, H. B., and Sommer, D. K., *ibid.*, **24**, 490(1963).
(5) Arvola, A., *ibid.*, **22**, 575(1961).
(6) Wallgren, H., and Savolainen, S., *Acta Pharmacol. Toxicol.*, **19**, 59(1962).
(7) Parisella, R. M., and Pritham, G. H., *Quart. J. Studies Alc.*, **25**, 248(1964).
(8) Wallgren, H., and Forsander, O., *Brit. J. Nutr.*, **17**, 453(1963).
(9) Myers, A. K., *J. Comp. Physiol. Phys.*, **55**, 606(1962).
(10) Mendelson, J. H., and Mello, N. K., *Quart. J. Studies Alc.*, **25**, 1(1964).
(11) Clay, M. L., *ibid.*, **24**, 36(1963).
(12) Mello, N. K., and Mendelson, J. H., *ibid.*, **24**, 226 (1963).
(13) Rodgers, D. A., and McClearn, G. E., *ibid.*, **23**, 26 (1962).
(14) Rodgers, D. A., and Thiessen, D. D., *ibid.*, **24**, 240 (1963).
(15) Korn, S. J., *ibid.*, **21**, 605(1960).
(16) LeiKola, A., *ibid.*, **23**, 369(1962).
(17) Moskowitz, H., and Wapner, M., *ibid.*, **25**, 619(1964).
(18) Hogans, A. F., Moreno, O. M., and Brodie, D. A., *Am. J. Physiol.*, **201**, 434(1961).
(19) Lester, D., *Quart. J. Studies Alc.*, **22**, 223(1961).
(20) Forsander, O. A., *ibid.*, **23**, 480(1962).
(21) Mendelson, J. H., Stein, S., and Mello, N. K., *Metab. Clin. Exptl.*, **14**, 1255(1965).
(22) Hawkins, R. D., Kalant, H., and Khanna, J. M., *Can. J. Physiol. Pharm.*, **44**, 241(1966).
(23) Thorpe, M. E. C., and Shorey, C. D., *Am. J. Pathol.*, **48**, 557(1966).
(24) Friedman, S. L., and Ingalls, J. W., *Quart. J. Studies Alc.*, **21**, 217(1960).
(25) Wallgren, H., Arvola, A., and Sammalisto, L., *ibid.*, **21**, 698(1960).
(26) Kosa, F., *ibid.*, **24**, 253(1963).
(27) Broadhurst, P. L., and Wallgren, H., *ibid.*, **24**, 476 (1963).

Micelle Size and Stability of Aqueous Amphotericin B (Polyene) Systems

By JOEL KIRSCHBAUM and SAMUEL G. KAHN

Concentrations of components were varied in aqueous amphotericin B systems containing cetyl dimethyl benzyl ammonium chloride, dimethylsulfoxide, amphotericin B, and water. Using the analytical ultracentrifuge and correcting for density and viscosity, the systems containing the smallest micelles were found by determination of sedimentation coefficients. This procedure proved valuable in subsequent formulations, containing amphotericin B for veterinary purposes.

AMPHOTERICIN B is a polyene antifungal antibiotic which has a molecular weight of approximately 960. It is relatively insoluble in water and other common solvents, but exceedingly soluble in dimethylsulfoxide (DMSO) (1). The preparation of an aqueous suspension, colloid, or solution of amphotericin B for use as a medicament would be very desirable. This study was undertaken to investigate how changes in concentration of each component in an aqueous DMSO–amphotericin B–cetyl dimethyl benzyl ammonium chloride system will affect the micelle size and stability of amphotericin in such a system.

EXPERIMENTAL

Solutions were prepared by dissolving the appropriate amounts of amphotericin B and cetyl dimethyl benzyl ammonium chloride in DMSO and diluting with deionized water to the desired concentration. The concentrations of amphotericin B (900 mcg./mg.) were varied from 12.5 to 400 mcg./ml. The alkyl quaternary ammonium salt, cetyl dimethyl benzyl ammonium chloride[1] was used in concentrations from 12.5 to 200 mcg./ml. DMSO (pharmaceutical grade) was varied in concentration from 0.25% to 8% of solution.

Sedimentation coefficients (S) were measured using 20-mm. centerpiece, 4° cells, and centrifuged at speeds of 12,590; 29,500; or 50,740 r.p.m. in the An-E rotor, the temperature being maintained at 20°C. The Schlieren phaseplate angles were from 50° to 65°. Images were recorded on Kodak metallographic plates and tenfold magnifications were traced using a Nikon shadowgraph. The areas under the Schlieren peaks or peak areas, were measured with a planimeter. These areas are proportional to the concentration of sedimenting substances.

Viscosities were measured at 20° C. employing capillary and Zimm viscometers (2). Densities were measured at 20° C. using a 10-ml. pycnometer. For molecular weight calculations, the partial specific volume of amphotericin B was determined in a 1-ml. pycnometer and found to be 0.64 ± 0.02.

Micelle weights were calculated using the Archibald approach to sedimentation equilibrium method (3). Concentration gradients were measured in 12 mm. interference cells at speeds of either 8,766 or 42,040 r.p.m. and evaluated using Engelberg's modification (4). Total concentration was measured with a synthetic boundary centerpiece in an interference cell. The Schlieren phaseplate angle was 75°. Concentration gradients at the meniscus and cell bottom were evaluated from enlarged images as previously described for the determination of S values.

RESULTS AND DISCUSSION

Table I presents data of peak areas and S values, corrected for viscosity and density. When the level of amphotericin B was maintained at 100 mcg./ml. and the concentrations of cetyl dimethyl benzyl ammonium chloride and DMSO varied, S values and peak areas were at a minimum when DMSO levels equalled 1% to 2% and cetyl dimethyl benzyl ammonium chloride concentrations were 25 to 100 mcg./ml. Interestingly, the S values for amphotericin B at these component concentrations did not change after 24 hr., thus indicating that at these concentrations cetyl dimethyl benzyl ammonium chloride and DMSO stabilized amphotericin B against increased micelle growth and precipitation. Increasing cetyl dimethyl benzyl ammonium chloride and/or decreasing DMSO concentrations increased the S values and permitted precipitation of the preparation. Very high concentrations of cetyl dimethyl benzyl ammonium chloride (200 mcg./ml.) and DMSO (8%) promoted large increases in S values and peak areas, indicating increased micelle size.

When DMSO was kept at 1% concentration and the levels of both cetyl dimethyl benzyl ammonium chloride and amphotericin B varied from 12.5 to 400 mcg., the S coefficients were minimum in the region where amphotericin B concentration was 100 mcg./ml. and the cetyl dimethyl benzyl ammonium chloride level was approximately 100 mcg./ml. (Table II). Similar results were obtained when amphotericin B and DMSO levels were varied and cetyl dimethyl benzyl ammonium chloride concentration was kept at a constant 100 mcg./ml. Preparations of greatest stability (lowest S value) were observed when the amphotericin level was 100 mcg./ml., the cetyl dimethyl benzyl ammonium chloride level was 50 to 100 mcg./ml., and the DMSO concentration equaled 1% to 2%. Micelle weight determinations gave parallel results and correlated positively with S values. In an aqueous amphotericin B system (amphotericin B, 100 mcg./ml.; cetyl dimethyl benzyl ammonium chloride, 50 mcg./ml.; DMSO, 1%), the minimal micelle weight of an amphotericin micelle was calculated to be 400,000 daltons. The micelle weight increased into the millions when the concentration of any component of the system was increased or decreased.

The molar ratio between cetyl dimethyl benzyl ammonium chloride (mol. wt. 395), amphotericin B (mol. wt. 960), and DMSO (mol. wt. 78) at minimal S value concentrations (or micelle size) is approximately 1:1:1000.

Received September 2, 1966, from the Squibb Institute for Medical Research, New Brunswick, N. J. 08903.
Accepted for publication October 21, 1966.

[1] Marketed as Cetol by Fine Organics, Inc., Lodi, N. J.

TABLE I.—SEDIMENTATION COEFFICIENTS (S) AND PEAK AREAS (cm.^{2}) FOR AMPHOTERICIN B MICELLES WHEN AMPHOTERICIN CONCENTRATION IS KEPT CONSTANT (100 mcg./ml.) AND CETYL DIMETHYL BENZYL AMMONIUM CHLORIDE AND DMSO LEVELS ARE VARIED

Cetyl Dimethyl Benzyl Ammonium Chloride, mcg./ml.	DMSO, %[a]					
	0.25	0.50	1	2	4	8
12.5	88[b] (0.008)[c]	38 (0.012)	23 (0.017)	31 (0.016)	60 (0.009)	662 (0.079)
25	43 (0.011)	21 (0.007)	13 (0.007)	9 (0.007)	28 (0.011)	437 (0.149)
50	23 (0.016)	12 (0.003)	23 (0.008)	20 (0.005)	24 (0.057)	58 (0.011)
100	34 (0.011)	31 (0.012)	24 (0.017)	46 (0.024)	76 (0.036)	42 (0.014)
200	47 (0.012)	37 (0.010)	41 (0.008)	67 (0.038)	139 (0.060)	140 (0.145)

[a] DMSO, dimethylsulfoxide. [b] S, sedimentation coefficient. [c] Peak area.

TABLE II.—SEDIMENTATION COEFFICIENTS (S) AND PEAK AREAS (cm.^{2}) FOR AMPHOTERICIN B MICELLES WHEN DMSO[a] CONCENTRATION IS KEPT CONSTANT (1%) AND CETYL DIMETHYL BENZYL AMMONIUM CHLORIDE AND AMPHOTERICIN B LEVELS ARE VARIED

Cetyl Dimethyl Benzyl Ammonium Chloride, mcg./ml.	Amphotericin B, mcg./ml.					
	12.5	25	50	100	200	400
12.5	160[b] (0.04)[c]					207 (0.020)
25		269 (0.007)			54 (0.014)	
50	91 (0.08)		58 (0.006)	24 (0.004)		49 (0.048)
100			53 (0.006)	25 (0.004)		
200		196 (0.011)			77 (0.008)	
400	485 (0.026)					331 (0.070)

[a] DMSO, dimethylsulfoxide. [b] S, sedimentation coefficient. [c] Peak area.

These results show that in formulating an aqueous system for a water-insoluble substance the concentrations of the substance and other components of the system are critical in determining the physical characteristics of a molecular species. The results suggest that physical chemical analysis, with an analytical ultracentrifuge, as used in these studies, can help select the more stable aqueous system for a relatively water-insoluble substance. This method proved practical when a stable water-miscible amphotericin B formulation was prepared as medication in the drinking water of poultry (5). Using this procedure, other water-insoluble substances, such as the polyene antifungal antibiotic nystatin, can be formulated into water-miscible systems.

REFERENCES

(1) Donovick, R., Steinberg, B. A., Dutcher, J. D., and Vandeputte, J., *Del Giornale Microbiologia*, **2**, 147(1956).
(2) Zimm, B. H., and Crothers, D. M., *Proc. Natl. Acad. Sci.*, **48**, 905(1962).
(3) Archibald, W. J., *J. Phys. Colloid Chem.*, **51**, 1204 (1947).
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By ALBERT T. AWAD, JACK L. BEAL, SUNIL K. TALAPATRA*, and MICHAEL P. CAVA†

The bark of *Zanthoxylum elephantiasis* (*Rutaceae*) was found to contain three major alkaloids which were identified as 6-canthinone, 5-methoxy-6-canthinone, and laurifoline.

THE GENUS *Zanthoxylum* (*Rutaceae*) contains some 200 species dispersed over the world, many of which have been studied because of their alkaloid content. Price (1) in an excellent review of the distribution of alkaloids in the *Rutaceae* alludes to the nomenclature confusion between the two genera *Zanthoxylum* and *Fagara* when he points out that a number of *Fagara* species are alternatively named as *Zanthoxylum* and vice versa. Indeed, the particular *Zanthoxylum* species which forms the subject of the present investigation was at one time placed in the genus *Fagara* (2). Fosberg (3) recently clarified the situation regarding the relationship of *Fagara* and *Zanthoxylum* in such a way that today less confusion exists.

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* Present address: Department of Chemistry, University College of Science, Calcutta, India.
† Present address: Department of Chemistry, Wayne State University, Detroit, Mich.

TABLE I.—SEDIMENTATION COEFFICIENTS (S) AND PEAK AREAS (cm.^{2}) FOR AMPHOTERICIN B MICELLES WHEN AMPHOTERICIN CONCENTRATION IS KEPT CONSTANT (100 mcg./ml.) AND CETYL DIMETHYL BENZYL AMMONIUM CHLORIDE AND DMSO LEVELS ARE VARIED

Cetyl Dimethyl Benzyl Ammonium Chloride, mcg./ml.	DMSO, %[a]					
	0.25	0.50	1	2	4	8
12.5	88[b] (0.008)[c]	38 (0.012)	23 (0.017)	31 (0.016)	60 (0.009)	662 (0.079)
25	43 (0.011)	21 (0.007)	13 (0.007)	9 (0.007)	28 (0.011)	437 (0.149)
50	23 (0.016)	12 (0.003)	23 (0.008)	20 (0.005)	24 (0.057)	58 (0.011)
100	34 (0.011)	31 (0.012)	24 (0.017)	46 (0.024)	76 (0.036)	42 (0.014)
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REFERENCES

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(2) Zimm, B. H., and Crothers, D. M., *Proc. Natl. Acad. Sci.*, **48**, 905(1962).
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THE GENUS *Zanthoxylum* (*Rutaceae*) contains some 200 species dispersed over the world, many of which have been studied because of their alkaloid content. Price (1) in an excellent review of the distribution of alkaloids in the *Rutaceae* alludes to the nomenclature confusion between the two genera *Zanthoxylum* and *Fagara* when he points out that a number of *Fagara* species are alternatively named as *Zanthoxylum* and vice versa. Indeed, the particular *Zanthoxylum* species which forms the subject of the present investigation was at one time placed in the genus *Fagara* (2). Fosberg (3) recently clarified the situation regarding the relationship of *Fagara* and *Zanthoxylum* in such a way that today less confusion exists.

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in Costa Rica, Mexico, Cuba, Hispaniola, and in Jamaica, where it is known popularly as "yellow sanders." It is a tree of moderate size, 15–40 ft. in height, which is conspicuous in having corky conical spiny knobs on the bark around the base of the trunk (2). This report concerns the isolation and identification of three alkaloids from the bark of *Z. elephantiasis*.

EXPERIMENTAL[1]

The bark used in this investigation was collected during the summer of 1962 at the foot of Long Mountain in Jamaica.[2] The coarsely ground bark (1.3 Kg.) was extracted in a modified Soxhlet with ethanol until a negative test was obtained with Valser's test solution. The ethanolic extract was concentrated *in vacuo* to a thick syrupy consistency and extracted 3 times with 750-ml. portions of 2% tartaric acid solution, leaving 35 Gm. of insoluble residue. The combined acid extracts were concentrated *in vacuo* to about 1 L. and then filtered. The filtrate was made alkaline (about pH 9.0) with ammonium hydroxide solution and then extracted with chloroform in a liquid–liquid continuous extractor. The alkaline aqueous solution containing quaternary salts was treated with ammonium reineckate solution according to the method described by Hogg *et al.* (4) to give 35 Gm. of crude alkaloid reineckate.

Isolation of Alkaloids from Tertiary Bases.—The chloroform extract containing the tertiary alkaloids was extracted with 50-ml. portions of 2% sodium hydroxide solution. The combined alkaline extracts were a deep yellowish-brown color and gave a negative test for alkaloids. The chloroform solution was washed with distilled water, dried over anhydrous sodium sulfate, and reduced to dryness *in vacuo* to give 4.8 Gm. of residue. The residue was digested in 150 ml. of 5% hydrochloric acid solution. The resulting solution was filtered and then made alkaline with ammonium hydroxide solution (pH 9.0) and extracted with ether in a small continuous liquid–liquid extractor. Partial evaporation of the ether at room temperature caused the separation of a white amorphous encrustation on the sides of the flask. The ether solution was decanted and the encrusted material was washed with acetone and then crystallized from methanol to give 280 mg. of long needles (5-methoxy-6-canthinone). The decanted ether solution was evaporated to give a viscous dark brown liquid, which after dissolving in boiling acetone, afforded an additional 120 mg. of 5-methoxy-6-canthinone.

The remaining acetone solution was evaporated to dryness and the residue was dissolved in chloroform which was then extracted with 0.1 *N* hydrochloric acid. The aqueous acid extract was concentrated and left at room temperature for 1 day when 150 mg. of an alkaloid hydrochloride separated. The hydrochloride was dissolved in distilled water and the solution was then made alkaline with ammonium hydroxide and extracted with ether. The ether extract was evaporated to dryness to give 105 mg. of amorphous residue. This residue was chromatographed on 5 Gm. of Woelm neutral alumina (grade III) using benzene as the eluant to obtain 90 mg. of crystals, m.p. 150–152°. Rechromatography as above followed by crystallization from methanol gave colorless needles, m.p. 159–160° (6-canthinone).

Treatment of Tartaric Acid Insoluble Residue.—The 35 Gm. of tartaric acid insoluble residue was digested with 5% hydrochloric acid solution. Work up of this acid solution in the same manner as the tartaric acid soluble fraction yielded an additional 80 mg. of 5-methoxy-6-canthinone and 220 mg. of 6-canthinone. A third alkaloid was demonstrated to be present in a small amount by paper and thin-layer chromatography. However, its isolation has been postponed until more plant material can be collected and processed.

Identification of 5-Methoxy-6-canthinone (I).—5-Methoxy-6-canthinone was crystallized from methanol as pale yellow needles, m.p. 237–238° dec. Positive identification of the alkaloid was made by comparison with an authentic sample[3] (mixed melting point determination, infrared, and ultraviolet spectra). In addition, the hydrochloride, the picrate, and the methiodide were prepared as derivatives and were found to have melting points corresponding to those reported in the literature (5).

Identification of 6-Canthinone (II).—6-Canthinone was crystallized from methanol as colorless needles which quickly became yellow on exposure to air (probably loss of solvent of crystallization), m.p. 159–160°. Positive identification of the alkaloid was made by comparison with an authentic sample[4] (mixed melting point determination, infrared, and ultraviolet spectra). In addition, the hydrochloride, the picrate, and the methiodide were prepared as derivatives and were found to have melting points corresponding to those in the literature (6).

Isolation of Laurifoline Chloride.—The 35-Gm. sample of alkaloid reineckate was divided into two portions. A 15-Gm. sample was converted to chloride by the method described by Hogg *et al.* (4), while a 20-Gm. sample was converted to chloride by utilizing an ion-exchange resin described as follows. The 20-Gm. sample was dissolved in a mixture of 600 ml. of acetone and water (1:1). The solution was filtered and then added dropwise to a suspension of 400 Gm. of ion-exchange resin[5] IRA 400 (chloride form) in 800 ml. of acetone and water (1:1) and the mixture was then mechanically stirred for 24 hr. At this time the mixture was filtered, giving a light, straw-colored filtrate which when evaporated to dryness *in vacuo*, gave 7.5 Gm. of residue. Extraction of the residue with methanol left 3.8 Gm. of methanol insoluble, nonalkaloidal material. Thin-layer chromatography of the methanolic solution of crude quaternary chlorides using Silica Gel G as the adsorbent and acetonitrile–methanol–ammonium hydroxide solution (equal parts) as the

[1] All melting points were determined using a Thomas-Hoover Uni-Melt capillary melting point apparatus. The infrared spectra were taken in KBr using a Perkin-Elmer model 237 infrared spectrophotometer. The ultraviolet spectra were determined using a Perkin-Elmer model 4000 Spectracord. Alkaloid tests employed were Valser's and Dragendorff's test solutions.

[2] The material was collected under the supervision of Michael P. Cava and was verified botanically by Dr. Dennis Adams, Department of Botany, University of the West Indies. A fresh bark sample has a local anesthetic effect on the lips and tongue when chewed; this effect is almost entirely lost in the dried bark (Cava, M. P., personal observations).

[3] The authors are grateful to Dr. J. R. Price, Division of Industrial Chemistry, C.S.I.R.O., Melbourne, Australia, for the authentic sample of 5-methoxy-6-canthinone.

[4] The authors are grateful to Dr. E. Ritchie, Department of Organic Chemistry, The University of Sydney, Sydney, New South Wales, Australia, for the authentic sample of 6-canthinone.

[5] Marketed as Amberlite by Rohm & Haas, Philadelphia, Pa.

solvent revealed three Dragendorff positive spots, R_f 0.22, 0.42, and 0.66.

In an attempt to separate the quaternary chlorides the methanolic solution was adsorbed on Woelm acid alumina, grade I, and chromatographed on a column (2.5 cm. × 38 cm.) containing 100 Gm. of the same type of alumina. The column was eluted successfully with benzene, benzene–chloroform (1:1), chloroform, chloroform containing 5% methanol, chloroform containing 10% methanol, and chloroform containing 15% methanol. Only nonalkaloidal material was removed until the chloroform–methanol solvent was introduced. The chloroform–methanol mixtures (2100 ml.) eluted the alkaloid components as a mixture.

The combined eluates containing alkaloid material when concentrated to a syrupy consistency and left for 3 days at room temperature deposited crystals which were recovered by filtration and washed with chloroform containing 1% methanol, yielding 130 mg. of crystalline quaternary chloride. The filtrate was evaporated to dryness and then dissolved in absolute ethanol, to yield an additional 60 mg. of the same crystalline material.

The 15-Gm. sample of alkaloid reineckate that was worked up according to the method of Hogg *et al.* (4) gave 6.6 Gm. of crude quaternary chloride.

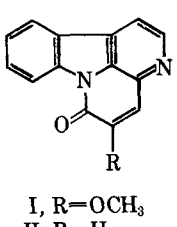
Identification of Laurifoline Chloride.—The above isolated quaternary chloride crystallized from ethanol as colorless prisms, m.p. 253° dec. $[\alpha]_D^{25}$ + 14° (c, 6.22 in methanol); $\lambda_{max.}^{methanol}$ 228 mμ (log ε 4.61), 282 (4.17), 308 (4.26). A strong bathochromic shift at the higher wavelength maxima was observed in an alkaline solution: $\lambda_{max.}^{methanol}$ 335 mμ (log ε 4.28).

Anal.—Calcd. for $C_{20}H_{24}NO_4Cl$: C, 63.56; H, 6.46; Cl, 9.40; N, 3.71; N—CH_3, 8.10; OCH_3, 16.52. Found: C, 63.56; H, 6.46; Cl, 9.26; N, 3.66; N—CH_3, 8.12; OCH_3, 16.06.

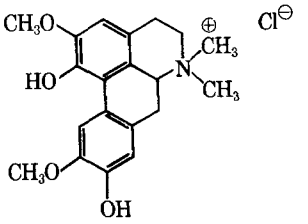
The compound was identified as laurifoline chloride (III) on the basis of infrared and ultraviolet spectral comparison with an authentic sample.[6] In addition, the picrate was prepared and found to have the same melting point (220°) as that reported in the literature (7).

DISCUSSION

The occurrence of alkaloids of the canthinone type in plants was first reported in 1952 when Haynes, Nelson, and Price described the isolation of 6-canthinone (6), 5-methoxy-6-canthinone (5), and 4-methylthio-6-canthinone (8) from *Pentaceras australis* Hook (*Rutaceae*). In a subsequent study of three Australian *Zanthoxylum* species, Cannon, Hughes, Ritchie, and Taylor reported the isolation of 6-canthinone from *Z. suberosum* C. T. White (9). The isolation of canthinones from the bark of *Z. elephantiasis* marks the second report of the occurrence of this type of alkaloid in a species of *Zanthoxylum*.



I, R=OCH_3
II, R=H



III

There have been recent reports of the isolation of canthinone alkaloids from plants belonging to a family other than the *Rutaceae*. In 1961, 4,5-dimethoxy-6-canthinone (10) was isolated from *Picrasma ailanthoides* (*Simaroubaceae*); in 1965, 4-methoxy-6-canthinone (11) was isolated from *Charpentiera obovata* Gand. (*Amaranthaceae*).

The quaternary aporphine alkaloid, laurifoline, was isolated for the first time from *Cocculus laurifolius* (*Menispermaceae*) by Tomita and Kusuda (7). The presence of aporphine alkaloids in the genus *Zanthoxylum* is not uncommon, but the isolation of laurifoline from *Z. elephantiasis* represents only the third report of the occurrence of laurifoline in a *Zanthoxylum* species. Tomita and Ishii (12) reported, in 1958, the isolation of laurifoline from the bark of *Z. ailanthoides*. The only other reported (13) isolation of laurifoline from any plant is from the bark of *Fagara pterota* L. (*Z. pterota* H.B.K.).

[6] The authors thank Dr. M. Tomita, Pharmaceutical Institute, Medical Faculty, University of Kyoto, Japan, and Dr. V. Deulofeu, Department of Organic Chemistry, Faculty of Sciences, Buenos Aires, Argentina, for authentic samples of laurifoline chloride.

REFERENCES

(1) Price, J. R., in "Chemical Plant Taxonomy," Swain, T., ed., Academic Press Inc., New York, N. Y., 1963, pp. 429–452.
(2) Fawcett, W., and Rendle, A. B., "Flora of Jamaica," vol. IV, British Museum, London, England, 1920, pp. 171–172.
(3) Fosberg, F. R., *Taxon*, **8**, 103(1959).
(4) Hogg, R., Beal, J. L., and Cava, M. P., *Lloydia*, **24**, 45(1961).
(5) Haynes, H. F., Nelson, E. R., and Price, J. R., *Australian J. Sci. Res.*, **A5**, 563(1952).
(6) *Ibid.*, **A5**, 387(1952).
(7) Tomita, M., and Kusuda, F., *Chem. Pharm. Bull.* (*Tokyo*), **1**, 1(1953).
(8) Nelson, E. R., and Price, J. R., *Australian J. Sci. Res.*, **A5**, 768(1952).
(9) Cannon, J. R., Hughes, G. K., Ritchie, E., and Taylor, W. C., *Australian J. Chem.*, **6**, 86(1953).
(10) Inamoto, N., Masuda, S., Shimamura, O., and Tsuyuki, T., *Bull. Chem. Soc. Japan*, **34**, 888(1961).
(11) Scheuer, P. J., and Pattabhiraman, T. R., *Lloydia*, **28**, 95(1965).
(12) Tomita, M., and Ishii, H., *Yakugaku Zasshi*, **78**, 1441 (1958).
(13) Albonico, S. M., Kuck, A. M., and Deulofeu, V., *Chem. Ind. London*, **1964**, 1580.

Evaluation of Antacid Activity for Metal Complexes of Tris(hydroxymethyl)aminomethane (THAM)

By NANCY CHEN and GABRIEL G. NAHAS

An ideal antacid should buffer in the physiological pH range of 3 to 5, and should have a high neutralizing capacity per unit weight. Also, its initial effect should be rapid and the ideal pH range should be maintained for a relatively prolonged period. The organic aluminum and bismuth salts of a weak organic base, tris(hydroxymethyl)aminomethane (THAM) were prepared and their relative buffer capacities were compared. THAM-gluconatodihydroxo-aluminate was found to be an effective soluble buffer antacid compound.

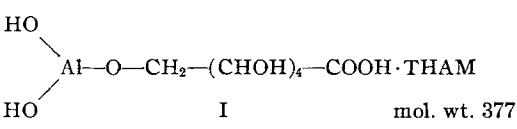
THE EXACT evaluation of antacids for *in vivo* titration is extremely difficult because of the many biological parameters involved. Therefore, *in vitro* methods must be used and these do not take into account individual physiological variations (*e.g.*, variations in composition of gastric juice, rate of secretion, rate of discharge from the stomach, etc.). However, such *in vitro* studies also present methodological difficulties. Hefferren *et al.* (1) compared the results obtained with different procedures and found that there were variations characteristic of a particular antacid and also of the method. Regardless of the procedure used, these *in vitro* studies indicate that an ideal antacid should buffer in the physiological pH range of 3 to 5, and should have a high neutralizing capacity per unit weight. Also, the initial effect should be rapid and the ideal pH range should be maintained for a relatively prolonged period.

The weak organic base, tris(hydroxymethyl)-aminomethane (THAM), is an unsatisfactory antacid because it fails to meet these criteria. Therefore, organic aluminum and bismuth salts of THAM (pH 7.0) were prepared and their relative buffering capacities were compared potentiometrically.

METHODS

Chemical and Physical Properties of Complexes and their Preparations (2).—The complexes with THAM were prepared from the aluminum and bismuth salts of several α-hydroxy-carboxylic acids (citric, gluconic, salicylic, *p*-aminosalicylic acids). Equivalent amounts of THAM were added to a suspension of the salt or to a solution obtained by heating the suspension to 70–80°. The complex was isolated by concentrating this aqueous solution to dryness *in vacuo*. Preparation of these compounds was performed in cooperation with Charra (2) as follows.

Aluminum–Gluconic Acid–THAM (1:1:1).—Gluconic acid (43.6 Gm. of 45% solution, 0.1 mole) is diluted in 200 ml. of water. In this solution, THAM (12.1 Gm., 0.1 mole) is dissolved, and the solution of aluminum isopropylate (20.4 Gm., 0.1 mole) in 100 ml. of isopropanol is added. Only a slight cloudiness appears. The mixture is stirred for 2 hr. at room temperature. It is then filtered and concentrated *in vacuo*. The solid residue is dried to constant weight in a vacuum desiccator. The analysis of the obtained product corresponds to formula I.

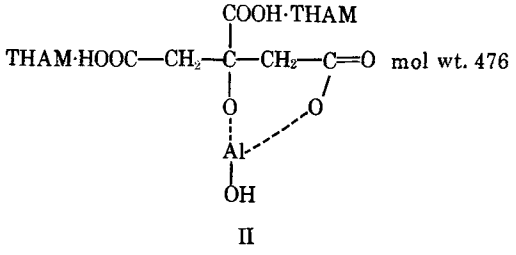


I mol. wt. 377

The pH of a 5% solution of this complex is 7.2.

Aluminum–Citric Acid–THAM (1:1:2).—Potassium alum [$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24\ H_2O$, 47.45 Gm., 0.05 mole] is dissolved in 350 ml. of lukewarm water. The solution is alkalized with ammonia. The precipitate of aluminum hydroxide is dried, filtered, and washed until there are no more $SO_4^=$ ions in the filtrate.

The aluminum hydroxide is suspended in 100 ml. of water. Hydrated citric acid (10.5 Gm., 0.05 mole) dissolved in 50 ml. of water is added and the mixture stirred for 1 hr. at room temperature. THAM (12.1 Gm., 0.1 mole) dissolved in 50 ml. of water is then added and the mixture heated for 1 hr. in a water-bath at 50°. The dissolution of aluminum hydroxide is now virtually complete. The solution obtained is filtered and concentrated *in vacuo* while stirring with a magnetic stirrer and heating in an oil bath at 80° at the maximum. The residue is made into paste with 50 ml. of absolute ethanol. It is dried and washed with 25 ml. of ethanol. The residue is then dried to constant weight in a vacuum desiccator. Analytical results of the product show that it corresponds to formula II.



II mol wt. 476

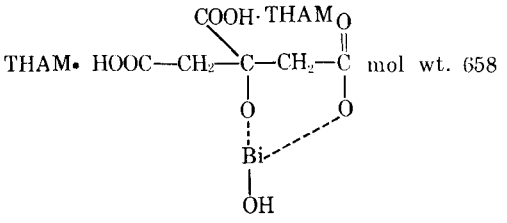
The pH of a 5% solution of this complex is 7.8.

Bismuth–Citric Acid–THAM (1:1:2).—Bismuth citrate (19.9 Gm., 0.05 mole) is suspended in 50 ml. of water. THAM (12.1 Gm., 0.1 mole) dissolved in 30 ml. of water is added. The mixture is heated for 1 hr. at 50°, while stirring, in order to obtain a complete dissolution. It is concentrated *in vacuo* while stirring magnetically. The white crystalline residue is dried to constant weight in a vacuum desiccator. The analysis of the product corresponds to formula III.

Received June 29, 1966, from the Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, N. Y. 10032.

Accepted for publication October 6, 1966.

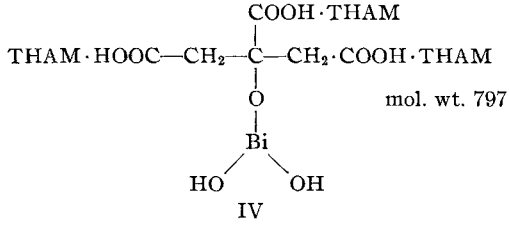
This work was supported in part by grant GM-09069-04 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and Army contract DA-49-193MD-2265.



III

The pH of a 5% solution of this complex is 6.70.

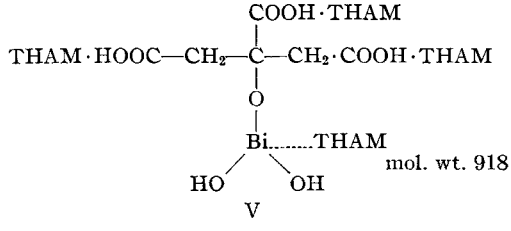
Bismuth–Citric Acid–THAM Complex (1:1:3).—The product is prepared according to the above described method, 0.15 mole of THAM being used. The analysis of the product corresponds to formula IV.



IV

The pH of a 5% solution of this complex is 7.55.

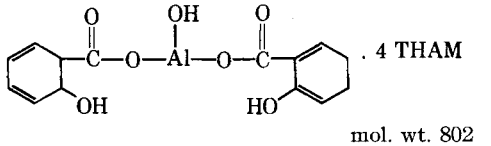
Bismuth–Citric Acid–THAM (1:1:4).—The product prepared by the method described above corresponds to formula V.



V

The pH of a 5% solution of this complex is 7.85.

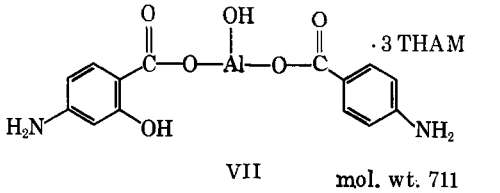
Aluminum–Salicylic Acid–THAM (1:2:4).—The product corresponds to formula VI.



VI

The pH of a 5% solution of this complex is 7.80.

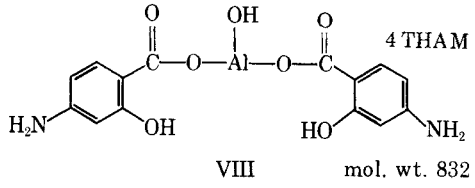
Aluminum–PAS Acid–THAM (1:2:3).—The analysis of the product corresponds to formula VII.



VII

The pH of a 5% solution of this complex is 7.4.

Aluminum–PAS Acid–THAM (1:2:4).—The analysis of the product corresponds to formula VIII.



VIII

The pH of a 5% solution of this complex is 7.5.

Antacid Activity Test.—The method of Gore, Martin, and Taylor (3) was used to determine antacid activity. An arbitrarily selected amount of the complex (calculated exactly to be 20% in excess of the neutralization value) (Table I) was added to 200 ml. of 0.015 *N* hydrochloric acid and the mixture was titrated in a double-walled vessel at 37 ± 0.1°. The pH was measured with a Knick model 35 pH meter and Beckman glass electrodes. Beckman standard buffer solutions at pH 4.00 and 7.00 were used to calibrate the meter. The pH was recorded for 30 min. To simulate the continuous gastric secretion of HCl, hydrochloric acid was then added to the mixture by means of a buret at the rate of 6 meq./hr. until an end point of pH 3.0 was reached.

RESULTS AND DISCUSSION

The relative buffering capacities of the various substances are given in Table II and illustrated in Figs. 1 and 2. A quantity (0.15%) of pepsin, peptone, and sodium chloride can be added to the hydrochloric acid as suggested by Brindle (4), Clemow and Lowry (5), and Grossmith (6). However, the use of such a "mock" gastric juice has little effect and comparable results are obtained with hydrochloric acid alone. In addition, there is some disagreement on the degree of acidity that would be most representative of *in vivo* conditions. However, most investigators recommend a pH of 3.0 to 5.0 and agree that carrying neutralization far beyond this range is undesirable.

TABLE I.—COMPOSITION OF COMPLEXES USED

Complexes	Unit Dose, Gm.
Aluminum–gluconic acid–THAM (1:1:1)	2.3
Aluminum–PAS acid–THAM (1:2:3)	2.5
Aluminum–PAS acid–THAM (1:2:4)	2.8
Aluminum–salicylic acid–THAM (1:2:4)	2.9
Bismuth–citric acid–THAM (1:1:4)	2.0
Bismuth–citric acid–THAM (1:1:3)	1.4
Aluminum–citric acid–THAM (1:1:2)	1.7
Bismuth–citric acid–THAM (1:1:2)	2.2

TABLE II.—BUFFERING CAPACITY

Complexes	0.1 *N* HCl ml. Added
Aluminum–gluconic acid–THAM (1:1:1)	117
Aluminum–PAS acid–THAM (1:2:3)	130
Aluminum–PAS acid–THAM (1:2:4)	140
Aluminum–salicylic acid–THAM (1:2:4)	140
Bismuth–citric acid–THAM (1:1:4)	60
Bismuth–citric acid–THAM (1:1:3)	38
Aluminum–citric acid–THAM (1:1:2)	31
Bismuth–citric acid–THAM (1:1:2)	27

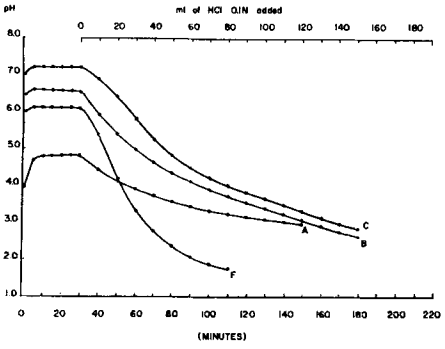


Fig. 1.—Acid consuming activity of four metal complexes of THAM. Key: A, aluminum–gluconic acid–THAM (1:1:1); B, aluminum–PAS acid–THAM (1:2:3); C, aluminum–PAS acid–THAM (1:2:4); F, bismuth–citric acid–THAM (1:1:3).

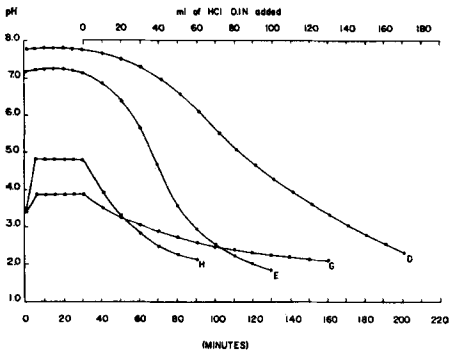


Fig. 2.—Acid consuming activity of four metal complexes of THAM. Key: D, aluminum–salicylic acid–THAM (1:2:4); E, bismuth–citric acid–THAM (1:1:4); G, aluminum–citric acid–THAM (1:1:2); H, bismuth–citric acid–THAM (1:1:2).

In the case of tris(hydroxymethyl)aminomethane–gluconatodihydroxo–aluminate (Al–gluconic acid–THAM, 1:1:1) the pH rose almost instantly to a value of 4.0 and after 5 min., reached a maximum pH of 5.0. The pH remained at about 5.0 for the next 30 min., during which time no additional acid was introduced. This suggests that *in vivo* an overdose of antacid would not increase the pH above 5.0. Moreover, when more acid was reintroduced, the pH did not fall below 3 until after approximately 12 meq. of HCl had been added also indicating that THAM–gluconatodihydroxoaluminate would be an effective soluble buffer antacid compound.

The complex of Bi–citric acid–THAM (1:1:1) had a smaller neutralizing capacity than Al–gluconic acid–THAM within the pH range 3 to 5 and the remaining antacids only have limited buffering capacities.

REFERENCES

(1) Hefferren, J. J., Schrotenboer, G., and Wolman, W., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 564(1956).
(2) Charra, A., Laboratoire Roger Bellon, Neuilly-Paris, France, personal communication.
(3) Gore, D. N., Martin, B. K., and Taylor, M. P., *J. Pharm. Pharmacol.*, **5**, 686(1953).
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Catharanthus Alkaloids X. Isolation of Lanceine and Vinosidine from *Catharanthus lanceus* Roots

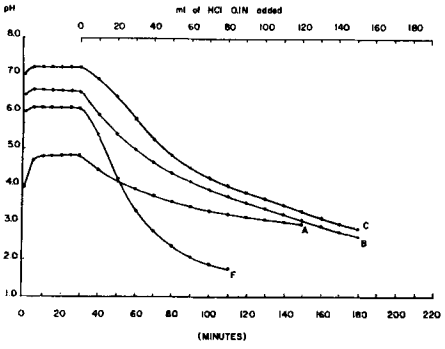
By RALPH N. BLOMSTER*, NORMAN R. FARNSWORTH, and DONALD J. ABRAHAM

In a continuing search for new biologically active entities in *Catharanthus lanceus*, additional work on the root (A) fraction has led to the isolation of two alkaloids. One of these, lanceine, was previously isolated from this plant by other workers. Vinosidine, however, although previously reported in a related species, is reported from this species for the first time.

EARLIER STUDIES on *Catharanthus lanceus*, as a part of a continued search for new and active antineoplastic principles from the various alkaloid fractions, and in an effort to elucidate, as completely as possible, the alkaloid composition of this plant, have resulted in the isolation of 12 crystalline alkaloids (1–4). Three of these, cathalanceine, pericyclivine, and periformyline proved to be new entities, with periformyline representing a new alkaloid of novel structure. Of the remaining nine, ajmalicine, yohimbine, and tetrahydroalstonine have been reported previously from *C. lanceus* roots by other workers (5–7). The additional six alkaloids have also been reported from the related *C. roseus* (8–12). Of these (leurosine, perivine, vindoline, pericalline, perimivine, and lochnerinine) leurosine is of major interest because of its high order of activity against the P-1534 leukemia, and because it was isolated initially from a *C. lanceus* crude alkaloid fraction that was devoid of activity against the P-1534 leukemia in DBA/2 mice (1).

This is a report on the isolation of two additional alkaloids, lanceine, previously reported from this plant by other investigators (6), and vinosidine, reported previously only from *C. roseus* (13).

Received April 26, 1966, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pa. 15213.
Accepted for publication September 26, 1966.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
* Lederle Pharmacy Faculty Award Recipient, 1966.
This investigation was supported by research grants H-06162, CA-08228, CA-08509, and FR-5455-04 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and from the Thaw Fund, University of Pittsburgh, Pittsburgh, Pa.



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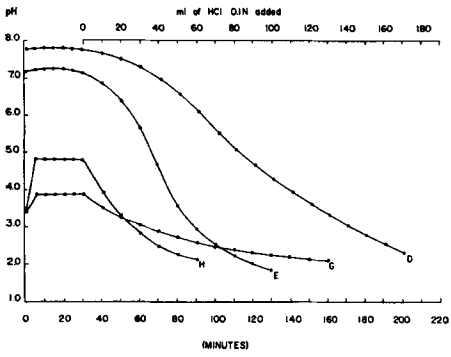

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REFERENCES

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TABLE I.—COLUMN CHROMATOGRAPHIC SEPARATION OF *C. lanceus* ROOT (A) FRACTION, 210 GM.

Eluent	Fraction	Fraction, Wt.,[a] Gm.	Alkaloid Isolated	Wt., Gm.
Benzene (fractions 1–40)	1–2	0.02	...	...
Benzene–chloroform (9:1)	3	2.81	...	...
(fractions 41–52)	4–5	32.70	Ajmalicine	0.920
Benzene–chloroform (5:1)	6–17	46.61	Ajmalicine	20.610
(fractions 53–65)	18–23	15.80	Ajmalicine	2.950
Benzene–chloroform (2:1)	24–33	20.40	Lanceine	0.026
(fractions 66–79)				
Benzene–chloroform (1:1)	34–78	6.70	...	...
(fractions 80–147)	79–104	8.80	Vinosidine	0.090
Chloroform	105–174	7.70	...	...
(fractions 148–210)	175–220	1.65	...	...
Chloroform–methanol (99:1)	221–226	2.10	...	...
(fractions 211–258)	227–238	3.20	...	...
Chloroform–methanol (9:1)	239–260	1.50	...	...
(fractions 259–284)	261–278	4.85	...	...
Chloroform–methanol (1:1)	279–316	3.10	...	...
(fractions 285–315)	317–348	11.40	...	...
Methanol				
(fractions 316–348)				

[a] All fractions gave positive tests for alkaloids except fractions 1–2.

EXPERIMENTAL[1]

Chromatographic Separation of C. lanceus Root (A) Fraction.—Two-hundred and forty-five grams of root (A) alkaloid fraction (1) was dissolved in benzene and this solution concentrated *in vacuo* to yield 29.05 Gm. of ajmalicine. Further concentration yielded 6.30 Gm. of benzene-insoluble, non-alkaloid precipitate. The remaining benzene-soluble material was placed at the top of an 8 × 86-cm. glass chromatographic column containing a benzene slurry of 4.0 Kg. of alumina (Alcoa F-20) partially deactivated with 144 ml. of 10% (v/v) acetic acid (1). Elution was accomplished initially with benzene, and then with eluting solvents having an increased polarity to yield 348 one-liter fractions. Each fraction was reduced to dryness, *in vacuo*, the residue dissolved in 20 ml. of the eluting solvent, and monitored by thin-layer chromatography on Silica Gel G plates using an ethyl acetate–absolute ethanol (3:1) eluent and the ceric ammonium sulfate spray reagent (CAS) (14, 15). Shifts in the alkaloid composition of the eluate, as determined by thin-layer chromatographic patterns, as well as differences in chromogenesis of the resolved alkaloids following treatment with the CAS reagent, were the criteria for determining the limits of each fraction (Table I).

Isolation of Lanceine.—Benzene eluate fractions 24–33 from the column chromatographic separation were taken to dryness, *in vacuo*, to yield 20.40 Gm. of residue, from which was obtained 3.60 Gm. of a crystalline material on direct crystallization from benzene. The R_f values of this material as well as its chromogenic reaction to the CAS detecting reagent on Silica Gel G thin-layer chromatography in three solvent systems (16) were identical with a reference sample of lanceine.[2] The melting point and infrared absorption spectrum of this material were identical with those found in the literature for lanceine (17). However, on alumina G thin-layer chromatography, using benzene–ethanol (98:2) as the eluent, the crystalline material proved to be a multicomponent mixture.

Nine grams of the crude alkaloid fraction 24–33 remaining after the removal of the crystals formed from benzene (*vide supra*), was dissolved in benzene and subjected to a gradient pH separation according to the method of Svoboda (18) in an attempt to separate lanceine. The initial pH of the acidic alkaloid solution was 3.3 and it was increased in 0.5 pH increments by the addition of NH_4OH until a final pH of 8.8 was reached. Each fraction was dried with anhydrous sodium sulfate, filtered, and reduced to about 10 ml. *in vacuo*, followed by thin-layer chromatography and treatment of the developed chromatograms with CAS reagent which served to point out those pH fractions to be combined prior to crystallization attempts. The fractions were combined to give two groups corresponding to pH 3.3–4.3 and 4.8–8.8. Fraction pH 3.3–4.3 yielded 0.110 Gm. of crystalline material on direct crystallization from ethanol. Again this material appeared to be identical with lanceine as regards melting point and infrared absorption spectrum, but on alumina G thin-layer chromatography, it proved to be a multicomponent mixture.

Repeated crystallization of the impure materials with several solvents failed to resolve the mixture, as did column chromatography on alumina (Woelm, neutral, activity grade II), utilizing benzene as the initial eluting agent and followed by solvents of increasing polarity. The mixture was finally resolved by repeated preparative thin-layer chromatography, utilizing 1 mm. thick alumina G plates, developed with anhydrous ether in a repetitive manner. In each case the solvent front was allowed to travel 170 mm., the plate air-dried, and rechromatographed. This was repeated 3 times. The area corresponding to lanceine, as determined by chromogenesis with the CAS reagent (16), was removed from the plate, eluted with methanol, filtered, evaporated to dryness *in vacuo*, and the residue was dissolved in a small amount of methanol and placed on the surface of a second

[1] Melting points are uncorrected and were determined using a Thomas-Hoover capillary melting point apparatus. Infrared absorption spectra were determined in a Beckman infrared spectrophotometer model IR-8.

[2] The authors express their appreciation to Dr. Jean LeMen for a sample of crude lanceine which was suitable for thin-layer chromatographic comparison only.

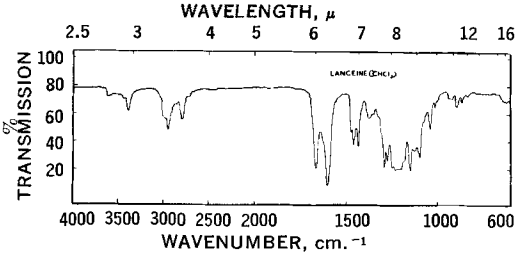

Fig. 1.—Infrared spectrum of isolated lanceine.

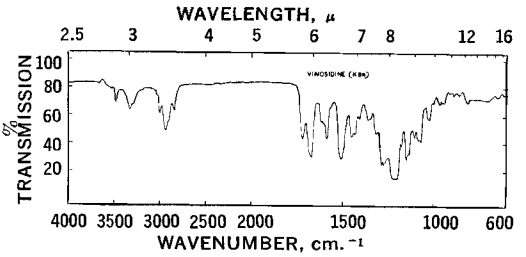

Fig. 2.—Infrared spectrum of isolated vinosidine.

preparative alumina G thin-layer plate. This plate was treated in the same manner as the first plate, and the material obtained on elution of the matrix was crystallized from cyclohexane at room temperature. This yielded 0.013 Gm. of crystalline lanceine, m.p. 143–145°, from each 0.100 Gm. of impure material utilized. The infrared absorption spectrum (Fig. 1) of the isolated lanceine was identical with that found in the literature (17). The R_f values for the isolated lanceine and a reference sample of this alkaloid, as well as chromogenic reactions to the CAS detecting agent after Silica Gel G thin-layer chromatography in three different solvent systems (16), and on alumina G thin-layer chromatography were also identical.

Isolation of Vinosidine.—Treatment of the dried benzene–chloroform (1:1) fractions 79–104 with methanol yielded crystals, which on recrystallization from methanol afforded 0.090 Gm. of vinosidine, m.p. 251–253° dec. The infrared absorption spectrum (Fig. 2) of isolated vinosidine and that of a reference sample[3] were identical, as were their R_f values and chromogenic response to the CAS reagent after thin-layer chromatography in three different solvent systems (16).

SUMMARY

The isolation of two alkaloids is reported. One, lanceine, was previously isolated from *C. lanceus* roots by other workers. Vinosidine was previously isolated from the related species *C. roseus*, but is reported herein for the first time from *C. lanceus*.

[3] A reference sample of vinosidine was supplied through the generosity of Dr. Gordon H. Svoboda.

REFERENCES

(1) Loub, W. D., Farnsworth, N. R., Blomster, R. N., and Brown, W. W., *Lloydia*, **27**, 470(1964).
(2) Blomster, R. N., Martello, R. E., Farnsworth, N. R., and Draus, F. J., *ibid.*, **27**, 480(1964).
(3) Farnsworth, N. R., Loub, W. D., Blomster, R. N., and Gorman, M., *J. Pharm. Sci.*, **53**, 1558(1964).
(4) Maloney, E. M., Farnsworth, N. R., Blomster, R. N., Abraham, D. J., and Sharkey, A. G., Jr., *ibid.*, **54**, 1166(1965).
(5) Janot, M.-M., and LeMen, J., *Compt. Rend.*, **239**, 1131(1954).
(6) Janot, M.-M., LeMen, J., and Gabbai, Y., *Ann. Pharm. Franc.*, **15**, 474(1957).
(7) Janot, M.-M., LeMen, J., and Hammouda, Y., *ibid.*, **14**, 341(1956).
(8) Svoboda, G. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 834(1958).
(9) Svoboda, G. H., Neuss, N., and Gorman, M., *ibid.*, **48**, 659(1959).
(10) Svoboda, G. H., *J. Pharm. Sci.*, **52**, 407(1963).
(11) Svoboda, G. H., Gorman, M., and Tust, R. H., *Lloydia*, **27**, 203(1964).
(12) Moza, B. K., and Trojanek, J., *Collection Czech. Chem. Commun.*, **28**, 1419(1963).
(13) Svoboda, G. H., Oliver, A. T., and Bedwell, D. R., *Lloydia*, **26**, 141(1963).
(14) Cone, N. J., Miller, R., and Neuss, N., *J. Pharm. Sci.*, **52**, 688(1963).
(15) Jakovljevic, I. M., Seay, L. D., and Shaffer, R. W., *ibid.*, **53**, 533(1964).
(16) Farnsworth, N. R., Blomster, R. N., Damratoski, D., Meer, W. A., and Cammarato, L. V., *Lloydia*, **27**, 302 (1964).
(17) Neuss, N., "Physical Data of Indole and Dihydroindole Alkaloids," 5th ed., Eli Lilly and Co., Indianapolis, Ind., 1961.
(18) Svoboda, G. H., *Lloydia*, **24**, 173(1961).

Steric Aspects of Adrenergic Drugs IV. Antagonism of Norepinephrine Effects by Ephedrine Isomers on the Isolated Rabbit Ileum

By P. N. PATIL, J. B. LAPIDUS, S. MOLINARI*, and A. TYE

The inhibition of the pendular movements of the isolated rabbit ileum, due to norepinephrine, was not antagonized in the presence of 10^{-5} *M* D(−)-ephedrine, L(+)-ephedrine, L(+)-pseudoephedrine, or D(−)-pseudoephedrine. However, a 10^{-3} *M* concentration of these isomers did antagonize the norepinephrine effects. The stereospecificity in such antagonism is not marked.

In the past a number of investigators have reported that in certain tissues such as rabbit ileum and cat uterus, epinephrine-induced relaxation can be blocked by ephedrine (1–4). This observation was recently confirmed by Van Rossum and Mujic (5). Furthermore, they have suggested that certain "indirectly" acting sympathomimetic amines do not of themselves cause inhibition of the rabbit jejunum, but antagonize the effects of "directly" acting sympathomimetic amines. These reports formed the basis for the present investigation, which was aimed at obtaining information regarding stereospecificity in such antagonistic effects.

EXPERIMENTAL

Twenty-four hour starved rabbits of either sex were sacrificed by a sharp blow on the head. The ileum was isolated and a small piece (about 2 cm.) was suspended in a 25-ml. jacketed tissue chamber which contained Tyrode's solution at 37°. A mixture of oxygen (95%) and carbon dioxide (5%) was continuously bubbled through the Tyrode's solution. The composition of the Tyrode's solution was as follows: NaCl, 8.0 Gm.; KCl, 0.2 Gm.; $CaCl_2$ ($2H_2O$), 0.26 Gm.; $MgCl_2$ ($6H_2O$), 0.188 Gm.; $NaHCO_3$, 1.0 Gm.; NaH_2PO_4, 0.05 Gm.; dextrose anhydrous, 1.0 Gm.; double distilled water to make 1000 ml. Pendular movements of ileum were recorded on a smoked drum *via* a light lever (magnification 1:20, tension approximately 500 mg.). The tissue was washed 3 or 4 times before cumulative dose-response curves were obtained. This method is described in detail by Van Rossum and Mujic (5). For each procedure six to nine pieces of ileum were obtained from three to four different animals. Only one dose response curve was obtained from each piece of ileum. The maximum height of the pendular movements was taken as 100% and inhibition expressed as the per cent of the initial activity. Ephedrine isomers were left in contact with the tissue for 2 min. before cumulative dose-response curves of norepinephrine were determined.

A stock solution of norepinephrine[1] was prepared in a solution containing 0.05% sodium metabisulfite. Ephedrine isomers were obtained by a method described previously (6).

RESULTS AND DISCUSSION

Norepinephrine produces a rapid inhibition of the pendular movements. The inhibition is complete in 10^{-6} *M* norepinephrine. The negative log molar concentration of norepinephrine for producing 50% inhibition is 6.9. This value is lower than that for jejunum which is 7.8 (5). Ephedrine isomers in concentrations of 10^{-5} *M* did not produce any intrinsic effects, nor did they exhibit any antagonism toward norepinephrine effects (Fig. 1 and Table I).

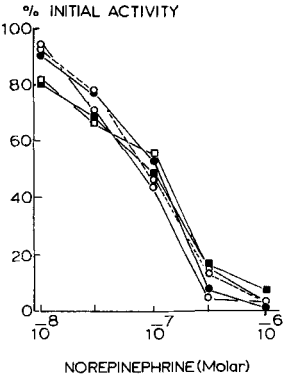

Fig. 1.—Cumulative dose-response curves of norepinephrine on rabbit ileum in the presence of 10^{-5} *M* D(−) - ephedrine (O- - -O); L(+)-ephedrine (O——O); L(+)-pseudoephedrine (□——□); D-(−) - pseudoephedrine (■——■). Norepinephrine control (●——●). Standard errors of the mean were 5 to 7% and showed a marked overlap.

Table I.—Lack of Inhibition of Norepinephrine Effects on Rabbit Ileum by 10^{-5} *M* Concentration of Ephedrine Isomers

	(−)-Norepinephrine −Log ED_{50} with S.E.M.			
Control	D(−)-Ephedrine, 10^{-5} *M*	L(+)-Ephedrine, 10^{-5} *M*	L(+)-Pseudoephedrine, 10^{-5} *M*	D(−)-Pseudoephedrine, 10^{-5} *M*
6.96	6.98	7.16	6.87	7.04
±0.08	±0.17	±0.11	±0.12	±0.14
$n^a = 9^b$	$n = 9^c$	$n = 7^c$	$n = 6^d$	$n = 7^d$
	$P >$ 0.05	$P >$ 0.05	$P >$ 0.05	$P >$ 0.05

[a] *n*, number of observations. [b] Nine animals. [c] Four animals. [d] Three animals.

In higher concentration (10^{-3} *M*), ephedrine showed variable intrinsic effects. D(−)-Ephedrine produced 34% (S.E. ± 4.2) inhibition; L(+)-ephedrine and D(−)-pseudoephedrine produced stimulant effects of 10% (S.E. ± 1) and 29% (S.E. ± 2), respectively, while L(+)-pseudoephedrine did not produce any observable effects.

The inhibitory effects of norepinephrine were antagonized by ephedrine isomers (Fig. 2). The dose-response curves were shifted to the right, *i.e.*, in the presence of ephedrine isomers a higher concentration of norepinephrine was required to

Received August 15, 1966, from the College of Pharmacy, Ohio State University, Columbus 43210.
Accepted for publication October 13, 1966.
Previous paper: Tye, A., Patil, P. N., and LaPidus, J. B., *J. Pharmacol. Exptl. Therap.*, to be published.
* National Science Foundation Pharmacy Undergraduate Research Participant, 1965.
[1] Regis Chemical Co.

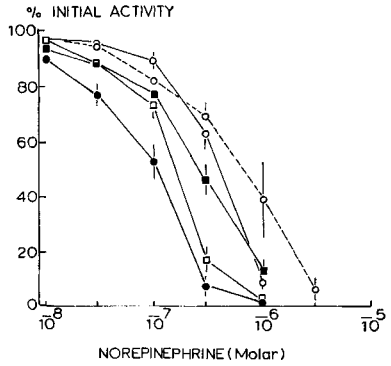

Fig. 2.—Illustrates the cumulative dose-response curves of norepinephrine on rabbit ileum in the presence of 10^{-3} *M* D(−)-ephedrine (○- - -○), L(+)-ephedrine (○——○), L(+)-pseudoephedrine (□——□), D(−)-pseudoephedrine (■——■). Norepinephrine control (●——●). Vertical lines, standard errors of the mean.

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TABLE II.—INHIBITION OF NOREPINEPHRINE EFFECTS ON RABBIT ILEUM BY 10^{-3} *M* CONCENTRATION OF EPHEDRINE ISOMERS

	(−)-Norepinephrine −Log ED_{50} with S.E.M.			
Control	D(−)-Ephedrine, 10^{-3} *M*	L(+)-Ephedrine, 10^{-3} *M*	L(+)-Pseudoephedrine, 10^{-3} *M*	D(−)-Pseudoephedrine, 10^{-3} *M*
6.96	6.16	6.41	6.78	6.49
±0.08	±0.13	±0.05	±0.04	±0.08
$n^a = 9^b$	$n = 6^c$	$n = 9^c$	$n = 8^c$	$n = 8^c$
	$P <$ 0.001	$P <$ 0.001	$P <$ 0.1	$P <$ 0.01

[a] *n*, number of observations. [b] Nine animals. [c] Three animals.

REFERENCES

(1) Thienes, C. H., *Proc. Soc. Expil. Biol. Med.*, **26**, 500 (1928–1929).
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3-Thenyl Nitrogen Mustards

By W. LEWIS NOBLES and CHARLES M. DARLING

As potential anticancer agents, three new 3-thenyl nitrogen mustards have been prepared from the corresponding diethanolamine derivatives. The NMR data for these compounds are reported.

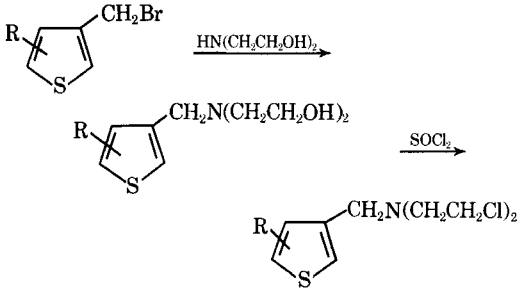
IN A SERIES of antihistaminic compounds, Clapp and co-workers (1) have suggested that the inclusion of a halogen atom on the thiophene ring improves the therapeutic ratio. This suggestion has not been exploited in thiophene nitrogen mustards. Indeed, besides dimethyl derivatives (2), no nuclear substituted thiophene nitrogen mustards have been reported.

Campaigne's comparative data (3) suggest that in testing thiophene analogs, there is a high probability that the 3-isomer will be at least as active as the 2-isomer, if not more active. Of the six thiophene nitrogen mustards previously reported (2, 4, 5), four are thenyl derivatives (2, 5) and, of these, only two are 3-thenyl isomers (2).

In this investigation, three 3-thenyl nitrogen mustard derivatives have been prepared as potential anticancer agents. These are denoted by structure I in which R = H, 2-bromo, and 2,5-dichloro.

Received October 3, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University 38677.
Accepted for publication October 14, 1966.
Taken in part from a dissertation presented by Charles M. Darling to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported in part by fellowship 5-F1-GM-19, 540-03 from the National Institute of General Medical Sciences and by grant CA 05131 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

As outlined in Scheme I, the synthetic route employed for the preparation of these compounds is a modification of that of Wilson and Tishler (5).

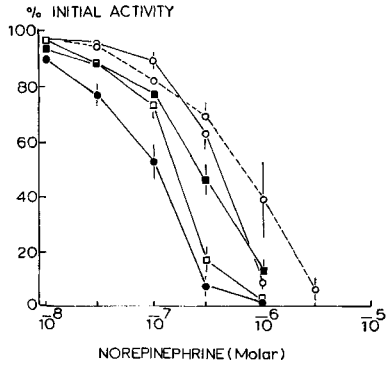


I

Scheme I

The 3-thenyl bromides were obtained by the reaction of *N*-bromosuccinimide with the respective methylthiophenes in the presence of catalytic amounts of benzoyl peroxide (6). The diethanolamine derivatives were prepared by treating the thenyl bromides with diethanolamine.

Preparation of the nitrogen mustard function by halogenation of the appropriate diethanolamine derivative with thionyl chloride (2, 5) or phosphorus oxychloride (4) has been reported in low yields, 12–27%. In each instance, the reaction mixture was cooled during the addition of the halogenating agent, and then it was heated to reflux. In the



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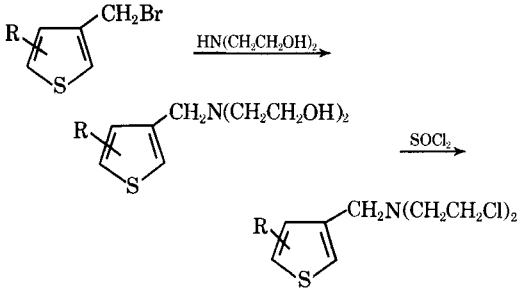

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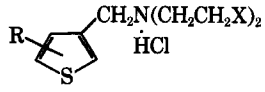
TABLE I.—3-THENYLAMINES



Compd.	R	X	Yield, %	M.p., °C.	Formula	Anal., %[a] Calcd.	Found
I	H	OH	26[b]	82.5–83.5	$C_9H_{15}NO_2S\cdot HCl$	C, 45.46	45.75
						H, 6.78	6.88
						N, 5.89	5.51
						S, 13.49	13.03
II	2-Br	OH	16.8[b]	70.5–71.5[c]	$C_{19}H_{14}BrNO_2S$	C, 38.58	38.77
						H, 5.04	5.18
						N, 5.00	5.13
III	2-Cl	OH	24.3[b]	143.5–144.5	$C_9H_{13}Cl_2NO_2S\cdot HCl$	C, 35.25	35.54
						H, 4.60	5.02
						N, 4.57	4.40
						S, 10.46	10.65
IV	H	Cl	78[d]	109–110.5	$C_9H_{13}Cl_2NS\cdot HCl$	C, 39.36	39.27
						H, 5.14	5.39
						N, 5.10	4.77
						S, 11.68	11.71
V	2-Br	Cl	83[d]	130.5–131.5	$C_9H_{12}BrCl_2NS\cdot HCl$	C, 30.57	31.31
						H, 3.71	3.97
						N, 3.96	3.93
						S 9.07	9.16
VI	2-Cl 5-Cl	Cl	61[d]	164–165.5	$C_9H_{11}Cl_4NS\cdot HCl$	C, 31.46	31.50
						H, 3.52	3.78
						N, 4.08	4.10
						S, 9.33	9.26

[a] Analyses by Dr. Alfred Bernhardt, Mulheim, Germany. [b] Yield calculated from corresponding 3-methylthiophene derivative. [c] Melting point of the free base. [d] Yield of halogenation reaction.

present work, the reaction mixture was allowed to reflux during the addition of thionyl chloride, and higher yields of the nitrogen mustard function were obtained consistently. The compounds along with pertinent data are listed in Table I.

EXPERIMENTAL[1]

3-Thenyl Diethanolamines.—The 3-thenyl bromides were prepared by known methods (6, 7) and were used as the crude lachrymatory oils. Each crude 3-thenyl bromide compound was refluxed with a 2 *M* ratio of diethanolamine in chloroform for 16 to 18 hr. After cooling the solution to room temperature, the mixture was poured over ice and the chloroform layer separated. The chloroform was removed with a flash evaporator. The residue of compound II was recrystallized from diethyl ether as the free base. The hydrochloride salt of compound I was prepared by saturating an ethereal solution of I with anhydrous hydrogen chloride and recrystallizing from acetone. The hydrochloride salt of compound III was prepared by the addition of concentrated hydrochloric acid to a solution of III in acetone; the salt was then recrystallized from absolute ethanol.

The NMR spectrum of I in deuterated pyridine was δ = 3.61 (t, probably C—CH_2—O, 4H), 4.33 (t, probably N—CH_2—C, 4H), 4.84 (s, thienyl—CH_2—N, 2H), 7.87 (s, O—H, disappeared in D_2O exchange), and 7.60 (m, undetermined) p.p.m.

[1] All melting points were taken on a Thomas-Hoover Uni-Melt melting point apparatus and are corrected. The NMR spectra were taken using a Varian model A60-A instrument and were measured at 60 Mc. The chemical shifts are reported as δ-values (p.p.m.) from tetramethylsilane (internal standard). The multiplicity is shown by s = singlet, d = doublet, t = triplet, and m = incompletely resolved multiplet. Coupling constants, *J*, are reported in c.p.s. Assignments are shown in parentheses.

The NMR spectrum of II in deuterated acetone was δ = 2.66 (t, probably C.CH_2—O, 4H), 3.57 (t, probably N—CH_2—C, 4H), 3.68 (s, thienyl—CH_2—N, 2H), 3.15 (s, O—H, 2H), 7.05 (d, probably 5-H of ring, 1H), and 7.39 (d, probably 4-H of ring, 1H) p.p.m. (Each J_{4-5} = 5.7 c.p.s. ± 0.2 c.p.s.)

The NMR spectrum of III in deuterated pyridine was δ = 3.48 (t, probably C—CH_2—O, 4H), 4.25 (t, probably N—CH_2—C, 4H), 4.48 (s, thienyl—CH_2—N, 2H), 7.80 (s, 4-H of ring, 1H), and 8.11 (s, O—H, disappeared on D_2 exchange) p.p.m.

2,2′-Dichlorodiethyl-3-thenylamines.—The general procedure for the preparation of the nitrogen mustard function is reported below. The free base of the appropriate diethanolamine derivative was dissolved in chloroform and a large excess (about 5 *M* ratio) of redistilled thionyl chloride was slowly added without cooling. Reflux began spontaneously and was continued for 5 to 7 hr. The reaction mixture was cooled to room temperature, and the chloroform and excess thionyl chloride were removed with a flash evaporator. In each instance, the residue was recrystallized from acetone using charcoal.

The NMR spectrum of IV in deuterated chloroform was δ = 3.52 (t, probably C—CH_2—Cl, 4H), 4.01 (t, probably N—CH_2—C, 4H), 4.50 (s, thienyl—CH_2—N, 2H), 7.36 (d, probably 4,5-H of ring, 2H) (J = 2.2 c.p.s.), and 7.72 (t, probably 2-H of ring, 1H) (J = 2.2 c.p.s.) p.p.m. (all J's ± 0.3 c.p.s.).

The NMR spectrum of V in deuterated chloroform was δ = 3.62 (t, C—CH_2—Cl, 4H), 4.15 (t, N—CH_2—C, 4H), 4.49 (s, thienyl—CH_2—N, 2H), 7.46 (d, probably 5-H of ring, 1H), and 7.78 (d,

probably 4-H of ring, 1H) p.p.m. (each J_{4-5} = 5.7 c.p.s. ± 0.2 c.p.s.).

The NMR spectrum of VI in deuterated chloroform was δ = 3.58 (t, probably C—CH_2—Cl, 4H), 4.08 (t, probably N—CH_2—C, 4H), 4.38 (s, thienyl—CH_2—N, 2H), and 7.54 (s, 4-H of ring, 1H) p.p.m.

REFERENCES

(1) Clapp, R. C., Clark, J. H., Vaughan, J. R., English, J. P., and Anderson, G. W., *J. Am. Chem. Soc.*, **69**, 1549 (1947).

(2) Goldfarb, Y. L., and Kondakova, M. S., *Zh. Obshchei Khim.*, **30**, 102(1960); through *Chem. Abstr.*, **54**, 21037 (1960).

(3) Campaigne, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 129(1957).

(4) Shirley, D. A., and Bell, G. R., *J. Med. Chem.*, **9**, 607(1966).

(5) Wilson, E., and Tishler, M., *J. Am. Chem. Soc.*, **73**, 3635(1951).

(6) Campaigne, E., and LeSuer, W. M., *ibid.*, **71**, 333 (1949).

(7) Campaigne, E., and Tullar, B. F., *Org. Syn.*, **33**, 96 (1953).

Pemoline and Magnesium Hydroxide *Versus* Pemoline: Enhancement of Learning and Memory of a Conditioned Avoidance Response in Rats

By N. PLOTNIKOFF and P. MEEKMA, JR.

Pemoline and magnesium hydroxide was found to be several times more potent than pemoline in enhancing the acquisition and retention of a conditioned avoidance response in rats.

NUMEROUS STUDIES in Europe indicated that the performance of fatigued humans was enhanced by pemoline under various test conditions (1–3). In previous studies Plotnikoff (4–7) reported that pemoline and magnesium hydroxide (PMH)[1] enhanced the acquisition and retention of a conditioned avoidance response in rats. The present study is a comparative study of PMH and pemoline on the same avoidance response.

EXPERIMENTAL

Methods—The test chamber as well as rates of acquisition and retention used for all conditioning studies were described earlier (4–7). The test equipment consisted of a wood chamber (11 × 12 in.) with a grid flooring. An escape platform was placed 11 in. above the grid floor outside of the test chamber. Male Sprague-Dawley rats (170 to 220 Gm.) were used. Only "slow learners" were used for all drug studies. Suspensions of the test drugs were prepared in 0.3% tragacanth. Acquisition trials consisted of the following 30-sec. sequence: 15 sec. inside the chamber without shock or buzzer, 10 sec. with buzzer, and finally 5 sec. of shock with buzzer. Retention trials consisted of a 30-sec. sequence without buzzer or shock stimulation. Criterion of learning was considered obtained when the mean jump-out time was 15 sec. or less for any given trial and succeeding trials.

Results—The principal difference observed between pemoline and PMH on the jump-out test was one of potency. As the data in Tables I and II illustrate, PMH is more potent in the enhancement of acquisition and retention of the jump-out response. In several studies, significant enhancement was observed with 0.3% tragacanth suspensions of PMH at doses of 1.25, 2.5, and 5.0 mg./Kg. p.o. In contrast, pemoline (0.3% tragacanth suspension) only showed significant enhancement at doses of 10 and 20 mg./Kg. p.o. Lower doses of pemoline (2.5 and 5.0 mg./Kg. p.o.) did not enhance acquisition or retention.

The approximate potency differences on acquisition between the two compounds is at least eightfold. Rats treated with PMH reached criterion of learning by the 9th trial at a dose of 1.25 mg./Kg., whereas pemoline-treated rats reached criterion of learning by the 4th trial at a dose of 10 mg./Kg.

The potency differences between the two compounds on retention were also approximately eightfold (1.25 mg./Kg. *versus* 10.0 mg./Kg.). Significant retention of the jump-out response was observed at a dose of 1.25 mg./Kg. in PMH-treated animals, whereas pemoline-treated animals only showed significant retention at a dose of 10 mg./Kg.

Control studies carried out with $Mg(OH)_2$ indicated there were no significant effects on acquisition or retention.

DISCUSSION

The present study has demonstrated that PMH is a more potent agent in enhancing acquisition and retention than pemoline. A similar difference between pemoline and PMH has been reported by Lange *et al.* (8) on anticonvulsant activity. The principal difference reported was the rate of absorption as determined by onset of anticonvulsant activity. PMH at a dose of 100 mg./Kg. exerted significant activity 15–30 min. after oral administration, whereas pemoline at the same dose exhibited activity only at 60 min. However, both PMH and pemoline have the same LD_{50} values in mice (oral LD_{50} 500 mg./Kg.). The effects of the two compounds are both similar resulting in overt stimulant effects at doses of 5–400 mg./Kg. and paradoxical depression (ataxia followed by coma) at toxic doses (500–1000 mg./Kg.). No convulsions were observed at toxic doses.

In the present study, PMH was demonstrated to be a more potent agent than pemoline in enhancing acquisition and, even more striking, retention of the jump response. Thus, it is possible that magnesium hydroxide component may be enhancing absorption. Clinically (9) PMH has been reported to have a faster onset of action and to have greater potency as an arousal agent than pemoline.

Received August 18, 1966, from the Pharmacology Department, Abbott Laboratories, North Chicago, Ill. 60064.

Accepted for publication October 19, 1966.

[1] Marketed as Cylert by Abbott Laboratories, North Chicago, Ill. Abbott-30400; an equimolar combination of 2-imino-5-phenyl-4-oxazolidinone (Abbott-13397) and magnesium hydroxide.

probably 4-H of ring, 1H) p.p.m. (each J_{4-5} = 5.7 c.p.s. ± 0.2 c.p.s.).

The NMR spectrum of VI in deuterated chloroform was δ = 3.58 (t, probably C—CH_2—Cl, 4H), 4.08 (t, probably N—CH_2—C, 4H), 4.38 (s, thienyl—CH_2—N, 2H), and 7.54 (s, 4-H of ring, 1H) p.p.m.

REFERENCES

(1) Clapp, R. C., Clark, J. H., Vaughan, J. R., English, J. P., and Anderson, G. W., *J. Am. Chem. Soc.*, **69**, 1549 (1947).

(2) Goldfarb, Y. L., and Kondakova, M. S., *Zh. Obshchei Khim.*, **30**, 102(1960); through *Chem. Abstr.*, **54**, 21037 (1960).

(3) Campaigne, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 129(1957).

(4) Shirley, D. A., and Bell, G. R., *J. Med. Chem.*, **9**, 607(1966).

(5) Wilson, E., and Tishler, M., *J. Am. Chem. Soc.*, **73**, 3635(1951).

(6) Campaigne, E., and LeSuer, W. M., *ibid.*, **71**, 333 (1949).

(7) Campaigne, E., and Tullar, B. F., *Org. Syn.*, **33**, 96 (1953).

Pemoline and Magnesium Hydroxide *Versus* Pemoline: Enhancement of Learning and Memory of a Conditioned Avoidance Response in Rats

By N. PLOTNIKOFF and P. MEEKMA, JR.

Pemoline and magnesium hydroxide was found to be several times more potent than pemoline in enhancing the acquisition and retention of a conditioned avoidance response in rats.

NUMEROUS STUDIES in Europe indicated that the performance of fatigued humans was enhanced by pemoline under various test conditions (1–3). In previous studies Plotnikoff (4–7) reported that pemoline and magnesium hydroxide (PMH)[1] enhanced the acquisition and retention of a conditioned avoidance response in rats. The present study is a comparative study of PMH and pemoline on the same avoidance response.

EXPERIMENTAL

Methods—The test chamber as well as rates of acquisition and retention used for all conditioning studies were described earlier (4–7). The test equipment consisted of a wood chamber (11 × 12 in.) with a grid flooring. An escape platform was placed 11 in. above the grid floor outside of the test chamber. Male Sprague-Dawley rats (170 to 220 Gm.) were used. Only "slow learners" were used for all drug studies. Suspensions of the test drugs were prepared in 0.3% tragacanth. Acquisition trials consisted of the following 30-sec. sequence: 15 sec. inside the chamber without shock or buzzer, 10 sec. with buzzer, and finally 5 sec. of shock with buzzer. Retention trials consisted of a 30-sec. sequence without buzzer or shock stimulation. Criterion of learning was considered obtained when the mean jump-out time was 15 sec. or less for any given trial and succeeding trials.

Results—The principal difference observed between pemoline and PMH on the jump-out test was one of potency. As the data in Tables I and II illustrate, PMH is more potent in the enhancement of acquisition and retention of the jump-out response. In several studies, significant enhancement was observed with 0.3% tragacanth suspensions of PMH at doses of 1.25, 2.5, and 5.0 mg./Kg. p.o. In contrast, pemoline (0.3% tragacanth suspension) only showed significant enhancement at doses of 10 and 20 mg./Kg. p.o. Lower doses of pemoline (2.5 and 5.0 mg./Kg. p.o.) did not enhance acquisition or retention.

The approximate potency differences on acquisition between the two compounds is at least eightfold. Rats treated with PMH reached criterion of learning by the 9th trial at a dose of 1.25 mg./Kg., whereas pemoline-treated rats reached criterion of learning by the 4th trial at a dose of 10 mg./Kg.

The potency differences between the two compounds on retention were also approximately eightfold (1.25 mg./Kg. *versus* 10.0 mg./Kg.). Significant retention of the jump-out response was observed at a dose of 1.25 mg./Kg. in PMH-treated animals, whereas pemoline-treated animals only showed significant retention at a dose of 10 mg./Kg.

Control studies carried out with $Mg(OH)_2$ indicated there were no significant effects on acquisition or retention.

DISCUSSION

The present study has demonstrated that PMH is a more potent agent in enhancing acquisition and retention than pemoline. A similar difference between pemoline and PMH has been reported by Lange *et al.* (8) on anticonvulsant activity. The principal difference reported was the rate of absorption as determined by onset of anticonvulsant activity. PMH at a dose of 100 mg./Kg. exerted significant activity 15–30 min. after oral administration, whereas pemoline at the same dose exhibited activity only at 60 min. However, both PMH and pemoline have the same LD_{50} values in mice (oral LD_{50} 500 mg./Kg.). The effects of the two compounds are both similar resulting in overt stimulant effects at doses of 5–400 mg./Kg. and paradoxical depression (ataxia followed by coma) at toxic doses (500–1000 mg./Kg.). No convulsions were observed at toxic doses.

In the present study, PMH was demonstrated to be a more potent agent than pemoline in enhancing acquisition and, even more striking, retention of the jump response. Thus, it is possible that magnesium hydroxide component may be enhancing absorption. Clinically (9) PMH has been reported to have a faster onset of action and to have greater potency as an arousal agent than pemoline.

Received August 18, 1966, from the Pharmacology Department, Abbott Laboratories, North Chicago, Ill. 60064.

Accepted for publication October 19, 1966.

[1] Marketed as Cylert by Abbott Laboratories, North Chicago, Ill. Abbott-30400; an equimolar combination of 2-imino-5-phenyl-4-oxazolidinone (Abbott-13397) and magnesium hydroxide.

TABLE I—EFFECT OF PMH ON THE ACQUISITION AND RETENTION OF THE JUMP-OUT RESPONSE (ORAL ROUTE)

Trial	Dose, mg./Kg.	Acquisition, Day 1	Retention, Day 2
1	1.25	30.0 ± 0.0[a]	7.0 ± 1.7[b]
2		29.3 ± 0.7	7.3 ± 1.7
3		29.0 ± 0.4	8.0 ± 1.9
4		21.0 ± 3.4	6.7 ± 1.2
5		18.8 ± 4.3	5.5 ± 0.8
6		16.7 ± 4.1	6.0 ± 1.3
7		16.7 ± 4.1	6.3 ± 1.1
8		16.2 ± 3.8	6.3 ± 0.9
9		9.0 ± 0.9[b]	5.7 ± 1.4
10		6.8 ± 1.4[b]	6.2 ± 1.1
1	2.5	29.8 ± 0.2	8.3 ± 3.4[b]
2		29.5 ± 0.2	8.2 ± 3.6
3		18.3 ± 3.9	7.2 ± 1.4
4		15.8 ± 3.9	6.8 ± 1.9
5		15.0 ± 4.4	6.3 ± 1.5
6		13.2 ± 3.1	6.7 ± 1.1
7		11.7 ± 3.4	6.3 ± 1.2
8		10.5 ± 2.1[b]	6.3 ± 0.4
9		8.2 ± 2.1[b]	6.2 ± 0.8
10		9.3 ± 3.0[b]	6.5 ± 0.4
1	5.0	29.2 ± 0.3	8.5 ± 2.9[b]
2		26.7 ± 2.0	5.7 ± 1.1
3		21.3 ± 2.8	3.8 ± 1.1
4		18.3 ± 3.5	4.2 ± 0.9
5		12.0 ± 3.4	3.3 ± 0.9
6		11.0 ± 3.5	4.5 ± 0.8
7		13.0 ± 2.5	4.7 ± 0.8
8		11.7 ± 3.3[b]	5.3 ± 1.1
9		9.8 ± 2.0[b]	5.0 ± 1.6
10		6.3 ± 2.2[b]	5.0 ± 1.3
1	Controls	29.8 ± 0.2	19.5 ± 3.2
2		29.3 ± 0.4	19.0 ± 3.1
3		25.8 ± 2.6	17.8 ± 2.6
4		22.2 ± 2.7	17.8 ± 2.9
5		21.0 ± 3.1	19.3 ± 3.5
6		18.5 ± 2.8	19.3 ± 3.6
7		19.3 ± 2.3	20.1 ± 3.4
8		21.5 ± 2.5	21.7 ± 3.3
9		20.8 ± 1.8	23.5 ± 3.2
10		21.7 ± 2.8	21.8 ± 3.8

[a] Mean jump-out time in seconds of six rats ± S.E.
[b] Mean jump-out time significantly different from controls p 0.05 (10).

TABLE II—EFFECT OF PEMOLINE ON THE ACQUISITION AND RETENTION OF THE JUMP-OUT RESPONSE (ORAL ROUTE)

Trial	Dose, mg./Kg.	Acquisition, Day 1	Retention, Day 2
1	2.5	28.7 ± 0.4[a]	24.2 ± 3.7
2		20.7 ± 2.6	27.3 ± 2.3
3		13.8 ± 3.9	25.8 ± 3.6
4		14.3 ± 2.9	25.8 ± 3.8
5		19.0 ± 1.7	27.3 ± 2.7
6		16.5 ± 0.2	27.7 ± 2.3
7		17.7 ± 1.7	27.7 ± 2.3
8		17.3 ± 0.8	27.5 ± 2.5
9		17.0 ± 2.2	27.2 ± 2.8
10		16.0 ± 2.4	27.7 ± 2.3
1	5.0	28.3 ± 0.6	21.6 ± 4.0
2		23.7 ± 4.0	19.7 ± 4.3
3		13.0 ± 3.3	19.8 ± 4.4
4		13.7 ± 3.4	19.5 ± 4.0
5		13.8 ± 3.3	19.2 ± 4.0
6		13.0 ± 2.9	18.7 ± 4.1
7		12.5 ± 2.7	20.0 ± 4.5
8		12.3 ± 2.6	20.0 ± 4.4
9		16.3 ± 3.5	17.2 ± 4.3
10		16.8 ± 3.7	20.0 ± 4.8
1	10.0	28.0 ± 0.6	6.5 ± 1.7[b]
2		23.0 ± 2.6	8.3 ± 2.1
3		18.5 ± 4.2	7.3 ± 1.9
4		8.8 ± 2.7[b]	5.3 ± 0.8
5		7.3 ± 1.9	6.0 ± 1.2
6		8.8 ± 2.5	4.5 ± 1.0
7		4.7 ± 0.8	4.0 ± 1.3
8		5.2 ± 1.7	4.0 ± 1.2
9		4.8 ± 1.3	4.0 ± 1.4
10		3.0 ± 0.6	4.7 ± 0.8
1	Controls	28.0 ± 0.5	18.2 ± 3.6
2		21.7 ± 3.0	17.2 ± 2.4
3		19.0 ± 3.4	17.3 ± 2.0
4		18.5 ± 3.0	20.0 ± 2.4
5		16.7 ± 3.2	18.7 ± 2.5
6		20.3 ± 3.6	18.8 ± 2.4
7		17.0 ± 3.3	21.3 ± 3.4
8		17.3 ± 2.8	22.8 ± 3.5
9		16.3 ± 2.0	23.5 ± 3.8
10		14.2 ± 1.2	23.5 ± 2.8

[a] Mean jump-out time in seconds of six rats ± S.E.
[b] Mean jump-out time significantly different from controls p 0.05 (8).

REFERENCES

(1) Dureman, I., *Clin. Pharmacol. Therap.*, **3**, 163(1962).
(2) Lienert, G. A., and Janke, W., *Arzneimittel-Forsch.*, **7**, 436(1957).
(3) Bugard, P., *Therapie*, **17**, 63(1962).
(4) Plotnikoff, N., *Science*, **151**, 703(1966).
(5) Plotnikoff, N., *Federation Proc.*, **25**, 262(1966).
(6) Plotnikoff, N., *Science*, **153**, 902(1966).
(7) Plotnikoff, N., *Life Sci.*, **5**, 1495(1966).
(8) Lange, W. E., Candon, B. H., and Chessin, M., *J. Pharm. Sci.*, **51**, 477(1962).
(9) U. S. pat. 3,108,045(October 22, 1963).
(10) Dunnett, C. W., *Am. Statistics Assoc. J.*, **50**, 1096 (1955).

Antiradiation Compounds VIII. Reaction of Thiols with Cyanogen Bromide

By WILLIAM O. FOYE, ALICIA M. HEBB, and JAMES MICKLES

The reaction of a thiol with cyanogen bromide provides a specific synthesis of disulfides without further oxidation. The reaction is apparently general, and was successful for MEA, MEG, and aliphatic, aromatic, and amino thiols. Oxidation of MEA with 3 per cent hydrogen peroxide also gave the disulfide, but similar oxidation of MEG gave the thiolsulfinate, distinguishable from the disulfide by infrared absorption in the 1100 cm.$^{-1}$ region.

THE REACTION of dithiols with cyanogen chloride has been found to result in the formation of cyclic imino dithioester hydrochlorides (1). With the assumption that 2-mercaptoethylamine hydrochloride (MEA) might react with cyanogen bromide in analogous fashion, either an imino dithioester hydrohalide or iminothiazolidine would be obtained, and provide possibly an active antiradiation agent. The former may be expected to liberate MEA *in vivo*, and in the latter case, some thiazolidines are known to be radioprotective (2). A stable, white, granular product was obtained from the proposed reaction (2 moles of MEA hydrochloride with 1 mole of cyanogen bromide) which did not reduce iodine and gave a positive test for the disulfide linkage with Grote's reagent (3). The melting point (221–222°) was somewhat higher than those recorded for the disulfide of MEA hydrochloride (203°, 210°, 212°, 216°) (4), however, and the carbon-hydrogen analysis was somewhat low for this compound.

A pure sample of the disulfide of MEA hydrochloride was accordingly required for comparison. By iodine oxidation of MEA hydrochloride, a product was obtained for which the melting point (217°) agreed with that reported by Schoberl (5). By use of alkaline peroxide (3%), a product was obtained which melted at 222–224° and gave the correct analytical data for the disulfide. The ultraviolet, infrared,[1] and Raman[2] spectra of the disulfide and the product of cyanogen bromide oxidation proved to be identical. (See Fig. 1 for infrared spectrum.) The Raman spectra showed strong absorption at 515 and 650 cm.$^{-1}$, expected for S-S and C-S stretching (6). It was suspected that the low C-H assay values might be due to the presence of bromide ion, but a correct analysis for chlorine was obtained, and no bromide ion was detected by the chlorine water–carbon tetrachloride test. In addition, a dibenzoyl derivative was obtained that was identical in yield and properties to that prepared from the disulfide.

The reaction of cyanogen bromide with other thiols was observed, and in each case the disulfide was formed. With 2-mercaptoethylguanidine (MEG), a compound corresponding to the disulfide (7) was obtained from cyanogen bromide oxidation, and a compound whose analytical data and infrared absorption spectra indicated the thiolsulfinate was obtained from oxidation by 3% hydrogen peroxide at 40°. A strong peak at 1115 cm.$^{-1}$ (Fig. 2), absent in the disulfide (Fig. 3), provided spectral evidence for the thiolsulfinate (8). This region of the spectrum (Fig. 2) was identical when observed in either Nujol or KBr.

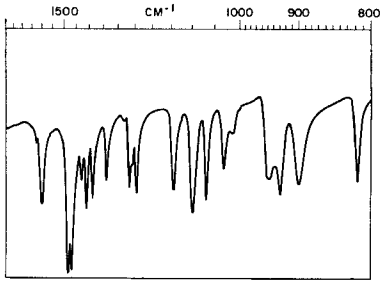


Fig. 1.—I.R. spectrum of 2-aminoethyl disulfide dihydrochloride in KBr.

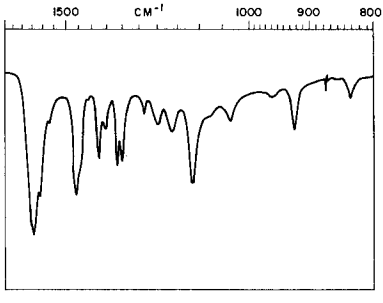


Fig. 2.—I.R. spectrum of 2-guanidinoethyl 2-guanidinoethanethiolsulfinate dihydrobromide in Nujol.

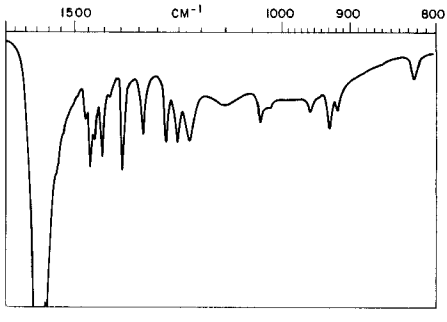


Fig. 3.—I.R. spectrum of 2-guanidinoethyl disulfide dihydrobromide in KBr.

Received July 13, 1966, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston 02115.
Accepted for publication September 28, 1966.
Abstracted in part from a thesis submitted by Alicia M. Hebb to the Massachusetts College of Pharmacy, Boston, in partial fulfillment of Master of Science degree requirements.
This project was supported by grant RH00297 from the Division of Radiological Health, Bureau of State Services, U. S. Public Health Service, Bethesda, Md.
Previous paper: Foye, W. O., LaSala, E. F., Georgiadis, M., and Meyer, W. L., *J. Pharm. Sci.*, **54**, 557(1965).

[1] Supplied by Dr. Winthrop E. Lange.

[2] Done through the courtesy of Professor R. C. Lord, Massachusetts Institute of Technology.

Other thiols subjected to cyanogen bromide oxidation included cysteine, homocysteine, 2-diethylaminoethanethiol hydrochloride,[3] 1-amino-2-propanethiol hydrochloride,[4] thiophenol, heptanethiol, dodecanethiol, *o*- and *p*-aminobenzenethiol hydrochloride, and *o*-, *p*-, and α-toluenethiol. The formation of a disulfide in each case was shown by melting point (except for two cases, the disulfides are known), elemental analysis, and infrared absorption spectra (two or three peaks at 1050–1250 cm.$^{-1}$ which were fairly strong in the case of the aromatic and aminoalkyl disulfides). These peaks are apparently due to CH_2 or aryl CH wag of CH_2 or CH adjacent to sulfur (6). Bredereck *et al.* (8) have shown two peaks near 1100 cm.$^{-1}$ for an aryl disulfide.

Antiradiation testing of MEA disulfide dihydrochloride at the Walter Reed Army Institute of Research, through the courtesy of Drs. D. P. Jacobus and T. R. Sweeney, showed the compound to possess good protective properties in mice *versus* 825 r., a finding which is in agreement with previous reports (9).

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass. Sulfur and chloride analyses were done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp apparatus and are corrected. Infrared absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

2-Aminoethyl Disulfide Dihydrochloride.—*By Cyanogen Bromide Oxidation.*—Cyanogen bromide (3.63 Gm., 0.034 mole) (Eastman Organic Chemicals) was added to a solution of 2-aminoethanethiol hydrochloride (7.78 Gm., 0.068 mole) (Aldrich Chemical Co.) in 20 ml. of absolute ethanol. A precipitate formed immediately, with liberation of heat, and was filtered, washed with ethanol, and air dried. A second crop was obtained by addition of ether to the filtrate. After recrystallization from 95% ethanol, a yield of 3.0 Gm. (78%) was obtained; m.p. 221–222°. [Lit. m.p. 216° (5)] λ_{max}. 245 mμ.

Anal.—Calcd. for $C_4H_{14}Cl_2N_2S_2$: C, 21.33; H, 6.27; Cl, 31.49; N, 12.43. Found: C, 19.87; H, 5.93; Cl, 31.30; N, 12.25.

Benzoylation in pyridine with exactly 2 moles of benzoyl chloride for 4 days at 25° gave a 71% yield of the dibenzoyl derivative, m.p. 132–133°. [Lit. m.p. 132° (10).]

By Alkaline Peroxide Oxidation.—A solution of 5.7 Gm. (0.5 mole) of 2-aminoethanethiol hydrochloride in 10 ml. of water was neutralized with a solution of 2.1 Gm. (0.05 mole) of lithium hydroxide in 20 ml. of water. The solution was cooled in an ice bath, and 3% hydrogen peroxide solution was added dropwise with stirring until the reaction solution gave a negative test for thiol with nitroprusside solution. The resulting solution was acidified with hydrochloric acid (1:1) solution to a pH of 2, concentrated to one-half its volume, and cooled. A semisolid resulted which was triturated in a mortar with 97.5% ethanol, washed with absolute ethanol, and dried *in vacuo*, giving 3.4 Gm. (60%) of crystalline product, m.p. 222–224°.

Anal.—Calcd. for $C_4H_{14}Cl_2N_2S_2$: C, 21.33; H, 6.27; Cl, 31.49; N, 12.43; S, 28.48. Found: C, 21.56; H, 6.32; Cl, 31.99; N, 12.21; S, 28.41.

The dibenzoyl derivative was prepared as above in 72% yield, m.p. 134–135°.

2-Guanidinoethyl Disulfide Dihydrobromide.—*S*-(2-Aminoethyl) isothiuronium bromide hydrobromide (11.2 Gm., 0.04 mole) (Matheson, Coleman and Bell) was dissolved in 30 ml. of water and 6 ml. of concentrated ammonium hydroxide and treated with cyanogen bromide (2.12 Gm., 0.02 mole) with stirring. After 2 hr., the orange solution was refrigerated, and a cream colored solid was isolated and recrystallized from ethanol. The yield was 1.8 Gm. (23%), m.p. 195–196°. [Lit. m.p. 195–197° (7).]

Anal.—Calcd. for $C_6H_{18}Br_2N_6S_2$: C, 18.1; H, 4.5; N, 21.1; S, 16.1. Found: C, 17.6; H, 4.5; N, 20.5; S, 16.0.

2-Guanidinoethyl 2-Guanidinoethanethiolsulfinate Dihydrobromide.—*S*-(2-Aminoethyl)isothiuronium bromide hydrobromide (14.0 Gm., 0.05 mole) was dissolved in 12.5 ml. of concentrated ammonium hydroxide, and 100 ml. of 3% hydrogen peroxide was added in 4 portions with stirring over a 2-hr. period. The solution was kept at 40° during the addition. It solidified on cooling, and the solid was triturated with absolute ethanol and recrystallized from ethanol. The yield was 3.5 Gm. (33%) of white crystalline solid, m.p. 175–176°.

Anal.—Calcd. for $C_6H_{18}Br_2N_6OS_2$: C, 17.4; H, 4.4; N, 20.3; S, 15.5. Found: C, 17.2; H, 4.2; N, 20.1; S, 15.4.

Other Thiols.—2-Diethylaminoethyl disulfide was obtained as the dihydrobromide, m.p. 233–235°, apparently by using an excess of cyanogen bromide.

Anal.—Calcd. for $C_{12}H_{30}Br_2N_2S_2$: C, 33.80; H, 7.11; N, 6.57; S, 15.05. Found: C, 34.81; H, 7.12; N, 6.53; S, 15.55.

o-Aminophenyl disulfide was obtained as the dihydrochloride, m.p. 225–227°, not previously reported.

Anal.—Calcd. for $C_{12}H_{14}Cl_2N_2S_2$: C, 44.89; H, 4.36; N, 8.72; S, 19.96. Found: C, 44.75; H, 4.71; N, 8.34; S, 19.40.

[3] Gift of Evans Chemetics, Inc.

[4] Supplied by T. R. Sweeney, Walter Reed Army Institute of Research.

REFERENCES

(1) Addor, R. W., *J. Org. Chem.*, **29**, 738(1964).
(2) Thomson, J. F., "Radiation Protection in Mammals," Reinhold Publishing Corp., New York, N. Y., 1962, p. 98.
(3) Grote, I. W., *J. Biol. Chem.*, **93**, 25(1931).
(4) Reid, E. E., "Organic Chemistry of Bivalent Sulfur," vol. III, Chemical Publishing Co., Inc., New York, N. Y., 1960, p. 402.
(5) Schoberl, A., Kawohl, M., and Hamm, R., *Chem. Ber.*, **84**, 571(1951).
(6) Colthup, N. B., Daly, L. H., and Wiberley, S. E., "Introduction to Infrared and Raman Spectroscopy," Academic Press Inc., New York, N. Y., 1964, pp. 306–307.
(7) Foye, W. O., Mickles, J., Duvall, R. N., and Marshall, J. R., *J. Med. Chem.*, **6**, 509(1963).
(8) Bredereck, H., Wagner, A., Beck, H., and Klein, R.-J., *Chem. Ber.*, **93**, 2736(1960).
(9) Thomson, J. F., *op. cit.*, p. 65.
(10) Coblentz, W., and Gabriel, S., *Chem. Ber.*, **24**, 1122 (1891).

Paper Chromatography Study of *In Vitro* and *In Vivo* Hydrolysis of Heroin in Blood

By GEORGE R. NAKAMURA* and TYUNOSIN UKITA†

A study was conducted using paper chromatography to elucidate the pathway of heroin hydrolysis in human blood *in vitro* and in dog's blood *in vivo*. Results indicated that heroin deacetylated rapidly to monoacetylmorphine which remained relatively stable in blood and left the bloodstream as such. Only a trace amount of morphine was detected *in vitro* and *in vivo*. Both compounds became undetectable after 30 min. in the *in vivo* experiment.

THIS LABORATORY has yet to report a positive finding of morphine or morphine derivatives in whole blood specimens. The problem seems to be in the limited sensitivity of the methods (1, 2) used or/and in the timing of blood collection. While the urine remains a preferred body fluid specimen for narcotic analysis since it acts as a reservoir of metabolic waste, the examination of blood specimens may yield information as to the origin of morphine eliminated in the urine.

For forensic consideration, it seems more convincing to inform the court that the subject had taken heroin, especially when he has had heroin in his possession. A finding of monoacetylmorphine in blood would strongly imply that heroin was taken.

Therefore, some experiments were performed to determine the nature of heroin hydrolysis in human blood and the length of time heroin and its hydrolysis products remain in the blood circulation, *in vivo*.

PROCEDURE

Preparations of diacetylmorphine, 6-monoacetylmorphine, and 3-monoacetylmorphine were the same as those described in a previous paper (3).

Ten milligrams of diacetylmorphine hydrochloride were dissolved in 20 ml. of fresh human serum in a 50-ml. conical flask and to this mixture was added 1 mg. of oxytetracycline HCl to suppress bacterial growth. The flask was fixed in a water bath at 37.5°. At various time intervals, a 1-ml. aliquot was removed and introduced into a small separator containing 0.5 ml. of saturated $NaHCO_3$. The alkaline mixture was immediately shaken with 20 ml. of 9:1 chloroform–ethanol mixture. Then, the extract was filtered through a pledget of cotton into a small beaker. The clear extract was evaporated to near dryness and the entire residue was applied onto a Whatman No. 1 chromatography paper. The chromatographic procedure was the same as that described by Genest and Farmilo (4) and was used as a standard method in this study.

To determine if nonenzymic hydrolysis, *i.e.*, autohydrolysis of heroin and 6-monoacetylmorphine could occur in human blood, serum was this time heated to 60° for 1 hr. and the substrate diacetylmorphine was introduced into the deactivated serum. The resulting mixture was incubated at 37°, extracted, and chromatographed under standard conditions. For further confirmation of possible autohydrolysis of heroin, the substrate was introduced into $^1/_{15}$ *M* phosphate buffer, pH 7.3, and the same standard procedure was followed for the hydrolysis study.

Three dogs, each weighing about 20 lb., were selected for the *in vivo* study of heroin hydrolysis. Each received an intramuscular injection of 80 mg. of pure diacetylmorphine hydrochloride. Twenty milliliters of blood was collected from the jugular vein at 10-min. intervals as shown in Fig. 3. The specimens were treated with heparin and chilled in an ice water bath. Plasma was separated from these specimens using a refrigerated centrifuge, adjusted to pH 8.0 with saturated $NaHCO_3$, and then shaken with 9:1 chloroform–ethanol mixture.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 showed that the *in vitro* hydrolysis of heroin in human serum proceeded rapidly and it disappeared within 1 hr. Furthermore, the hydrolysis proceeded by pathway of 6-monoacetylmorphine. 3-Monoacetylmorphine was not a product of hydrolysis indicating the more rapid cleavage of the phenolic acetyl group. The amount of morphine liberated at the end of 7 hr. was comparatively small. 6-Monoacetylmorphine was still present in the serum in a large proportion after 7 hr. of incubation, indicating its stability and its possible role as an important mediator for the pharmacological activity of heroin (5). Also this stability in blood offered a possibility for its forensic detection in human blood.

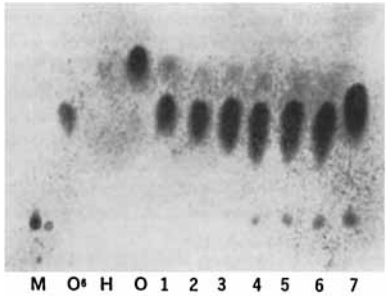


Fig. 1.—*In vitro* hydrolysis of heroin in human serum at 37.5°. Aliquots from reaction mixture examined at 1-hr. intervals. Key: M, morphine; 0^6, 6-monoacetylmorphine; and H, heroin.

Received August 12, 1966, from the U. S. Army Military Police Crime Laboratory, San Francisco, Calif. 96343.
Accepted for publication October 7, 1966.
The authors are grateful to Dr. Yutaka Furusho, Department of Veterinary Medicine, U. S. Army 406th Medical Laboratory, Camp Zama, Japan, for his assistance in conducting the animal experiments; and to Dr. E. Leong Way, Department of Pharmacology, University of California Medical Center, San Francisco, for reading the manuscript and suggesting improvements.
In the research reported here, the "Principles of Laboratory Animal Care," as established by the National Society for Medical Research, were adhered to.

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Control experiments using enzyme-deactivated human serum (Fig. 2) indicated that a rapid hydrolysis of the phenol acetyl group occurred without enzymic catalysis. The same chromatographic pattern was obtained by using phosphate buffer instead of deactivated serum. This observation further indicated that heroin could be degraded mostly to 6-monoacetylmorphine in the human body by autohydrolysis. Autohydrolysis seems to hold true for further degradation of 6-monoacetylmorphine to morphine since the amount of morphine liberated from both the heat-treated and untreated serum was approximately the same. Ellis (6) demonstrated by a manometric method of analysis that human blood was devoid of 6-monoacetylmorphine esterase activity. However, in the authors' study, the rate of morphine liberation was slightly faster in nontreated serum, therefore, the possible occurrence of enzyme catalysis could not be entirely precluded.

Cochin *et al.* (7) has shown that the plasma level of morphine in dogs after a subcutaneous injection attained a peak level at 45 min. The authors' paper chromatographic study of heroin metabolism in dogs showed that 6-monoacetylmorphine was the principal breakdown product of heroin in blood and that it persisted on the chromatogram for a 30-min. period. The chromatogram in Fig. 3 showed strong spots for 6-monoacetylmorphine and weaker spots for morphine for a 30-min. period, indicating again the relatively stable monoacetylmorphine in blood even under *in vivo* conditions. Only a faint spot for morphine could be detected after 30 min. suggesting that most of the degraded products of heroin had left the blood stream by that time. 6-Monoacetylmorphine and morphine were characterized by ultraviolet absorption spectra examination and color tests described by the authors previously (3).

SUMMARY

In vitro and *in vivo* study of heroin in blood revealed that monoacetylmorphine was the principal product and it was relatively stable *in vitro* for at least 7 hr. in blood at 37°. The study further indicated that heroin entered the blood stream and was rapidly deacetylated to monoacetylmorphine, leaving the circulation virtually in this form. The detection of monoacetylmorphine was, therefore, indicated for forensic purpose. However, the study showed that blood must be collected within a 30-min. period after administration.

Morphine was detected on the chromatogram upon *in vitro* and *in vivo* hydrolysis at a much lower level.

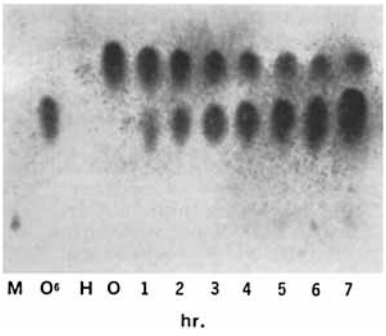

Fig. 2.—*In vitro* hydrolysis of heroin in enzyme-deactivated serum at 37.5°. Same key and conditions as those in Fig. 1.

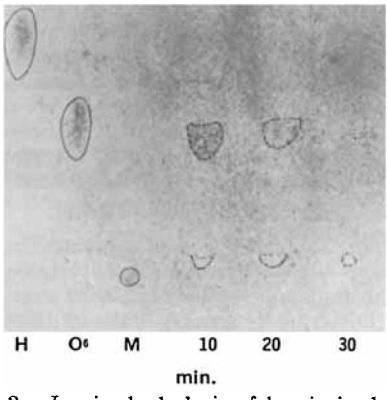

Fig. 3.—*In vivo* hydrolysis of heroin in dog, administered intramuscularly 80 mg. of diacetylmorphine hydrochloride; 20 ml. of blood collected at 10-min. intervals and examined for metabolites. Key same as in Fig. 1.

REFERENCES

(1) Mannering, G. J., Dixon, A. C., Carroll, N. V., and Cope, O. B., *J. Lab. Clin. Med.*, **44**, 292(1954).
(2) Cochin, J., and Daly, J. W., *Experientia (Basel)*, **18**, 294(1962).
(3) Nakamura, G. R., and Ukita, T., *J. Forensic Sci.*, **7**, 465(1962).
(4) Genest, K., and Farmilo, C. G., *U.N. Bull. Narcotics*, **11**, 20(1959).
(5) Way, E. L., Young, J. M., and Kemp, J. W., *ibid.*, **17**, 25(1965).
(6) Ellis, S., *J. Pharmacol. Expil. Therap.*, **94**, 130(1948).
(7) Cochin, J., Haggart, J., Woods, L. A., and Seevers, M. H., *ibid.*, **111**, 74(1954).

Effect of Certain Drugs on Perfused Human Placenta VII. Serotonin *Versus* Angiotensin-II

By RONALD F. GAUTIERI and RALPH T. MANCINI*

Serotonin produced vasoconstriction in perfused human placental vessels primarily *via* stimulation of α-adrenergic receptors and secondarily *via* direct stimulation of the vascular smooth muscle, whereas angiotensin-II produced vasoconstriction primarily *via* direct stimulation of the vascular smooth muscle and secondarily *via* stimulation of α-adrenergic receptors. In this system, serotonin was shown to cause a wider variation in its pressor action than angiotensin-II.

PREVIOUS INVESTIGATIONS in this laboratory have shown that serotonin is the most powerful vasoconstrictor of human placental vessels (1–6) and that part of this action is due to α-adrenergic cell stimulation (4). Recently, in testing other known pressor agents in this preparation, it was observed that angiotensin-II produced vasoconstriction which frequently was comparable in degree to that produced by serotonin. Therefore, the purpose of the present investigation was to compare the vasoconstrictor activity of angiotensin-II and serotonin as well as to discern whether angiotensin-II, like serotonin, caused part of its vasoconstricting action *via* stimulation of α-adrenergic receptors.

MATERIALS AND METHODS

Full term human placentas, obtained from the hospital 15–20 min. after normal delivery, were used throughout this investigation. Each was transported to the laboratory in a glass container filled with 1 L. of Tyrode's solution maintained at 38°.

The apparatus employed and the procedures involved in the preparation and perfusion of the placentas, in recording and maintaining the perfusion pressure, and in measuring the inflow and outflow volumes of the perfusate have been described in previous investigations (3, 4). As in previous papers, umbilical cords of placentas measuring more than 5.5 cm. in length were designated as long cords; those under 5.5 cm. as short cords. In 56 successful placental preparations, the duration of each experiment was approximately 1 hr.

The dosage of the agonist serotonin chosen to obtain the desired pressor response was always 50 mcg., because this dosage was formerly shown to yield consistent results (3, 4). The same dosage was used for the agonist angiotensin-II in order to be able to compare its vasoconstricting properties with those of serotonin.

Astrom and Samelius (7) have reported that 1–3 mg. of phentolamine abolished the pressor response of serotonin. However, this laboratory has shown that 3 mg. of phentolamine only partially blocked the vasoconstricting action of serotonin (4). Subsequently, a dosage of 3 and 6 mg. of the antagonist, phentolamine, was utilized after each agonist not only to confirm our previous results with serotonin and observe the effects of the lower dosage of phentolamine upon the activity of angiotensin-II, but also to observe whether doubling the dosage could affect more profoundly the pressor action of either of the agonists.

The experimental regimen was divided into three parts which were: (*a*) obtaining a control response with either agonist, (*b*) administering one of the phentolamine doses, when the agonist's initial pressor action subsided, and (*c*) administering two additional doses of either agonist approximately 3 and 15 min. after the antagonist.

The following drugs were injected into the arterial side of the perfusion: 5-hydroxytryptamine creatinine sulfate (serotonin), 0.01%; angiotensin-II,[1] 0.04%; and phentolamine methanesulfonate,[2] 0.2%.

RESULTS AND DISCUSSION

The following results, which are summarized in Tables I and II, were obtained on full-term placental vasculature perfused at the pressure range of 50–90 mm. Hg which corresponded to inflow rates of 30–60 ml. of perfusion fluid (Tyrode's solution modified by the addition of 0.525% polyvinylpyrrolidone[3]) per minute.

In a total of 42 placental preparations, regardless of the cord length, phentolamine always inhibited the normal pressor action of serotonin, with the 6-mg. dose producing the greatest degree of serotonin antagonism (Table I, Figs. 1 and 2). Angiotensin-II produced increases in perfusion pressure that, based on the mean responses in short cord placental preparations, were not significantly different from those caused by serotonin (Tables I and II). Phentolamine always reduced the vasoconstriction produced by angiotensin-II, though the 6-mg. dose was not more effective in producing antagonism than the 3 mg. dose (Table II, Fig. 3). In addition, within 15 min. after the phentolamine treatment, serotonin and angiotensin-II again produced their normal responses (Figs. 1–3). Also, it can be observed from Tables I and II, that, of the two pressor agents utilized, serotonin caused the widest variation in its pressor action.

Angiotensin-II has been shown to constrict innervated blood vessels (aorta and carotid arteries of pigs) *via* the release of norepinephrine from its stores at sympathetic nerve endings (8). Also, angiotensin-II has been shown to constrict guinea pig intestine both indirectly by stimulating ganglion

Received July 18, 1966, from the School of Pharmacy, Temple University, Philadelphia, Pa.

Accepted for publication September 30, 1966.

The authors express sincere thanks to the members of the Obstetrical Staff, Temple University Hospital, for the generous supply of placentas, to Dr. David E. Mann, Jr., for his valuable suggestions and discussions, and to Mr. J. C. Tatnall for his technical assistance.

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Previous paper: Ward, C. O., and Gautieri, R. F., *J. Pharm. Sci.*, **55**, 474(1966).

[1] Hypertensin. Valyl-5-angiotensin-II amide, lot No. B-5578, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N. J.

[2] Marketed as Regitine by Ciba Pharmaceutical Co., Summit, N. J.

[3] Marketed as Plasdone C by General Aniline and Film Corp.

TABLE I.—EFFECTS OF PHENTOLAMINE ON THE ACTION OF SEROTONIN IN THE PLACENTAL VASCULATURE

Drug and Dose	Cord Length[a]	No. Expt.	Pressure Changes, mm. Hg: Increase	Mean Change	S.E.[b]	*P* Value
Before phentolamine, 3 mg.	S	16	+4 to +108	+35	±7.8	...
Serotonin, 50 mcg.	L	15	+43 to +145	+83	±9.5	...
After phentolamine, 3 mg.	S	16	+1 to +93	+26	±8.1	.25
Serotonin, 50 mcg.	L	15	+10 to +165	+62[c]	±11.9	.1
Before phentolamine, 6 mg.	S	9	+13 to +58	+33	±5.6	...
Serotonin, 50 mcg.	L	2	+35 to +66	+50	±15.2	...
After phentolamine, 6 mg.	S	9	+1 to +36	+7[c]	±3.5	.005
Serotonin, 50 mcg.	L	2	+4 to +20	+12	±7.8	...

[a] S, short; L, long. [b] S.E., standard error. [c] Significant reduction in pressor response compared to control serotonin.

cells causing the release of acetylcholine and directly by an action on the intestinal smooth muscle (9). Recently, Vogin and Buckley (10), using a β-adrenergic receptor blocking agent to potentiate the activity of angiotensin-II, noted α receptor cell stimulation as another mechanism whereby angiotensin-II produced vasoconstriction. The present

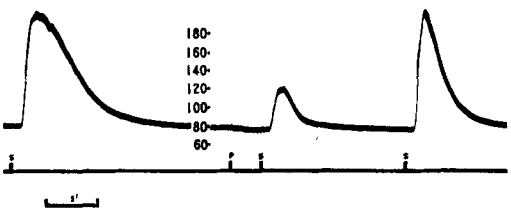


Fig. 1.—Effect of phentolamine on the action of serotonin in placental vasculature. Key: S, serotonin, 50 mcg.; P, phentolamine methanesulfonate, 3 mg.

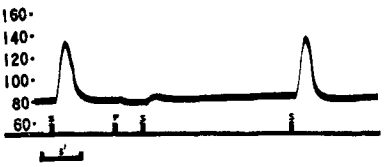


Fig. 2.—Effect of phentolamine on the action of serotonin in placental vasculature. Key: S, serotonin, 50 mcg.; P, phentolamine, 6 mg.

TABLE II.—EFFECTS OF PHENTOLAMINE ON THE ACTION OF ANGIOTENSIN-II IN THE PLACENTAL[a] VASCULATURE

Drug and Dose[b]	Pressure Changes, mm. Hg: Increase	Mean Change	S.E.[c]	*P* Value
Before phentolamine, 3 mg. Angiotensin, 50 mcg.	+21 to +51	+34	±4.2	...
After phentolamine, 3 mg. Angiotensin, 50 mcg.	+12 to +33	+23[d]	±2.4	.025
Before phentolamine, 6 mg. Angiotensin, 50 mcg.	+12 to +36	+25	±3.3	...
After phentolamine, 6 mg. Angiotensin, 50 mcg.	+11 to +30	+19[d]	±2.5	.1

[a] Short cord length in each case. [b] Seven experiments in each case. [c] S.E., standard error. [d] Significant reduction in pressor response compared to control angiotensin-II.

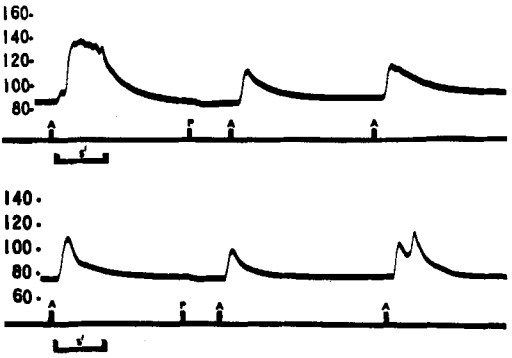


Fig. 3.—Effect of phentolamine on the action of angiotensin-II in placental vasculature. Key: A, angiotensin-II, 50 mcg.; P, phentolamine methanesulfonate. Top, 3 mg.; bottom, 6 mg.

investigation has confirmed the action of angiotensin-II on α adrenergic receptors in a noninnervated organ (11) by a different procedure—namely, the partial blockade of angiotensin-II's vasoconstrictor action by the use of the α-adrenergic receptor blocking agent, phentolamine.

Because phentolamine can diminish markedly the pressor action of serotonin, it follows that serotonin must produce most of its vasoconstriction, in these vessels, *via* stimulation of α receptors, with the remainder being due to direct stimulation of the vascular smooth muscle. On the other hand, angiotensin-II, whose vasoconstriction can be antagonized only partially by phentolamine, must produce most of its vasoconstriction, in these vessels, by a direct action on the vascular smooth muscle, with the remainder being due to stimulation of α receptors.

REFERENCES

(1) Gautieri, R. F., and Ciuchta, H. P., *J. Pharm. Sci.*, **51**, 55(1962).
(2) Ciuchta, H. P., and Gautieri, R. F., *ibid.*, **52**, 974 (1963).
(3) *Ibid.*, **53**, 184(1964).
(4) Mancini, R. T., and Gautieri, R. F., *ibid.*, **53**, 1476 (1964).
(5) *Ibid.*, **54**, 1443(1965).
(6) Ward, C. O., and Gautieri, R. F., *ibid.*, **55**, 474(1966).
(7) Astrom, A., and Samelius, U., *Brit. J. Pharmacol.*, **12**, 410(1957).
(8) Distler, A., Liebau, H., and Wolff, H. P., *Nature*, **207**, 764(1965).
(9) Khairallah, P. A., and Page, I. H., *Am. J. Physiol.*, **200**, 51(1961).
(10) Vogin, E. E., and Buckley, J. P., *J. Pharm. Sci.*, **53**, 1483(1964).
(11) Spivack, M., *Anat. Rec.*, **85**, 85(1943).

Effects of Temperature on Convulsive Liability of Pentylenetetrazol, Strychnine Sulfate, and Thebaine Hydrochloride

By JACK A. HALL*, ROBERT B. NELSON, and ALBERT I. EDLIN

The CD_{50}'s of pentylenetetrazol, strychnine, and thebaine were determined by intraperitoneal injection into white mice after 1-hr. exposure to ambient temperatures of 6, 16, 22, 24, 30, and 35°. Rectal temperatures were also measured. The data are interpreted on the basis of the pharmacology of the compounds used and the observed effects of temperature on the actions of these compounds.

THE INFLUENCE of body temperature and environmental temperature on the potency of central nervous system (CNS) stimulants has been studied by several investigators (1–5). The amount of information available on the effect of temperature on drug action is considerable; however, due to the variations in experimental design the correlation between the work of different researchers is not so clear cut as would be desirable. Indeed, there are more than a few cases of apparent contradiction. To cite but a few: Chen *et al.* (1) reported that the convulsive dose 50 (CD_{50}) of strychnine sulfate in mice was unaltered by changes in environmental temperature from 20 to 40°. Bogdanovic (2) found the toxicity of strychnine to be decreased in rats which had been cooled to 16–18° by the Giaja method. Bogdanovic also reported that the toxicity of pentylenetetrazol (PTZ) was unaltered by hypothermia. Bogdanovic suggests that the different responses to drugs produced by hypothermia may be related to the different levels of the CNS at which the drugs act. This would explain in part his observation that pentylenetetrazol, which acts at numerous levels of the neural axis, is unaltered by a decrease in body temperature.

However, Swinyard and Toman (3) found the susceptibility to convulsions induced by pentylenetetrazol to be increased by lowering the body temperature (*cf. References 4, 5*). This does not necessarily mean that Bogdanovic is wrong in his contention that the temperature-potency relationship is influenced by the level(s) of the CNS at which drugs act. Should his contention be true, it would be expected that two agents which act in a similar manner such as strychnine and thebaine (7) would produce similar responses upon a change in environmental temperature. Furthermore, both compounds should differ from pentylenetetrazol. This study tests such an hypothesis.

Fuhrman and Fuhrman (6) in their excellent review on the effects of temperature on the action of drugs attribute the importance of environmental temperature mainly to the changes it invokes in body temperature. Assuming this to be true, it should be possible to determine the minimum change in body temperature which will result in a significant variation in response to a given drug. If this minimum change were consistent over a wide range of body temperatures, a predictability would exist that would simplify considerably the interpretation of any data which might be gathered over a wide environmental temperature range. This study was undertaken to see if such a predictability exists.

Received April 25, 1966, from the College of Pharmacy, Idaho State University, Pocatello 83201.

Accepted for publication October 7, 1966.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

Supported by an Idaho State University Faculty Research Grant.

* National Science Foundation Undergraduate Research Participant.

EXPERIMENTAL

Pentylenetetrazol, thebaine hydrochloride, and strychnine sulfate were dissolved separately in distilled water and injected intraperitoneally into male mice of the Swiss albino strain weighing between 14 and 37 Gm. The mice were exposed to environmental temperatures of 6, 16, 22, 24, 30, and 35° for 1 hr. prior to injection of the compounds. Fifty mice/compound/temperature were used to determine the convulsive dose 50 (CD_{50}). Ten additional mice/environmental temperature were employed in determining the mean rectal temperature. All data were evaluated by the statistical method of Litchfield and Wilcoxon (8).

RESULTS AND DISCUSSION

This experiment was designed to study the relationship between temperature (environmental and rectal) and convulsive liability of PTZ, strychnine sulfate, and thebaine hydrochloride. The relatively short time of exposure (1 hr.) was used to minimize the adaptation of the animals to the stresses of the environmental temperatures employed in this study. The results are shown in Table I. Across temperature comparisons are presented in Table II.

Pentylenetetrazol.—Table I shows that the CD_{50} of PTZ rose progressively with the temperature. As can be seen in Table II, the CD_{50} of PTZ was not significantly different at environmental temperatures of 16 through 30°. All other combinations of environmental temperatures compared revealed significant differences in the CD_{50}'s.

Table II shows that the minimum change in rectal temperature which was accompanied by a significant alteration of the CD_{50} of PTZ was 1°. However, an increase in ambient temperature from 6 to 16° raised the rectal temperature 1.7° but did not alter significantly the CD_{50}. In all cases where rectal temperatures differed by 1.8° or more there were significant changes in the CD_{50} of PTZ.

The data for the PTZ portion of this experiment are not in accord with those of Bogdanovic (2) and Braun and Lusky (5), but do agree with those of Swinyard and Toman (3).

Strychnine.—The CD_{50} of strychnine sulfate differed significantly only between the extremes of the ambient temperatures studied and was significantly larger at 35° than at 6°. As expected, the greatest change in rectal temperature (4.2°) occurred between these two environmental temperatures (Table II).

In the case of strychnine the results of the present experiment are at variance with the work of Chen *et al.* (1), who found the CD_{50} and LD_{50} of strych-

TABLE I.—EFFECTS OF TEMPERATURE ON THE CD_{50} OF PENTYLENETETRAZOL, STRYCHNINE SULFATE, AND THEBAINE HYDROCHLORIDE

No.	Temp., °C. Environmental	Temp., °C. Rectal	CD_{50}, mg./Kg. Pentylenetetrazol	CD_{50}, mg./Kg. Strychnine	CD_{50}, mg./Kg. Thebaine
1	6	33.5	48.0	0.96	16.8
2	16	35.2	50.0	1.19	17.0
3	22	35.3	52.0	1.28	16.5
4	24	36.5	52.0	1.29	19.0
5	30	36.7	53.0	1.29	20.5
6	35	37.7	62.5	1.58	17.0

TABLE II.—ACROSS TEMPERATURE COMPARISON OF THE EFFECT OF CHANGES IN TEMPERATURE ON THE CD_{50} OF PENTYLENETETRAZOL, STRYCHNINE SULFATE, AND THEBAINE HYDROCHLORIDE

Temp. Range, °C. Environmental	Change in Rectal Temp., °C.	Remarks PTZ	Remarks Strychnine	Remarks Thebaine
6–16	1.7	0[a]	0	0
6–22	1.8	+[b]	0	0
6–24	3.0	+	0	+
6–30	3.2	+	0	+
6–35	4.2	+	+	0
16–22	0.1	0	0	+
16–24	1.3	0	0	+
16–30	1.5	0	0	+
16–35	2.5	+	0	0
22–24	1.2	0	0	+
22–30	1.4	0	0	+
22–35	2.4	+	0	0
24–30	0.2	0	0	+
24–35	1.2	+	0	0
30–35	1.0	+	0	+

[a] 0, Indicates no statistically significant alteration of the CD_{50}. [b] +, Indicates a statistically significant alteration of the CD_{50}.

nine to be unaltered by changes in temperature from 20 to 40°. This disagreement may be due in part to the use of a smaller temperature range on the part of Chen *et al.*

Thebaine.—The CD_{50} of thebaine hydrochloride was significantly larger at 30° than at all other environmental temperatures studied. Any change in ambient temperature from this point resulted in a decrease in the CD_{50} of the compound. A change in rectal temperature of 1.7° which occurred between environmental temperatures of 6° and 16° was not accompanied by a significant change in the CD_{50}. On the other hand, a significant difference in the CD_{50} did exist between ambient temperatures of 16 and 22°, although rectal temperature differed by only 0.1° between these two points (Table II). The maximum change in the CD_{50} was obtained between ambient temperatures of 22 and 30° with the CD_{50} being significantly greater at the latter temperature. No references were found concerning the effect of temperature on the CD_{50} of thebaine.

Strychnine and thebaine did not respond to temperature changes in a qualitatively similar manner, although both are reported to act at the same level of the CNS (7). This does not agree with Bogdanovic, who contends that CNS stimulants respond to changes in temperature in accordance with their levels of activity (2).

SUMMARY AND CONCLUSIONS

A study was conducted in order to determine the effects of environmental and body temperatures on the CD_{50} of pentylenetetrazol (PTZ), strychnine sulfate, and thebaine hydrochloride in mice. No correlation was found between the levels of action of the compounds in the CNS and the influence of temperature on their CD_{50}'s. Furthermore, no predictability was found to exist with regard to the minimum change in body temperature which resulted in a significant change in the CD_{50} of any of the compounds studied. The CD_{50}'s of pentylenetetrazol and strychnine sulfate increased as the temperature rose. Thebaine on the other hand had a maximum CD_{50} at an environmental temperature of 30°. The CD_{50} dropped sharply at temperatures both above and below this point.

In conclusion, changes in body temperature produced by varying the ambient temperature are of fundamental importance. However, no predictability exists with regard to the minimum change in body temperature which will result in a significant alteration in the CD_{50} of the compounds studied in this experiment. Finally, little can be said from this experiment about the nature of the influence of temperature on the action of these compounds.

REFERENCES

(1) Chen, K. K., Anderson, R. C., Steldt, F. A., and Mills, C. A., *J. Pharmacol. Expil. Therap.*, **79**, 127(1943).
(2) Bogdanovic, S. B., *Abstr. Commun. Congr. Intern. Physiol., 20th Congr.*, 109, Bruxelles, Belgium, 1956.
(3) Swinyard, E. A., and Toman, J. E. P., *Am. J. Physiol.*, **154**, 207(1948).
(4) Owens, R., *ibid.*, **193**, 560(1958).
(5) Braun, H. A., and Lusky, L. M., *Toxicol. Appl. Pharmacol.*, **2**, 458(1960).
(6) Fuhrman, G. J., and Fuhrman, F. A., *Ann. Rev. Pharmacol.*, **1**, 65(1961).
(7) Corrado, A. P., and Longo, V. G., *Arch. Intern. Pharmacodyn.*, **132**, 255(1961).
(8) Litchfield, J. T., and Wilcoxon, T., *J. Pharmacol. Expil. Therap.*, **96**, 99(1949).

Effect of Reserpine and Chlorpromazine on Some Cold Stress-Induced Biochemical Alterations

By P. C. DANDIYA, R. R. VARMA, R. K. SOGANI, and K. P. KHUTETA

The effects of reserpine and chlorpromazine on cold stress-induced changes in adrenal ascorbic acid of rats and blood glucose, serum cholesterol, and serum sodium levels of rabbits were studied. While pretreatment with chlorpromazine minimized many of these stress-induced alterations, reserpine pretreatment aggravated them. Reserpine was found to be effective in preventing the stress-induced reduction of serum cholesterol level. Pretreatment with chlorpromazine minimized the adrenal ascorbic acid depletion due to cold stress. It also significantly prevented rise in blood sugar and fall in serum sodium produced by cold stress.

STRESS stimulates the hypothalamo-adrenal axis and produces a number of functional and biochemical alterations. It is a well known fact that most types of stresses have associated psychic components, and a number of stress-induced changes are mediated through the hypothalamus. Tranquilizers, by calming the mind, could possibly reduce the psychic component of stress and thus minimize the stress-induced alterations. A survey of the past literature shows that only few sporadic studies have been made so far to study the effect of tranquilizers on stress-induced biochemical changes (1–4). Though a considerable amount of work has been done to study the changes in the adrenal ascorbic acid and blood glucose levels during stress, difference of opinion exists regarding the effectiveness of tranquilizers to counteract these changes (1–3, 5, 6), and the present study is aimed at clarifying the divergent views expressed by different workers in this regard. No previous attempt has been made to study the influence of tranquilizers on stress-induced changes in the circulating ions and cholesterol, and therefore, this virgin field of exploration was selected with the hope that with such lucid exposition of the beginning stages of altered metabolism, an insight into the diverse disturbances caused by stress could be attained which will hasten the development of appropriate therapeutic agents for the prevention or amelioration of some of the stress-induced abnormalities.

MATERIAL AND METHODS

Albino rats (125 to 175 Gm.) and rabbits (1.25 to 1.75 Kg.) were subjected to cold stress by keeping them at an environmental temperature of 2° ± 1° for 1 hr. In order to keep the animals in identical conditions the day before the experiment (12 hr. before experimentation) they were isolated, during which period no food was given. The effects of drugs and stress were determined on rat adrenal ascorbic acid and rabbit blood glucose, serum cholesterol, and sodium. The levels were studied in normal controls, after stress, after tranquilizers, and after producing stress in animals pretreated with tranquilizers. Reserpine and chlorpromazine were administered in doses of 1 mg./Kg. intraperitoneally in rats. In rabbits these drugs were administered intramuscularly in a dose of 0.3 mg./Kg. and 3 mg./Kg., respectively. The animals were subjected to stress 45 min. after chlorpromazine and 4 hr. after reserpine pretreatments. The rats were decapitated and the rabbit's blood was collected immediately after stress or after an equivalent interval following the drug administration in those animals not subjected to stress, for the biochemical studies.

Adrenal ascorbic acid was extracted in 4% trichloroacetic acid and estimated by the method of Roe and Kuether (7). Blood glucose was estimated by Somogyi-Nelson's method (8, 9), serum cholesterol by Bloor's method, as given in the handbook of "Lumetron Photoelectric Colorimeter," model 400-A, p. 536, and serum sodium by using a flame photometer (10).

RESULTS

The findings are summarized in Fig. 1, which shows control levels and alterations in various biochemical parameters after drug, after stress, and after drug plus stress.

The depletion of adrenal ascorbic acid after stress was increased in reserpine-pretreated animals and decreased in chlorpromazine-pretreated animals. While reserpine produced a significant depletion in the control adrenal ascorbic acid level, the change due to chlorpromazine treatment was insignificant. Significant hyperglycemia was seen after cold stress and also after reserpine and chlorpromazine treatment. Stress-induced hyperglycemia was less in chlorpromazine and exaggerated in reserpine-pretreated animals. Stress produced a fall in serum cholesterol level. Reserpine pretreatment reduced this fall, but pretreatment with chlorpromazine was ineffective in this respect. Neither drug altered the prestress control cholesterol level. A moderate reduction in serum sodium level occurred after stress. Pretreatment with reserpine aggravated this stress-induced alteration while pretreatment with chlorpromazine minimized it. The prestress control sodium level was unaltered after reserpine or chlorpromazine.

DISCUSSION

Acute cold stress produces a fall in adrenal ascorbic acid, serum cholesterol, and serum sodium levels and a rise in blood glucose level. Most actions of reserpine are stress-like and a number of stress-induced alterations are aggravated by its pretreatment. Stress-like effects of reserpine have been reported by others (11–13). It may be a nonspecific action or an action mediated *via* hypothalamus as proposed by Maickel *et al.* (12). Most effects of chlorpromazine pretreatment on stress are just opposite to those seen after reserpine.

Received August 1, 1966, from the Departments of Pharmacology and Physiology, S. M. S. Medical College, Jaipur, India.

Accepted for publication October 12, 1966.

This investigation was supported by the Indian Council of Medical Research.

Chlorpromazine and reserpine were supplied, respectively, by May and Baker Ltd., Dagenham, England, and Ciba Pharmaceutical Products Ltd., Bombay, India.

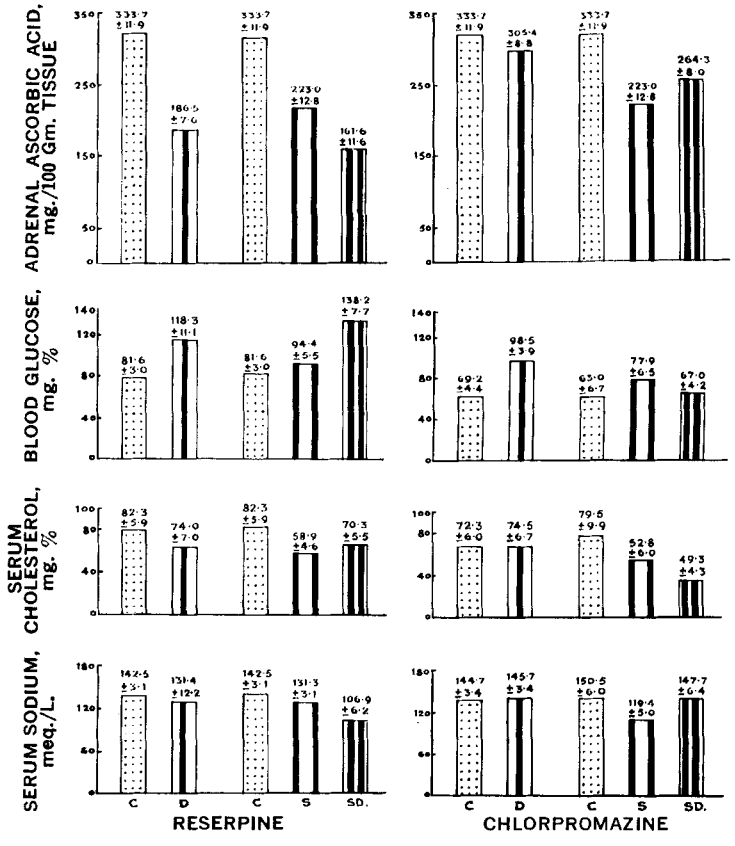


Fig. 1.—Effect of reserpine and chlorpromazine on cold stress-induced biochemical alterations. Key: C, control; D, drug alone; S, stress alone; SD, stress after drug pretreatment. The number of animals used for the adrenal ascorbic acid estimation was 36 (control), 15 (cold stress alone), and 8 each in other groups; while in the case of all other experiments the number of animals was 8 in each group. In some of these experiments control of animals was taken more than once. For easy comprehension, the control values have been shown along with corresponding observation. The figures denote mean values ± standard error in each group.

These opposing actions of reserpine and chlorpromazine point to their different mechanisms of action. There could be some relationship of this difference to their different actions on ergotropic and trophotropic systems of Hess (14), as reported by Brodie *et al.* (15). Work on these lines is in progress.

REFERENCES

(1) Mahfouz, M., and Ezz, E. A., *J. Pharmacol. Exptl. Therap.*, **123**, 39(1958).
(2) Deshpande, V. R., and Tiwaskar, H. V., *Ind. J. Med. Res.*, **48**, 478(1960).
(3) Ashford, A., and Shapero, M., *Brit. J. Pharmacol.*, **19**, 458(1962).
(4) Olling, C. Jh., and deWield, D., *Acta Endocrinol. (Kbh).*, **22**, 283(1956).
(5) Gupta, S. K., Patel, M. A., and Joseph, A. D., *Arch. Intern. Pharmacodyn.*, **128**, 82(1960).
(6) Courvoisier, S., Fournel, J., Ducrot, A., Kolsky, M., and Koetscher, P., *ibid.*, **92**, 305(1953).
(7) Roe, J. W., and Kuether, C. A., *J. Biol. Chem.*, **147**, 399(1943).
(8) Nelson, N., *ibid.*, **153**, 375(1944).
(9) Somogyi, M., *ibid.*, **195**, 19(1952).
(10) Hawk, P. B., Oser, B. L., and Summerson, W. H., "Practical Physiological Chemistry," Blakiston Co., New York, N. Y., 1954, p. 650.
(11) Khazan, N., Sulman, F. G., and Winnik, H. Z., *Proc. Soc. Exptl. Biol. Med.*, **106**, 579(1961).
(12) Maickel, R. P., Westermann, E. O., and Brodie, B. B., *J. Pharmacol. Exptl. Therap.*, **134**, 167(1961).
(13) Saffran, M., and Vogt, M., *Brit. J. Pharmacol.*, **15**, 165(1960).
(14) Hess, W. R., "Monograph of Biology and Medicine," vol. 3, Grune and Stratton, New York, N. Y., 1954; through Brodie, B. B., Spector, S., and Shore, P. A., *Pharmacol. Rev.*, **11**, 548(1959).
(15) Brodie, B. B., Spector, S., and Shore, P. A., *Pharmacol. Rev.*, **11**, 548(1959).

Tissue Levels of Chloroamphetamines in Rats and Mice

By RAY W. FULLER and C. W. HINES

During the first 4 hr. after i.p. injection of equimolar amounts of the two drugs, more 4-chloroamphetamine than amphetamine was present in the brain and other tissues of rats and mice. The higher levels resulted from a slower disappearance of 4-chloroamphetamine. Other amphetamines substituted with chlorine in the *para* position disappeared more slowly from rat brain than did amphetamine. This may be due to blocking of a major metabolic pathway for amphetamine—*para* hydroxylation—by the *para* substitution, since amphetamines with chlorine substituted in other positions disappeared from tissues at rates comparable to that for amphetamine.

PLETSCHER *et al.* (1) first reported that the levels of 4-chloro-*N*-methylamphetamine were higher than those of amphetamine in the brains of rats given equimolar doses of the two compounds. This has led to the study of the effect of different chloro substitutions on the tissue distribution and disappearance rate of amphetamine. The results of this study, which have been presented in a preliminary form,[1] are reported in detail in this paper.

EXPERIMENTAL

The animals used were male albino rats from the Harlan strain, body weight approximately 150 Gm., or Cox standard albino mice, body weight approximately 16 Gm. Dextroamphetamine sulfate[2] was obtained from Smith Kline & French Laboratories. *l*-Amphetamine sulfate was from the Ott Chemical Co. The chloroamphetamines were synthesized as the hydrochlorides in the Lilly Research Laboratories. In all experiments, the compounds were injected intraperitoneally in aqueous solution at a dose of 0.1 mmole/Kg. (18–24 mg./Kg.). Four rats or six mice per group were used except as otherwise noted. Animals were decapitated at the time intervals indicated. The organs were quickly removed, frozen on dry ice, and stored at −15° prior to analysis. Drug levels were determined by the methyl orange assay as used by Dubnick *et al.* (2). Hydroxylated amphetamines that might be formed metabolically are not measured by this assay. An internal standard curve for each drug and each tissue was used to calculate drug levels. This standard curve was determined from control animal tissues to which drug had been added *in vitro*. Tissue blanks were low. Typical blank values were, in mμmoles/Gm.: heart and spleen, 0; blood, 1; brain, 2; liver, 6; kidney, 18.

RESULTS AND DISCUSSION

The levels of dextroamphetamine and of *dl*-4-chloroamphetamine in the brain of rats and mice after i.p. injection are shown in Fig. 1. In both species, the 4-chloro compound disappeared much more slowly than the unsubstituted amphetamine. 4-Chloroamphetamine and 4-chloro-*N*-methylamphetamine lower serotonin in the brain of rats but not in mice (1, 3). This species difference is not due to differences in concentrations of the compounds in the brain, since the levels and disappearance rates of 4-chloroamphetamine were almost identical in rats and mice. The species difference thus rests on some basis other than tissue levels of the drugs. On the other hand, drug levels may explain why 4-chloroamphetamines lower serotonin in rat brain while amphetamine does not. The former compounds persist in the brain for longer times than does amphetamine.

Disappearance rates (from rat brain) for other substituted amphetamines were calculated from plots of the type shown in Fig. 1. The results are shown in Table I. All of the compounds with a chloro substituent in the 4 position disappeared much more slowly (9–11%/hr.) from brain than did those with no chloro substitution in this position (49–73%/hr.). There seemed to be no steric effect in either group of compounds. Since amphetamine is *para* hydroxylated in rats (4–6) and mice,[3] the slower disappearance rate of the 4-substituted compounds may be closely related to the fact that this metabolic pathway has been blocked.

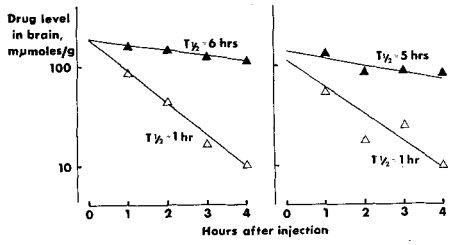


Fig. 1.—Brain levels of dextroamphetamine and *dl*-4-chloroamphetamine in rats (left) and mice (right). Key: ▲, *dl*-4-chloroamphetamine; Δ, dextroamphetamine.

To determine if binding of the drugs in the brain played a role in their rate of disappearance, the drug concentrations were measured in the supernatant and precipitated fractions after centrifugation of brain homogenates from treated rats. The centrifugation was carried out in a Spinco model L ultracentrifuge at a force of 105,000 × g. Drug levels were then determined in both supernatant and precipitated fractions. Results are shown in Fig. 2. The supernatant fraction was taken to represent "free" drug and the precipitated fraction "bound" drug. Unsubstituted amphetamine was found primarily in the soluble fraction, whereas all chlorinated amphetamines were bound to a greater degree. The association of chloroamphetamines with the

Received August 31, 1966, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind. 46206.
Accepted for publication October 14, 1966.

[1] Federation of American Societies for Experimental Biology, Atlantic City, N. J., meeting, April 1966.

[2] Marketed as Dexedrine sulfate by Smith Kline & French Laboratories, Philadelphia, Pa.

[3] After injection of ^{3}H-amphetamine into mice, the authors have isolated from the urine of the mice an ether-soluble substance that was identified by paper chromatography as *p*-hydroxyamphetamine. It was not established whether this represented a major or minor metabolite.

TABLE I.—RATE OF DISAPPEARANCE OF SUBSTITUTED AMPHETAMINES FROM RAT BRAIN

Substitution	Isomer	Disappearance Rate, %/hr.
None	*d*	53
	l	49
2-Chloro-*N*-methyl	*dl*	73
2,6-Dichloro	*dl*	58
3-Chloro	*dl*	53
4-Chloro	*dl*	9
4-Chloro-*N*-methyl	*dl*	11
3,4-Dichloro	*d*	11
3,4-Dichloro	*l*	11

particulate fraction may reflect their greater lipid solubility. The degree of binding may influence the length of time that the drugs remain in the brain. Kuntzman and Tsai (7) found that the chloro derivative of norcyclizine had a longer *in vivo* half-life than did cyclizine, perhaps because of more extensive binding to plasma proteins. However, Fig. 1 and Fig. 2 show that 2,6-dichloroamphetamine had a high bound/free ratio and still disappeared rapidly. This would suggest that drug binding is not the major factor that determines disappearance rate in this case, consistent with the idea that blockade of metabolism accounts for the slower disappearance of chloroamphetamines.

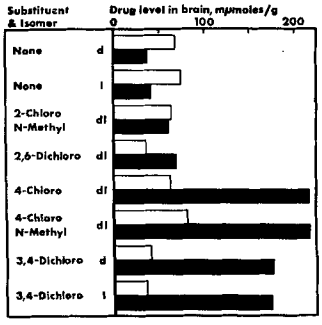


Fig. 2.—Occurrence of substituted amphetamines in free and bound forms in rat brain. Brains were analyzed 1 hr. after administration of the drugs. Key: open bar, free; closed bar, bound.

The levels of amphetamine and of 4-chloroamphetamine in other tissues of the rat, measured at 1 and 4 hr. after injection, are shown in Fig. 3. The relative distribution between the various tissues was similar for the two compounds. Large amounts of the compounds were present in the lung and kidney. (Drug levels in the lung and epididymal fat pads were not determined at 1 hr.) An important difference between the drugs existed in the blood levels. In

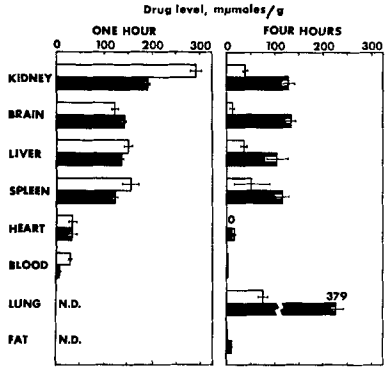


Fig. 3.— Tissue levels of dextroamphetamine and *dl*-4-chloroamphetamine in the rat. Amphetamine levels are represented by open bars, 4-chloroamphetamine by closed bars. Standard errors of the means of determinations in 5 rats per group are shown.

addition to the 60-min. time shown in Fig. 3, blood levels were determined at 15, 30, and 45 min. Levels of amphetamine in blood at these times ranged from 4 to 10 times as high as the levels of 4-chloroamphetamine. Tissue/plasma ratios were therefore much higher for 4-chloroamphetamine than for amphetamine, suggesting that the over-all tissue uptake and binding of the chloroamphetamine was greater. This is also revealed by the fact that at 4 hr., when blood levels of both drugs had dropped to the limits of detection, tissue concentrations of 4-chloroamphetamine were still amost as high as at 1 hr. In all of the tissues, the levels of 4-chloroamphetamine at 4 hr. were substantially higher than those of amphetamine, indicating that a slower disappearance rate of the chloroamphetamine occurs in other tissues as well as in the brain. Tissue distribution of the drugs in mice was very similar to that shown in Fig. 3 for the rat.

The data presented show that substitution of chlorine in the *para* position of amphetamine markedly slows the disappearance rate of the drug from tissues. It is suggested that an important reason may be the blocking of one of the metabolic pathways—namely, *para* hydroxylation—by which amphetamine is normally transformed prior to excretion from the body.

REFERENCES

(1) Pletscher, A., Bartholini, G., Bruderer, H., Burkard, W. P., and Gey, K. F., *J. Pharmacol. Exptl. Therap.*, **145**, 344 (1964).
(2) Dubnick, B., Leeson, G. A., Leverett, R., Morgan, D. F., and Phillips, G. E., *ibid.*, **140**, 85(1963).
(3) Fuller, R. W., Hines, C. W., and Mills, J., *Biochem. Pharmacol.*, **14**, 483(1965).
(4) Axelrod, J., *J. Pharmacol. Exptl. Therap.*, **110**, 315 (1954).
(5) Dring, L. G., Smith, R. L., and Williams, R. T., *J. Pharm. Pharmacol.*, **18**, 404(1966).
(6) Ellison, T., Gutzait, L., and Van Loon, E. J., *J. Pharmacol. Exptl. Therap.*, **152**, 383(1966).
(7) Kuntzman, R., and Tsai, I., *Federation Proc.*, **25**, 418 (1966).

Absorption of Ethanol by the Common Guppy (*Lebistes reticulatus*)

By NATHAN A. HALL and WILLIAM L. HAYTON*

The uptake of ethanol by guppies (*Lebistes reticulatus*) from solutions of varying concentrations has been observed at 25°. Results of the observations were compared with those obtained with goldfish (*Carassius auratus*) at 20° and 25°. Both time of death and time of overturn were used as criteria of pharmacological effect. Plots of the reciprocal time of effect against ethanol concentration failed to intersect the origin in all cases, except for goldfish at 20° where time of death was observed. The experimental demonstration of a threshold concentration for each fish implies that the actual plot should be a sigmoid curve. The results are discussed in relation to the theory of Levy and Gucinski. Guppies appear to be suitable for drug absorption studies.

A THEORY describing the uptake of drugs and toxic chemicals by fish from their aqueous environment has been developed by Levy and Gucinski (1). They have tested the theory with goldfish and have shown it to be useful in the study of factors influencing biological membrane permeation by passive diffusion of drugs (2–4). This investigation represents an attempt to apply the theoretical concepts of Levy to another experimental fish, the common guppy.

The guppy has been used extensively in water pollution studies (5), is easily kept under laboratory conditions, and is sensitive to many toxic substances. Since the usual adult is small (200–500 mg.), it was thought that this fish might be suited for the study of drugs available in limited quantities or for drugs which give poorly predictable responses with other fish.

EXPERIMENTAL

Fish—Common guppies (*Lebistes reticulatus*) were obtained from a local supplier of tropical fish in lots of 100 fish. They were kept in an aerated aquarium filled with tap water and fitted with a submersion filter and a thermostatically controlled heater set at 25°. Regulation of the relative size for use in experiments was accomplished by allowing the fish to escape from polystyrene cups punctured by holes of known size. Only those fish able to pass through 5-mm. holes but unable to pass through 3-mm. holes were used.

Goldfish (*Carassius auratus*) were purchased at a local pet shop.

Method—A single adult guppy was transferred by net from the aquarium to a 100-ml. beaker containing 30 ml. of the drug solution, and a stopwatch was started. Beaker sizes and solution volumes for goldfish were those of Levy and Gucinski (1). The fish was observed for drug effect and the time recorded. Prior to use all drug solutions were equilibrated in a constant-temperature water bath at 25°, and the beaker containing the drug solution was placed inside a 600-ml. beaker suspended in a 25° constant-temperature water bath. The temperature of the test medium was thus maintained at 24 to 25°. A similar procedure was used for studies at 20° with a refrigerated constant-temperature bath.

At each drug concentration several fish were run. None of the solutions were re-used, thus each fish was exposed to fresh solution. Results were expressed as the average time for a group of fish to show an effect, and the standard deviations of means were routinely calculated. Ethanol solutions were prepared by pipeting the calculated quantity of absolute ethanol into a volumetric flask and bringing to volume with distilled water.

Absorption of Ethanol

To gain experience in the use of fish and to test the reproducibility of the previously reported method, a series of experiments with graded concentrations of ethanol were conducted with goldfish at two different temperatures, 25° and 20°. Results were plotted as the reciprocal time of death *versus* percentage by volume of ethanol. Table I lists the regression equations obtained for the plotted lines and those of Levy and Gucinski for comparison. Six fish were used to determine each point at 25° and four at 20°.

The variation among lots of fish was expected and has been previously reported. The higher temperature was observed to increase the slope of the plotted line and to cause a larger negative intercept than previously reported.

A series of experiments was then conducted in a similar manner with guppies. Since these fish have a natural tropical habitat and preliminary experiments showed them sensitive to low temperatures, absorption at only 25° was determined. Six fish were run for each point and at least 5 points were plotted for each run. The regression equations for the plotted experimental data are listed in Table II. The data indicated a greater sensitivity or rate of absorption for guppies than for goldfish, and a lot to lot variation was evident. All plots showed negative intercepts.

Criteria of Drug Effect—Considerable difficulty was encountered in determining the end point of death with guppies because of their size. Although a magnifying lens was helpful in observing the cessation of mouth and gill movements, optical difficulties were complicated by the beakers housing the fish. Investigators in river pollution studies have utilized the loss of balance equilibrium or "overturn" as an indication of toxic effect (6), especially where the death end point is uncertain. Accordingly, observations were made using the overturn time as the criterion of drug effect. Figure 1 is a representative plot of the experimental data for goldfish and guppies with both overturn and death end points at 25°, and Table III lists regression equations for the plotted lines with overturn as the end point.

Received September 6, 1966, from the College of Pharmacy, University of Washington, Seattle 98105.
Accepted for publication November 10, 1966.
* National Science Foundation Undergraduate Research Participant.

TABLE I—REGRESSION EQUATIONS FOR RECIPROCAL TIME OF DEATH ($1/T_L$) *Versus* ETHANOL CONCENTRATION (C) WITH GOLDFISH

Source	Eq.	Temp.	No. of Points Determining Line
Levy and Gucinski (1)	$1/T_L = 0.62\ C - 0.01$	20°	6
Levy and Gucinski (1)	$1/T_L = 0.64\ C + 0.36$	20°	4
Levy and Gucinski (1)	$1/T_L = 0.63\ C - 0.098$	20°	3
This study	$1/T_L = 0.48\ C + 0.036$	20°	4
This study	$1/T_L = 0.89\ C - 0.93$[a]	25°	5

[a] Plotted in Fig. 1.

TABLE II—REGRESSION EQUATIONS FOR RECIPROCAL TIME OF DEATH *Versus* ETHANOL CONCENTRATION WITH GUPPIES

Fish Lot No.	Eq.	Ethanol Concn. Range, % by Vol.
I G	$1/T_L = 1.81\ C - 1.91$	2–16
IV G	$1/T_L = 2.26\ C - 3.50$	2–16
VII G	$1/T_L = 2.04\ C - 4.12$[a]	3–7

[a] Plotted in Fig. 1.

TABLE III—REGRESSION EQUATIONS FOR RECIPROCAL OVERTURN TIME ($1/T_{OT}$) *Versus* ETHANOL CONCENTRATION (C)

Fish Lot No.	Eq.	Temp.
I F (Goldfish)	$1/T_{OT} = 7.65\ C - 17.24$[a]	25°
I F (Goldfish)	$1/T_{OT} = 4.82\ C - 12.68$	20°
II G (Guppies)	$1/T_{OT} = 5.74\ C - 13.45$	25°
IV G (Guppies)	$1/T_{OT} = 5.87\ C - 14.70$	25°
VII G (Guppies)	$1/T_{OT} = 5.70\ C - 15.07$[a]	25°

[a] Plotted in Fig. 1.

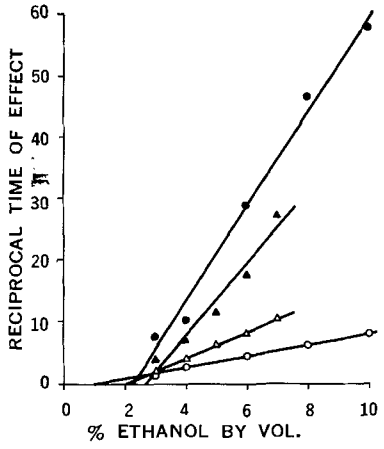


Fig. 1—A plot of reciprocal time of effect as a function of ethanol concentration at 25° for goldfish and guppies. Key: ○, goldfish, death end point; ●, goldfish, overturn end point; △, guppies, death end point; ▲, guppies, overturn end point.

DISCUSSION

The theory developed by Levy and Gucinski (1) to describe the goldfish as an experimental animal in drug absorption states $1/T_L = (K/L)\ C$, in which T_L is time of death, L the lethal dose, K the apparent absorption rate constant, and C the concentration of the drug in the medium, offers a number of attractions including the avoidance of involved chemical assays and tedious lethal dose determinations. It seems, however, that the theoretical model may possess certain limitations in application due to the simplifying assumptions upon which it is based, as pointed out by Levy and Gucinski.

Levy states that a number of assumptions are necessary for the model to be applicable. These include: (*a*) absorptiono ccurs only by passive diffusion; (*b*) the concentration gradient is essentially constant during the experiment; (*c*) the membrane permeability characteristics are unchanging under the experimental conditions; (*d*) drug elimination, including biotransformation, is negligible during the experiment; and (*e*) the pharmacologic end point occurs without significant delay after the lethal dose of the drug has been absorbed. He recommends using the experimental data themselves to indicate if the requirements are met. A plot of the reciprocal death time *versus* concentration should be linear and intersect the origin (2).

Initial experiments with goldfish showed that our experimental techniques would yield results conforming to the theory for ethanol with death as the observed pharmacological effect. (See Table I.) The lower slope of our plot as compared to those of Levy and Gucinski at 20° was ascribed to variations among lots of goldfish. The increase in temperature to 25° increased the slope of the plot and resulted in a negative intercept; the increase in slope would be expected from both more rapid diffusion and greater lethality of the ethanol for the fish at the higher temperature.

When guppies were used as the experimental fish, there was observed a three to fourfold greater slope than with goldfish, but most notable was the consistent negative intercept of the plotted line. Initially, this intercept was thought to be due to the lesser precision of experimental points where death occurred under 10 min.; therefore, a run was designed to obtain a plot where all points were derived from data in which death times were greater than 10 min. (Table II, lot VII G). The negative intercept appeared to be consistent.

The plots of the experimental data for both goldfish and guppies when overturn time was used as the criterion of drug action (Table III) showed a greater slope and greater negative intercept than when death was used. The increased slope was expected, for all fish lost their sense of balance before expiring. The greater slope for goldfish at 25° (lot I F) than for guppies (lots II G, IV G, VII G) was somewhat surprising since guppies seemed more

sensitive to ethanol lethality than goldfish (Tables I, II, and Fig. 1). Goldfish, however, are considerably deeper (measuring from ventral to dorsal surface) than are guppies; thus, their sense of balance is evidently more sensitive to slight CNS depression.

The repeated negative intercepts for plots of all the guppy experiments, for the goldfish experiment at 25°, and for the goldfish experiments where overturn time was observed seem to indicate that the theory fails to describe the experimental observations. Although plots of the data are apparently linear, they do not intersect the origin. Consideration of the assumptions upon which the theory is based may indicate that the failure of guppies to produce plots in agreement with the theory might be due to unknown characteristics of the fish. However, it does not appear reasonable that with guppies factors such as biotransformation, delay of pharmacological effect, and changing membrane permeability would be greatly different from goldfish when the plots exhibit similar linearity. Furthermore, it is difficult to ascribe deviation of the goldfish data for overturn times to factors much different from those functioning where time of death is observed.

One consistency is apparent. Whenever the slope of the plot increases, the negative intercept increases. This observation demonstrates that the kinetic model does not apply under conditions where one or more of the simplifying assumptions is not fulfilled. In 1917 Powers (7) concluded, after extensive experiments with goldfish and various toxicants, that the survival time is inversely related to the concentration only over a limited concentration range. In both low and high concentrations considerable deviation from linearity is evident and the true curve has a sigmoid form. Extrapolation of the linear portion of the curve to zero on either axis will tend to obscure the real behavior of the fish. A plot which intersects the origin cannot show a threshold concentration—that concentration which causes no observable effect. Most toxic substances studied in fish exhibit a threshold concentration (8).

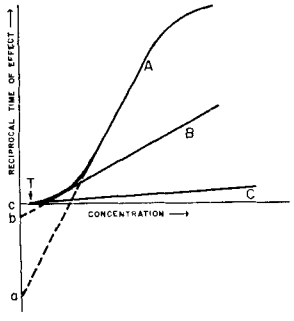


Fig. 2—A hypothetical plot of reciprocal time of effect versus *concentration of drug. Curves* A, B, *and* C *have a common threshold concentration* T. *Extrapolation of the linear portion of each curve intersect the ordinate at* a, b, *and* c.

Both goldfish and guppies have been kept in this laboratory for over 24 hr. in appreciable ethanol concentrations without loss of balance or death. For guppies the threshold at 25° lies between 0.25 and 0.50% and for goldfish between 1.0 and 2.0%. Therefore, the true plot of reciprocal time of effect *versus* concentration should intersect the concentration axis at some point other than the origin; the curves should assume a sigmoid form. If the hypothetical situations depicted in Fig. 2 are considered, the three illustrated cases parallel the results obtained in this study. Extrapolation of the linear part of curve A will give a large negative intercept, a. Curve B will give a smaller negative intercept, b, and curve C will give a negligible intercept, c. The regression equation obtained for goldfish with the time-of-death end point (Table I, 20°) corresponds to hypothetical curve C; equations for goldfish and guppies with the time-of-death end point at 25° (Table I, 25°, Table II) are similar to hypothetical curve B; and equations for goldfish and guppies with the overturn time end point (Table III) resemble curve A.

Since threshold concentrations can be demonstrated experimentally, a deviation from linearity for all plots is implied. The theory in its original form was not designed for low concentrations of ethanol, and the observed deviations from linearity therefore are to be expected. Similar behavior might be expected for other chemicals. The experimental system upon which the theory was based was stated to be useful "due to the relatively large volumes of aqueous medium and high drug concentrations" (1), however, a later publication stated that plots of the data "should be linear and intersect the origin" as a test of the validity of the mathematical model for a particular set of experimental conditions (2). From the foregoing considerations it seems unwise to assign theoretical or physiological significance to the intercept values.

This investigation points up some of the limitations of the original theory which can be encountered, some of which would be expected from the simplifying assumptions. Levy and co-workers have utilized the kinetic model with agents other than ethanol and have found goldfish absorption to correlate well with other biological data (1–3). Italian investigators, working with three different drugs, have also verified the usefulness of the model by making direct comparisons between goldfish absorption and oral absorption in rats (9).

The theory of Levy and Gucinski (1) appears to be applicable to guppies as well as goldfish, provided that the selected drug concentrations are well above the threshold levels.

REFERENCES

(1) Levy, G., and Gucinski, S. P., *J. Pharmacol. Exptl. Therap.*, **146**, 80(1964).
(2) Levy, G., and Miller, K. E., *J. Pharm. Sci.*, **53**, 1301 (1964).
(3) *Ibid.*, **54**, 1319(1965).
(4) Levy, G., Miller, K. E., and Reuning, R. H., *ibid.*, **55**, 394(1966).
(5) Jones, J. R. E., "Fish and River Pollution," Butterworths Scientific Publications, Washington, D. C., 1964, p. 42.
(6) *Ibid.*, p. 40.
(7) Powers, E. B., *Illinois Biol. Monographs*, **4**, 127(1917).
(8) Jones, J. R. E., *ibid.*, **4**, 45(1917).
(9) Coppi, G., and Bonardi, G., *Boll. Chim. Farm.*, **105** 462(1966).

Potential Antiradiation Drugs: α-Alkyloximinonitriles

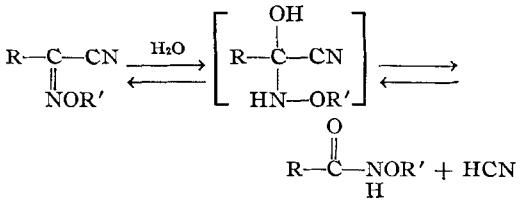
By DOMINICK A. COVIELLO

Of three α-alkyloximinonitriles tested for radiation protection, two were very toxic and inactive, while one with low toxicity gave slight protection.

MANY classes of compounds including nitriles have shown varying degrees of protection against radiation damage in mammals. It has been suggested that conceivably the nitriles act by the slow sustained-release of HCN which is protective (1). If the theory has merit, it was postulated that α-alkyloximinonitriles, reacting with water in the cellular milieu, could yield an intermediate which would decompose to yield HCN and an *O*-alkylhydroxamic acid according to Scheme I.

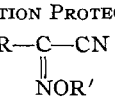
It is interesting to note (Table I) that 1-benzyloximinobutanecarbonitrile gives slight protection, and has an LD_{50} of about 1000 mg./Kg. while the other two, 1-benzyloximinopropanecarbonitrile and 1-benzyloximinoheptanecarbonitrile, give no protection with LD_{50}'s of less than 50 mg./Kg.

Some of the nitriles were previously reported (2). The new compounds were characterized by the characteristic nitrile absorption in the infrared (3) and by alkaline hydrolysis back to the starting acids. They are shown in Table II.



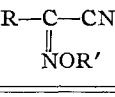
Scheme I

TABLE I.—α-ALKYLOXIMINONITRILES TESTED FOR RADIATION PROTECTION



Compd. R	Compd. R′	LD_{50}, mg./Kg.	Test Dose, mg./Kg.	Test Evaluation
CH_3	$C_6H_5CH_2$	Less than 50	50–150	No protection
			150–350	No protection
C_2H_5	$C_6H_5CH_2$	1000–1500	350–700	No protection
			700–1000	Slight protection
C_5H_{11}	$C_6H_5CH_2$	Less than 50	150–350	No protection
			350–700	No protection

TABLE II.—α-ALKYLOXIMINONITRILES PREPARED



Compd. R	Compd. R′	B.p.	$n_D/t°$	Yield	C—N Stretch cm.$^{-1}$
Methyl	Methyl	132–133/760 mm.	1.4336/20°	54	2230
Methyl	Ethyl	147–148/760 mm.	1.4241/24°	58	2230
Methyl	Benzyl	80–85/0.03 mm.	1.3815/26°	21	2236
Ethyl	Ethyl	166–167/760 mm.	1.4258/28°	60	2230
Ethyl	Benzyl	102–105/0.5 mm.	1.5179/25°	17	2235
Butyl	Methyl	84–85/15 mm.	1.4300/25°	55	2240
Butyl	Ethyl	87–89/15 mm.	1.4325/26°	50	2240
Butyl	Benzyl	96–98/0.01 mm.	1.5087/25°	45	2240
Amyl	Benzyl	123–125/0.1 mm.	1.475/6°	29	2248

The amide precursors for the nitrile preparation have been reported (4).

Received August 12, 1966, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago 60612.
Accepted for publication October 26, 1966.
This investigation was supported in part by grant RH 00293-01 (MCHB) and RH 00293-02 (MCHB) from the Division of Radiological Health, Bureau of State Services (EH), U. S. Public Health Service, Bethesda, Md.
Biological testing and test data have been made available through the courtesy of Dr. David Jacobus, Walter Reed Institute of Research, Washington, D. C.
The technical assistance of J. C. Ziebell and W. L. Jacobson is also acknowledged.

REFERENCES

(1) Thomson, J. F., "Radiation Protection in Mammals," Reinhold Publishing Corp., New York, N. Y., 1962.
(2) Coviello, D. A., *J. Pharm. Sci.*, **53**, 972(1964).
(3) Nakanishi, K., "Infrared Absorption Spectroscopy—Practical," Holden-Day, Inc., San Francisco, Calif., 1962.
(4) Coviello, D. A., *J. Med. Chem.*, **7**, 121(1964).

New Compounds:

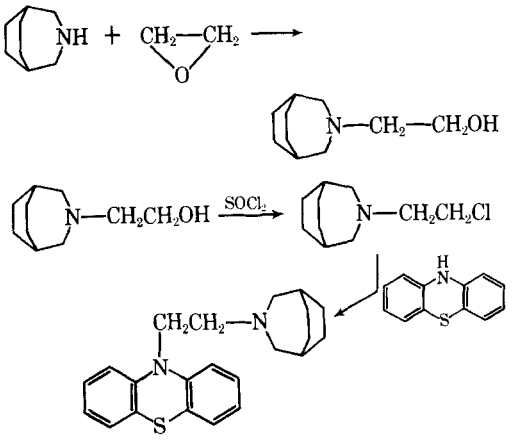
3-[2-(2-Chloro-10-phenothiazinyl)ethyl]-3-azabicyclo[3.2.2]nonane

By WILLIAM T. KING, CHARLOTTE H. BRUENING, and W. LEWIS NOBLES

The synthesis of 3-[2-(2-chloro-10-phenothiazinyl)ethyl] - 3 - azabicyclo[3.2.2]nonane is reported; the pharmacological evaluation of this agent, an analog of promethazine, has not yet been reported.

PREVIOUSLY, Anderson and his associates (1) reported the synthesis of 3 [3-(2-chloro-10-phenothiazinyl)propyl] - 9 - methyl - 3,9 - diazabicyclo - [3.3.1]nonane dihydrochloride in the course of preparing synthetic relatives of various 2-substituted phenothiazines and their 10-aminoalkyl derivatives. Almost simultaneously, the authors were experimenting with the use of an analogous heterocyclic amine, 3-azabicyclo[3.2.2]nonane, as a possible intermediate in the synthesis of potential medicinals (2–5). Thus, the present report is to indicate the extension of these studies to the preparation of the 3-azabicyclo[3.2.2]nonane analog of existing phenothiazines which are of significant value as tranquilizers.

This new compound was prepared by means of the outline shown in Scheme I.



Scheme I

The results of the pharmacological testing of this compound will be reported elsewhere.

EXPERIMENTAL

3-Azabicyclo[3.2.2]nonane-3-ethanol.—To 37.5 Gm. (0.3 mole) of 3-azabicyclo[3.2.2]nonane dissolved in 50 ml. of methanol was added 17.8 Gm. (0.4 mole) of ethylene oxide over a 1-hr. period. The temperature of the reaction mixture was maintained below 47° by keeping the mixture in ice water. The reaction mixture was stirred for 1 hr. and 45 min. after the completion of the addition; the methanol was then removed under reduced pressure. Distillation of the product then yielded 32.3 Gm. (60%) of 3-azabicyclo[3.2.2]nonane-3-ethanol, b.p. 94° (1.8 mm.).

Anal.—Calcd. for $C_{10}H_{19}NO$: C, 70.96; H, 11.31; N, 8.28. Found: C, 70.93; H, 11.06; N, 8.29.

2-(3-Azabicyclo[3.2.2]nonyl)ethyl Chloride Hydrochloride.—A solution of 32.3 Gm. (0.19 mole) of 3-azabicyclo[3.2.2]nonane-3-ethanol in 175 ml. of benzene was placed in a dry, three-necked flask. The solution was stirred while 29.7 Gm. (0.25 mole) of thionyl chloride in dry benzene was added dropwise. The reaction was slightly exothermic. After the addition of the thionyl chloride, the mixture was refluxed for 3 hr. and then cooled. The solid product was collected by filtration and washed with benzene to yield 35.6 Gm. (54%) of the desired product, m.p. 225° (sublimation).

Anal.—Calcd. for $C_{10}H_{18}ClN.HCl$: C, 53.50; H, 8.54; N, 6.24. Found: C, 53.42; H, 8.64; N, 6.18.

3-[2-(2-Chloro-10-phenothiazinyl)ethyl]-3-azabicyclo[3.2.2]nonane.—The hydrochloride salt, indicated above, was converted to the free base by the addition of a saturated solution of sodium carbonate until the solution was slightly basic to litmus. This basic solution was extracted with three 200-ml. portions of ether. The ether layer was dried over anhydrous sodium carbonate for 24 hr. and the free base obtained by distillation after the evaporation of the ether. To 4 Gm. (0.1 mole) of sodamide, dissolved in diethyleneglycol dimethyl ether in an atmosphere of nitrogen, was added 20.3 Gm. (0.11 mole) of 2-(3-azabicyclo[3.2.2]nonyl)ethyl chloride; then 25 Gm. (0.9 mole) of 2-chlorophenothiazine (dissolved in 125 ml. of diethyleneglycol dimethyl ether) was added. After the reaction mixture was stirred and heated for 2 hr. at 135°, it was cooled to room temperature. Filtration produced a muddy semisolid mass which liquefied on exposure to air. Small quantities of this material were dissolved in butanol and precipitated with anhydrous ether. Recrystallization from butanol–hexane yielded a white powder, m.p. 205–206°.

Anal.—Calcd. for $C_{22}H_{25}ClN_2S$: C, 62.69; H, 6.22; N, 6.25. Found: C, 62.29; H, 6.50; N, 6.47.

Received August 15, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University 38677.

Accepted for publication October 27, 1966.

Abstracted in part from an exhibit prepared by William T. King as an entry in the Mississippi Science Fair, Spring 1964.

REFERENCES

(1) Anderson, E. L., Bellinzona, G. B., Craig, P. N., Jaffe, G. E., Janeway, K. P., Kaiser, C., Lester, B. M., Nikawitz, E. J., Pavloff, A. M., Reiff, H. E., and Zirkle, C. L., *Arzneimittel-Forsch.*, **12**, 937(1962).

(2) Blanton, C. D., Jr., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 878(1962).

(3) *Ibid.*, **52**, 46(1963).

(4) *Ibid.*, **53**, 521(1964).

(5) *Ibid.*, **53**, 1130(1964).

Rapid Method of Obtaining the Clear Aqueous Filtrate of an Oil/Water Emulsion

Sir:

In most reports on experiments requiring the assay of one or both phases of an emulsion, the technique used to separate these phases involves centrifugation. Very often this technique is useless without the aid of a high-speed (or ultra-) centrifuge. Due to the nature of centrifugation, it is almost impossible to perform any short-term kinetic studies dealing with the partitioning of a drug from one phase of an emulsion to the other.

The authors have found in these laboratories that most o/w emulsions can be filtered, yielding a clear filtrate of the aqueous phase. The technique makes use of a vacuum pump, membrane filter assembly, and membrane filters, such as those manufactured by the Millipore Filter Corp., Bedford, Mass. The pore size required varies according to the droplet size of the internal phase and the interfacial tension between the drop and the external phase. For emulsions of very small particle size ($< 1\ \mu$), it was found that passing the emulsion through a .22-μ filter, and then passing the filtrate through a 100-mμ filter was adequate in most cases. It is difficult to filter large quantities, due to clogging (we assume) of the pores of the filter. This is overcome by changing the filter. Prefilters were tested, but proved ineffective.

In the filtering of preparations containing high amounts of surface-active agents, foaming occurs, but can be controlled by judicious use of the vacuum. Pressure filtration was not attempted. Emulsions (o/w) prepared from nonionic, anionic, and cationic emulsifiers were all capable of being filtered. When the per cent of internal phase was about 8–10%, clogging occurred faster than with more dilute emulsions.

The equation of Young and Laplace (1), $\Delta P = 2\gamma/r$, where ΔP is the pressure difference, inside and out of the drop, γ is the interfacial tension, and r is the radius of the droplet, can be used to determine the filtering requirements. If ΔP of the emulsion is greater than 1×10^6 dynes cm.$^{-2}$, and if the pore of the filter has a significantly smaller diameter than that of the droplet, the droplet will not be appreciably distorted, and can be filtered from the external phase.

This method is currently being employed in studying the rate of transport of a drug from the aqueous phase to an emulsified oil phase. The oil content is about 2%, with the droplet size being approximately 1.5 μ in diameter. The emulsion is sampled initially at 60-sec. intervals, and the disappearance of the drug from the aqueous phase is measured.

It was also determined that the oil phase, if external, can be obtained with the internal phase (water) being left on the filter.

This filtration method was also tested as a means of rapidly determining partition coefficients. Reese *et al.* (2) reported in 1964 a nonemulsion method for determining partition coefficients. They reported the True Partition Coefficient of benzoic acid in cyclohexane as being 1.18; the Apparent Partition Coefficient (their method) was 1.05, with 6 hr. required to be within 1% of equilibrium. Repeating their work, using an emulsion prepared by ultrasonification for 20 min., gave an Apparent Partition Coefficient of 1.27.

This method of filtering emulsions carries with it the limitations of membrane filters, *i.e.*, sorption and/or leaching of the filter materials. This may be minimized by use of the fluorinated hydrocarbon filters. It also permits assay of only the external phase, with the implicit assumption that what is not in the external phase is in the internal.

(1) Adamson, A. W., "Physical Chemistry of Surfaces," Interscience Publishers, New York, N. Y., 1963.
(2) Reese, D. R., Irwin, G. M., Dittert, L. W., Chong, C. W., and Swintosky, J. V., *J. Pharm. Sci.*, **53**, 591(1964).

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Received October 24, 1966.
Accepted for publication December 5, 1966.

This investigation was supported by a fellowship from the Eli Lilly Foundation, in part by fellowship 1-F1-GM-34,237-01 from the National Institute of General Medical Sciences, and in part by grant GM-13368-01 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

REVIEWS

Pharmaceutical Chemistry. Vol. 1: Theory and Application. Edited by L. G. CHATTEN. Marcel Dekker, Inc., 95 Madison Avenue, New York, N. Y. 10016, 1966. xii + 504 pp. Price $14.50.

This text is a contributive volume with 13 chapters by 13 different authors. The purpose of the volume, according to the preface, is "to provide a broad basis on which the senior undergraduate and graduate student can build." The editor writes that "Although it is anticipated that the student has a fundamental knowledge of chemistry, each of the chapters deals with some basic principles which will serve as a review." If the advanced student is to use this book and it is assumed that he has studied quantitative analysis and organic chemistry, in the reviewer's opinion, the text has not met these objectives.

A detailed examination of the book supports this contention. The introductory chapter introduces the concept of quality control and sampling; in addition, the subjects of mass and volume determination and laboratory techniques which are usually treated in a first course in analysis are reviewed. Although the calculation of titration curve points and titration of mixtures is treated very well, some twenty pages are used to review equilibria topics which should be familiar to students at this level (or at least they can review on their own). The chapter dealing with precipitation, complex formation, and oxidation-reduction methods is an extensive consideration of the very numerous analyses of this type with some theoretical comment. Similarly, the chapter on acidimetry and alkalimetry deals with rudimentary analytical topics, *e.g.*, methods of expressing solution concentrations, and then treats many different acid-base analyses. This type of material would be better presented in a reference rather than a textbook. Nonaqueous titrimetry is presented at too practical a level without use of topics such as glacial acetic acid equilibria. The chapter on complexometry is good, but rigorous calculations involving complex equilibria are not adequately developed. Although the chapter on alkaloidal assay is interesting, it fails, *e.g.*, to use structures and calculations to point out acid-base behavior and to employ the concept of partition coefficients to explain solvent distribution behavior. However, the use of tables to summarize the many analytical methods is excellent. The miscellaneous methods chapter is very good in its treatment of topics such as the Karl Fischer titration, gas analysis, and nitrosation. Likewise, the section on ion-exchange separations is very well done. Enough theory is presented to permit the student to make simple calculations describing column behavior. Column, thin-layer, and paper chromatography are treated well in a single chapter but certain organizational lapses are disturbing, *viz.*, partition column chromatography and two-dimensional thin-layer chromatography are introduced in sections discussing detection methods. The experiments at the end of this chapter are good. The last two chapters deal with the analysis of (*a*) fixed oils, fats and waxes, and (*b*) volatile oils. It is assumed that since the substances are complex mixtures whose analysis necessitates the use of many methods, they are used as examples. Both chapters are disappointing in that they are listings of physical and chemical procedures with some explanatory comment. The chemical methods are not adequately explained with equations and the calculations are represented by formulas which do not invite reasoning. The section on functional group analysis is not done well.

The following observations may be made in summary. The treatment in several of the chapters is too elementary. No use or mention is made of statistics, nor is enough use made of organic reaction mechanisms. Instrumental topics are introduced with little or no background (thereby requiring another book and additional expense). Not all chapters have problems and questions. If this text is to be used by advanced students, in spite of several good chapters, some good experiments, and an admirable use of literature references, it falls short.

Reviewed by Thomas Medwick
College of Pharmacy
Rutgers—The State University
Newark, N. J.

Sulfur Dioxide. Applications in Foods, Beverages and Pharmaceuticals. By LOUIS C. SCHROETER. Pergamon Press, Inc. 44-01 21st St., Long Island City, N. Y. 11101, 1966. xiv + 342 pp. 14 × 22 cm. Price $11.

"Sulfur Dioxide" is an excellent monograph containing both the applications of this commercially important compound in pharmaceuticals, foods, and beverages and theoretical reference material useful to medicinal, inorganic, analytical, and pharmaceutical chemists.

The first chapter contains a complete review of the preparation and properties of sulfur dioxide. The properties of this compound are described in a sophisticated physicochemical manner and are well referenced. An outstanding characteristic of the book is the number of references (over 1100) that are cited.

The second chapter, dealing with the oxidation of sulfur dioxide, is particularly good. Sulfite oxidation in aqueous systems is discussed in detail from a kinetic-mechanistic standpoint reflecting the author's expertness in this area. Many of the key references are to the published work of Dr. Schroeter.

Chapter three contains the inorganic reactions, complexes and clathrates of sulfurous acid species. The reactions are limited to those typical of aqueous sulfurous acid systems. The complexes and clathrates are considered in a quantitative manner.

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chapter. Of the many reactions covered, the discussions on the reactivity of sulfite with aromatic nitro compounds and with benzyl alcohols are especially thorough.

In the fifth chapter, standard quantitative and qualitative methods of detection for sulfurous acid salts are presented together with the special methods needed for sulfite estimation in foods and beverages.

In the next chapter, the food and beverage applications are discussed. The importance of understanding the chemical reactivity of sulfurous acid salts with sugars, proteins, vitamins, and the like is effectively stressed, and the use of sulfurous acid in specific food preservation is extensively covered.

"Pharmaceutical Applications" comprises chapter seven. There are essentially three major topics in this chapter: (*a*) practical aspects, (*b*) theoretical considerations, and (*c*) an extensive listing of specific applications to various drugs.

The monograph ends with a chapter on the toxicity of sulfur dioxide. Plant, human, and animal toxicity is reported.

As T. Higuchi states in the foreword, this simple compound touches us all—from the preparation of dried apricots to free radical chemistry. Under one cover, Dr. Schroeter has provided us with a reference covering all aspects of sulfur dioxide—from utility to chemical reactivity.

Reviewed by Theodore Sokoloski
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Ohio State University
Columbus

Clinical Pharmacy: A Text for Dispensing Pharmacy. By GLENN L. JENKINS, GLEN J. SPERANDIO, and CLIFTON J. LATIOLAIS. McGraw-Hill Book Co., 330 West 42nd St., New York, N.Y., 1966. viii + 379 pp. 15 × 22.5 cm. Price $11.95.

In their preface, the authors state that their text is to be a departure from the conventional text of compounding and dispensing. The use of the term "clinical" in the title implies the intent of the authors to create a text for a course in dispensing pharmacy given immediately before graduation. It indicates a modern text which will help prepare the student to practice his profession not only as a capable pharmacist today, but also as an informed pharmacist tomorrow.

It is the opinion of this reviewer, however, that "Clinical Pharmacy" falls far short of its worthy goal. Indeed, instead of being a text for advanced students, it more appropriately belongs to that textbook class suitable for the beginning pharmacy student.

In addition to the topics normally covered in a dispensing text of pharmacy, it is heartening to note the chapters on allergenic extracts, investigational drugs, clinical research, the pharmacy as a drug information center, and pharmacy service in small hospitals and nursing homes. A chapter on the ethical and moral aspects of the profession and the relationship between a pharmacist and his patron, should have been added to complete the text. While the material covered in these chapters is necessary, more emphasis could have been placed on the relationship of the community pharmacist with the activities described.

The balance of the text is a shallow treatment of the subjects normally discussed in other dispensing texts. The depth to which a senior student ought to be taken in these subjects is never achieved.

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"Pharmaceutical Applications" comprises chapter seven. There are essentially three major topics in this chapter: (*a*) practical aspects, (*b*) theoretical considerations, and (*c*) an extensive listing of specific applications to various drugs.

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As T. Higuchi states in the foreword, this simple compound touches us all—from the preparation of dried apricots to free radical chemistry. Under one cover, Dr. Schroeter has provided us with a reference covering all aspects of sulfur dioxide—from utility to chemical reactivity.

Reviewed by Theodore Sokoloski
College of Pharmacy
Ohio State University
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Clinical Pharmacy: A Text for Dispensing Pharmacy. By GLENN L. JENKINS, GLEN J. SPERANDIO, and CLIFTON J. LATIOLAIS. McGraw-Hill Book Co., 330 West 42nd St., New York, N.Y., 1966. viii + 379 pp. 15 × 22.5 cm. Price $11.95.

In their preface, the authors state that their text is to be a departure from the conventional text of compounding and dispensing. The use of the term "clinical" in the title implies the intent of the authors to create a text for a course in dispensing pharmacy given immediately before graduation. It indicates a modern text which will help prepare the student to practice his profession not only as a capable pharmacist today, but also as an informed pharmacist tomorrow.

It is the opinion of this reviewer, however, that "Clinical Pharmacy" falls far short of its worthy goal. Indeed, instead of being a text for advanced students, it more appropriately belongs to that textbook class suitable for the beginning pharmacy student.

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Distillation Products Industries
Rochester, N. Y.

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the several chapters on the "common-sense" aspects of infrared by the editor. This book is not over-all a practical manual on infrared spectroscopy nor is it an effective introductory textbook; it is instead a valuable collection of authoritative information on the practice and use of infrared spectroscopy for the non-specialist in infrared. The book is recommended as an addition to the collection of volumes on infrared spectra which should be available in the laboratory.

Reviewed by Lester Chafetz
Pharmaceutical Research and Development Laboratories
Warner-Lambert Research Institute
Morris Plains, N. J.

Food Chemicals Codex. First Edition. Prepared by the Committee on Specifications of the Food Chemicals Codex of the Food Protection Committee. National Academy of Sciences-National Research Council. National Academy of Sciences-National Research Council, 2101 Constitution Ave., N. W., Washington, D. C. 20418, 1966. xv + 846 pp. 15.5 × 23 cm. Price $25.

The "Food Chemicals Codex" promises to develop an importance comparable to the "U. S. Pharmacopeia" and the "National Formulary" as a compendium of chemical identity and specifications.

As an activity of the Food Protection Committee, the First Edition was prepared under the direction of Dr. Justin L. Powers, assisted by various expert committees. Originally published as a series of loose-leaf parts, the present first edition appears in bound form. It is the result of approximately five years of organization and development.

The "Food Chemicals Codex" is designed to provide standards for food grade chemicals, and its status in this respect is established by an endorsement appearing in each book indicating that the Food and Drug Administration will regard the specifications in the "Food Chemicals Codex" as defining an "appropriate food grade" within the meaning of the Food Additives Amendment and regulations. The "Food Chemicals Codex" will, therefore, in the future serve the food industry and its various activities in the same way that the "U. S. Pharmacopeia" and the "National Formulary" have served the drug and pharmaceutical industries.

The first edition consists of over 800 pages of text embracing 518 food chemicals. As would be expected, the makeup is analogous to that of its comparable compendia for drugs. Following the specific monographs there is a section of general tests and apparatus which the quality control chemist will find extremely useful. The policy of the "Food Chemicals Codex" is that other analytical methods with equal or greater accuracy may be used, but that only the assays and tests described therein are authoritative.

The first edition recognizes that in order to keep pace with the use of chemicals in food continuing revision will be necessary. The Governing Board of the National Academy of Sciences has approved a plan for sponsoring the Codex through its next revision, scheduled for publication in 1971. During the interim there will be revision supplements whenever necessary.

The "Food Chemicals Codex" should be available to every chemist who has responsibility for the manufacture, distribution, and quality control of chemicals which fall under the general category of food additive chemicals. It will serve as a valuable reference and text in schools and libraries.

Reviewed by L. W. Hazleton
Hazleton Laboratories, Inc.
Falls Church, Va.

British National Formulary 1966. Published jointly by the British Medical Association and the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17 Bloomsbury Square, London W.C.1, England, 1966. 356 pp. 11 × 17.5 cm. Price $1.75.

The "British National Formulary" now is published in only one edition which combines the essential features of the former Standard and Alternative editions in order to retain the information required by both prescribers and pharmacists. Monographs which formerly followed the Notes for Prescribers are replaced with a listing of preparations and page references to the Classified Formulary section of the book.

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Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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VOLUME 56

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JANUARY TO DECEMBER, 1967

JOURNAL OF **Pharmaceutical Sciences**

March 1967 volume 56, number 3

Review Article

Diffusional Models Useful in Biopharmaceutics

Drug Release Rate Processes

By WILLIAM I. HIGUCHI

THE EVOLUTION of a given body of knowledge from a catalog of apparently unrelated facts to an exact science at the molecular level generally follows a path in which somewhere along the way the consideration of quantifiable *physical* models becomes necessary. Such models, when they enter upon the scene, play many roles in the course of the development of the area of knowledge. Initially they serve to provide the primitive quantitative interrelationships among the variables that considered intuition alone had been unable to do. From these, suitable *in vitro, in situ,* or *in vivo* experiments are designed. With refinement of the models and with continued experimentation, knowledge is efficiently built up to a rather sophisticated level.

This review is intended to cover some of those stiuations in biopharmaceutics where physical models involving drug transport have materially helped or are helping in answering complex questions. Some situations are also discussed where such methods have not yet been applied but are indicated. Attention is given only to those problems in which physical models have been applied in contrast to those where empirical or arbitrary mathematical kinetic models have been employed.

DISSOLUTION RATES OF SOLIDS

General Considerations—Wurster and Taylor (1) have already presented a comprehensive review of this subject. Wagner (2), in an earlier review article, also discussed this subject and related it to biological availability. The readers are referred to both of these excellent reviews which serve to provide more than adequate background for the present discussion.

From the standpoint of rate mechanisms involving pure substances there are basically three processes that have been accessible to the physical model treatment. These have been employed alone or in combinations in setting up the models for describing dissolution rate mechanisms. Figure 1 schematically illustrates those cases when each of these alone is rate determining.

Case *A* represents the diffusion layer showing a crystal or a polycrystalline solid dissolving into pure solvent. This model in its earliest

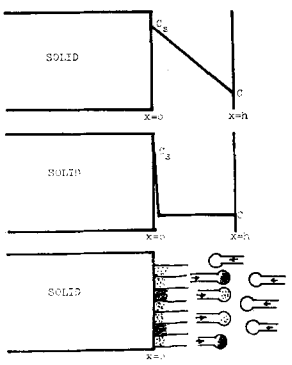

Fig. 1—Illustration of the situations for the three mechanisms of dissolution rate behavior of solids. Concentration profiles are shown for cases A *and* B, *and packet transport is depicted for case* C. *Case* A, *diffusion layer model (top); case* B, *interfacial barrier model (middle); case* C, *Danckwerts' model (bottom).*

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form was probably first discussed by Nernst (3). Here it is assumed that there is a liquid layer ("film") of thickness, h, in which there is a negligible velocity component in the x-direction (perpendicular to the surface). At $x > h$, it is assumed that rapid mixing is present. Therefore, no gradients of concentration may exist in this region. At $x = 0$ (the solid–liquid interface) it is assumed that solid–solution equilibrium exists. Then the rate of solute movement and therefore the dissolution rate is determined entirely by Brownian motion diffusion of the molecules in the liquid "film" ($x = 0$ to $x = h$).

Case B illustrates the interfacial barrier model where, because of a high activation free energy for the interfacial transport step, diffusion across the interface is much slower than diffusion across the "film" or transport by eddy packets to be discussed below. As a result, crystal–solution equilibrium at $x = 0$ may not be assumed, and this consideration must be included in the model.

Finally, we have case C representing the Danckwerts' model (4), where one imagines macroscopic packets of solvent reaching the solid–liquid interface by eddy diffusion in some random fashion. During its residence at the interface the packet is able to absorb solute according to the usual laws of diffusion. These surface elements are continuously replaced by new packets of solvent. This surface renewal process may then be related to the solute transport rate.

Rate Laws Predicted by the Different Mechanisms—Let us consider the situation where a one component, one phase, solid of macroscopic ($\gtrsim 1$ mm.) dimensions and of low to moderate ($\simeq 5\%$) solubility dissolves without disintegration and without chemical reaction into a solvent under mild to high agitation conditions. Then the dissolution rate per unit area, G, according to the diffusion layer model for this case will be

$$G = \frac{D}{h}(C_s - C) \qquad \text{(Eq. 1)}$$

where D is the solute molecule diffusion coefficient, C_s is the solubility, C is the solute concentration, and h as defined in Fig. 1 is the effective diffusion layer ("film") thickness.

The Danckwerts' model for this case gives

$$G = S^{1/2}D^{1/2}(C_s - C) \qquad \text{(Eq. 2)}$$

where S is the mean rate of production of fresh surface.

When an interfacial barrier is important, it is much more difficult to derive a relationship for G in physically significant terms. First of all, the true (or microscopic) surface area rather than the geometric external area (of a tablet) should be considered. Furthermore, the different faces of a crystal should have different interfacial barriers. However, one often writes for this case,

$$G = k_i(C_s - C) \qquad \text{(Eq. 3)}$$

where k_i is the effective interfacial transport rate constant. The first-order dependence on $(C_s - C)$ implies that the process is a unimolecular one. This is reasonable as one would expect that the step movement rate on a crystal surface would be usually unimolecular. However, perhaps at large undersaturations cooperative molecular effects may become important. More experimental studies are needed before the $(C_s - C)$ part of Eq. 3 can be regarded as the usually expected form in a surface controlled situation. For the present we shall take Eq. 3 as the best at the moment to represent this case.

When the interfacial barrier concept is combined with the diffusion layer case we have a double-barrier model

$$G = \frac{D(C_s - C)}{h\left[1 + \frac{D}{hk_i}\right]} \qquad \text{(Eq. 4)}$$

which reduces to Eqs. 1 or 3, respectively, in the limits, $k_i \gg D/h$ and $k_i \ll D/h$.

When the interfacial barrier idea is combined with Danckwerts' surface renewal mechanism we have

$$G = \frac{S^{1/2}D^{1/2}(C_s - C)}{\left[1 + \frac{S^{1/2}D^{1/2}}{k_i}\right]} \qquad \text{(Eq. 5)}$$

which reduces to Eqs. 2 or 3 in the limits.

The diffusion layer and the Danckwerts theories may also be combined (5, 6). Surface elements of a finite length, L, in the x-direction must be taken. This combination leads to expressions which reduce to Eqs. 1 and 2, respectively, in the limits, $L \to 0$ and $L \to \infty$.

Experimental Approach to an Examination of the Theories for One Component—One Phase Case—The reviewer believes[1] that there has been relatively convincing experimental work showing which theory is applicable and under what conditions. Part of this stems from the fact that the researchers frequently have been more interested in establishing other aspects than in differentiating the three basic models described above.

[1] Readers are referred to Wurster and Taylor's discussion (1). (See also *References 7* and *8*.)

The classical Noyes-Whitney law (9), which states that

$$G = \text{constant } x(C_s - C) \qquad \text{(Eq. 6)}$$

really, by itself, reveals very little mechanistically when a single substance dissolves in a pure solvent under constant hydrodynamic conditions. This is because all theories predict the same first-order dependence upon $\Delta C = C_s - C$.

When the dissolution behavior of many substances in the same solvent is compared under the same hydrodynamic conditions, it may be possible to separate those which follow Eqs. 1 or 2 from those following Eqs. 3, 4, or 5. If, furthermore, compounds with a sufficiently wide range of D values are included in the study while maintaining everything else but C_s constant, one should be able to distinguish data conforming to Eq. 1 from those following Eq. 2 or the diffusion layer–Danckwerts combination theory (6). The reviewer is not aware of work on this latter aspect that has led to unambiguous results. The work of Desai *et al.* (10), to be discussed later, probably provides a more promising approach to this problem.

Finally by varying the rate of agitation one should be able to distinguish between those following Eq. 3 from those following Eqs. 4 or 5, provided that sufficient data are available with other substances obeying Eqs. 1 or 2 under the same hydrodynamic conditions. Data obeying Eqs. 4 and 5 may then be distinguished by employing the method used for Eqs. 1 and 2.

Wurster and Taylor (11) in their studies of the dissolution rate behavior of prednisolone employed Eq. 4 and Eq. 1. These investigators suggested that the double-barrier mechanism (Eq. 4) better described their data on the influence of agitation upon the dissolution rates. Some recent single crystal studies by Tawashi (12) with cholesterol monohydrate and by Mehta (13) with methylprednisolone show that under certain conditions single crystals of these substances of about 100-μ dimensions dissolve by some interfacially controlled process in aqueous media.

Small Particle Problems—Goyan (14) derived a relationship for the dissolution rate of a small spherical particle involving the surface renewal mechanism. In one form it may be written

$$G = \left[\frac{D}{a} + S^{1/2}D^{1/2}\right](C_s - C) \qquad \text{(Eq. 7)}$$

where a is the particle radius (equivalent volume sphere) and the other symbols have been defined. Goyan has suggested that Eq. 7 rather than Eq. 1 better describes the data (15) on the dissolution rate of salicylamide particles in water at high agitation rates.

Equation 7 nicely reduces to the purely diffusion controlled case when $S \to 0$ or when $a \to 0$, *viz.*,

$$G = \frac{D}{a}(C_s - C) \qquad \text{(Eq. 8)}$$

It is noteworthy (compare Eq. 8 with Eq. 1) that, for small particles, $h \simeq a$ even with *no* agitation.

For micron size particles at low to moderate agitation rates, Eq. 8 should be applicable if interfacial barrier effects are absent. The dissolution rate behavior of dibutyl phthalate droplets in water agreed very well with this theory (16).

If interfacial barrier effects are not negligible, a double-barrier relation may be written,

$$G = \frac{D}{a}\left[\frac{C_s - C}{1 + \dfrac{D}{ak_i}}\right] \qquad \text{(Eq. 9)}$$

which is identical to Eq. 4 when $a = h$.

Particle size distribution effects may be taken into account by selecting a particle size distribution function (17, 18) that is time dependent and by combining it with the appropriate Eqs. 7, 8, or 9. For example, if the particle size distribution may be expressed by the function $f(a)$, where a is a function of time and where $f(a)da$ is the number of particles between the sizes a and $a + da$, we may write for the total dissolution rate, J, of the suspension,

$$J(t) = \int_{a_s}^{a_e} f(a)G(a)da \qquad \text{(Eq. 10)}$$

where a_s and a_e are, respectively, the smallest and the largest particles in the distribution. If $f(a)$ is known by a particle size distribution measurement, then Eqs. 7, 8, or 9 may be employed for G to provide an equation for J explicit in time. This procedure was used employing Eq. 8 to analyze data on the dissolution rate of a steroid suspension in water (18).

Dissolution Rates with Simultaneous Solution Interactions Involving Additives in the Solvent—In many practical situations the dissolving drug may become involved in an acid–base reaction, in complex formation, or in some other kind of solubilization interaction. In these instances the three basic models (Fig. 1) still apply in a qualitative sense. However, detailed consideration of the move-

ment of all solution species becomes necessary, resulting in equations that are generally different from those for the one component problem.

As an illustrative example, let us choose the case where the dissolving molecule, A, reacts with an additive molecule, B, present in the solvent to form a complex, AB, according to $A + B = A \cdot B$, with

$$K = \frac{(A \cdot B)}{(A)(B)} \qquad \text{(Eq. 11)}$$

Assume that equilibrium is rapid compared to diffusion over distances of importance ("film" thickness, packet dimensions, etc.). This is a reasonable assumption for most acid–base reactions and for most interactions leading to complex formation. Assume also that no new phases may precipitate. Then the diffusion layer theory gives for this situation (19–21)

$$G = \frac{D_A}{h}\left[1 + \frac{D_B B_h}{D_A\left(A_0 + \frac{D_B}{D_{AB}K}\right)}\right](A_0 - A_h) \qquad \text{(Eq. 12)}$$

D_A, D_B, and D_{AB} are the diffusion coefficients for A, B, and AB. A_0 and A_h are the concentrations of A at $x = 0$ and $x = h$. B_h is the concentration of B in the solvent ($x \geq h$).

Physically the situation regarding the species leading to Eq. 12 may be described as follows. Species A is diffusing from $x = 0$, where $A_0 =$ solubility of A, to $x = h$, where $A = A_h$. Species B diffuses in the opposite direction from $x = h$, where $B = B_h$, to $x = 0$. Along the way B complexes with A according to Eq. 11 and the complex $A \cdot B$ diffuses out along with free A in the same direction as A. The net effect is a "facilitation" of the transport of A into solution, resulting in the factor

$$\left[1 + \frac{D_B B_h}{D_A\left(A_0 + \frac{D_B}{D_{AB}K}\right)}\right]$$

multiplying the rate given by Eq. 1.

The Danckwerts theory does not give differential equations that can be solved analytically for the situation (19) involving this equilibrium between A, B, and $A \cdot B$. However, Olander has solved the special case, when $D_A = D_B = D_{A \cdot B} = D$, in which

$$G = S^{1/2}D^{1/2}\left[1 + \frac{B_h}{A_0 + \frac{1}{K}}\right](A_0 - A_h) \qquad \text{(Eq. 13)}$$

It is noteworthy that, when $D_A = D_B = D_{A \cdot B} = D$, the diffusion layer theory (Eq. 12) reduces to

$$G = \frac{D}{h}\left[1 + \frac{B_h}{A_0 + \frac{1}{K}}\right](A_0 - A_h) \qquad \text{(Eq. 14)}$$

As Olander points out (20), Eqs. 13 and 14 are identical except for the dependence on D and the hydrodynamics. It is further noteworthy that both Eqs. 13 and 14 reduce to Eqs. 1 and 2, respectively, if one notes that

$$(C_s - C) = (A_0 - A_h)\left[1 + \frac{B_h}{A_0 + \frac{1}{K}}\right] \qquad \text{(Eq. 15)}$$

where $C_s - C$ is the "total" concentration differential for A between the solid–solvent interface and the bulk solvent.

It follows from the above that, when all the diffusion coefficients are equal, the Noyes-Whitney law should apply regardless of whether the diffusion layer theory or the Danckwerts theory is appropriate. These conclusions are not restricted to the reaction, $A + B = A \cdot B$. Situations involving other kinds of equilibria have also been worked out (19–21). Because the diffusion coefficients of molecules and ions vary approximately as the cube root of the molecular weight, the Noyes-Whitney law should be frequently applicable as long as interfacial barrier effects or the precipitation of new phases are absent. Extensive experimental studies by Nelson (22, 23) and by Hamlin *et al.* (24) substantiate this.

Situations where significant deviations from the Noyes-Whitney law should occur, except when phase changes take place, are those involving either very small or very large diffusion coefficients. A case in point is the dissolution rate behavior of a basic amine drug in HCl solutions (25). Here the large diffusion coefficient of the HCl apparently caused the dissolution rate to be as much as 3 times greater than the Noyes-Whitney law predictions.

The studies by Desai *et al.* (10) on the initial dissolution rate behavior of benzocaine in aqueous polysorbate 80[2] solutions are particularly noteworthy. Their experiments carried out under constant hydrodynamic conditions provided data that could be used to simultaneously test the diffusion layer theory, the Danckwerts theory, and the Noyes-Whitney law. For this situation the diffusion layer theory gives the following equation (10, 26)

$$G = \frac{D}{h}C_s + \frac{D^1}{h}C^1_s \qquad \text{(Eq. 16)}$$

where D^1 is the diffusion coefficient of the micelle-

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

solubilized drug and C_s^1 is the solubility increase due to solubilization. All other terms are the same as in Eq. 1.

In contrast to Eq. 16, Danckwerts theory (10) leads to the following equation for the dissolution rate of benzocaine in polysorbate solutions.

$$G = S^{1/2} \times \sqrt{(C_s + C^1_s)(DC_s + D^1C^1_s)} \quad \text{(Eq. 17)}$$

The symbols have the same meanings as before.

Finally, according to the Noyes-Whitney law, we have for this situation

$$G = \text{constant } x(C_s + C^1_s) \quad \text{(Eq. 18)}$$

The comparison of the experimental data with Eqs. 16, 17, and 18 is shown in Fig. 2. Clearly, in this example, the diffusion layer theory agrees best with the experimental data. Both Eqs. 17 and 18 show discrepancies with data that are far greater than the scatter of the data.

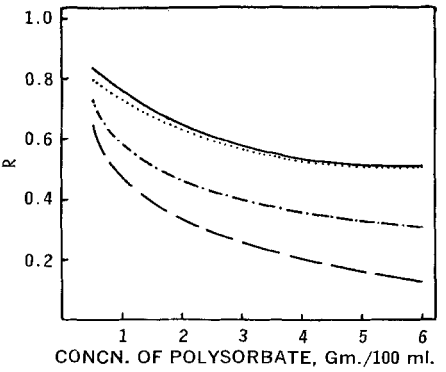


Fig. 2—Data and theory on the influence of polysorbate 80 solubilization on the dissolution rate behavior of benzocaine. R is ratio of rate in polysorbate solution to that without polysorbate (from Desai et al.). *Key: ———, experimental; · · · · · · · , diffusion layer theory; – · – · –, Danckwerts theory; – – – –, Noyes-Whitney theory.*

The reviewer believes that studies by Desai *et al.* represent the first clear-cut demonstration of mechanism differentiation. This technique should have extensive application in the future—particularly in studying the effects of hydrodynamic factors on mechanisms.

Some Comments on the Influence of Additives at the Interface—An interfacially adsorbing agent may help in increasing the dissolution rate. A surfactant may improve the wetting of the surface and effectively increase the available area (27, 28). It may also increase the interfacial rate constant k_i (Eq. 3) by assisting in the release of surface molecules through interfacial tension lowering.

Surface-active agents may play other roles. For example, the formation of a surface adsorbed layer of aliphatic long chain ammonium ions retards the dissolution rate of apatite crystals (29). Also it is expected on theoretical grounds (30) that for very water insoluble drugs ($C_s \lesssim 10^{-8}$ Gm. ml.$^{-1}$) the intrinsic rate of micellar solubilization of a drug may be very low, and the dissolution rate may be much slower than that predicted by Eq. 16. This might occur if, for example, the surfactant micelles have the same charge as the crystal surface (due to, say, the adsorbed surfactant), and the close approach of the micelle to the surface is improbable.

Dissolution Rates Involving Simultaneous Phase Changes—There have been a number of reports in the literature (31, 32) where the formation of a new surface phase was noted during the dissolution of the drug. Invariably the formation of such phases leads to slower dissolution rates than if it did not occur.

The physical model approach involving the consideration of simultaneous diffusion, chemical equilibria, and new phase precipitation has now been employed in a number of instances. The dissolution rate behavior in phosphate buffers of the sodium salt of a relatively water-insoluble weak acid drug was analyzed (33) by this technique. The data were found to be consistent with the model in which a surface coating of the weak acid was formed under certain conditions.

The pamoate salt of an amine drug forms a coating of pamoic acid during its dissolution in HCl solutions (32). Assuming that the diffusion of the protonated amine through this coating is rate determining, a model was constructed and compared to data (34).

The dissolution rate behavior of apatites is important because of its relation to bone and tooth mineral dynamics (35, 36). The possibility of dicalcium phosphate surface phase deposition during apatite dissolution in aqueous lactate buffers was considered (35). More recently (37, 38) the influence of the fluoride ion on the dissolution rate behavior of apatites has been examined by means of models involving surface phases of fluorapatite and CaF_2. The questions of what effects phosphate, calcium, and fluoride ions and pH have on these mechanisms are being answered by these models and the appropriate experiments.

Recently Bernardo (39) examined the problem of the anomalous dissolution rate behavior (40) of the polymorphs of methylprednisolone. A model was considered in which it was assumed that simultaneous surface reversion of polymorph II (the higher energy one) to polymorph I

occurred during the dissolution of polymorph II. This led to equations which were consistent with the experimental observation that at high stirring rates the dissolution rates for forms I and II were indistinguishable.

Dissolution Rates from Mixtures—Recently a mathematical analysis based on a physical model was presented (41) to describe the dissolution rate behavior of polyphase mixtures. For a two phase mixture in which the two components, A and B, do not interact in any way with each other, we may describe the model in the following way. Upon exposure to solvent, both components of the mixture should tend to dissolve at rates proportional to their solubilities and their diffusion coefficients. After some time, usually one of the phases would become depleted at the solid–liquid interface region because N_A/N_B may not be equal to $(D_A C_{SA})/(D_B C_{SB})$, where N_A and N_B are the original amounts of A and B in the mixture, D_A and D_B are the diffusion coefficients, and C_{SA} and C_{SB} are the respective solubilities. As a result, a surface layer is formed that is composed of only one of the phases. The three possible situations after zero time are illustrated in Fig. 3 for the one-dimensional, two-phase mixture problem.

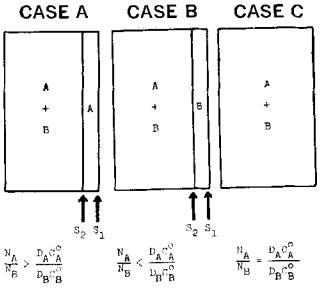


Fig. 3—Dissolution behavior of two-phase mixture of A *and* B. *In case* A, *phase* B *dissolves fast enough to leave a layer of pure* A *behind; in case* B, *the reverse is true; while in case* C, *dissolution rates of* A *and* B *are proportional to their relative amounts in the mixture.*

The following dissolution rate equations based on this model were derived[3] for each of the situations given in Fig. 3.

When

$$N_A/N_B > (D_A C_{SA})/(D_B C_{SB}),$$

$$\left.\begin{aligned} G_A &= \frac{D_A C_{SA}}{h} \\ \text{and} \\ G_B &= \frac{N_B}{N_A} G_A \end{aligned}\right\} \quad \text{(Eq. 19)}$$

When

$$N_A/N_B < (D_A C_{SA})/(D_B C_{SB}),$$

$$\left.\begin{aligned} G_B &= \frac{D_B C_{SB}}{h} \\ \text{and} \\ G_A &= \frac{N_A}{N_B} G_B \end{aligned}\right\} \quad \text{(Eq. 20)}$$

When

$$N_A/N_B = (D_A C_{SA})/(D_B C_{SB}),$$

$$\left.\begin{aligned} G_A &= \frac{D_A C_{SA}}{h} \\ \text{and} \\ G_B &= \frac{D_B C_{SB}}{h} \end{aligned}\right\} \quad \text{(Eq. 21)}$$

Equations 19–21 were applied (41) to the data on benzoic acid–salicylic acid tablet mixtures dissolving in water. Excellent agreement between data and theory was found in this case.

The above equations have a restriction in that they are quantitatively applicable in the steady state only. This requires that the C_{SA} and C_{SB} do not differ too greatly, say no more than a factor of 100 if, for example, the tablet thickness is on the order of millimeters.

If one of the components is very much less soluble than the other, *i.e.*, if either $C_{SA}/C_{SB} \to 0$ or ∞, then the problem reduces to that of solute release from an inert matrix which is discussed under *Drug Release Out of Matrix Systems*. The intermediate ranges have been treated also (41).

This two-phase model has been extended (41) to the case in which the components, A and B, may interact to form a complex, $A \cdot B$, in solution. The resulting equations for this case very nicely agreed with data on the dissolution rate behavior of caffeine–benzocaine mixtures in water. The same basic model was used (39) to derive equations that accurately described the dissolution rate behavior of mixtures of different crystalline forms of drugs. Experimental data with sulfathiazole forms I and II and with methylprednisolone polymorphs agreed well with the theory. The model was also employed in the re-examination of Nelson's data (42) on the dissolution rate behavior of benzoic acid–trisodium phosphate mixtures in water.

The simple nature of this model should allow extension to mixture problems involving more than two phases.

DRUG RELEASE OUT OF MATRIX SYSTEMS

General Considerations—This portion of the

[3] While the diffusion layer theory was used to express the dissolution rate behavior of the surface phases in Eqs. 19–21, the Danckwerts theory could have been used instead.

review[4] is concerned with drug release problems in which the external geometry of the dosage form remains essentially unchanged during drug release. Therefore, cases in this category include drug release from ointments, nondisintegrating tableted matrices, and perhaps from injections of the depot type. The discussion is directed toward the recent *in vitro* type work where attempts have been primarily made to understand the roles played by those factors associated with the dosage form itself. The question of whether conclusions from studies such as these truly reflect the *in vivo* availability is not under scrutiny here.

Special mention should be made that the most significant applications of the physical model approach in this area in recent times are those presented in the reports by T. Higuchi (44–47). These studies form the basis for much of the discussion here. They are also reflected in much of the thinking and research being done today on diffusion controlled release of drugs.

Diffusion Controlled Solute Release Obeying Fick's Law—When Fick's law is obeyed, the diffusion coefficient, D, is constant. For the one-dimensional diffusion of a single solute, we may write

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \text{(Eq. 22)}$$

Here C is the solute concentration at time t and at position x.

Various mathematical solutions to Eq. 22 have provided useful quantitative methods for studying the drug release problem. For planar diffusion to a perfect sink at $x = 0$ from a region, $0 \angle x \angle h$, at a uniform initial ($t = 0$) concentration of C_0, the solution to Eq. 22 is (45, 48–50)

$$Q = hC_0\left[1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty} \times \frac{1}{(2m+1)^2}\exp.\left(\frac{-D(2m+1)^2\pi^2 t}{4h^2}\right)\right] \qquad \text{(Eq. 23)}$$

where Q = amount of drug released to the sink per unit area. It can be shown (51) that Eq. 23 may be approximated rather well by Eq. 24 up to about 30 to 50% drug release,

$$Q = 2C_0\left(\frac{Dt}{\pi}\right)^{1/2} \qquad \text{(Eq. 24)}$$

where Eq. 24 is the solution to the case for h at infinity.

Figure 4 shows how the drug concentration profiles may change with time in the region, $0 < x < h$. It is noteworthy that the slopes of these curves at $x = 0$ give the rate of release at the various times, *i.e.*,

$$\text{rate} = \frac{dQ}{dt} = D\left(\frac{dC}{dx}\right)_{x=0} \qquad \text{(Eq. 25)}$$

and may be obtained by simply differentiating Eqs. 23 or 24 with times.

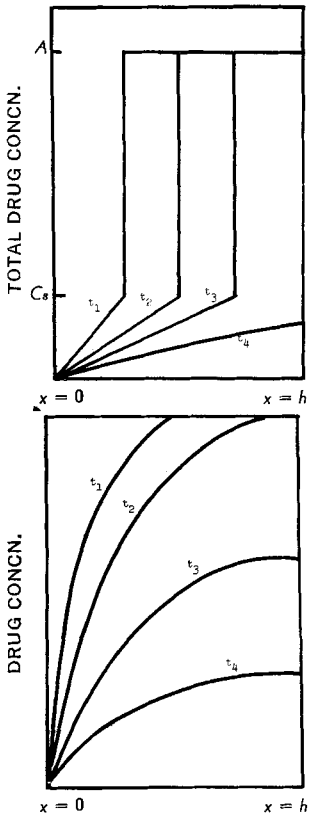


Fig. 4—Concentration profiles for the two situations during drug release to a perfect sink. Solid in matrix (top); solution in matrix (bottom).

It can be seen that if D is known we may easily predict the release rate behavior by means of Eqs. 23–25. While D may always be determined experimentally, it is of interest from the mechanism standpoint to consider the various theoretical methods for predicting D for the different situations.

First of all, there is the well known Stokes-Einstein relation,

$$D = \frac{kT}{6\pi na} \qquad \text{(Eq. 26)}$$

where k is the Boltzmann constant, T is temperature, n is the viscosity of the matrix, and a is the hydrodynamic radius of the diffusing drug molecule. This equation is practically quantitatively applicable when the matrix is composed

[4] The readers are referred to an earlier review (43) on this subject for background.

of solvent molecules that are comparable to or smaller than the diffusing drug molecules so that the microscopic viscosity is close to the macroscopic viscosity. The case when the situation is reversed, *i.e.*, drug diffusion out of polymer solutions or gels, will be discussed later.

The meaning of D when the matrix is heterogeneous has received much attention recently (51–57). A number of approximate relationships have been considered and compared with experimental data.

When the matrix is a suspension or an emulsion, the effective diffusion coefficient, D_e, must be used instead of D in Eqs. 23 and 24. D_e may be obtained from mixture formulas based on analogous electrostatic situations (52, 58–61). Equations 27 and 28 are, respectively, the Bruggeman (59) and the Maxwell-Rayleigh-Lorentz-Clausius-Mosotti-Wagner-Wiener relations for the effective permeability constant, P_e, for a two-phase system consisting of an internal phase of spherical particles dispersed in a continuous phase.

$$\left(\frac{P_e - P_i}{P_c - P_i}\right)\left(\frac{P_c}{P_e}\right)^{1/3} = V_c \qquad \text{(Eq. 27)}$$

and

$$\frac{P_e - P_c}{P_e + 2P_c} = \left(\frac{P_i - P_c}{P_i + 2P_c}\right) V_i \qquad \text{(Eq. 28)}$$

Here the P's are the permeability constants and the V's are the volume fractions of the phases. The subscripts i and c refer to the internal phase and the continuous phase, respectively. Equations 27 and 28 are the two most popular mixture formulas discussed in the literature. Other formulas are available (52), including those which take into account shape effects and the effects of a "coating" of the internal phase. Higuchi (52) considered a semiquantitative mixture relationship that correlated well with literature data.

Now D_e may be calculated (52, 53) with either Eqs. 27 or 28 if we note further that

$$D_e = \frac{P_e}{K_e} \qquad \text{(Eq. 29)}$$

and

$$K_e = K_i V_i + V_c \qquad \text{(Eq. 30)}$$

where K_i is the partition coefficient of the solute between the internal and the external phases.

Koizumi (53) has tested Eqs. 23 and 24 and 27–30 with data on pyridine release from water-in-oil emulsions into an aqueous sink. Agreement of both Eqs. 27 and 28 with data was satisfactory. However, Koizumi's more critical tests were carried out with solute mixtures of pyridine and pyridine hydrochloride to be discussed later.

Higuchi analyzed data on drug release from oil-in-water emulsions. Good consistency of the data (62) with Eqs. 23 and 24 and with the Higuchi mixture formula (52) was found in this instance.

Drug release from inert plastic matrices initially saturated with a drug solution of concentration, C_0, may follow equations similar to Eqs. 23 and 24. Here, for example, instead of Eq. 24 we may write (54)

$$Q = 2\epsilon C_0 \left(\frac{Dt}{\tau\pi}\right)^{1/2} \qquad \text{(Eq. 31)}$$

where $\epsilon \equiv V_c$ = porosity, and τ = tortuosity. The quantity τ may be calculated by means of Eqs. 27 or 28 if the internal phase particles are spherical and impermeable. Thus, if $V_i = V_c = 0.5$, we get $P_e = 0.35P_c$ and $\tau = 1.43$, according to Eq. 27, and $P_e = 0.40P_c$ and $\tau = 1.25$ according to Eq. 28. The Higuchi mixture formula (52) gives $P_e = 0.35P_c$ and $\tau = 1.43$ for this situation.

Desai *et al.* (55) employing Eq. 31 found experimental τ values mainly in the range of 1.3 to 3.0 for polyvinyl chloride compressed tablet matrices when $V_i \simeq 0.60$ to 0.70. This is in very good agreement with the expected values of about 1.4 to 2.0 according to Eqs. 27, 28, and the Higuchi formula (52). When polyethylene plastic tablet matrices were used (55),[5] higher τ values of 6 to 10 were found. This difference in general behavior has been explained (55) on the basis of the much greater plasticity of the polyethylene and the much greater elasticity of the polyvinyl chloride influencing the structures of their respective matrices when the materials are subjected to compaction.

It is worthwhile to point out that both Eqs. 27 and 28 lead to Eq. 32 when V_i is small.

$$D_e = \frac{D_1}{V_c + K_i V_i}\left[1 + 3V_i \frac{K_i D_i - D_c}{K_i D_i + 2D_c}\right] \qquad \text{(Eq. 32)}$$

This equation is not only useful in estimating accurate values of D_e for dilute suspensions and emulsions but also when the matrix is a dilute polymer solution or a gel.

If a gel forming polymer does not bind the drug but acts only to provide some "mechanical" resistance to drug diffusion, Eq. 22 becomes

$$D_e = \frac{D_1}{V_c}\left(1 - \frac{3}{2} V_i\right) \qquad \text{(Eq. 33)}$$

Equation 33 appears to accurately describe (63)

[5] See also Desai, S. J., Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1966.

the diffusion of sodium and cesium ions in aqueous agar gels.

Suppose a viscous but dilute polymer solution is the matrix with the polymer binding the drug according to a linear law, *i.e.*,

$$M = k_0 C_c \qquad \text{(Eq. 34)}$$

where M = amount of drug bound per unit volume of polymer, C_c = concentration of the drug in the "continuous" phase, and k_0 = constant. Then Eq. 32 should be used with $D_i = 0$ and

$$K_i = \frac{M}{C_c} \qquad \text{(Eq. 35)}$$

If the binding does not obey Eq. 34, then Fick's law will not be obeyed and other methods must be considered. (See under *Diffusion Controlled Release Not Obeying Fick's Law.*)

If a suspension type matrix with $D_i = 0$ is able to adsorb drug according to a linear relation similar to Eq. 34, then equations analogous to 34 and 35 may be used (43) with Eqs. 32, 27, or 28 and Q calculated with Eqs. 23 or 24. Again if the adsorption isotherm is not linear, then the mathematics cannot be handled analytically and numerical methods become necessary. (See under *Diffusion Controlled Release Not Obeying Fick's Law.*)

In all of the preceding discussion involving Eqs. 23 and 24 and the mixture relations, Eqs. 27–30, we have assumed that local equilibrium (52) is maintained, *i.e.*, partitioning or binding occurs rapidly. If local equilibrium is not maintained the equations may not be correct. In this connection, Koizumi (64) has theoretically treated the case where the rate of oil–water interfacial transport becomes important in influencing the rate of drug release from emulsions. It appears safe to state (52) that in most situations the assumption of local equilibrium will be a good one for the small particle sizes normally involved in most emulsions.

Diffusion Controlled Release Not Obeying Fick's Law—For many situations in drug release rate problems, Eq. 22 alone cannot describe the process. In the following we shall consider some of those cases that have been studied.

Diffusion controlled drug release when the drug is dispersed as a solid in a matrix was first studied by Higuchi (45–47). If the matrix is a homogeneous liquid, the one-dimensional theory (planar release) gives

$$Q = \sqrt{D(2A - C_s)C_s t} \qquad \text{(Eq. 36)}$$

where D is the drug molecule diffusion coefficient in the matrix, A is the total amount of drug present in the matrix per unit volume, and C_s is the solubility of the drug in the matrix substance.

If the matrix is heterogeneous and diffusion takes place in the intergranular pores, *e. g.*, drug dispersed in an inert plastic matrix with aqueous pores, instead of Eq. 36 we get

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 37)}$$

where D is the diffusion coefficient in the solvent, ϵ = porosity of the matrix, and τ, as before, is the tortuosity.

Figure 4 shows the drug concentration profiles in the matrix at different times in accordance with the theory leading to Eqs. 36 and 37. The concentration gradients from the solid-drug boundary ($x = s$) to the matrix boundary ($x = 0$) are linear as long as $2A \gg C_s$ (for Eq. 36) or $\epsilon C_s \ll 2A$ (for Eq. 37) which is the key assumption in the derivation of these two equations.

Desai *et al.* (54, 55) and Singh *et al.* (56) have made extensive experimental and further theoretical studies of Eq. 37. In their approach several different plastics and several drugs were involved. They independently determined the appropriate D (diffusion cell experiments), the solubility C_s, the appropriate ϵ (two ways), and τ by an independent experiment with the same matrix utilizing Eq. 31. Under the expected conditions (good penetration of the pores and when $\epsilon C_s \ll 2A$) Eq. 37 agreed quantitatively with the experimental data over a wide range of variables.

These investigators also showed (55) that in some instances (polyethylene matrices without surfactant in the solvent) wetting may be poor and therefore the effective τ values may be in the tens of thousands. More recent studies (56) show that in these cases the diffusion controlled model probably fails, and the release rate is determined by the channel penetration rate of the solvent. In other instances (polyvinyl chloride matrices in water) wetting was efficient, but air removal from the matrix significantly altered the drug release pattern.

Desai *et al.* (55) have extended Eq. 37 to take into account the hydrodynamic flow of the solvent in the pores. This refinement was necessary to explain the behavior of moderately soluble solutes.

These investigators have also further extended the same model taking into account the effect of a concentration dependent D and the possibility of linear binding of the drug onto the plastic during the release of drug.

Very recently Singh *et al.* (56) extended the theory to describe the release from mixtures of drugs in an inert matrix. They have derived equations which also take into account the possibility of complex formation between the

components of the phases. The theory is being used to evaluate the data on mixtures currently being gathered in these laboratories.

A very important situation where Eq. 22 fails is in the release of drugs when the partition coefficient, K_i (see Eqs. 30 and 35), is not constant but is dependent on the drug concentration. K_i may vary with drug concentration if (*a*) the drug is a weak acid or a weak base and therefore K_i would be pH dependent, (*b*) the drug molecule associates as carboxylic acids do in mineral oil, or (*c*) the drug binds or complexes according to some nonlinear law, *e.g.*, Langmuir binding, to some component in the system.

Koizumi (53) has investigated the theoretical and experimental methods for handling this situation. By applying Boltzmann's method (48, 49) and employing Eqs. 27 and 28, he numerically computed the drug release rates from emulsions into aqueous sinks when Eq. 37

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial C}{\partial x}\right) \qquad \text{(Eq. 37)}$$

rather than Eq. 22 was obeyed. The theoretical predictions agreed very well with experimental data on the release of solute from pyridine–pyridine hydrochloride solute mixtures in water-in-oil emulsions. As expected, the Bruggeman Eq. 27 agreed better with the data than Eq. 26 over a wide range of the solute mixture ratio. The agreement of theory with data was particularly significant because all parameters entering into the theory were determined independently of the rate experiments themselves.

Koizumi also investigated theoretically and experimentally the effect of a membrane (*e.g.*, cellophane) separating the emulsion from the aqueous sink. This is an important practical factor in many *in vitro* experimental methods. This modification gave better agreement of the data with theory under certain expected conditions.

The reviewer believes that with the use of such numerical methods in conjunction with modern computers there is practically no problem that is inaccessible to theoretical analysis. There will be many examples of such attacks on diffusion problems in the future.

REFERENCES

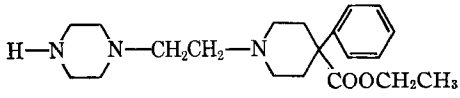
(1) Wurster, D. E., and Taylor, P. W., *J. Pharm. Sci.*, **54**, 169(1965).
(2) Wagner, J. G., *ibid.*, **50**, 359(1961).
(3) Nernst, W., and Brunner, E., *Z. Physik. Chem.*, **47**, 52(1904); **47**, 56(1904).
(4) Danckwerts, P. V., *Ind. Eng. Chem.*, **43**, 1460(1951).
(5) Toor, H. L., and Marchello, J. M., *A. I. Ch. E. J.*, **4**, 97(1958).
(6) Huang, C. J., and Kuo, C. H., *ibid.*, **9**, 161(1965); **11**, 901(1965).
(7) Johnson, A. I., and Huang, C. S., *ibid.*, **2**, 412(1956).
(8) Hanratty, T. J., *ibid.*, **2**, 359(1956).
(9) Noyes, A., and Whitney, W., *J. Am. Chem. Soc.*, **19**, 930(1897); Noyes, A., and Whitney, W., *Z. Physik. Chem.*, **23**, 689(1897).
(10) Desai, S. J., Singh, P., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, unpublished data.
(11) Wurster, D. E., and Taylor, P. W., *ibid.*, **54**, 670 (1965).
(12) Tawashi, R., and Higuchi, W. I., unpublished data.
(13) Mehta, S. C., and Higuchi, W. I., unpublished data.
(14) Goyan, J. E., *J. Pharm. Sci.*, **54**, 645(1965).
(15) Niebergall, P. J., Milosovich, G., and Goyan, J. E., *ibid.*, **52**, 236(1963).
(16) Higuchi, W. I., *ibid.*, **53**, 405(1964).
(17) Higuchi, W. I., and Hiestand, E. N., *ibid.*, **52**, 67 (1963).
(18) Higuchi, W. I., Rowe, E. L., and Hiestand, E. N., *ibid.*, **52**, 162(1963).
(19) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 376(1958).
(20) Olander, D. R., *A. I. Ch. E. J.*, **6**, 233(1960).
(21) Higuchi, W. I., Nelson, E., and Wagner, J. G., *J. Pharm., Sci.*, **53**, 333(1964).
(22) Nelson, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 607 (1957).
(23) *Ibid.*, **48**, 96(1959).
(24) Hamlin, W. E., Northam, J. I., and Wagner, J. G., *J. Pharm. Sci.*, **54**, 1651(1965).
(25) Hamlin, W. E., and Higuchi, W. I., *ibid.*, **55**, 205 (1966).
(26) Higuchi, W. I., *ibid.*, **53**, 532(1964).
(27) Wurster, D. E., and Seitz, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 335(1960).
(28) Taylor, P. W., and Wurster, D. E., *J. Pharm. Sci.*, **54**, 1654(1965).
(29) Roseman, T. J., and Higuchi, W. I., to be published.
(30) Higuchi, W. I., unpublished data.
(31) Levy, G., and Procknal, J. A., *J. Pharm. Sci.*, **51**, 294 (1962).
(32) Morozowich, W., Chulski, T., Hamlin, W. E., Jones, P. M., Northam, J. I., Purmalis, A., and Wagner, J. G., *ibid.*, **51**, 933(1962).
(33) Higuchi, W. I., Mir, N. A., Parker, A. P., and Hamlin, W. E., *ibid.*, **54**, 8(1965).
(34) Higuchi, W. I., and Hamlin, W. E., *ibid.*, **52**, 575 (1963).
(35) Higuchi, W. I., Gray, J. A., Hefferren, J. J., and Patel, P. R., *J. Dental Res.*, **44**, 330(1965).
(36) Higuchi, W. I., Patel, P. R., and Hefferren, J. J., *J. Pharm. Sci.*, **54**, 587(1965).
(37) Mir, N. A., and Higuchi, W. I., to be published.
(38) Nelson, K. G., and Higuchi, W. I., to be published.
(39) Higuchi, W. I., Mehta, S. C., and Bernardo, P. D., *J. Pharm. Sci.*, **56**, 200(1967).
(40) Hamlin, W. E., Nelson, E., Ballard, B. E., and Wagner, J. G., *ibid.*, **51**, 432(1962).
(41) Higuchi, W. I., Mir, N. A., and Desai, S. J., *ibid.*, **54**, 1405(1965).
(42) Nelson, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 300 (1958).
(43) Higuchi, W. I., *Proc. Am. Assoc. Colleges Pharm. Teachers' Seminar*, **1961**, 13.
(44) Higuchi, T., "Permeation of Mechanical Barriers by Chemical Agents," Medical Laboratories contract report No. 32, contract No. DA-18-108-CML-2576, May 1954, Chemical Corps Medical Laboratories, Army Chemical Center, Md.
(45) Higuchi, T., *J. Soc. Cosmetic Chemists*, **11**, 85(1960).
(46) Higuchi, T., *J. Pharm. Sci.*, **52**, 1145(1963).
(47) *Ibid.*, **50**, 874(1961).
(48) Barrier, R. M., "Diffusion In and Through Solids," Cambridge University Press, London, England, 1951.
(49) Jost, W., "Diffusion in Solids, Liquids, and Gases," Academic Press Inc., New York, N. Y., 1952.
(50) Crank, J., "Mathematics of Diffusion," Oxford University Press, London, England, 1964.
(51) Higuchi, W. I., *J. Pharm. Sci.*, **51**, 802(1962).
(52) Higuchi, W. I., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 598(1960).
(53) Koizumi, T., and Higuchi, W. I., *J. Pharm. Sci.*, unpublished data.
(54) Desai, S. J., Simonelli, A. P., and Higuchi, W. I., *ibid.*, **54**, 1495(1965).
(55) Desai, S. J., Singh, P., Simonelli, A. P., and Higuchi, W. I., *ibid.*, **55**, 1230(1966).
(56) Singh, P., Desai, S. J., Simonelli, A. P., and Higuchi, W. I., *ibid.*, unpublished data.
(57) Schwartz, J., Simonelli, A. P., and Higuchi, W. I., to be published.
(58) Fricke, H., *Physiol. Rev.*, **24**, 575(1924).
(59) Bruggeman, D. A. G., *Ann. Physik.*, **24**, 636(1935).
(60) Hanai, T., Koizumi, N., and Gotoh, R., *Kolloid Z.*, **167**, 41(1959).
(61) Higuchi, W. I., *J. Phys. Chem.*, **62**, 649(1958).
(62) Patel, K. C., Banker, G. S., and DeKay, H. G., *J. Pharm. Sci.*, **50**, 300(1961).
(63) Slade, A. L., Cremers, A. E., and Thomas, H. C., *J. Phys. Chem.*, **70**, 2840(1966).
(64) Koizumi, T., to be published.

Synthesis of Some *N,N'*-Disubstituted Piperazines as Potential Analgesics

By DOUGLAS C. KRIESEL and OLE GISVOLD

The synthesis and pharmacological screening of some *N,N'*-disubstituted piperazinonorpethidines is described. Certain members were prepared by *N*-alkylating norpethidine, while other analogs were made by condensing an appropriate *N*-monosubstituted piperazine with *N*-(2-chloroethyl)-norpethidine. Two compounds possessed significant analgesic activity although of a lower order than pethidine.

RECENT INVESTIGATIONS have demonstrated that certain *N,N'*-disubstituted piperazines possessed antihypertensive, adrenolytic, and anti-inflammatory properties (1). Some bicyclic structures containing a piperazine moiety have been shown to have analgesic activity (2). It previously had been reported that the *N*-piperazinoethyl analog of norpethidine (I) had ex-



I

hibited no demonstrable analgesic activity when tested in doses of at least 50 mg./Kg. in rats. However, hetero atoms in the four position of a six membered ring analog do not necessarily lead to lowered activity, for *N*-morpholinoethylnorpethidine and *N*-1,4-thiazinoethylnorpethidine were found to be more potent than pethidine (3).

This communication deals with the synthesis of certain *N,N'*-disubstituted piperazines in an attempt to prepare suitable analgesics. The rationale for introducing some selected groups to form some of the desired analogs was drawn from the area of the phenothiazine tranquilizers. The substitution of β-acetoxyethyl and β-hydroxyethyl moieties on one nitrogen of the piperazino group in the 2-chloro-10-piperazinopropylenephenothiazines gave drugs possessing both ataractic and antiemetic properties. The incorporation of these substituents into the piperazinonorpethidines was carried out in order to determine their contributions to analgesic and other activities.

Received August 24, 1966, from the School of Pharmacy, Southwestern State College, Weatherford, OK 73096
Accepted for publication January 3, 1967.
Abstracted from a portion of the thesis submitted by Douglas C. Kriesel to the Graduate School, University of Minnesota, Minneapolis, Minn., in partial fulfillment of Doctor of Philosophy degree requirements.

DISCUSSION

Ethylene oxide was distilled into a solution of *N*-benzylpiperazine in methanol to yield 1-(2-hydroxyethyl)-4-benzylpiperazine, which has been reported by Baltzly (4). The synthesis of 1-(2-chloroethyl) - 4 - benzylpiperazine dihydrochloride (II) was completed by refluxing the substituted alcohol with thionyl chloride. Amination with norpethidine in dimethylformamide afforded the wanted product (XI) which was characterized as the trimaleate. All of the 1-substituted norpethidines were isolated as their trimaleates.

The preparation of 1-(3-hydroxy-*n*-propyl)-4-methylpiperazine was accomplished by following the procedure of McElvain and Bannister (5), *i.e.*, monoalkylation of piperazine with trimethylene chlorohydrin, followed by reductive formylation. Treatment of this product with 48% HBr gave the desired alkyl halide, 1-(3-bromo-*n*-propyl)-4-methylpiperazine (III). Condensing this product with norpethidine yielded *N*-(4-methyl-1-*n*-propylenepiperazino)-norpethidine (XII).

Alkylation of norpethidine with 1-(2-chloroethyl)-4-methylpiperazine[1] in dimethylsulfoxide at 100° for 18 hr. yielded *N*-(4-methyl-1-ethylenepiperazino)-norpethidine (XIII).

Some of the desired compounds were prepared *via* the condensation of β-chloroethylnorpethidine with an *N*-monosubstituted piperazine. A modification of the procedure of Anderson *et al.* (6) gave the desired β-haloamine. One of the monosubstituted piperazine intermediates, 1-(2-hydroxyethyl)-piperazine, was prepared according to the procedure of McElvain and Bannister (5), *i.e.*, by reacting piperazine with ethylene oxide.

A second *N*-substituted piperazine intermediate, 1-(2-ethoxyethyl)-piperazine, was synthesized in two steps as follows: *N*-benzylpiperazine was refluxed in benzene with an equivalent amount of β-bromoethyl ethyl ether to give 1-(2-ethoxyethyl)-4-benzylpiperazine (IV). It was obtained as an oil by fractional distillation and was analyzed as the dihydrochloride (V). Catalytic hydrogenation debenzylated the substituted piperazine yielding the dihydrochloride of 1-(2-ethoxyethyl)-piperazine (VI).

The synthesis of 1-(2-acetoxyethyl)-piperazine (IX) was carried out in a manner similar to that of

[1] Purchased as the dihydrochloride from Aldrich Chemical Co., Milwaukee, Wis.

TABLE I—*N,N'*-DISUBSTITUTED PIPERAZINES AND THEIR SALTS

Compd.	R	R'	B.p., or M.p., °C.	Salt	Formula	Anal., % Calcd.	Found
II	Benzyl	CH_2CH_2Cl	214–216	.2 HCl	$C_{13}H_{21}Cl_3N_2$	C, 51.07	51.29
						H, 6.92	6.94
III	CH_3	$CH_2CH_2CH_2Br$	253–354 dec.	.2 HBr	$C_8H_{19}Br_3N_2$	C, 25.09	25.19
						H, 5.00	5.06
IV	Benzyl	$CH_2CH_2OCH_2CH_3$	145–146 at 2.5 mm.				
V	Benzyl	$CH_2CH_2OCH_2CH_3$	234–236	.2 HCl	$C_{15}H_{26}Cl_2N_2O$	C, 56.07	56.35
						H, 8.16	8.01
VI	H	$CH_2CH_2OCH_2CH_3$	151–153	.2 HCl	$C_8H_{20}Cl_2N_2O$	C, 55.35	55.55
						H, 6.42	6.54
VII	Benzyl	$CH_2CH_2OOCCH_3$	144–147 at 0.7 mm.				
VIII	Benzyl	$CH_2CH_2OOCCH_3$	196–198	.2 $C_4H_4O_4$	$C_{23}H_{30}N_2O_{10}$	C, 55.86	56.14
						H, 6.12	6.28
IX	H	$CH_2CH_2OOCCH_3$	81–84 at 0.8 mm.				
X	H	$CH_2CH_2OOCCH_3$	136–139	.2 $C_4H_4O_4$	$C_{16}H_{24}N_2O_{10}$	C, 47.52	47.39
						H, 5.98	6.27

the ethoxyethyl analog with the exception that debenzylation by catalytic hydrogenation was done in the absence of any aqueous acid in order to prevent any possible hydrolysis of the ester.

β-Chloroethylnorpethidine was condensed with 1-(2-hydroxyethyl)-piperazine, 1-(2-ethoxyethyl)-piperazine, and 1-(2-acetoxyethyl)-piperazine to yield, respectively, products XIV, XV, and XVI.

EXPERIMENTAL

All melting points were taken with a Thomas-Hoover Uni-Melt apparatus. The melting points and the boiling points are uncorrected. With the exception of products XI, XII, and XIII, which were microanalyzed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y., all of the elemental analyses were performed by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, Minn.

1 - (2 - Chloroethyl) - 4 - benzylpiperazine Dihydrochloride (II)—A stirred ice-cold solution of 1-(2-hydroxyethyl)-4-benzylpiperazine (4.0 Gm., 0.018 mole) in 50 ml. of benzene was treated with dropwise addition of thionyl chloride (4.6 Gm., 0.038 mole). The mixture was refluxed for 30 min., cooled, 50 ml. of dry ether added, and the tan solid filtered. After recrystallization from methanol–ether, white crystals resulted. Yield: 2.6 Gm. (46.5%). (Table I.)

1 - (3 - Bromo - n - propyl) - 4 - methylpiperazine Dihydrobromide (III)—A solution of 1-(3-hydroxypropyl)-4-methylpiperazine (3.5 Gm., 0.022 mole) in 160 ml. of 48% aqueous hydrogen bromide was slowly distilled from an all-glass apparatus until 80 ml. of distillate had been collected. After refluxing overnight, the reaction mixture was concentrated to dryness under reduced pressure, and the oily precipitate was triturated in acetone, which rapidly transformed it into a fine brownish powder. The solid was recrystallized from 95% ethanol giving white crystals. Yield: 8.0 Gm. (95.0%). (Table I.)

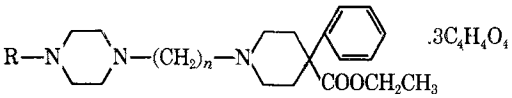
1 - (2 - Ethoxyethyl) - piperazine Dihydrochloride (VI)—1-(2-Ethoxyethyl)-4-benzylpiperazine (IV) (5.0 Gm., 0.02 mole) was dissolved in a mixture of 15 ml. of 10% aqueous HCl and 45 ml. of 95% ethanol and to it was added 1 Gm. of 10% palladium on charcoal. The mixture then was hydrogenated at 40 p.s.i. for 24 hr. The reaction mixture was filtered through a diatomaceous earth[2] filter bed, and the filtrate was concentrated to dryness *in vacuo*. The resulting white powder was crystallized from a mixture of absolute ethanol and ether. Yield: 2.25 Gm. (48.5%). (Table I.)

1 - (2 - Acetoxyethyl) - 4 - benzylpiperazine Dimaleate (VIII)—To 20 ml. of dimethylformamide was added *N*-benzylpiperazine (10 Gm., 0.057 mole) and β-chloroethyl acetate (7.0 Gm., 0.057 mole). Five grams of anhydrous sodium carbonate was added and the mixture was stirred at 100° for 16 hr. After cooling, 50 ml. of water was added, and the aqueous mixture was extracted three times with ether. The ethereal extracts were dried over anhydrous sodium sulfate, the solvent removed on a rotating evaporator, and the residue fractionally distilled. Yield: 10.0 Gm. (67.5%) (VII). (Table I.) The dimaleate salt was prepared by dissolving the product (0.5 Gm., 0.0019 mole) in 5 ml. of absolute ethanol and adding it to a solution of maleic acid (0.44 Gm., 0.0038 mole) in 5 ml. of absolute ethanol. The white crystals that formed on standing were recrystallized from 85% ethanol. Yield: 0.5 Gm. (53%). (Table I.)

1 - (2 - Acetoxyethyl) - piperazine Dimaleate (X)—1 - (2 - Acetoxyethyl) - 4 - benzylpiperazine (VII) (7.0 Gm., 0.019 mole) was dissolved in 50 ml. of absolute ethanol and to it was added 1 Gm. of 10% palladium on charcoal. The mixture was hydrogenated in a Parr hydrogenating apparatus at 40 p.s.i. for 24 hr. The reaction mixture was filtered through a diatomaceous earth filter bed, and the ethanol removed on a rotating evaporator. The residual oil was fractionally distilled under reduced pressure. Yield: 3.0 Gm., (65.2%) (IX). (Table I.) A portion of the oil (0.5 Gm., 0.0029 mole) was dissolved in 5 ml. of absolute ethanol and added to a solution of maleic acid (0.68 Gm., 0.006 mole) in 5 ml. of absolute ethanol. The white crystals that resulted upon cooling were recrystallized from absolute ethanol–ether. (Table I.)

[2] Marketed as Celite by Johns Manville, New York, N. Y.

TABLE II—TRIMALEATE SALTS OF *N*-SUBSTITUTED PIPERAZINONORPETHIDINES



Compd.	R	*n*	M.p., °C.	Formula	Anal., % Calcd.	Found	ED_{50}, mg./Kg.[a]
XI	Benzyl	2	205–207	$C_{39}H_{49}N_3O_{14}$	C, 59.76	59.47	34.8 ± 4.1
					H, 6.30	6.21	
XII	CH_3	3	198–200	$C_{34}H_{47}N_3O_{14}$	C, 56.58	56.95	NA
					H, 6.56	6.52	
XIII	CH_3	2	195–197	$C_{33}H_{45}N_3O_{14}$	C, 56.10	55.69	14.5 ± 2.4
					H, 6.35	6.49	
					N, 5.94	5.95	
XIV	CH_2CH_2OH	2	192–195	$C_{34}H_{47}N_3O_{15}$	C, 55.35	55.55	NA
					H, 6.42	6.54	
					N, 5.70	5.98	
XV	$CH_2CH_2OCH_2CH_3$	2	187–189	$C_{36}H_{51}N_3O_{15}$	C, 56.46	56.75	NA
					H, 6.71	6.95	
					N, 5.49	5.45	
XVI	$CH_2CH_2OOCCH_3$	2	180–182	$C_{36}H_{49}N_3O_{16}$	C, 55.45	55.17	NA
					H, 6.33	6.63	

[a] Calculated as the free base; NA, no activity at 50 mg./Kg.

N - (4 - Methyl - 1 - n - propylenepiperazino)-norpethidine Trimaleate (XII)—Norpethidine[3] (0.5 Gm., 0.002 mole) was dissolved in 10 ml. of dimethylformamide and to it was added 1-(3-bromo-*n*-propyl)-4-methylpiperazine dihydrobromide (III) (0.83 Gm., 0.0022 mole) dissolved in 1 ml. of 20% aqueous sodium hydroxide. The mixture was dried over anhydrous sodium sulfate, anhydrous sodium carbonate (0.39 Gm., 0.003 mole) was added, and the mixture was stirred at 100° for 18 hr. After filtration, the solvent was removed *in vacuo*. The residue, dissolved in 10 ml. of absolute ethanol, was added to maleic acid (0.75 Gm., 0.006 mole) in 5 ml. of absolute ethanol. The white crystals which separated were recrystallized from 95% ethanol. Yield: 0.45 Gm. (31%). (Table II.)

N - [4 - (2 - Ethoxyethyl) - 1 - ethylenepiperazino] - norpethidine Trimaleate (XV)—*N* - (2-Chloroethyl)-norpethidine hydrochloride (0.5 Gm., 0.0015 mole) and 1-(2-ethoxyethyl)-piperazine dihydrochloride (VI) (0.35 Gm., 0.0015 mole) were dissolved in 3 ml. of water and the solution was basified with 5% aqueous sodium hydroxide. Dimethylformamide (10 ml.) was added along with anhydrous sodium carbonate (2.0 Gm.) and the mixture was stirred at 100° for 18 hr. After filtration of the solid, the filtrate was concentrated on a rotating evaporator to an oily residue. The oil was dissolved in 10 ml. of absolute ethanol and added to maleic acid (0.52 Gm., 0.0045 mole) dissolved in 5 ml. of absolute ethanol. The white solid was filtered and recrystallized from 85% ethanol. The yield of white microcrystals was 0.45 Gm. (39%). (Table II.)

[3] Prepared according to the procedure of Thorp, R., and Walton, E., *J. Chem. Soc.*, **1948**, 559.

BIOLOGICAL DATA

Preliminary screening for analgesic activity was carried out using a modification of the procedure described by Eddy and Leimbach (7). Mixed white mice were placed on a metal plate kept at 53° by floating it on top of a circulating hot water bath. Those animals whose reaction time increased to at least twice the mean reaction time for the control animals were assumed to show significant pain relief. The reaction time was the interval from the time the animal was placed on the plate to the time it licked its hind feet or attempted to jump off the container. The test was conducted 30 min. after the required dose was given subcutaneously. The test animals were kept on the plate for a maximal period of 60 sec. The ED_{50} levels were calculated according to the method of Miller and Tainter (8). Pethidine, according to this method, had an ED_{50} of 9 mg./Kg.

REFERENCES

(1) Mull, R., Tannenbaum, C., Dapero, M., Bernier, M., Yost, W., and de Stevens, C., *J. Med. Chem.*, **8**, 332(1965).
(2) Cignarella, G., Occelli, E., and Testa, E., *ibid.*, **8**, 326 (1965).
(3) Millar, R., and Stephenson, R., *Brit. J. Pharmacol.*, **11**, 27(1956).
(4) Baltzly, R., Buck, J. S., Lorz, E., and Schön, W., *J. Am. Chem. Soc.*, **66**, 263(1944).
(5) McElvain, S., and Bannister, L., *ibid.*, **76**, 1126(1954).
(6) Anderson, R., Frearson, P., and Stearn, E., *J. Chem. Soc.*, **1956**, 4088.
(7) Eddy, N., and Leimbach, D., *J. Pharmacol. Exptl. Therap.*, **107**, 385(1953).
(8) Miller, L., and Tainter, M., *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1944).

Syntheses of Some New Mannich Bases Using 2,6-Dimethylmorpholine as Amine Moiety

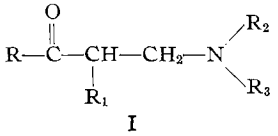
By K. K. KHULLAR and L. G. CHATTEN

The use of 2,6-dimethylmorpholine as an amine moiety in Mannich reaction has been investigated. Nine new Mannich bases have been synthesized using nine different ketones. The salts of β-amino esters have also been synthesized by Mannich reaction using *p*-nitrophenylacetic acid, formaldehyde solution (37 per cent), and three different amines (2,6-dimethylmorpholine, piperidine, and 4-methylpiperidine) followed by esterification. The structures of these compounds have been established by infrared spectra, mass spectra, and elemental analyses.

In their studies on the synthesis of antispasmodics by Mannich reaction (1), Denton and co-workers (2) observed that the most effective amino group in their series from a pharmacological standpoint was piperidyl.

Compounds containing this amine moiety possessed outstanding properties when the acyl group belonged to the propiophenone series which possessed no ring or side chain substituents. In addition, piperidyl was as effective as any other amino group in the propionaphthone series. On the other hand, morpholinyl derivatives generally were the least active. The changes in activity which resulted from structural variations prompted the investigation of the use of substituted morpholines in the Mannich reaction to determine whether the resulting compounds possessed improved activity.

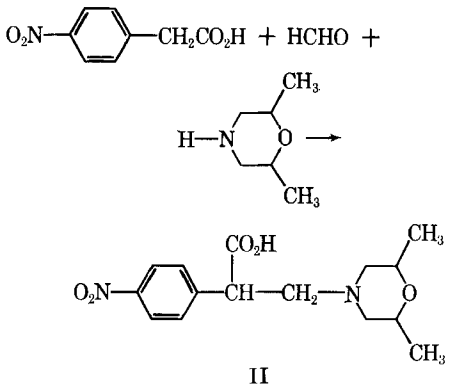
Although Mannich bases containing the morpholine ring as in structure I are known to possess low activity (2), their basic esters have pronounced antispasmodic activity and in general are less toxic than their diethylamine analogs (3). Synthesis of the β-amino acid (Scheme I) has been successfully attempted by Mannich reaction of *p*-nitrophenylacetic acid, formaldehyde (37%), and 2,6-dimethylmorpholine in aqueous medium (4), and the salt of the β-amino ester has been synthesized (Scheme II) by esterification of the appropriate acid by thionyl chloride and methanol (5).



I

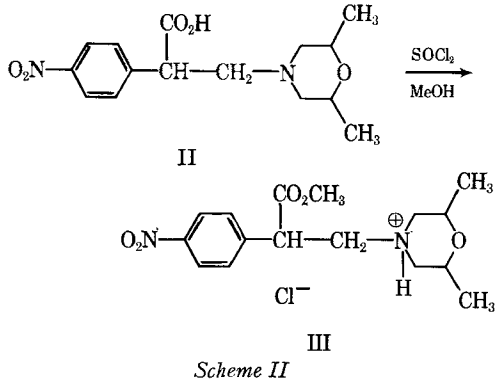
For the purpose of comparison of pharmacological activities, salts of β-amino esters have also been synthesized using piperidine and 4-methylpiperidine as amine moieties.

Received August 16, 1966, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.
Accepted for publication November 7, 1966.
The authors thank Mrs. Pauline Mudry for determining the infrared spectra of the compounds reported here.



II

Scheme I



II

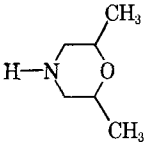
III

Scheme II

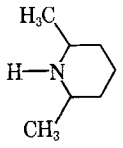
DISCUSSION

2,6-Dimethylmorpholine, b.p. 144–146°/758 mm., was converted into its hydrochloride by passing dry hydrogen chloride gas through a solution of 2,6-dimethylmorpholine in ether. The Mannich bases of type I were obtained by *Method A* (6) (see under *Experimental*). Where a solid product did not separate on cooling, the solvent and unreacted acetone were removed on the evaporator. The resulting residue was recrystallized from acetone. These reactions, in addition to their use in the preparation of compounds which may have antispasmodic activity, will permit speculation on the steric requirements of the Mannich reaction. It is noteworthy that, whereas the reaction proceeds easily and gives crystalline products with sharp melting points with 2,6-dimethylmorpholine, the reactions

fail with 2,6-dimethylpiperidine under similar conditions.



2,6-Dimethylmorpholine



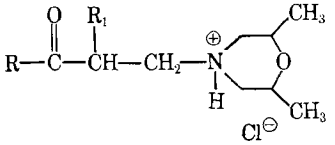
2,6-Dimethylpiperidine

Furthermore, as illustrated by Scheme III, it is intended to convert the morpholino Mannich bases into compounds of type IV by stirring the Mannich base with potassium cyanide and piperidine hydrochloride at room temperature (7). The resulting compounds would appear to possess interesting possibilities as antiperistaltic agents (8) and/or analgesics (9).

Mass spectra of the salts of Mannich bases of type I, recorded by direct probe introduction method using the MS9 mass spectrometer, permit ready elucidation of their structure. In addition, it is hoped to be able to correlate mass spectral data of these bases with their structure-activity relationship as antispasmodics. Since more information is required to substantiate our viewpoint, this report will appear at a later date.

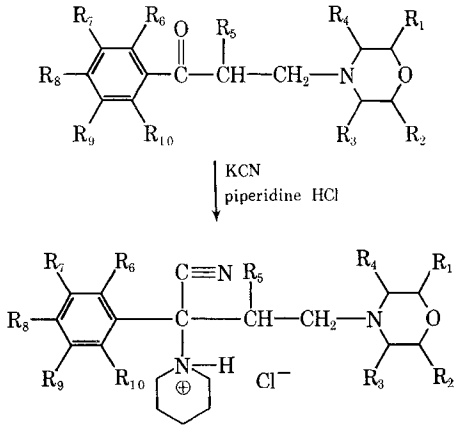
Infrared spectra (10) of the salts of Mannich bases of type I performed on a Perkin-Elmer instrument using Nujol mull appear in Table I. The

TABLE I—MANNICH BASES TYPE I[a]



Compd.	R	R_1	Yield, %	M.p., °C.	Formula	Anal. Calcd.	Anal. Found	Infrared
1	Phenyl	H	70	195–196.5	$C_{15}H_{21}NO_2 \cdot HCl$	C, 63.48 H, 7.81 N, 4.93	63.11 7.69 4.95	ν_{max}. 2870 (—CH str), 2670, 2530, 2450 (NH), 1690, (Ph—C), 1600, 1580 (phenylgr.), 1460, 747, 695 (monosub. ben. ring), 1087 (=CH—O—CH<) cm.$^{-1}$.
2	*p*-Nitrophenyl	H	61	203–204	$C_{15}H_{20}N_2O_4 \cdot HCl$	C, 54.79 H, 6.44 N, 8.52	54.95 6.02 8.79	ν_{max}. 2900 (—CH str), 2670, 2530, 2450 (NH$^+$), 1700 (Ph—C—) 1610 (phenylgr.) 1530, 1350 (=C—NO_2), 1087 (>CH—O—CH<), and 860–800 (1:4 disub. ben. ring) cm.$^{-1}$.
3	Methyl	H	42	156–157	$C_{10}H_{19}NO_2 \cdot HCl$	C, 54.17 H, 9.09 N, 6.32	54.22 9.09 6.50	ν_{max}. 2900 (—CH str), 2670, 2550, 2410 (NH$^+$), 1720 (carbonyl gr), 1087 (>CH—O—CH<) cm.$^{-1}$.
4	Naphthyl	H	55	221–223	$C_{19}H_{23}NO_2 \cdot HCl$	C, 68.36 H, 7.25 N, 4.19	68.27 6.97 4.02	ν_{max}. 2900 (—CH str), 2670, 2530, 2450 (NH$^+$), 1680 (ph—C—) 1600, 1575 (α-sub. naphthalene ring), 1100–1070 (>CH—O—CH<), 798–770, 770 (4 adj. H atoms on aro. ring) cm.$^{-1}$.
5	Phenyl	CH_3	68	187–188.5	$C_{16}H_{23}NO_2 \cdot HCl$	C, 64.52 H, 8.12 N, 4.70	64.53 8.18 4.50	ν_{max}. 2900 (—CH str), 2700, 2570 (NH$^+$), 1680 (carbonyl-Ph—C), 1600, 1587 (phenylgr.), 1455, 765, 704 (monosub. ben. ring), and 1087 (>CH—O—CH<) cm.$^{-1}$.
6	*p*-Methoxyphenyl	H	60	203–205	$C_{16}H_{23}NO_2 \cdot HCl$	C, 61.23 H, 7.70 N, 4.46	60.98 7.70 4.39	ν_{max}. 2900 (—CH str.), 2680, 2560, 2480 (NH$^+$), 1680 (Ph—C—) 1605, 1578 (phenylgr.), 1265 (=C—O—), 1087 cm.$^{-1}$ (>CH—O—CH<), 850–800 (1:4 disub. ben. ring) cm.$^{-1}$.
7	*p*-Chlorophenyl	H	63	203.5–204.5	$C_{15}H_{21}NO_2Cl \cdot HCl$	C, 56.61 H, 6.65 N, 4.40	56.47 6.69 4.33	ν_{max}. 2910 (—CH str.), 2690, 2570, 2490 (NH$^+$), 1695 (Ph—C—), 1595, 1577 (phenylgr.), 1095 (>CH—O—CH<), 860–800 (1:4 disub. ben. ring) cm.$^{-1}$.
8	*p*-Methylphenyl	H	80	207–208	$C_{16}H_{23}NO_2 \cdot HCl$	C, 64.64 H, 8.08 N, 4.71	64.63 8.29 4.49	ν_{max}. 2900 (—CH str), 2680, 2550, 2470 (NH$^+$), 1690 (Ph—C—), 1613, 1580 (phenylgr.), 1090 (>CH—O—CH<), 860–798 (1:4 disub. ben. ring) cm.$^{-1}$.
9	*m*-Nitrophenyl	H	53	196–197	$C_{15}H_{20}N_2O_4 \cdot HCl$	C, 54.79 H, 6.40 N, 8.52	54.55 6.60 8.58	ν_{max}. 2900 (—CH str.), 2560, 2430, 2360 (NH$^+$), 1700 (Ph—C—), 1620, 1590, (phenylgr.), 1537, 1350 (=C—NO), 1093 (>CH—O—CH<), 804, 790, 697 (metasub. ben. ring) cm.$^{-1}$.

[a] Prepared by *Method A*.



IV

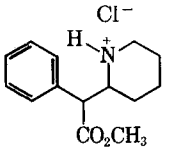
Scheme III

Mannich bases of type II were obtained by *Method B* (see under *Experimental*) (4). Infrared spectra of this type of compound are reported in Table II.

The β-amino acids formed from *p*-nitrophenylacetic acid through the Mannich reaction (type II) are insoluble in water and most organic solvents. These compounds exist as zwitterions, and since zwitterions are usually rather inert pharmacologically, no special attempt was made to purify these amino acids.

The salts of the β-amino esters (type III) were prepared from the amino acids by *Method C* (see under *Experimental*) (5). (Table III.) This type of compound, in addition to being antispasmodic, may also have mild psychomotor stimulant properties for minor neuroses, psychoneuroses, psychoses, and also for narcolepsy, as indicated by its structural similarity to methylphenidate HCl.

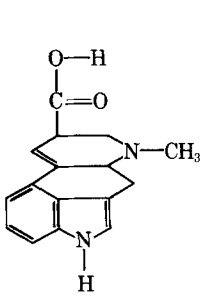
The $-NO_2$ group may increase the toxicity, but the compound without the nitro group may easily



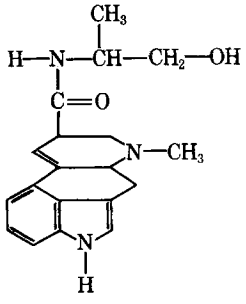
Methylphenidate HCl

be obtained by using phenylmalonic acid in place of *p*-nitrophenylacetic acid.

All the naturally occurring ergot alkaloids are derivatives of lysergic acid. They have been widely



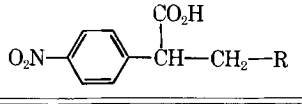
Lysergic Acid



Ergometrine

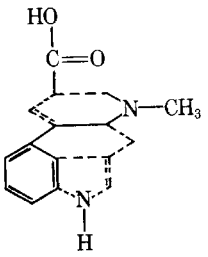
used as oxytocics, vasoconstrictors, and sympatholytics (11). Bovet and his co-workers (12), through the ingenious design of a series of analogs utilizing the method of disjunction, have provided an answer to the very important and interesting question—whether the three actions of these alkaloids depend on a single pharmacomorphic moiety or whether there are distinct moieties responsible for each type of action. According to them, these actions are indeed separable. They visualized the presence of a sympathomimetic amine structure embedded in the lysergic acid nucleus as in V and VI.

TABLE II—MANNICH BASES TYPE II[a]

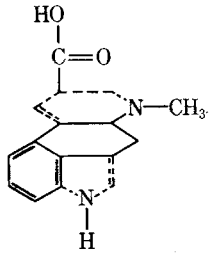


Compd.	R	Yield, %	M.p., °C.	Formula	Infrared[c]
1[b]	Piperidyl	58	136	$C_{16}H_{18}N_2O_4$	$\nu_{max.}$ 2900 (—CH str.), 2500–2000 ($^+$NH), 1630 (—C(=O)—O$^-$), 1605 (phenylgr.), 1515, 1330–150 (=C—NO_2), 843 cm.$^{-1}$.
2	4-Methyl-piperidyl	56	143	$C_{16}H_{20}N_2O_4$	$\nu_{max.}$ 2900 (—CH str.), 2500–2100 ($^+$NH), 1625 (—C(=O)—O$^-$), 1600 (phenyl gr.), 1515, 1350 (=C—NO_2), 860-800 (1:4 disubst. benzene ring), 740, 695 cm.$^{-1}$.
3	2,6-Dimethyl-morpholinyl	88	142–143	$C_{16}H_{20}N_2O_5$	$\nu_{max.}$ 2900 (—CH str.), 2500–1800 ($^+$NH), 1640 (—C(=O)—O$^-$), 1605 (phenylgr.), 1520, 1340 (=C—NO_2), 1095 (>CH—O—CH<), 770–680 cm.$^{-1}$.

[a] Prepared by *Method B*. Converted to ester; hence acids not submitted for elemental analysis. [b] Compound *1* has been synthesized previously in literature (6). [c] $\nu_{max.}$ 2900 cm.$^{-1}$ may be due to Nujol as well.

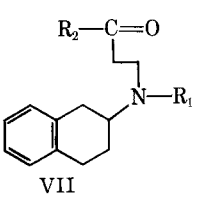


V

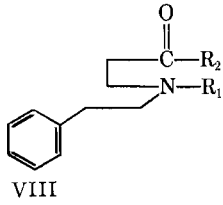


VI

On the basis of this type of reflection, various series of products were designed, synthesized, and evaluated for the above-mentioned activities. These series included the types of disjunction analogs shown in VII and VIII.



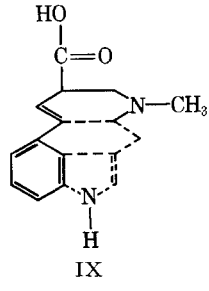
VII



VIII

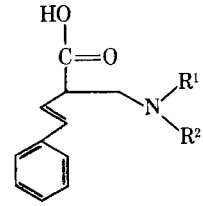
where R_1 = various alkyl, and
R_2 = various alkylamide groups.

Careful examination of the structure of lysergic acid shows the presence of another type of structure (IX).



IX

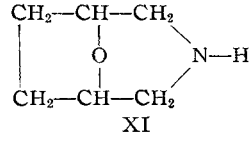
Upon writing the delineated portion separately (X),



X

it is observed to be a simple vinylog of the type of β-amino acid II which has been synthesized in this laboratory. It is intended to convert this acid into amide derivatives of various ethanol and propanolamines which may be of special interest (as found in ergometrine).

The successful use of substituted morpholines in Mannich reaction would pave a way for the use of 8-oxa-3-azabicyclo[3.2.1]octane (13) which has fused tetrahydrofuran and morpholine rings (XI).



XI

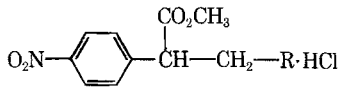
This would provide an interesting addition to the study of co-relating the structure of Mannich bases with antispasmodic activity.

Additional modifications to these Mannich bases, some of which may incorporate suggestions by other authors (14–18), will be reported in subsequent publications, together with the results of pharmacological screening.

EXPERIMENTAL

All melting points were determined on a Thomas-Hoover capillary apparatus and are corrected. Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer model 21. Elemental analyses were performed by Dr. G. Weiler, Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England. All the yields reported were calculated on the basis of amine used. All the chemicals used were

TABLE III—SALTS OF BASIC ESTERS[a]



Compd.	R	Yield, %	M.p., °C.	Formula	Anal. Calcd.	Anal. Found	Infrared
1	Piperidyl	Quan.	211	$C_{16}H_{20}N_2O_4 \cdot HCl$	C, 54.79 H, 6.44 N, 8.52	54.68 7.21 8.44	ν_{max}. 2900 (—CH str.), 2570, 2470 (NH⁺), 1730 (—C(=O)—) 1600 (phenylgr.), 1530, 1345 (=C—NO_2), 862–837 cm.⁻¹.
2	4-Methyl-piperidyl	Quan.	148.5–149.5	$C_{16}H_{22}N_2O_4 \cdot HCl$	C, 56.14 H, 6.72 N, 8.19	56.29 6.72 8.19	ν_{max}. 2900 (—CH str.) 2600, 2440, 2375 (NH⁺), 1750 (carbonyl group), 1612, 1605 (phenyl gr.), 1520 1350 (=C—NO_2), 860–800 (1:4 disubst. benzene ring), 735, 685 cm.⁻¹.
3	2,6-Dimethyl-morpholinyl	68	150–152	$C_{16}H_{22}N_2O_5 \cdot HCl$	C, 53.56 H, 6.46 N, 7.80	53.73 6.67 7.62	ν_{max}. 2900 (—CH str.) 2400 (NH⁺), 1737 (—C(=O)—), 1600 (phenylgr.), 1530, 1343 (=C—NO_2), 1082 (>CH—O—CH<), 860, 930 cm.⁻¹.

[a] Prepared by *Method C*.

obtained from commercial sources unless otherwise reported.

Method A—A mixture of a ketone (0.10 mole), 2,6-dimethylmorpholine hydrochloride (0.10 mole), paraformaldehyde (0.20 mole), and concentrated hydrochloric acid (0.50 ml.) in 50 ml. of absolute ethanol was heated under reflux. After refluxing for 3 hr., another 0.10 mole of paraformaldehyde was added and refluxing continued for an additional 6 hr. Boiling acetone (200 ml.) was added to the hot mixture with shaking. The resulting solution was allowed to cool overnight, and then in the refrigerator for 3 hr. The crystalline product thus precipitated was filtered. Ethanol–acetone is a suitable solvent pair for crystallization.

Method B—The β-amino acids were prepared according to the procedure of Mannich and Stein (4). A white solid started separating after about 4 hr. The crystalline product thus precipitated after 24 hr. was filtered. The residue was washed twice with absolute ethanol (50-ml. portions) and dried. Some of these acids turn red on standing, probably due to air oxidation.

Method C—Thionyl chloride (20 ml.) was added to the β-amino acid (0.025 mole) dropwise with cooling and shaking. After the completion of addition, the mixture was allowed to stand at room temperature for about 16 hr. During this period, the entire solid went into solution. The unreacted thionyl chloride then was removed on the evaporator. The residue then was taken up in carbon tetrachloride (100 ml.), methanol (20 ml.) was added slowly, and the resulting mixture was refluxed on a steam bath. After about 6 hr., the solvent and unreacted methanol were removed on the evaporator, and a portion of the resulting residue was recrystallized from acetone.

REFERENCES

(1) Blicke, F. F., in "Organic Reactions," John Wiley & Sons, Inc., New York, N. Y., 1942, vol. 1, p. 303.
(2) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, **71**, 2048(1949).
(3) Cheney, L. C., and Bywater, W. G., *ibid.*, **64**, 970 (1942).
(4) Mannich, C., and Stein, L., *Ber.*, **58**, 2659(1925).
(5) Barnes, R. A., and Fales, H. M., *J. Am. Chem. Soc.*, **75**, 975(1953).
(6) Mannich, C., and Lammering, D., *Ber.*, **55**, 3510 (1922).
(7) Janssen, P. A. J., U. S. pat. 3,041, 344 (June 26, 1962); through *Chem. Abstr.*, **59**, 6417(1963).
(8) "New Drugs," American Medical Association, Chicago, Ill., 1965, p. 104.
(9) Casadio, S., Pala, G., Crescenzi, E., Bruzzese, T., Marazzi-Uberti, E., and Coppi, G., *J. Med. Chem.*, **8**, 589 (1965).
(10) Thompson, W. E., Warren, R. J., Eisdorfer, I. B., and Zarembo, J. E., *J. Pharm. Sci.*, **54**, 1819(1965).
(11) Schueler, F. W., "Chemobiodynamics and Drug Design," The Blakiston Division, McGraw-Hill Book Co., Inc., Toronto, Ontario, Canada, 1960, pp. 417–419.
(12) Bovet, D., *Rend. Inst. Super. Sanita*, **13**, 541(1949).
(13) Newth, F. H., and Wiggins, L. F., *J. Chem. Soc.*, **1948**, 155.
(14) Denton, J. J., Schedl, H. P., Neier, W. B., and Lawson, V. A., *J. Am. Chem. Soc.*, **71**, 2054(1949).
(15) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, **53**, 521(1964).
(16) Duvoisin, R. C., *Bull. N.Y. Acad. Med.*, **41**, 898 (1965).
(17) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 878(1962).
(18) Janssen, P. A. J., *et al.*, *J. Med. Pharm. Chem.*, **1**, 105(1959).

Stability of Metal Complexes of Salicylic Acid Derivatives and Analogs III

3,6-Dialkyl Derivatives and Pyridine Analogs

By WILLIAM O. FOYE, MARTIN D. BAUM, and DAVID A. WILLIAMS

Stability constants are reported for the complexation of a series of 3,6-dialkylsalicylic acids and pyridine analogs of salicylic acid with Cu (II), Fe (III), and Al (III) ions. These compounds were selected in an attempt to increase the affinity of salicylates for ferric ion. The 3,6-dialkyl substituents lowered metal complex stabilities, but the presence of a ring-nitrogen adjacent to the phenolic group increased complex stabilities considerably. The 3,6-dialkylsalicylic acids revealed somewhat greater analgesic effects than salicylic acid, but less anti-inflammatory action.

THE AVIDITY of salicylic acid for complex formation with transition metals has suggested that some, if not all, of the biological effects of salicylates may be due to complexation of metalloenzymes (1). Ample evidence may be found in the literature that salicylic acid or its derivatives become involved in enzymatic reactions; some references of this nature were cited in a previous paper (1), and other examples have appeared since (2–6). Since salicylates apparently affect cellular oxidations, salicylate derivatives which show a greater affinity for iron might be expected to show enhanced biological effects. Increased affinity for iron could be realized in two ways. Introduction of bulky substituents in the 3,6-positions of salicylic acid would tend to crowd the chelating groups together and thus provide a better fit for the ferric ion, which is a relatively small ion (7). Or the introduction of nitrogen into the ring in positions adjacent to the chelating groups might also provide molecules

Received October 31, 1966, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115
Accepted for publication December 8, 1966.
Abstracted from the theses submitted by D. A. Williams (1962) and M. D. Baum (1964) in partial fulfillment of Master of Science degree requirements.
This project was supported in part by a grant awarded by The Dow Chemical Co., Midland, Mich.

obtained from commercial sources unless otherwise reported.

Method A—A mixture of a ketone (0.10 mole), 2,6-dimethylmorpholine hydrochloride (0.10 mole), paraformaldehyde (0.20 mole), and concentrated hydrochloric acid (0.50 ml.) in 50 ml. of absolute ethanol was heated under reflux. After refluxing for 3 hr., another 0.10 mole of paraformaldehyde was added and refluxing continued for an additional 6 hr. Boiling acetone (200 ml.) was added to the hot mixture with shaking. The resulting solution was allowed to cool overnight, and then in the refrigerator for 3 hr. The crystalline product thus precipitated was filtered. Ethanol–acetone is a suitable solvent pair for crystallization.

Method B—The β-amino acids were prepared according to the procedure of Mannich and Stein (4). A white solid started separating after about 4 hr. The crystalline product thus precipitated after 24 hr. was filtered. The residue was washed twice with absolute ethanol (50-ml. portions) and dried. Some of these acids turn red on standing, probably due to air oxidation.

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REFERENCES

(1) Blicke, F. F., in "Organic Reactions," John Wiley & Sons, Inc., New York, N. Y., 1942, vol. 1, p. 303.
(2) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, **71**, 2048(1949).
(3) Cheney, L. C., and Bywater, W. G., *ibid.*, **64**, 970 (1942).
(4) Mannich, C., and Stein, L., *Ber.*, **58**, 2659(1925).
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(6) Mannich, C., and Lammering, D., *Ber.*, **55**, 3510 (1922).
(7) Janssen, P. A. J., U. S. pat. 3,041, 344 (June 26, 1962); through *Chem. Abstr.*, **59**, 6417(1963).
(8) "New Drugs," American Medical Association, Chicago, Ill., 1965, p. 104.
(9) Casadio, S., Pala, G., Crescenzi, E., Bruzzese, T., Marazzi-Uberti, E., and Coppi, G., *J. Med. Chem.*, **8**, 589 (1965).
(10) Thompson, W. E., Warren, R. J., Eisdorfer, I. B., and Zarembo, J. E., *J. Pharm. Sci.*, **54**, 1819(1965).
(11) Schueler, F. W., "Chemobiodynamics and Drug Design," The Blakiston Division, McGraw-Hill Book Co., Inc., Toronto, Ontario, Canada, 1960, pp. 417–419.
(12) Bovet, D., *Rend. Inst. Super. Sanita*, **13**, 541(1949).
(13) Newth, F. H., and Wiggins, L. F., *J. Chem. Soc.*, **1948**, 155.
(14) Denton, J. J., Schedl, H. P., Neier, W. B., and Lawson, V. A., *J. Am. Chem. Soc.*, **71**, 2054(1949).
(15) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, **53**, 521(1964).
(16) Duvoisin, R. C., *Bull. N.Y. Acad. Med.*, **41**, 898 (1965).
(17) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 878(1962).
(18) Janssen, P. A. J., *et al.*, *J. Med. Pharm. Chem.*, **1**, 105(1959).

Stability of Metal Complexes of Salicylic Acid Derivatives and Analogs III

3,6-Dialkyl Derivatives and Pyridine Analogs

By WILLIAM O. FOYE, MARTIN D. BAUM, and DAVID A. WILLIAMS

Stability constants are reported for the complexation of a series of 3,6-dialkylsalicylic acids and pyridine analogs of salicylic acid with Cu (II), Fe (III), and Al (III) ions. These compounds were selected in an attempt to increase the affinity of salicylates for ferric ion. The 3,6-dialkyl substituents lowered metal complex stabilities, but the presence of a ring-nitrogen adjacent to the phenolic group increased complex stabilities considerably. The 3,6-dialkylsalicylic acids revealed somewhat greater analgesic effects than salicylic acid, but less anti-inflammatory action.

THE AVIDITY of salicylic acid for complex formation with transition metals has suggested that some, if not all, of the biological effects of salicylates may be due to complexation of metalloenzymes (1). Ample evidence may be found in the literature that salicylic acid or its derivatives become involved in enzymatic reactions; some references of this nature were cited in a previous paper (1), and other examples have appeared since (2–6). Since salicylates apparently affect cellular oxidations, salicylate derivatives which show a greater affinity for iron might be expected to show enhanced biological effects. Increased affinity for iron could be realized in two ways. Introduction of bulky substituents in the 3,6-positions of salicylic acid would tend to crowd the chelating groups together and thus provide a better fit for the ferric ion, which is a relatively small ion (7). Or the introduction of nitrogen into the ring in positions adjacent to the chelating groups might also provide molecules

Received October 31, 1966, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115
Accepted for publication December 8, 1966.
Abstracted from the theses submitted by D. A. Williams (1962) and M. D. Baum (1964) in partial fulfillment of Master of Science degree requirements.
This project was supported in part by a grant awarded by The Dow Chemical Co., Midland, Mich.

with an increased affinity for iron, which has a greater affinity for nitrogen than oxygen (7).

Both types of salicylate derivatives were therefore prepared, and their complex forming abilities with Fe (III), Al (III) (a still smaller ion), and Cu (II) (a larger ion, but also involved in cellular oxidations) ions have been determined. Some of the characteristic biological effects of salicylates were then determined, and possible correlations with metal-binding strengths were sought. In this way, evidence for a possible mode of salicylate action might be obtained, or more potent salicylate derivatives found.

METHODS

Analytical reagent grade $Fe(NO_3)_3 \cdot 9H_2O$, $AlCl_3 \cdot 6H_2O$, and $CuCl_2 \cdot 2H_2O$ were used, and carbonate-free 0.01 *M* sodium or potassium hydroxide solution was prepared according to Armstrong (8).

The solutions were stored in polyethylene bottles under nitrogen and diluted quantitatively with boiled distilled water just prior to use. The normality was checked against potassium diphthalate. The organic ligands were prepared as described below, and their purity was ascertained by thin-layer chromatography. The method of Reio (9) was used, in which the developing solvent contained methyl ethyl ketone, acetone, formic acid, and water in the ratio of 40:2:1:6. The plates were sprayed with a solution of bromophenol blue in ethanol (0.06%) containing 1 drop of 10% sodium hydroxide solution, and the presence of one spot confirmed the absence of isomers or other impurities.

Melting ranges were determined with a Mel-Temp apparatus and are corrected. Elemental analyses were obtained from Weiler and Strauss, Oxford, England. 3,4-Pyridinedicarboxylic acid was obtained from Aldrich Chemical Co. and was recrystallized to constant melting point (260°) from water.

2 - Hydroxy - 3,6 - dimethylbenzoic Acid—The method of Moody (10) was modified. 2,5-Dimethylphenol (25 Gm., 0.204 mole) dissolved in 200 ml. of xylene was placed in a 1000-ml. Parr autoclave. Granular sodium (9.5 Gm., 0.41 mole) was added and the bomb was sealed and heated at 160° for 3 hr. Xylene (300 ml.) and dry ice (50 Gm.) were added to the cooled contents, and the bomb was resealed and heated at 160° for 10 hr. with periodic shaking. Water (300 ml.) was then cautiously added to the cooled contents, and the mixture was heated, cooled, and separated. The aqueous layer was acidified, and the resulting precipitate was collected and dried. The crude product was purified by complexation with copper sulfate in aqueous solution, filtering, acidifying the filtrate, and collecting the precipitate. The yield was 24 Gm. (90%), m.p. 197–198°. [Lit. m.p. 193° (10).] Further purification resulted by dissolving the product in aqueous sodium bicarbonate, boiling with activated charcoal, filtering, acidifying, and collecting the precipitate, m.p. 200–201°.

Anal.—Calcd. for $C_9H_{10}O_3$: C, 65.05; H, 6.08. Found: C, 65.36; H, 6.41.

2 - Hydroxy - 3 -methyl - 6 - isopropylbenzoic Acid—A modification of the method of Sondern (11) was used. 2-Methyl-5-isopropylphenol (50 Gm., 0.33 mole) was dissolved in 300 ml. of xylene, which was stirred and refluxed, and carbon dioxide was admitted continuously. Granular sodium (15.3 Gm., 0.67 mole) was added during 1 hr., and the mixture was stirred for 5 hr. and cooled. Water was then added slowly until all sodium was spent. The mixture was heated, cooled, and separated, and the aqueous layer was acidified. The precipitate was collected, dissolved in hot alcohol, and the phenol was removed by addition of water. The hot supernate was allowed to cool, and crystals were collected and dried. After recrystallization from ethanol–acetic acid (1:1), a 16% yield was obtained, m.p. 157–158°. [Lit. m.p. 134° (12).]

Anal.—Calcd. for $C_{11}H_{14}O_3$: C, 68.10; H, 7.22 Found: C, 67.81; H, 7.22.

2 - Hydroxy - 3 - tert - butyl - 6 - methylbenzoic Acid—The above procedure was followed, and from 50 Gm. (0.304 mole) of 2-*tert*-butyl-5-methylphenol was obtained a 19% yield of product, m.p. 183–184°. [Lit. m.p. 182° (10).]

Anal.—Calcd. for $C_{12}H_{16}O_3$: C, 69.28; H, 7.75. Found: C, 69.09; H, 7.69.

2 - Hydroxy - 3,6 - dimethyl - 5 - nitrobenzoic Acid—Ten grams (0.06 mole) of 2-hydroxy-3,6-dimethylbenzoic acid was nitrated according to the method of Sondern (11). The crude product was washed several times with ethylene dichloride, dissolved in aqueous sodium bicarbonate, boiled with activated charcoal, filtered, acidified, and collected. A 16% yield was obtained, m.p. 202–205° dec. [Lit. m.p. 182–184° dec. (10).]

Anal.—Calcd. for $C_9H_9NO_5$: C, 51.30; H, 4.26; N, 6.64. Found: C, 51.36; H, 4.47; N, 6.70.

2 - Hydroxy - 3,6 - dimethyl - 5 - aminobenzoic Acid Hydrochloride—The previous product (1 Gm., 0.0047 mole) was dissolved in absolute ethanol (50 ml.) in a hydrogenation bottle, and 1.1 equivalents of dry hydrogen chloride were added. Ten per cent palladium-on-charcoal (0.5 Gm.) was added, and the compound was hydrogenated at 2–3 atm. After reduction was complete, the catalyst was removed, and the alcohol was evaporated. The residue was washed with ethyl acetate, dissolved in hot glacial acetic acid, and precipitated with benzene. The resulting crystals (0.4 Gm., 40%) melted at 245–246° dec.

Anal.—Calcd. for $C_9H_{12}ClNO_3$: C, 49.66; H, 5.56; N, 6.44. Found: C, 49.80; H, 5.27; N, 6.18.

2 - Hydroxypyridine - 3 - carboxylic Acid—The method of Feibel and Spoerri (13) was used. The product melted at 256–257°. [Lit. m.p. 255° (13).]

Anal.—Calcd. for $C_6H_5NO_3$: C, 51.80; H, 3.62; N, 10.07. Found: C, 51.32; H, 3.75; N, 10.20.

3 - Hydroxypyridine - 4 - carboxylic Acid—The method of Bachman and Barker (14) was used. The product melted at 315–316° dec. [Lit. m.p. 315° dec. (14).]

Anal.—Calcd. for $C_6H_5NO_3$: C, 51.80; H, 3.62; N, 10.07. Found: C, 51.52; H, 3.90; N, 10.26.

Ionization Constants—The methods employed were a spectrophotometric procedure for groups having a pKa value below 2 or above 11 and a potentiometric procedure for groups having a pKa

value between 2 and 11. Both methods are fully described by Albert and Serjeant (15).

Stability Constants—Potentiometric titrations were carried out under nitrogen either in 95% ethanol or in distilled water. Fifty-milliliter volumes of 0.001 *M* solutions of the organic acid were titrated with 0.01 *N* sodium or potassium hydroxide in 0.5-ml. portions first in the absence of metal ions and then in the presence of 0.000025 mole of a divalent metal salt or 0.0000167 mole of a trivalent metal salt. Fifty-milliliter volumes of the same quantities of the metal salts were also titrated with 0.01 *N* alkali. The aqueous solutions titrated were first brought to a constant ionic strength with 0.1 *M* potassium chloride. In several instances, titration with alkali did not uncover all the values necessary for calculation of the constants, so resort was made to back titration with 0.01 *N* hydrochloric acid.

In the case of the titrations done in 95% alcohol, pH values were recorded with a Vibron Electrometer (model 33B, Electronic Instruments Ltd.) with a pH attachment and glass (type GG 33) and calomel (type RJ 23) electrodes, as described by Albert and Serjeant (16). After each addition of alkali, the solution was mixed for 2 min. before the reading was made. For the titrations in water, pH values were measured with a Beckman research pH meter using glass and calomel electrodes.

When 3-hydroxypyridine-4-carboxylic acid was mixed with cupric chloride in the above concentrations, precipitation occurred. This required a 0.0002 *M* solution of the acid and 0.0001 *M* solution of cupric chloride to be titrated with 0.002 *N* alkali.

Calculations were done as previously (17), except that $[L^-]$ has been substituted for [Sc] (concentration of free chelating species), βnm for Ks, and P and Q for β and α, respectively, in conformance with present convention. This gives the following equations for the solution of values of $[L^-]$:

$$\log [L^-] = \log([Lo] - [KOH] - [H^+] + [OH^-]) - \log P \quad \text{(Eq. 1)}$$

$$\log [L^-] = \log(2[Lo] - [KOH] - [H^+] + [OH^-]) - \log P \quad \text{(Eq. 2)}$$

The term $[OH^-]$ was generally dropped, since the titrations were not usually carried to a pH of 10, and the term was therefore insignificant. Values of $\bar{n}$ were calculated from:

$$\bar{n} = \frac{[Lo] - Q[L^-]}{[Mo]} \quad \text{(Eq. 3)}$$

If the pH in the presence of metal ion more closely approximated the pH value obtained with the organic acid alone at one equivalent, then Eq. 1 was used; at two equivalents, Eq. 2. Equation 1 was used for the 2-hydroxypyridine-3-carboxylic acid systems, where one proton was apparently picked up by the ring nitrogen, and only one proton was liberated; Eq. 2 was used for the others. Formation curves were also plotted ($\bar{n}$ against $-\log [L^-]$) to show whether stepwise complexation had taken place (18).

Values of K_1, K_2, and K_3 were calculated as before (17) where the difference between log K_1 and log K_2 was at least 2.5. In those cases where the values of log K_1 and log K_2 were closer, K_1 and β_2 were evaluated from Eq. 4 using the method of least squares (15).

$$\frac{\bar{n}}{(\bar{n} - 1)[L^-]} = \frac{(2 - \bar{n})[L^-]}{(\bar{n} - 1)} \beta_2 - K_1 \quad \text{(Eq. 4)}$$

RESULTS AND DISCUSSION

Table I lists the ionization constants determined for the salicylate analogs and derivatives, and Tables II–IV list the stability constants for the complexes with Cu (*II*), Fe (*III*), and Al (III) ions. The values of the stability constants obtained in aqueous alcohol (final concentration approximately 79% ethanol) cannot be considered valid for some purposes, but for comparison of metal-binding abilities with biological effects, these values should suffice. (For a discussion of the irregularities in ionization constants, and consequently in metal-binding constants, to be expected in nonaqueous or mixed solvents, see *Reference 15*, pp. 66–68.) However, the same sequence of complexing abilities should be found in water as in aqueous alcohol. The complexes of these dialkyl-substituted salicylic acids were too insoluble in water to permit pH measurements in meaningful concentrations, despite the use of the more sensitive instrument employed.

Not all values of K_1, K_2, or K_3 could be obtained by titration with alkali, either because of precipitation of the metal complexes, or because in several cases values of $\bar{n}$ less than 1 were not realized. In the latter cases, high affinity of the metal for the ligand probably prevented part of the titration curve from being revealed, and no complexation was evident until one equivalent of alkali had been added (as in the case of the cupric complexes of the 3,6-dimethyl and 5-nitro-3,6-dimethyl derivatives in water). The latter situation was corrected by back titration with acid.

Table I—Ionization Constants (25°)

Acid	Solvent	pKa^1	pKa^2	pKa^3
2-Hydroxy-3,6-dimethylbenzoic	EtOH	6.07	13.20	
2-Hydroxy-3-*tert*-butyl-6-methylbenzoic	EtOH	5.77	13.05	
2-Hydroxy-3-methyl-6-isopropylbenzoic	EtOH	5.70	13.00	
2-Hydroxy-5-amino-3,6-dimethylbenzoic	EtOH	6.37	11.55	
2-Hydroxybenzoic[a]	H_2O	3.00	13.82	
2-Hydroxy-3,6-dimethylbenzoic	H_2O	3.23	12.32	
2-Hydroxy-5-nitro-3,6-dimethylbenzoic	H_2O	2.78	8.54	
2-Hydroxypyridine-3-carboxylic[b]	H_2O	3.28	5.33	12.62
3-Hydroxypyridine-4-carboxylic	H_2O	0.10	4.83	11.30
3,4-Pyridinedicarboxylic	H_2O	1.50	2.95	5.07

[a] *Reference 15*, p. 134. [b] Canic, V., *et al.*, *Glasnik Khem. Drushtva Beograd*, **21**, (No. 2) 65(1956); through *Chem. Abstr.*, **54**, 8 15(1960).

TABLE II—STABILITY CONSTANTS OF THE CUPRIC COMPLEXES (25°)

Acid	Solvent	Log K_1	Log K_2	Log β_2
2-Hydroxy-3,6-dimethylbenzoic	EtOH–H_2O	13.93	9.48	23.41
2-Hydroxy-3-*tert*-butyl-6-methylbenzoic	EtOH–H_2O	13.21	9.82	23.03
2-Hydroxy-3-methyl-6-isopropylbenzoic	EtOH–H_2O	12.66	8.22	20.88
2-Hydroxy-5-amino-3,6-dimethylbenzoic	EtOH–H_2O	10.51		
2-Hydroxybenzoic[a]	0.1 *M* KCl	10.60	7.85	18.45
2-Hydroxy-3,6-dimethylbenzoic	0.1 *M* KCl	9.06		17.83
2-Hydroxy-5-nitro-3,6-dimethylbenzoic	0.1 *M* KCl	5.87	3.55	9.42
2-Hydroxypyridine-3-carboxylic	0.1 *M* KCl	18.29	15.04	33.33
3-Hydroxypyridine-4-carboxylic	0.1 *M* KCl	9.98		18.47
3,4-Pyridinedicarboxylic	0.1 *M* KCl	3.07		6.52

[a] Sillén, L. G., and Martell, A. E., "Stability Constants of Metal-Ion Complexes," The Chemical Society, Burlington House, London, England, 1964, p. 534.

TABLE III—STABILITY CONSTANTS OF THE FERRIC COMPLEXES (25°)

Acid	Solvent	Log K_1	Log K_2	Log K_3	Log β_3
2-Hydroxy-3,6-dimethylbenzoic	EtOH–H_2O	16.93	14.83		
2-Hydroxy-3-*tert*-butyl-6-methylbenzoic	EtOH–H_2O	16.17	13.86		
2-Hydroxy-3-methyl-6-isopropylbenzoic	EtOH–H_2O	15.57	13.89		
2-Hydroxy-5-amino-3,6-dimethylbenzoic	EtOH–H_2O	12.26	9.40		
2-Hydroxybenzoic[a]	0.1 *M* KCl	16.48	11.68	8.68	36.84
2-Hydroxy-3,6-dimethylbenzoic	0.1 *M* KCl	14.76	11.70		
2-Hydroxy-5-nitro-3,6-dimethylbenzoic	0.1 *M* KCl	8.98	8.31	6.04	23.33
2-Hydroxypyridine-3-carboxylic	0.1 *M* KCl	17.53	15.50		
3-Hydroxypyridine-4-carboxylic	0.1 *M* KCl	11.97	9.16		
3,4-Pyridinedicarboxylic	0.1 *M* KCl	5.68	4.55		

[a] Sillen, L. G., and Martell, A. E., *op. cit.*

TABLE IV—STABILITY CONSTANTS OF THE ALUMINUM COMPLEXES (25°)

Acid	Solvent	Log K_1	Log K_2	Log K_3	Log β_3
2-Hydroxy-3,6-dimethylbenzoic	EtOH–H_2O	16.12	14.67		
2-Hydroxy-3-*tert*-butyl-6-methylbenzoic	EtOH–H_2O	15.12	13.97	11.85	40.94
2-Hydroxy-3-methyl-6-isopropylbenzoic	EtOH–H_2O	15.86	13.89	11.93	41.58
2-Hydroxy-5-amino-3,6-dimethylbenzoic	EtOH–H_2O	12.78	10.65	8.94	32.37
2-Hydroxybenzoic[a]	0.1 *M* KCl	14.00	10.7	8.6	33.3
2-Hydroxy-3,6-dimethylbenzoic	0.1 *M* KCl	11.62	11.06		
2-Hydroxy-5-nitro-3,6-dimethylbenzoic	0.1 *M* KCl	8.64	6.64	4.62	19.90
2-Hydroxypyridine-3-carboxylic	0.1 *M* KCl	16.97	14.90		
3-Hydroxypyridine-4-carboxylic	0.1 *M* KCl	12.00	8.01	7.39	27.40
3,4-Pyridinedicarboxylic	0.1 *M* KCl	4.06	4.02	3.73	11.81

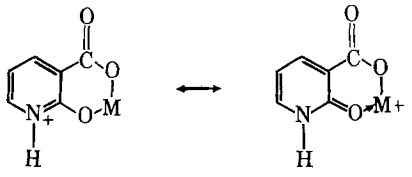
[a] *Reference 17.*

The sequence of complexing abilities found was essentially the same for either the cupric, ferric, or aluminum complexes.

With the 3,6-dialkyl-substituted salicylic acids, the 3,6-dimethyl compound showed more avidity for the metal ions than the more bulky isopropyl or *tert*-butyl derivatives. It also showed less metal-complexing ability in water than salicylic acid itself, so the entire series of 3,6-dialkylsalicylic acids may be considered as having lower complexing ability not only for iron but for the other metal ions observed. Apparently, increased bulk around the chelating carboxyl and hydroxyl functions did not contribute to increased stability, but led instead to increased steric hindrance. Introduction of the 5-amino group also lowered stability, possibly by quinoid contributions by the phenolic group which lowered its basicity. The 5-nitro group lowered stability as would be predicted from its electron withdrawing effect. The presence of a ring-nitrogen atom adjacent to the phenolic group did, however, augment complex stability considerably.

Comparison of the complex stability constants with the ionization constants shows the complex stabilities paralleled by the phenolic ionizations, the greater the basicity of the phenolic group, the greater the stability of the complex. An apparent exception to this may be seen with 2-hydroxypyridine-3-carboxylic acid, where the presence of the ring nitrogen adjacent to the phenolic group would be expected to increase its acidity and thus lower, in some cases, the complex stability constants. The fact that both the phenolic ionization and complex stabilities are greater than those of the 3-hydroxypyridine-4-carboxylic acid suggests that the 2-hydroxy group may undergo tautomerism leading to increased resonance (see structures I and II) and thus increased stability, in the chelate ring. The 3-hydroxy compound is structurally incapable of this tautomerism. That the 5-amino group in the 3,6-dimethylsalicylic acid, also capable of tautomerism, did not show the same enhancement of complex stability may be attributed to the π-excessive character of the 5-amino-substituted ring

and the π-deficient character of the pyridine ring.



I II

Biologic Activities—Results of analgesic testing[1] of the 3,6-dialkyl-substituted salicylic acids showed that both the 3,6-dimethyl and 3-methyl-6-isopropyl derivatives had about 40% greater activity than aspirin, while the 3-*tert*-butyl-6-methyl derivative had about 20% more activity. Analgesic testing was carried out in rats (20 per compound) at compound dosages of 300 mg./Kg. by heating the rat's tail. Because of the lower metal-binding abilities of these acids in water in comparison to that of salicylic acid, it cannot be concluded that greater avidity for metal ions confers greater analgesic effects. It is significant, however, that *o*-thymotic acid also claimed to have a greater analgesic effect than salicylate (19) has also somewhat lower metal-binding constants (1).

Tests for anti-inflammatory and antierythemic effects[2] were also positive for 3,6-dimethylsalicylic acid. In this test, carried out by the method of Winder *et al.* (20), a dosage level of 80 mg./Kg. in guinea pigs produced a decrease in response to U.V.-induced erythema in $^7/_8$ animals, compared to the same response from 100 mg./Kg. of aspirin. In comparison, 3,5-diisopropylsalicylic acid (1) reduced these effects in only $^2/_8$ animals, and both 2-hydroxypyridine-3-carboxylic acid and 3-hydroxypyridine-4-carboxylic acid showed no effects at 80 mg./Kg.

[1] Carried out under the direction of Dr. Howard J. Jenkins.
[2] These results were supplied through the courtesy of Dr. Blaine M. Sutton, Smith Kline & French Laboratories.

REFERENCES

(1) Foye, W. O., and Turcotte, J. G., *J. Pharm. Sci.*, **51**, 329(1962).
(2) Bollet, A. J., *Arthritis Rheumat.*, 4, 624(1961).
(3) Smith, M. J. H., Bryant, C., and Hines, W. J. W., *Nature*, **202**, 96(1964).
(4) Bizzi, A., Codegoni, A. M., and Garattini, S., *ibid.*, **204**, 1205(1964).
(5) Gould, J., and Smith, M. J. H., *J. Pharm. Pharmacol.*, **17**, 83(1965).
(6) Foye, W. O., Solis, M. C. M., Schermerhorn, J. W., and Prien, E. L., *J. Pharm. Sci.*, **54**, 1365(1965).
(7) Albert, A., *Federation Proc., No. 3-Suppl. 10*, **20**, 141(1961).
(8) Armstrong, D. M. G., *Chem. Ind.*, **1955**, 1405.
(9) Reio, L., *J. Chromatog.*, **2**, 338(1958); **4**, 458(1960).
(10) Moody, J. E., Jr., Ph.D. Thesis, University of Connecticut, Storrs, Conn., 1960.
(11) Sondern, C., *Arch. Pharm.*, **11**, 28(1940).
(12) Kekulé, A., *Ber.*, **6**, 1089(1873).
(13) Feibel, L. R., and Spoerri, P. E., *J. Am. Chem. Soc.*, **70**, 3908(1948).
(14) Bachman, G. B., and Barker, R. S., *J. Org. Chem.*, **14**, 97(1947).
(15) Albert, A., and Serjeant, E. P., "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962.
(16) Albert, A., and Serjeant, E. P., *Biochem. J.*, **76**, 621 (1960).
(17) Pecci, J., and Foye, W. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 411(1960).
(18) Albert, A., *Biochem. J.*, **54**, 646(1953).
(19) O'Brien, J. E., and Thoms, R. K., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 258(1958).
(20) Winder, C. V., Wax, J., Burr, V., Benn, M., and Rosiere, C. E., *Arch. Intern. Pharmacodyn.*, **116**, 221(1958).

Constituents of the Rhizome of *Asarum canadense*

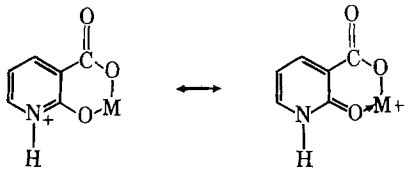
By LUDWIG BAUER, CHARLES L. BELL, JAMES E. GEARIEN, and HIROSHI TAKEDA

A pentane extract from the rhizomes of *Asarum canadense* was separated first by means of steam distillation, and then by column and gas chromatography. From the steam-volatile oil the following constituents were isolated (percentages are shown in parentheses): methyleugenol (44.5), linalyl acetate (41.1), geraniol (7.4), linalool (5.3), limonene (0.8), α-terpineol (0.4), bornyl acetate (0.3), aristolone (0.1), elemicin (0.1), 2,3,4,5-tetramethoxyallylbenzene (0.05), 2,4-dimethoxycinnamaldehyde (0.05), and two unidentified compounds (0.01 each). The steam-nonvolatile residue contained some of the above and also β-sitosterol.

THE FRAGRANT essential oil of *Asarum canadense* (Canada snakeroot, wild ginger) has not been investigated since 1902 when Power reported it to contain mainly methyleugenol (3,4-dimethoxyallylbenzene), pinene, and (after saponification) linalool, borneol, terpineol, geraniol, and several unidentified compounds (1, 2). This work set out to re-examine this oil and, in particular, search for minor constituents. Instead of subjecting the ground rhizomes immediately to steam distillation (1, 2), it was advantageous to obtain a pentane extract first which then was separated into a steam-volatile fraction (oil *A*) and a residue (oil *B*). Examination of oil *A*, by gas chromatography (GC) revealed the presence of at least 13 components (Fig. 1, Table

Received September 1, 1966, from the Department of Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680
Accepted for publication November 7, 1966.
Abstracted in part from a dissertation presented by H. Takeda to the University of Illinois at the Medical Center, Chicago, June 1966, in partial fulfillment of Doctor of Philosophy degree requirements.
The authors acknowledge with thanks receipt of the following authentic samples: aristolone from Dr. N. Soma, Takamine Laboratory, Osaka, Japan; ferulone from Dr. A. Marsili, University of Pisa, Pisa, Italy; elimicin from Dr. A. T. Shulgin, Dow Chemical Co., Walnut Creek, Calif.; 2,3,4,5-tetramethoxyallylbenzene from Dr. E. Stahl, Universität des Saarlandes, Saarbrücken, Germany; β-sitosterol from Dr. Percy L. Julian, Julian Associates, Inc., Franklin Park, Ill.

and the π-deficient character of the pyridine ring.



I II

Biologic Activities—Results of analgesic testing[1] of the 3,6-dialkyl-substituted salicylic acids showed that both the 3,6-dimethyl and 3-methyl-6-isopropyl derivatives had about 40% greater activity than aspirin, while the 3-*tert*-butyl-6-methyl derivative had about 20% more activity. Analgesic testing was carried out in rats (20 per compound) at compound dosages of 300 mg./Kg. by heating the rat's tail. Because of the lower metal-binding abilities of these acids in water in comparison to that of salicylic acid, it cannot be concluded that greater avidity for metal ions confers greater analgesic effects. It is significant, however, that *o*-thymotic acid also claimed to have a greater analgesic effect than salicylate (19) has also somewhat lower metal-binding constants (1).

Tests for anti-inflammatory and antierythemic effects[2] were also positive for 3,6-dimethylsalicylic acid. In this test, carried out by the method of Winder *et al.* (20), a dosage level of 80 mg./Kg. in guinea pigs produced a decrease in response to U.V.-induced erythema in $^7/_8$ animals, compared to the same response from 100 mg./Kg. of aspirin. In comparison, 3,5-diisopropylsalicylic acid (1) reduced these effects in only $^2/_8$ animals, and both 2-hydroxypyridine-3-carboxylic acid and 3-hydroxypyridine-4-carboxylic acid showed no effects at 80 mg./Kg.

[1] Carried out under the direction of Dr. Howard J. Jenkins.
[2] These results were supplied through the courtesy of Dr. Blaine M. Sutton, Smith Kline & French Laboratories.

REFERENCES

(1) Foye, W. O., and Turcotte, J. G., *J. Pharm. Sci.*, **51**, 329(1962).
(2) Bollet, A. J., *Arthritis Rheumat.*, 4, 624(1961).
(3) Smith, M. J. H., Bryant, C., and Hines, W. J. W., *Nature*, **202**, 96(1964).
(4) Bizzi, A., Codegoni, A. M., and Garattini, S., *ibid.*, **204**, 1205(1964).
(5) Gould, J., and Smith, M. J. H., *J. Pharm. Pharmacol.*, **17**, 83(1965).
(6) Foye, W. O., Solis, M. C. M., Schermerhorn, J. W., and Prien, E. L., *J. Pharm. Sci.*, **54**, 1365(1965).
(7) Albert, A., *Federation Proc., No. 3-Suppl. 10*, **20**, 141(1961).
(8) Armstrong, D. M. G., *Chem. Ind.*, **1955**, 1405.
(9) Reio, L., *J. Chromatog.*, **2**, 338(1958); **4**, 458(1960).
(10) Moody, J. E., Jr., Ph.D. Thesis, University of Connecticut, Storrs, Conn., 1960.
(11) Sondern, C., *Arch. Pharm.*, **11**, 28(1940).
(12) Kekulé, A., *Ber.*, **6**, 1089(1873).
(13) Feibel, L. R., and Spoerri, P. E., *J. Am. Chem. Soc.*, **70**, 3908(1948).
(14) Bachman, G. B., and Barker, R. S., *J. Org. Chem.*, **14**, 97(1947).
(15) Albert, A., and Serjeant, E. P., "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962.
(16) Albert, A., and Serjeant, E. P., *Biochem. J.*, **76**, 621 (1960).
(17) Pecci, J., and Foye, W. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 411(1960).
(18) Albert, A., *Biochem. J.*, **54**, 646(1953).
(19) O'Brien, J. E., and Thoms, R. K., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 258(1958).
(20) Winder, C. V., Wax, J., Burr, V., Benn, M., and Rosiere, C. E., *Arch. Intern. Pharmacodyn.*, **116**, 221(1958).

Constituents of the Rhizome of *Asarum canadense*

By LUDWIG BAUER, CHARLES L. BELL, JAMES E. GEARIEN, and HIROSHI TAKEDA

A pentane extract from the rhizomes of *Asarum canadense* was separated first by means of steam distillation, and then by column and gas chromatography. From the steam-volatile oil the following constituents were isolated (percentages are shown in parentheses): methyleugenol (44.5), linalyl acetate (41.1), geraniol (7.4), linalool (5.3), limonene (0.8), α-terpineol (0.4), bornyl acetate (0.3), aristolone (0.1), elemicin (0.1), 2,3,4,5-tetramethoxyallylbenzene (0.05), 2,4-dimethoxycinnamaldehyde (0.05), and two unidentified compounds (0.01 each). The steam-nonvolatile residue contained some of the above and also β-sitosterol.

THE FRAGRANT essential oil of *Asarum canadense* (Canada snakeroot, wild ginger) has not been investigated since 1902 when Power reported it to contain mainly methyleugenol (3,4-dimethoxyallylbenzene), pinene, and (after saponification) linalool, borneol, terpineol, geraniol, and several unidentified compounds (1, 2). This work set out to re-examine this oil and, in particular, search for minor constituents. Instead of subjecting the ground rhizomes immediately to steam distillation (1, 2), it was advantageous to obtain a pentane extract first which then was separated into a steam-volatile fraction (oil *A*) and a residue (oil *B*). Examination of oil *A*, by gas chromatography (GC) revealed the presence of at least 13 components (Fig. 1, Table

Received September 1, 1966, from the Department of Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680
Accepted for publication November 7, 1966.
Abstracted in part from a dissertation presented by H. Takeda to the University of Illinois at the Medical Center, Chicago, June 1966, in partial fulfillment of Doctor of Philosophy degree requirements.
The authors acknowledge with thanks receipt of the following authentic samples: aristolone from Dr. N. Soma, Takamine Laboratory, Osaka, Japan; ferulone from Dr. A. Marsili, University of Pisa, Pisa, Italy; elimicin from Dr. A. T. Shulgin, Dow Chemical Co., Walnut Creek, Calif.; 2,3,4,5-tetramethoxyallylbenzene from Dr. E. Stahl, Universität des Saarlandes, Saarbrücken, Germany; β-sitosterol from Dr. Percy L. Julian, Julian Associates, Inc., Franklin Park, Ill.

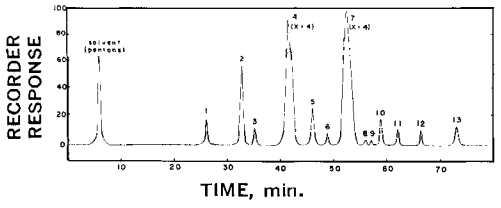


Fig. 1—Vapor-phase chromatogram of the steam-volatile oil, A, from a pentane extract of the rhizomes of Asarum canadense *(20 ft. × 3/8-in. o.d. coiled aluminum column), packed with 20% SE-30 on Chromosorb W, nonlinearly programmed from 90 to 250° over 54 min. using the Autoprep model A-700, the helium flow rate being 200 ml. per minute measured at the outlet of the apparatus. Table I lists the constituents associated with the numbered peaks.*

I) of which two were present in about equal amounts and composed 85% of the oil. Although column chromatography failed to separate the components of oil *A*, this technique served to concentrate the minor constituents somewhat more effectively (see Table II). Each eluate was resolved by means of preparative GC, and the pure components were identified by comparing their various spectra with those of authentic samples and those in the literature. The constituents of the steam-volatile oil *A* are discussed first (Fig. 1, Table I).

DISCUSSION

Monoterpene Hydrocarbons—The first fraction in the GC of *A* was identified as limonene by its mass spectrum (molecular ion *m/e* 136, base peak *m/e* 68), identical to the published spectrum (3), and to that of an authentic sample of dipentene. Since pinene was reported to be present in this oil (2), attempts were made to find this hydrocarbon by examining the mass spectra of all GC fractions before and after chromatography of *A*. Although the retention times (under the conditions for the separation of *A*) and mass spectra of pure α- and β-pinenes were in hand, these hydrocarbons could not be detected. It was observed that after chromatography of *A*, β-myrcene was found in fraction *A*-I which was identified by its NMR (4, 5) and mass spectra (3, 5, 6). This hydrocarbon was definitely not present in oil *A* and it was found that at the same time, the amount of linalyl acetate in *A* decreased from 44 in *A* to 1.8% after chromatography. The suspicion that linalyl acetate underwent elimination was confirmed when a sample of the ester was chromatographed under similar conditions to give β-myrcene, linalool, and unchanged ester in the ratio of 2:1:2. It was interesting to note that alumina acted as a basic catalyst promoting the elimination of acetic acid from linalyl acetate to give β-myrcene only, uncontaminated by *cis*- and *trans*-β-ocimene (5). This reaction is so different from the catalytic pyrolysis of linalyl acetate (7) over acid-washed Chromosorb P at 140–150° to give β-myrcene, *cis*- and *trans*-β-ocimene, and dipentene in the ratio of 43:20:35:2. The conversion of the linalyl acetate to linalool during the chromatography can be explained by either a hydrolytic process due to adsorbed water or by ester interchange during elution with a mixture of chloroform and ethanol.

TABLE I—COMPONENTS OF THE VAPOR-PHASE CHROMATOGRAM SHOWN IN FIG. 1

Peak No.	Component	Retention Time, min.	Compn. Vol., %
1	Limonene	25.4	0.8
2	Linalool	32.5	5.3
3	α-Terpineol	35.0	0.4
4	Linalyl acetate	41.6	41.1
5	Geraniol	46.0	7.2
6	Bornyl acetate	48.5	0.3
7	Methyleugenol	52.0	44.5
8	Unidentified	56.0	0.01
9	Unidentified	57.0	0.01
10	Elemicin	58.5	0.1
11	3,4-Dimethoxycinnamaldehyde	62.0	0.05
12	2,3,4,5-Tetramethoxyallylbenzene	66.6	0.05
13	Aristolone	73.0	0.1

TABLE II—COLUMN CHROMATOGRAPHY OF OIL *A*

Fraction	Eluting Solvent	Yield, Gm.	Components	Percentage, from GC
A-I	Pentane	23	β-Myrcene	30
			Limonene	25
			Geraniol	10
			Bornyl acetate	5
			Methyleugenol	25
A-II	Pentane–benzene (9:1)	7	Geraniol	1
			Bornyl acetate	1
			Methyleugenol	95
			3,4-Dimethoxycinnamaldehyde	3
A-III	Benzene	5	Linalool	10
			Geraniol	2.5
			Bornyl acetate	2.5
			Methyleugenol	80
			3,4-Dimethoxycinnamaldehyde	5
A-IV	Benzene–ether (9:1)	3.9	Linalool	30
			Methyleugenol	60
			Elemicin	6
			2,3,4,5-Tetramethoxyallylbenzene	4
A-V	Ether	2.0	Linalool	45
			α-Terpineol	35
			Methyleugenol	5
			Aristolone	5
A-VI	Ether–chloroform (9:1)	1.0	Linalool	40
			α-Terpineol	35
			Aristolone	5
A-VII	Chloroform	1.0	Linalool	80
			α-Terpineol	15
A-VIII	Chloroform–ethanol (9:1)	8.0	Linalool	40
			α-Terpineol	10
			Linalyl acetate	10
			Geraniol	40

Monoterpene Alcohols and Esters—In identifying these components, it was possible to make use of the recently published mass spectra of a number of monoterpene alcohols and esters (8, 9). Cognizance was taken of the fact that in a number of instances the mass spectra of alcohols or their esters did not show the parent peak (8), the first peak for the alcohols being at M-18 and for the esters being either (M-RCO-1) or at (M-RCO_2H) (9).

Of the terpene alcohols in the steam-volatile oil *A*, geraniol was found in largest abundance and

was identified by its mass spectrum, which was identical to that of a purified authentic specimen and to the published spectrum (6, 8). Its NMR spectrum ($CDCl_3$) also matched the published one (10), except it was possible to observe the exchangeable OH proton as a singlet at 2.53 δ. α-Terpineol was also present and was identified by its mass spectrum (8) and its NMR spectrum ($CDCl_3$) which showed the vinyl proton at 5.48 δ, a singlet for one CH_3 at 1.75 δ, one for two CH_3's at 1.27 δ. In the steam-volatile oil *A*, linalool was found only in about 4%, but after chromatography its percentage rose to 15.6% (see above). The NMR spectrum of linalool in $CDCl_3$ showed three CH_3 singlets at 1.26, 1.60, and 1.68 δ, the exchangeable OH proton at 2.05 δ, and the alkene protons as complex multiplets between 4.9 and 6.3 δ. The mass spectrum of this sample of linalool also showed a peak at *m/e* 155 (M + 1) which had been noted previously (8) and been the subject of considerable discussion.[1]

Linalyl acetate was the major ester fraction of this essential oil and was found to be quite stable to steam distillation and to GC under the conditions described, but did react on alumina (see above). Its mass spectrum was not included among the terpene esters studied by Sydow (9) and is appended (Fig. 2). Like most terpene esters, it lacked the molecular ion peak at *m/e* 196, and the first fragment appeared at *m/e* 136 (M-60, M-CH_3-CO_2H). At first glance the fragmentation pattern appeared almost like that of β-myrcene—with some additional peaks and changes in relative intensities (see Table III). The peak *m/e* 60 is attributed to acetic acid, a fragment which was observed sometimes in the mass spectra of terpene acetates (9).

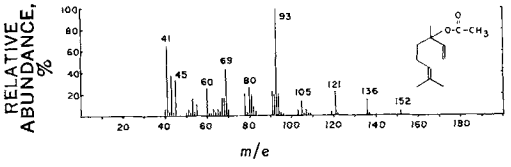


Fig. 2—The mass spectrum of linalyl acetate.

The only other ester which was isolated was bornyl acetate whose NMR and mass spectra matched those of an authentic sample. Although the mass spectra of bornyl and isobornyl acetates are almost identical (9), Biemann (11) was able to distinguish between these two isomers on the basis of the ratio of the abundance of the M and (M-60) ions. The identity of the ester isolated here was established here on the basis of its NMR spectrum ($CDCl_3$), which showed the methine proton as a doublet of quartets at 4.91 δ [previously reported at 5.19 δ, in CCl_4 (12)] which distinguished this ester from isobornyl acetate. Furthermore, the infrared spectrum of the isolated ester matched that of bornyl rather than isobornyl acetate, particularly due to differences in the 1000–1100 cm.$^{-1}$ region.

Table III—Mass Spectra of Linalyl Acetate, Linalool, and β-Myrcene

Linalyl Acetate (Mol. Wt. 196)		Linalool (Mol. Wt. 154)		β-Myrcene (Mol. Wt. 136)	
m/e	% Σ_{40}	*m/e*	% Σ_{40}	*m/e*	% Σ_{40}
40	0.6	41	8.1	40	1.3
41	10.4	43	6.3	41	14.6
42	0.3	53	1.7	42	1.4
43	6.1	54	1.1	43	2.6
44	0.3	55	6.3	44	2.6
45	5.2	56	1.5	51	1.7
50	0.3	57	0.3	53	2.3
51	0.6	58	0.3	55	1.7
52	0.3	59	2.5	64	0.8
53	2.4	67	1.7	65	1.4
54	0.3	68	2.0	67	2.9
55	1.6	69	7.8	68	2.0
60	4.0	71	10.8	69	12.0
61	0.3	72	0.5	70	1.7
62	0.3	77	2.0	77	4.1
63	0.8	79	2.5	78	0.8
64	0.4	80	4.5	79	5.8
65	0.8	81	2.0	80	3.5
66	0.4	82	0.3	81	0.8
67	2.4	83	1.7	82	0.8
68	2.4	91	1.0	91	4.4
69	6.9	92	2.5	92	3.5
70	0.6	93	12.6	93	13.1
78	3.2	94	2.5	94	3.5
79	0.3	95	0.7	96	1.1
80	4.0	96	1.6	105	1.0
81	3.2	97	0.3	107	1.0
82	1.3	105	2.2	121	3.2
83	0.4	106	0.3	136	2.9
91	3.5	107	1.7	137	0.5
92	3.2	108	0.5	...	...
93	16.0	109	1.2	...	...
94	3.2	111	0.7	...	...
95	0.4	120	0.3	...	...
96	0.3	121	3.0	...	...
103	0.6	135	0.3	...	...
105	2.1	136	2.5	...	...
106	0.3	139	0.3	...	...
107	0.8	155	0.5	...	...
108	0.3	...	...	...	...
109	0.3	...	...	...	...
119	0.4	...	...	...	...
121	3.5	...	...	...	...
122	0.3	...	...	...	...
136	2.4	...	...	...	...
137	0.3	...	...	...	...
152	0.6	...	...	...	...

Aromatic Compounds—Since a number of different alkoxy substituted allyl- and propenylbenzenes have been discovered in several *Asarum* species (13), *e.g.*, asarone [*trans*-1-(2,4,5-trimethoxyphenyl)-1-propene] and *trans*-methylisoeugenol, the predominant ethers in *Asarum europeum* (14), safrole, the principal component of *A. arifolium*, and mainly methyleugenol in *A. caduatum* and *canadense*, a particular effort was made to determine the nature of the aromatic constituents in *A. canadense*.

The major component of the steam-volatile oil *A* was proved to be methyleugenol whose spectra matched those of an authentic sample. It was oxidized by potassium permanganate to veratric acid (see under *Experimental* for the oxidation of elemicin). Its NMR spectrum (neat) showed the benzyl protons as a doublet (with fine structure) at 3.27 δ (J = 6 c.p.s.), the two OCH_3 resonances at

[1] It was suggested by Willhalm, B., Thomas, A. F., and Stoll, M., *Acta Chem. Scand.*, **18**, 1573(1964), that the peak *m/e* 155 stems from contamination of samples of linalool by (*cis* and *trans*) linalool oxides which are almost impossible to separate from the alcohol by chromatography. Since the mass spectra of the oxides [Felix, D., Melera, A., Seibl, J., and Kovats, E., *Helv. Chim. Acta*, **46**, 1513(1963)] contain fragments also present in the mass spectrum of linalool, the origin of the *m/e* 155 peak remains to be discovered.

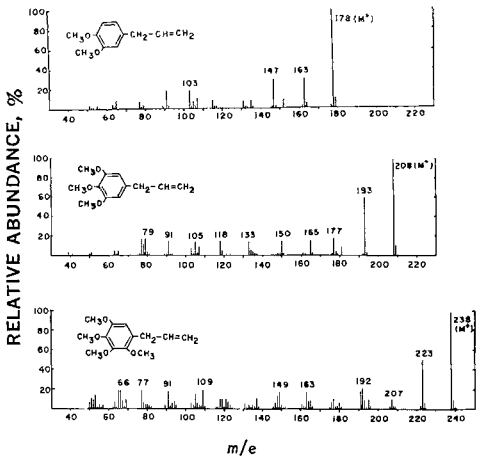


Fig. 3—The mass spectra of methyleugenol (top), elemicin (middle), and 2,3,4,5-tetramethoxyallylbenzene (bottom).

m/e 135.[2] The loss of a methoxy radical from the molecular ion showed the peak at *m/e* 147 (confirmed by the metastable ion at 69.5). The formation of a fragment *m/e* 151 by the loss of a vinyl radical from the molecular ion (M-27) would be expected to produce a substituted tropylium ion whose stability would overcome the relatively unstable vinyl radical. It should be noted that the mass spectrum of methylisoeugenol was quite similar to that of methyleugenol except for the absence of the peak at *m/e* 151 (M-27) in the former.

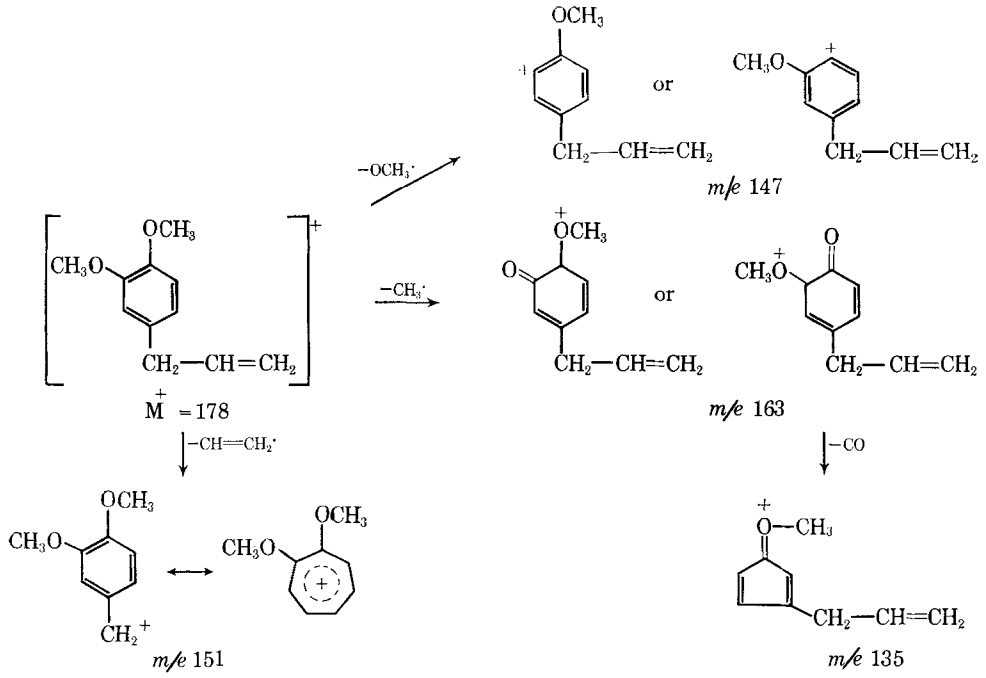
A small amount of elemicin (3,4,5-trimethoxyallylbenzene) was isolated from oil *A* and was identical to an authentic sample (15). Its structure was proved also by permanganate oxidation to 3,4,5-trimethoxybenzoic acid. Its NMR spectrum ($CDCl_3$) showed one aromatic proton at 6.41δ and the nine OCH_3 protons as a singlet at 3.82δ. The mass spectrum of elemicin (Fig. 3, Table IV) showed that the parent peak (208) was the base peak and the next most intense peak (61%) was *m/e* 193 (M-15) due to loss of a methyl radical,

TABLE IV—MASS SPECTRA OF METHYLEUGENOL, ELEMICIN, 2,3,4,5-TETRAMETHOXYALLYLBENZENE, AND 3,4-DIMETHOXYCINNAMALDEHYDE

Methyleugenol		Elemicin		2,3,4,5-Tetramethoxyallylbenzene		3,4-Dimethoxycinnamaldehyde	
m/e	% Σ_{40}	*m/e*	% Σ_{40}	*m/e*	% Σ_{40}	*m/e*	% Σ_{40}
51	1.8	51	1.3	51	1.5	51	0.9
65	1.8	63	1.3	65	2.7	52	2.5
77	2.4	65	1.3	66	3.0	53	1.1
79	1.8	77	3.5	67	1.4	63	0.9
91	4.8	78	1.7	77	2.7	64	1.7
103	4.8	79	3.9	78	1.1	66	1.1
105	1.8	91	3.5	91	2.1	77	4.3
107	3.3	103	1.5	94	1.1	78	2.7
115	2.1	105	2.8	103	1.1	89	1.7
131	1.8	107	1.5	105	1.9	91	3.6
135	2.4	118	3.5	109	2.2	102	1.1
147	8.4	119	1.3	118	1.1	103	2.5
151	4.2	133	3.2	119	1.1	106	1.4
163	8.4	134	1.3	121	1.6	118	1.7
178M+	30.0	135	1.3	137	1.8	121	3.6
179	3.6	150	3.0	148	1.8	123	1.1
180	0.6	151	1.1	149	2.1	132	0.9
...	...	161	1.1	163	3.0	133	1.7
...	...	165	3.0	164	1.4	138	2.1
...	...	177	3.5	165	1.4	149	3.6
...	...	178	1.1	177	1.8	151	1.1
...	...	181	1.7	191	2.5	161	12.5
...	...	193	13.5	193	1.4	162	2.3
...	...	194	1.3	195	1.2	163	1.7
...	...	208M+	21.6	207	1.4	164	1.1
...	...	209	2.4	223	6.3	177	3.6
...	...	210	0.4	238M+	13.7	191	3.3
...	...	...	...	239	1.6	192M+	17.9
...	...	...	...	240	0.3	193	2.3
...	...	...	...	...	...	194	0.5

3.66 and 3.67 δ, and the aromatic protons as a singlet at 6.72 δ. The vinyl protons formed an ABC spin system, and that portion of the spectrum resembled the pattern for eugenol and safrol (10). The mass spectrum of methyleugenol (Fig. 3 and Table IV) showed that the parent peak, $M^+ = 178$, was the base peak. Several schemes are suggested to explain some of the peaks observed (Scheme I). Loss of a CH_3 radical produced the peak at *m/e* 163 (confirmed by a metastable ion at 149.2) which might be expected to lose CO to give a fragment (corresponding metastable peak at 179). The peak at *m/e* 184 (M-27) indicated the loss of a vinyl radical (4%), and *m/e* 177 (M-31) also appeared in 15% which is considerably lower compared to that of methyleugenol (25%). The successive loss of a methyl group and carbon monoxide, *m/e* 165 (M-15-28), was confirmed from the metastable peak at 141. A further loss of a methyl radical

[2] It was reported that the mass spectra of *o*- and *p*-dimethoxybenzenes showed peaks at *m/e* 123 (M-15) and *m/e* 95 (N-15-28) due to the successive loss of a CH_3 radical and then CO (*Reference 17*, p. 179).



Scheme I

from 165 produces a peak *m/e* 150, and the corresponding metastable peak at 136.3 was also observed. The initial fragmentation of elemicin may proceed by an analogous path to that of methyleugenol.

A minute quantity of 2,3,4,5-tetramethoxyallylbenzene was isolated whose NMR and mass spectra (Fig. 3, Table IV) were identical to an authentic sample obtained from parsley (16). Its NMR spectrum ($CDCl_3$) showed only three OCH_3 singlets at 3.80, 3.87, and 3.93δ, (in the ratio of 2:1:1) and the aromatic proton at 6.48δ and the pattern usually associated with the allyl group.

The only other aromatic compound which was isolated at this time was methylconiferaldehyde (3,4-dimethoxycinnamaldehyde), which was identical to an authentic sample. Its NMR spectrum ($CDCl_3$) showed the OCH_3 protons as a singlet at 3.80δ, the aldehyde proton at 9.6δ (J = 8 c.p.s.), and the rest of the spectrum similar to that of cinnamaldehyde. Its mass spectrum (Fig. 4, Table IV) showed the base peak at M^+ = 192, and the successive loss of a CH_3 radical (M-15, *m/e* 177, metastable peak at 163.1) followed by the loss of CO (M-15-28, *m/e* 149 metastable peak at 125.4). The successive loss of a OCH_3 radical (M-31, *m/e* 161, metastable at 135) and then CO (M-31-28, *m/e* 133, metastable at 109.1) was also confirmed, this CO stemming probably from the aldehyde side chain, a behavior observed previously in aromatic aldehydes (17).

Safrole could not be detected in any of the fractions collected, nor could there be found any propenylbenzenes. The latter would have shown up readily in the NMR spectra, since the CH_3 doublet of the CH_3CH=CH—group would have been readily discernible.

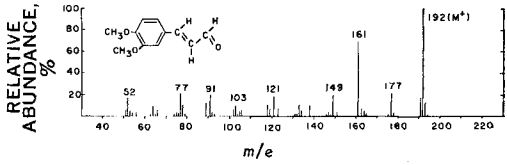


Fig. 4—The mass spectrum of 2,4-dimethoxycinnamaldehyde.

Aristolone—This sesquiterpene ketone was isolated from the steam volatile oil and the nonvolatile residue. It was shown to be identical to a sample previously isolated from *Aristolochia debilis* (18) and to an enantiomer, ferulone, obtained after air-oxidation of ferulene which was found in *Ferula communis* (19).

The structure and stereochemistry of aristolone had been established and the ketone shown to possess a cyclopropane ring (18). Although the cyclopropane protons were identified clearly in the NMR spectra of a number of hydrocarbons possessing the aristolane skeleton (19, 20), these protons were not clearly discernible in our 60-Mc. spectra of aristolone but were identified clearly in the 100-Mc. NMR spectrum[3] in C_6D_6 (Fig. 5, see under *Experimental*).

The mass spectrum of aristolone (Fig. 6) showed the molecular ion peak to be the base peak at *m/e* 218. The major fragmentation peaks were those resulting from loss of 15 (CH_3), 29 (C_2H_5), 43 (C_3H_7), 57 (C_4H_9), 71 (C_5H_{11}), 85 (C_6H_{13}), and 99 (C_7H_{15}) from the molecular ion, the charge remain-

[3] Determined on a Varian HA-100 NMR spectrometer by LeRoy F. Johnson, Varian Associates, Inc., Palo Alto, Calif. The authors thank Mr. Johnson for obtaining this spectrum and also for performing the decoupling experiments.

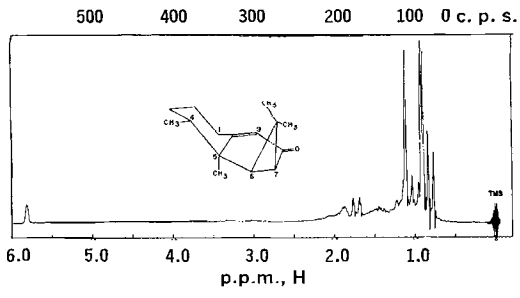


Fig. 5—The NMR spectrum of aristolone in C_6D_6 at 100 Mc.

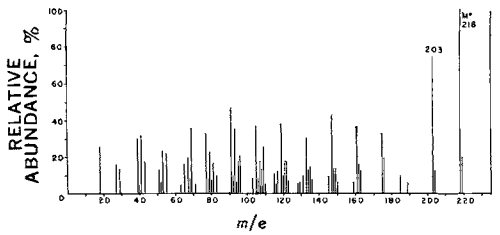


Fig. 6—The mass spectrum of aristolone.

ing with the oxygen-containing fragment. The corresponding metastable peaks for production of the seven fragments were also observed.

β-Sitosterol—This steroid was isolated from the steam-nonvolatile fraction *B* and identified by comparison with an authentic sample. This steroid is widely distributed in plants (21) and interestingly enough has just been found in *A. europeum* (22).

EXPERIMENTAL

Boiling and melting points are uncorrected. Mass spectra were obtained by means of the Hitachi RMU-6D mass spectrometer, ionizing voltage was 75 e.v., inlet temperature for the terpene derivatives being kept to a minimum. In recording the spectra, relative intensities of each fragment above 5% of the strongest line are shown except in the region of the molecular ion where all lines are included. NMR spectra were determined by means of the Varian A-60 spectrometer using tetramethylsilane (TMS) as internal reference, all signals being recorded in parts per million (δ) downfield from TMS. Infrared spectra were taken between salt plates or in $CHCl_3$ solution on a Perkin-Elmer model 337 spectrophotometer.

Gas Chromatography—Preparative GC was carried out by means of the Aerograph Autoprep model A-700 chromatograph using 20-ft. × $^3/_8$-in. o.d. coiled aluminum columns packed with either 20% silicon gum rubber (SE-30) on Chromosorb W (40–60 mesh), or with 20% diethyleneglycol succinate (DEGS) on firebrick (40–60 mesh), both purchased from Wilkins Instrument and Research Co., Inc. All runs were nonlinearly temperature programmed at the "50" power setting, helium was used as the carrier gas, the flow rate was 200 ml./min. as measured at the outlet. For preparative runs, 0.4 to 0.6-ml. aliquots were injected repeatedly, the fractions trapped in 5-ml. glass traps, and cooled in dry ice and 2-propanol. After collection, the fractions were permitted to come to room temperature and reinjected in 1–5-μl. aliquots into a 150-ft. × 0.01-in. o.d. analytical column coated with castorwax (isothermal runs) in a Perkin-Elmer model 800 chromatograph, the flow of helium being 5 ml./min. as measured at the outlet.

Authentic Samples—The following samples were obtained commercially: β-myrcene (Aldrich Chemical Co.), α-pinene and dipentene (K and K Laboratories), geraniol and linalool (Eastman Chemicals), α-terpineol and linalyl acetate (Matheson Scientific Co.), bornyl acetate (Baker Chemical Co.). These were either distilled *in vacuo* or steam distilled and then purified by preparative GC (SE-30 column, Autoprep) under the general conditions described above and the required fraction collected. If the cut was not pure, it was then passed through a column of 30% FFAP on Chromosorb W column (Autoprep) until a pure fraction was obtained.

Methyleugenol was prepared from eugenol in a fashion described for the synthesis of anisole (23), b.p. 156–159° at 40 mm. [Lit. (24) b.p. 237–239°.] Isomethyleugenol was prepared by the isomerization of methyleugenol (10 Gm.) with hot saturated alcoholic KOH (10 ml.) instead of sodium (25) at 200° for 1 hr., b.p. 163–168° at 38 mm. [Lit. (26) b.p. 263°.]

3,4-Dimethoxycinnamaldehyde was prepared (27) from veratraldehyde and acetaldehyde and purified finally by GC, m.p. 84–85°. [Lit. (27) m.p. 82–83°.]

Isolation of Steam-Volatile Oil A—The plants grew in dense patches in shaded areas along the Des Plaines River in the Dam IV Forest Preserve (about 25 miles northwest of Chicago, Ill.) and were harvested (in small samples from each patch) during May 1960 and 1961.[4] Dry rhizomes were also purchased from the Meer Corp. in 1964.[5]

Eight kilograms of dried milled rhizomes were extracted continuously with hot pentane (b.p. 35–36°) for 1 week and the solvent removed (Vigreux column) on the steam bath. The residue (210 Gm., 2.6%) was steam distilled (5 hr.), and the distillate extracted with pentane. Evaporation of the solvent yielded 100 Gm. of a fragrant light yellow oil (*A*) which was examined immediately by GC. (Fig. 1, Table I).

Column Chromatography of Steam-Volatile Oil A—The oil *A* (50 Gm.) was placed on neutral alumina (750 Gm., Woelm, grade I) and eluted with various solvents until no further oil was obtained with a particular solvent (usually about 1.5 L.). Pentane was distilled through a Vigreux column at 760 mm., but other solvents were removed *in vacuo* using a rotatory flash evaporator at the lowest possible temperature, and the residue weighed. Each fraction then was subjected to preparative

[4] The authors thank Mr. Roland F. Eisenbeis, Superintendent of Conservation, Forest Preserve District, for permission to harvest the plants. The authors thank Mr. Floyd A. Swink, Morton Arboretum, Lisle, Ill., who identified the specimens as *Asarum canadense*, the plants usually found in this area, and who pointed out to us that *Asarum canadense* is polymorphic, *sensu latu*. A detailed discussion of the minute differences distinguishing four varieties of *A. canadense* L. is given by J. Steyermark ("Flora of Missouri," Iowa State University Press, Ames, Iowa, 1963, p. 572).

[5] Dr. William A. Meer reported to us that this material was collected during the spring and summer in northwestern Ohio and met the requirements of the N.F. VII.

GC under the conditions described in Fig. 1. The results of these separations are summarized in Table II.

Oxidation of Elemicin—A suspension of elemicin (0.1 Gm.) in aqueous $KMnO_4$ (0.5 Gm. in 10 ml.) was stirred and boiled under reflux for 1 hr. On cooling, $NaHSO_3$ (0.4 Gm.) was added, the mixture acidified with concentrated HCl, and extracted with $CHCl_3$. There was isolated 3,4,5-trimethoxybenzoic acid (0.05 Gm., 50%), m.p. 165°, M^+ 212, and whose infrared spectrum was identical to an authentic sample, m.p. 168° (Eastman Chemicals).

Chromatography of Linalyl Acetate—The ester (1 Gm.) was placed on neutral alumina (20 Gm.), and the following components were eluted and their identity proved by GC (enrichment method): β-myrcene (0.2 Gm., by pentane), unchanged ester (0.2 Gm., by ether), and linalool (0.1 Gm., by chloroform–ethanol, 9:1).

Separation of Steam-Nonvolatile Oil B—The steam-nonvolatile residue (after 5-hr. distillation) was taken up in pentane and the solvent removed. The residual oil (50 Gm.) was placed on basic alumina (750 Gm., Alcoa grade F-20) and eluted by each of the solvents shown in Table V. Some of the fractions then were distilled *in vacuo* and the distillates examined by GC.

Aristolone—The ketone was conveniently isolated from fraction *B*-VI. Short-path distillation of a portion of this fraction (1.5 Gm.) between 100–105° at 0.15 mm. afforded a viscous oil which slowly solidified. Sublimation of this solid at 100–110° at 0.075 mm. yielded the ketone (0.4 Gm.), m.p. 100–101° [lit. (18) m.p. 100–101°], undepressed on admixture with an authentic sample (18). $[\alpha]_D^{25}$ −321.3° (c, 1.5, $CHCl_3$), ν C=O in $CHCl_3$ 1640 $cm.^{-1}$. [Lit. (18) ν C=O in $CHCl_3$ 1658 $cm.^{-1}$.] $\lambda_{max.}^{EtOH}$ 234 (log ϵ 4.18), 310 mμ (log ϵ 2.15). [Lit. (18) $\lambda_{max.}^{EtOH}$ 235 (log ϵ 4.11), 310 mμ (log ϵ 2.07).]

The chemical shifts of aristolone in CCl_4 did not quite agree with those reported (20) (see Table VI), and in particular the cyclopropane resonances were not clearly discernible. However, the 100 Mc. spectrum[4] in C_6D_6 (Fig. 5) showed the resonances of H-6 and H-7 as two doublets, and, as expected, H-7 was considerably more shielded due to the inductive and anisotropic effect of the C=O group. The chemical shifts of H-7 and H-6 compared well with those observed for the α- and β-protons of cyclopropyl ketones (28, 29), and the magnitude of the coupling constant was of the order expected for adjacent *cis* cyclopropane protons (29).

The signal arising from the cyclopropane proton adjacent to the C=O group (H-7) showed fine splitting (J = 1.1 c.p.s.) which was traced to spin–spin interaction with the vinyl proton. This coupling through the carbonyl group (30) was proved by irradiating the vinyl signal (H-9) at 5.77 δ (in C_6D_6) while sweeping the rest of the spectrum which changed the signal at 1.72 δ to a simple doublet. Irradiation of the signal at 1.72 δ (H-7) sharpened the unresolved multiplet at 5.77 δ, and turned the doublet at 0.99 δ to a singlet, thus establishing the chemical shift of H-6. The only other change which was observed by irradiating at 1.72 δ was the collapse of the methyl doublet at 0.80 δ to an ill-defined broad signal, which suggests that the chemical shift of the methine proton at C-4 was in the vicinity of 1.72 δ. A considerable solvent effect

TABLE VI—NMR SPECTRAL DATA FOR ARISTOLONE

Protons and Coupling Constants Involved	C_6D_6 100 Mc.	C_6H_6 60 Mc.	$CDCl_3$ 60 Mc.	CCl_4 60 Mc.	CCl_4[a]
CH_3 Doublet at C-4	0.80	0.81	1.10	1.07	0.93
$J_{CH_3,H-4}$	(6.7)	(6.5)	(6.2)	(6.0)	(6)
CH_3 Singlets at C-5, C-11	0.90	0.90	1.20	1.20	1.21
	0.92	0.92	1.20	1.20	1.28
	1.12	1.12	1.25	1.24	—
H-7	0.99	[b]	1.38	(1.30)[c]	1.38
H-6	1.72	1.72	1.72	1.60	1.74
$(J_{6,7})$	(8.5)	(8.4)	(8.2)	(8.0)	(8)
$(J_{7,9})$	(1.1)	(1.1)	(1.2)	(1.4)	(1)
H-9	5.77	5.78	5.72	5.60	5.73

[a] Data from *Reference 20.* [b] Doublet not clearly observable. [c] This chemical shift was calculated on the assumption that in this solvent H-6 and H-7 were observed as an AB quartet. However, the fourth line necessary to compute this value would be hidden underneath the CH_3 singlets at 1.22 δ.

TABLE V—CHROMATOGRAPHY OF OIL *B*

Fraction	Eluting Solvent	Yield, Gm.	Comments
B-I	Pentane	9.4	Fractions, b.p. 70–135° at 0.4 mm. (5 Gm.), contained varying quantities of linalool, bornyl acetate, methyleugenol, and elemicin; fractions, b.p. 130–170° at 0.1 mm. (4.0 Gm.), could not be separated and identified.
B-II	Pentane–benzene (9:1)	6.7	Methyleugenol (99%)
B-III	Benzene	12	Fractions, b.p. 80–105° at 0.1 mm. (3 Gm.), consisted of varying mixtures of methyleugenol, elemicin, and 2,3,4,5-tetramethoxyallylbenzene; b.p. 106–125° at 0.1 mm. (1 Gm.) contained aristolone in addition to the ethers.
B-IV	Benzene–ether (9:1)	5.5	Aristolone (see under *Aristolone*)
B-V	Ether	0.5	Not examined further.
B-VI	Ether–chloroform (9:1)	2.0	β-Sitosterol (see under *β-Sitosterol*)
B-VII	Chloroform	2.0	β-Sitosterol (see under *β-Sitosterol*)
B-VIII	Chloroform–ethanol (9:1)	3.0	Linalool (88%)
B-IX	Ethanol	—	

was noted for the chemical shifts of one of the cyclopropane protons (H-7) and that of the CH_3 resonances in benzene and chlorinated solvents. (Table VI). These shifts are attributed to benzene–carbonyl solvent complexes (31) but in the absence of model compounds, no assignment for the chemical shifts of different CH_3 groups is made at present.

Anal.[6]—Calcd. for $C_{15}H_{22}O$: C, 82.51; H, 10.15. Found: C, 82.26; H, 10.29.

Its 2,4-dinitrophenylhydrazone crystallized from ethanol, m.p. 165°. [Lit. (18) m.p. 169–170°.]

Anal.[7]—Calcd. for $C_{21}H_{26}N_4O_4$: N, 14.06. Found: N, 13.90.

β-Sitosterol—Rechromatography of fraction *B*-VI (2 Gm.) on basic alumina (50 Gm.) gave in the ether–chloroform (9:1) eluent a light yellow solid (1 Gm.) which was extracted with hot pentane. The pentane solution was evaporated and the residue recrystallized from ethanol to give the product (0.5 Gm.), m.p. 137°. [Lit. (32) m.p. 137°.] It was undepressed by an authentic sample. $[\alpha]_D^{24}$ −37.07° (c, 0.47 in $CHCl_3$). [Lit. (32) $[\alpha]_D^{25}$ −37.0 ($CHCl_3$).] ν OH in $CHCl_3$ at 3600 cm.$^{-1}$. Its NMR spectrum showed one vinyl proton at 5.38 δ and the >CH—O— proton as a multiplet at 3.50 δ, and the rest of the spectrum was like that published previously (33). Its mass spectrum is reproduced in Fig. 7 and showed *m/e* 414.

Anal.[6]—Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15. Found: C, 83.83; H, 12.26.

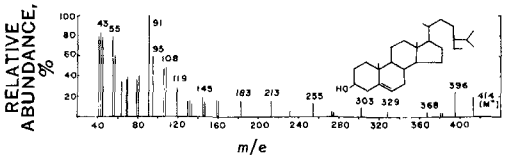


Fig. 7—The mass spectrum of β-sitosterol.

β-Sitosterol Benzoate—A solution of β-sitosterol (0.2 Gm.) in pyridine (1.5 ml.) was treated with benzoyl chloride (0.2 Gm.) and heated over a free flame for 1 min. The solution was poured into ice water, the solid filtered, and washed with 5% K_2CO_3 solution. After crystallization, the solid melted at 143°. [Lit. (32) m.p. 146°.] ν C=O in $CHCl_3$ at 1740 cm.$^{-1}$, M^+ at *m/e* 518.

[6] Microanalysis by Micro-Tech Laboratories, Skokie, Ill.

[7] Microanalysis by Mr. Leo Horner, Department of Chemistry, University of Illinois at the Medical Center, using a Coleman nitrogen analyzer, model 29.

REFERENCES

(1) Power, F. B., *Proc. Am. Pharm. Assoc.*, **28**, 464(1880).
(2) Power, F. B., and Lees, F. H., *J. Chem. Soc.*, **81**, 58 (1902).
(3) Ryhage, R., and von Sydow, E., *Acta Chem. Scand.*, **17**, 2025(1965).
(4) Mitzner, B. M., Theimer, E. T., Steinbach, L., and Wolt, J., *J. Org. Chem.*, **30**, 646(1965).
(5) Ohloff, G., Seibl, J., and Kováts, E., *Ann.*, **675**, 83 (1964).
(6) Budzikiewicz, H., Djerassi, C., and Williams, D. H., "Structure Elucidation of Natural Products by Mass Spectrometry," vol. II, Holden-Day, Inc., San Francisco, Calif., 1964.
(7) Mitzner, B. M., Lemberg, S., Theimer, E. T., *Can. J. Chem.*, **44**, 1090(1966).
(8) von Sydow, E., *Acta Chem. Scand.*, **17**, 2504(1963).
(9) *Ibid.*, **19**, 2083(1965).
(10) Bhacca, N. S., Hollis, D. P., Johnson, L. F., Pier, E. A., and Shoolery, J. N., "NMR Spectra Catalog," Varian Associates, Inc., Palo Alto, Calif., 1962, 1963.
(11) Biemann, K., and Seibl, J., *J. Am. Chem. Soc.*, **81**, 3149(1959).
(12) Flautt, T. J., and Erman, W. F., *ibid.*, **85**, 3212(1963).
(13) Burlage, H. M., and Lynn, E. V., *J. Am. Pharm. Assoc.*, **16**, 407(1927).
(14) Stahl, E., and Jork, H., *Arch. Pharm.*, **299**, 670 (1966); Gracza, L., Csizér, É., and Tatár, J., *Acta Pharm. Hung.*, **35**, 169(1965).
(15) Shulgin, A. T., *Can. J. Chem.*, **43**, 3437(1965).
(16) Stahl, E., and Jork, H., *Arch. Pharm.*, **297**, 273(1964).
(17) Budzikiewicz, H., Djerassi, C., and Williams, D. H., "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1964, p. 191.
(18) Furukawa, S., and Soma, N., *J. Pharm. Soc. Japan*, **81**, 559(1961).
(19) Carboni, S., Settimo, A. D., Malaguzzi, V., Marsili, A., and Pacini, P. L., *Tetrahedron Letters*, **1965**, 3017.
(20) Büchi, G., Greuter, F., and Tokoroyama, T., *ibid.*, **1962**, 827.
(21) Robinson, T., "The Organic Constituents of Higher Plants," Burgess Publishing Co., Minneapolis, Minn., 1963, p. 139.
(22) Gracza, L., *Plant Medica*, **12**, 440(1964).
(23) Hiers, G. S., and Hager, F. D., *Org. Syn., Coll. Vol. I*, **1932**, 58.
(24) Graebe, C., and Borgmann, E., *Ann.*, **158**, 282(1871).
(25) Eykman, J. F., *Ber.*, **23**, 855(1890).
(26) Ciamician, G., and Silber, P., *ibid.*, **23**, 1164(1890).
(27) Frudenberg, K., Orthner, L., and Fikentscher, H., *Ann.*, **436**, 286(1924).
(28) Pittman, C. V., and Olah, G. A., *J. Am. Chem. Soc.*, **87**, 5123(1965).
(29) Pierre, J. L., and Arnaud, P., *Bull. Soc. Chim. France*, **1966**, 1040.
(30) Frei, J., *et al.*, *Helv. Chim. Acta*, **49**, 1049(1966).
(31) Bhacca, N. S., and Williams, D. H., "Application of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, p. 159.
(32) Bernstein, S., and Wallis, E. S., *J. Org. Chem.*, **2**, 341 (1937).
(33) Slomp, G., and Mackellar, F. A., *J. Am. Chem. Soc.*, **84**, 204(1962).

Absorbance-Stat Method for the Study of Reaction Kinetics in Solution

By KENNETH A. CONNORS and JULIUS H. FAVILLA

A method is described for the kinetic study of reactions in which the concentration of one reactant is held essentially constant by addition of that reactant throughout the course of the reaction; this condition of constancy is attained by maintaining the solution absorbance, which is made a function of reactant concentration, at a constant value. This technique, the absorbance-stat method, is exemplified by alkaline ester hydrolysis, an acid-base indicator being added to the solution. By titrating with alkali to keep the absorbance of the indicator constant, the hydroxide-ion concentration is held constant. The principles of this technique are developed, and the method is compared with the conventional pH-stat procedure. The second-order rate constants of alkaline hydrolysis of methyl and ethyl acetates have been determined. The method is satisfactorily accurate and precise.

In the "continuous-titration" method of studying reactions that produce or consume hydrogen or hydroxide ion, a poorly buffered solution of the reacting substrate is titrated with acid or alkali to keep the pH at an essentially constant value. The rate of addition of titrant is related to the rate of the chemical reaction. As commonly carried out, the procedure utilizes potentiometric detection of the solution pH by means of a glass electrode–saturated calomel electrode system. The titrant can be added manually while its volume and the corresponding time are recorded. During the past decade, however, instruments have been designed to carry out the pH measurement, titration, and recording steps automatically; several of these instruments, called pH-stats, are available commercially. The pH-stat method for studying reaction rates has been reviewed by Jacobsen *et al.* (1). Throughout the present paper the term "pH-stat" will be applied to the continuous-titration method with potentiometric pH detection, whether carried out manually or instrumentally.

The continuous-titration technique appears to have been introduced in 1922 by Knaffl-Lenz (2), who studied rates of enzyme-catalyzed ester hydrolyses. Knaffl-Lenz added an acid-base indicator to the reaction solution to function as a visual pH detector, by titrating to keep the solution a definite color, the pH was maintained constant. Later development of this technique utilized electrometric pH determination, as described above. With the present availability of highly sensitive and stable spectrophotometers, the original colorimetric procedure regains its attractiveness, and in this paper the theory and practice of this technique, which will be called the "absorbance-stat" method, are explored.

Consider the reaction between a substrate S and a reagent R, in which reagent is consumed on a stoichiometric basis.

$$S + R \rightarrow \text{products}$$

The velocity of this reaction is given by the rate equation $v = k\,[S][R]$, where k is the second-order rate constant. When [R] is maintained constant, this takes the first-order form $v = k_{app.}\,[S]$, where $k_{app.}$, the apparent first-order rate constant, is equal to $k[R]$. The absorbance-stat method permits the evaluation of $k_{app.}$ for reactions of two general types. (*a*) Reactions in which R possesses an absorption spectrum such that at a selected wavelength R is the only reaction component that absorbs radiation. Then the absorbance due to R is maintained constant by continuous titration with a solution of R. (*b*) Reactions in which an indicator is added whose absorption intensity is a function of the R concentration; the reaction mixture is titrated with a solution of R to keep the absorbance constant.

The first of these possibilities might be exemplified by oxidations with permanganate or by bromine-addition reactions. The second class includes, as its most important examples, some reactions in which R is hydrogen ion or hydroxide ion. Thus acid-catalyzed amide hydrolysis or alkali-catalyzed ester hydrolysis might be studied by the absorbance-stat method.

In this paper the potentialities of this technique have been investigated by applying it to hydroxide ion-catalyzed ester hydrolyses. The theoretical treatment is also based upon this reaction. With suitable modification the theory can be adapted to other reactions.

PRINCIPLES

Dependence of Titrant Volume on Time—The relationship between the rate of addition of titrant and the rate of chemical reaction must be established to permit evaluation of the reaction rate constant. The reaction is written

Received September 19, 1966, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication December 7, 1966.

$$E + OH^- \xrightarrow{k_{OH}} \text{products}$$

with 1 equivalent of hydroxide being consumed per mole of ester hydrolyzed. The solution buffer capacity is assumed to be negligible and the volume change of mixing upon the addition of titrant is neglected. The volume change resulting from conversion of E and OH^- to reaction products also is neglected. The temperature is assumed constant. Then the ester concentration can change through chemical reaction and by dilution with titrant.

$$d[E] = (\delta[E]/\delta t)_V dt + (\delta[E]/\delta V)_t dV \quad \text{(Eq. 1)}$$

The first of these derivatives is given by the rate equation:

$$(\delta[E]/\delta t)_V = -k_{OH}[E][OH] \quad \text{(Eq. 2)}$$

The dilution effect is found by differentiating the definition of concentration, $[E] = q_E/V$, where q_E is the milliequivalents of ester contained in V milliliters of solution. The result is

$$(\delta[E]/\delta V)_t = -[E]/V \quad \text{(Eq. 3)}$$

The differential dV in Eq. 1 can be evaluated from a consideration of the effects of the reaction and the titration on the hydroxide-ion concentration.

$$d[OH] = (\delta[OH]/\delta t)_V dt + (\delta[OH]/\delta V)_t dV = 0 \quad \text{(Eq. 4)}$$

In Eq. 4 the differential $d[OH]$ is set equal to zero, expressing the experimental objective of the continuous-titration method. From the rate equation, the time effect can be found:

$$(\delta[OH]/\delta t)_V = -k_{OH}[E][OH] \quad \text{(Eq. 5)}$$

To find the titration effect, the hydroxide-ion concentration is written

$$[OH] = (\text{meq. of } OH^- \text{ present initially} + \text{meq. of } OH^- \text{ added})/V$$

$$[OH] = (q_{OH} + NV_t)/V \quad \text{(Eq. 6)}$$

where q_{OH} represents the milliequivalents of hydroxide present in the initial sample solution, N is the titrant normality, V_t is the volume of titrant added, and V is the total volume of reaction solution. Therefore, $V = V_0 + V_t$, if V_0 is the volume of sample solution before any titrant is added. Equation 6 is differentiated to give

$$(\delta[OH]/\delta V)_t = 1/k'V \quad \text{(Eq. 7)}$$

where $k' = 1/(N - [OH])$. Equations 4, 5, and 7 can be combined, yielding Eq. 8.

$$dV = k'k_{OH}V[E][OH]dt \quad \text{(Eq. 8)}$$

Substitution of Eqs. 2, 3, and 8 into Eq. 1 gives

$$\frac{d[E]}{[E](1 + k'[E])} = -k_{obs.}dt \quad \text{(Eq. 9)}$$

where a later result has been anticipated by defining $k_{obs.} = k_{OH}[OH]$. Equation 9 gives, on integration,

$$\ln \frac{[E](1 + k'[E]_0)}{[E]_0(1 + k'[E])} = -k_{obs.}t \quad \text{(Eq. 10)}$$

in which $[E]_0$ represents the ester concentration at time zero, and $[E]$ is its concentration at time t. An equation similar to Eq. 10 has been presented by Breuer and Jenkins (3), who, however, give $1/N$ as the value of k'.

The number of milliequivalents of ester initially present is equal to $V_0[E]_0$, which is equal to the number of milliequivalents of hydroxide consumed in the entire reaction, or $V_\infty N$.

$$[E]_0 = V_\infty N/V_0 \quad \text{(Eq. 11)}$$

V_∞ is the volume of titrant added at $t = \infty$. The number of milliequivalents of ester unreacted at time t is equal to the total meq. added minus the meq. hydrolyzed, leading to Eq. 12 for the concentration $[E]$.

$$[E] = N(V_\infty - V_t)/V \quad \text{(Eq. 12)}$$

With Eqs. 11 and 12, Eq. 10 can be expressed in terms of titrant volumes:

$$\ln \left[\frac{V_\infty - V_t}{V_\infty}\right] \left[\frac{V_0 + k'NV_\infty}{(V_0 + k'NV_\infty) + V_t(1 - k'N)}\right] = -k_{obs.}t \quad \text{(Eq. 13)}$$

When $V_t(1 - k'N) <<< (V_0 + k'NV_\infty)$, Eq. 13 becomes

$$\log \left[\frac{V_\infty - V_t}{V_\infty}\right] = -\frac{k_{obs.}t}{2.303} \quad \text{(Eq. 14)}$$

Equation 14 is therefore a satisfactory approximation when $(N - [OH]) \cong N$ or when $V_t <<< V_0$. Though these are separate conditions, they are usually both satisfied by employing a very concentrated titrant solution.

The above considerations apply to both the pH-stat and the absorbance-stat methods. According to Eq. 14, a plot of $\log(V_\infty - V_t)$ against time should be linear with slope equal to $-k_{obs.}/2.303$.

Effect of Volume Change on Indicator Concentration—During the course of the reaction a volume change in the range 1–5% will usually result from the addition of titrant. The case for keeping this change as small as possible was presented in the preceding paragraphs. In the absorbance-stat method this volume change can exert a secondary effect by reducing the total indicator concentration in the reaction solution. This reduction in indicator concentration will lead to an absorbance decrease. Since the absorbance is the quantity that is maintained constant in this technique, the reduction in indicator concentration will be compensated for by a further addition of titrant, which will convert some of the indicator to the more highly absorbing form and thus restore the absorbance to its initial value. (This description supposes that the basic form of the indicator is responsible for the light absorption.) But this means that the pH has changed, because an alteration in the ratio of acid to base forms of the indicator is a reflection of a pH change. Thus, the hydroxide-ion concentration will undergo a small but possibly significant shift during the reaction because of this dilution effect on the indicator.

The possible error from this source has not been analyzed quantitatively because it is easily eliminated. The remedy is simply to add to the titrant the same concentration of indicator that is present in the initial sample solution. Then no matter how much titrant is added, the total indicator concentration will remain constant. By holding the absorbance at a constant value, the pH will

remain unchanged. The experimental problem is not difficult. Proportional amounts of an indicator stock solution are added to the titrant and to the sample solution. The new titrant normality can be calculated from the initial normality and volumes.

Calculation of Second-Order Rate Constants—The preceding considerations provide a basis for the evaluation of the first-order rate constant, $k_{obs.}$ The goal of the kinetic measurements usually is the second-order constant, k_{OH}, which is given by $k_{OH} = k_{obs.}/[OH]$. An estimate must therefore be made of [OH] in the reaction solution. This can be made in two general ways. (*a*) The *concentration* of hydroxide ion, which will be symbolized as c_{OH}, can be obtained; this is based ultimately upon titration data. (*b*) The conventional *activity* of hydroxide ion, a_{OH}, may be found; this is ultimately based upon a potentiometric determination of pH. Writing for the experimental first-order constant the two equivalent expressions

$$k_{obs.} = (k_{OH})^c c_{OH}$$

$$k_{obs.} = (k_{OH})^a a_{OH}$$

shows that the second-order rate constants expressed on the concentration and activity scales are related by Eq. 15,

$$(k_{OH})^c/(k_{OH})^a = a_{OH}/c_{OH} = y_{OH} \quad \text{(Eq. 15)}$$

where y_{OH} is the apparent molar activity coefficient, which in its present use simply has the significance that it is a factor relating the rate constants calculated on these two bases. y_{OH} depends upon the ionic strength of the solution. In most practical situations y_{OH} is appreciably different from unity, and it is essential to distinguish between the rate constants $(k_{OH})^c$ and $(k_{OH})^a$.

pH can be measured potentiometrically at any time during the reaction; then the quantity a_{OH} can be calculated with the use of pKw at the appropriate temperature. (It is assumed here that the reaction mixture is a fully aqueous solution.) In the calculation of second-order hydrolytic rate constants based on pH measurements, the pH determination usually contributes a greater share of error than does the measurement of $k_{obs.}$ The accuracy inherent in the kinetic measurements may be preserved by basing the second-order constant on the concentration, c_{OH}, which can often be established with greater accuracy than can a_{OH}.

If c_{OH} is not to be calculated for the absorbance-stat reaction solution, the exact value of absorbance selected as the static value is not of great importance, and immediately after the reaction is initiated a suitable value can be adopted. But if c_{OH} is to be estimated, the absorbance becomes critical. Suppose the sample solution is prepared with V_S ml. of solvent, V_I ml. of indicator stock solution, and V_A ml. of a standard solution of alkali of normality N_A. At the cell path length and wavelength selected for the kinetic experiment, this solution possesses an absorbance, A'. The reaction is next initiated by adding V_E ml. of ester (either neat or in a stock solution). The absorbance immediately changes because of three effects: (*a*) dilution of the indicator; (*b*) conversion of some indicator from the base to the acid form because of the dilution effect on the hydroxide ion; (*c*) hydrolysis of the ester to reduce the hydroxide-ion concentration. The third effect does not enter the argument because the present concern is to select the proper absorbance value at $t = 0$ before any ester has hydrolyzed, this then being the absorbance value at which the solution will be maintained throughout the reaction. The correct absorbance is obtained by taking the dilution of the indicator into account (Eq. 16),

$$A = A'(V_S + V_I + V_A)/V_0 \quad \text{(Eq. 16)}$$

where $V_0 = V_S + V_I + V_A + V_E$. Even in the absence of the ester hydrolysis, however, the observed absorbance would be less than A because of the dilution effect on hydroxide ion, so the solution is immediately titrated to achieve the absorbance, A, and this value is maintained through the reaction. In effect, this procedure restores c_{OH} to the value it had before addition of the ester. Then the quantity, c_{OH}, to be used in converting $k_{obs.}$ to $(k_{OH})^c$, is obtained with Eq. 17.

$$c_{OH} = V_A N_A/(V_S + V_I + V_A) \quad \text{(Eq. 17)}$$

Sensitivity of the Absorbance-Stat Method—The acid-base indicator equilibrium is

$$HI \rightleftarrows H^+ + I^-$$

and the acid dissociation constant for the indicator may be written $K_I = a_H[I]/[HI]$. The indicator ratio is given by

$$[I]/[HI] = (A - A_{HI})/(A_I - A)$$

where A_{HI} is the absorbance of a solution containing indicator transformed totally to its acid form, A_I is the absorbance when the indicator is in its base form, and A is the absorbance when it is present in any mixture of the two forms; all of these quantities refer to the same total concentration of indicator. The hydrogen-ion activity can be expressed in terms of these absorbances as in Eq. 18.

$$a_H = K_I(A_I - A)/(A - A_{HI}) \quad \text{(Eq. 18)}$$

From Eq. 18 the derivative da_H/dA can be found, and this can be transformed with the relation $d\text{pH} = -da_H/2.3a_H$ to give (in incremental form) Eq. 19.

$$\Delta\text{pH} = \frac{(A_I - A_{HI})\Delta A}{2.3(A_I - A)(A - A_{HI})} \quad \text{(Eq. 19)}$$

This equation provides a basis for evaluating the relative sensitivities of the pH-stat and absorbance-stat methods. Sensitivity in these continuous-titration procedures may be expressed as the smallest change in detector signal that will elicit a response from the titrator mechanism. It will be clear from Eq. 19 that the sensitivity in the absorbance-stat method depends upon the solution absorbance, increasing with concentration of the indicator; moreover, sensitivity is maximal when $\text{pH} = \text{pK}_I$, which corresponds to the condition $A = (A_I - A_{HI})/2$. The relationship of A to pH for any indicator suggests, however, that good sensitivity can be achieved in the approximate range $\text{pH} = \text{pK}_I \pm 1$.

The comparative sensitivities of potentiometric and spectrophotometric control of pH can be demonstrated with Eq. 19 and some hypothetical but reasonable values for the experimental quantities. Suppose $A_I = 2.00$, $A_{HI} = 0.00$, $A = 0.90$, and

ΔA = 0.005. Then Eq. 19 yields ΔpH = 0.0044. Since ΔA = 0.005 seems to be a conservative estimate of attainable spectrophotometric sensitivity, while ΔpH = 0.0044 probably is an optimal sensitivity for the pH-stat, it appears that the absorbance-stat should be capable of more sensitive response to the changing solution conditions than is the pH-stat.

EXPERIMENTAL

Materials—Ethyl acetate (Baker and Adamson reagent grade) was distilled, b.p. 76–77°. Methyl acetate (Matheson Coleman & Bell) was distilled, b.p. 55–56°. Alizarin yellow R (Coleman & Bell) was used directly. Other chemicals were of reagent or primary standard grade. All water was redistilled from alkaline permanganate.

Indicator stock solution was prepared by dissolving 25 mg. of alizarin yellow R in 0.5 ml. of 1.0 *N* sodium hydroxide; this was diluted to 50 ml. with water, the solution was allowed to stand overnight in a refrigerator, it was filtered, and 25 ml. of the filtrate was diluted to 50 ml. with water.

Apparatus—Reactions were carried out in a flask originally designed for photometric titrations (4). The flask (capacity about 120 ml.) was constructed with a jacket through which water was circulated from a constant-temperature water bath. This apparatus was mounted directly over an air-driven magnetic stirrer, the entire assembly being placed near the thermostated cell compartment of a Cary model 14 recording spectrophotometer. Short lengths of rubber tubing connected the inlet and outlet of the reaction flask to a flow-through spectrophotometer cell that was firmly positioned in a cell holder in the sample cell compartment. A Teflon-covered stirring bar provided the stirring and pumping action. The reaction flask was tightly closed with a rubber stopper, through which passed the tip of a 2-ml. micrometer buret (Roger Gilmont Instruments); the buret tip extended below the surface of the reaction solution.

pH measurements were made with a Sargent model DR pH meter equipped with a wide-range glass electrode.

Procedure—A typical experiment was conducted as follows. To the reaction flask 75.0 ml. of freshly boiled water, 3.0 ml. of indicator stock solution, and 5.0 ml. of standard 0.1 *N* sodium hydroxide were added. This solution was allowed to reach temperature equilibrium. The spectrophotometer wavelength selector was set at 495 mμ (the absorption maximum of the base form of the indicator), and the recorder was activated. About 2 meq. of ester was added to initiate the reaction. From the buret 1 *N* NaOH was added to keep the absorbance as constant as possible; the chart tracing is a helpful aid in minimizing the fluctuations about the mean absorbance value. The titrant volume was recorded as a function of time; reactions were followed until no further change in absorbance was observed. All reactions were carried out at 25.0°.

RESULTS AND DISCUSSION

Ethyl acetate was selected as the ester for the developmental stage of this investigation because its hydrolysis is uncomplicated, it is water soluble, and many other studies of its alkaline hydrolysis have been made; its volatility is a disadvantage. Alizarin yellow R was found to be a suitable indicator in the pH range required to give a convenient rate of reaction. This indicator possesses good sensitivity from pH 9.8 to 11.8, and it is adequately stable under these conditions.

The volatility of ethyl acetate prevented the use of a nitrogen stream as protection against atmospheric contamination, and it was necessary to stopper tightly the reaction flask to prevent absorption of carbon dioxide from the atmosphere as well as loss of ethyl acetate to the atmosphere. After such sources of error were eliminated, very well-behaved kinetic data were obtained. Figure 1 shows a characteristic plot of titrant volume as a function of time. The first-order plot according to Eq. 14 is shown in Fig. 2; the line is straight over about 4 half-lives, and this behavior could be reproduced at will. The V_∞ value tended to rise slightly with unrealistically long periods of waiting, probably because of a slight decomposition of the indicator, so the V_∞ value used in the calculations was taken after the expiration of 7 to 9 half-lives. With the conditions described here, it was found unnecessary to incorporate indicator in the titrant.

The mean value of $(k_{OH})^c$ for ethyl acetate at 25.0° was found, from such measurements, to be 0.103 L./mole-sec., with a standard deviation of 0.0013 L./mole-sec. This must be considered

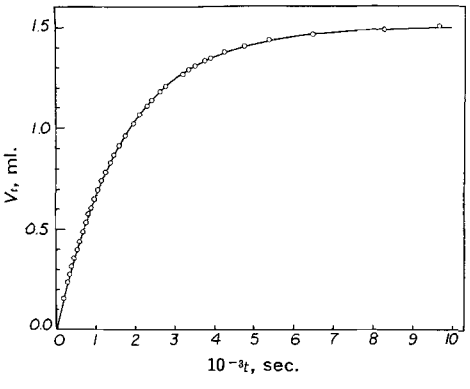


Fig. 1—Plot of titrant volume against time for the absorbance-stat study of the alkaline hydrolysis of ethyl acetate at 25°; c_{OH} = *0.00569* N, *absorbance = 0.64.*

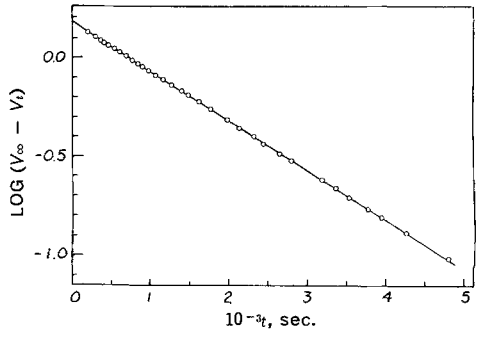


Fig. 2—First-order plot of the data shown in Fig. 1.

excellent precision, and it reflects the very good reproducibility of the observed first-order rate constants. The mean value of $(k_{OH})^a$, from these same determinations, was 0.126 L./mole-sec., with a standard deviation of 0.012 L./mole-sec., and this poorer precision is a result of the difficulty in obtaining precise pH measurements. In these studies the pH was in the range 11.6–11.8.

Literature values of $(k_{OH})^c$ for ethyl acetate at 25° vary widely. Terry and Stieglitz (5) give the value 0.113 L./mole-sec. with a claimed accuracy of 0.75%; they also review earlier reports, with the mean value of six workers being 0.110 L./mole-sec. (ranging from 0.106 to 0.114). Salmi and Kantola (6) found 0.109 L./mole-sec., Shrivastava (7) reported 0.091 L./mole-sec., Myers *et al.* (8) gave 0.111 L./mole-sec., and Flom and Elving (9) found 0.110 L./mole-sec. (standard deviation 0.010). Wilson and Terry (10) observed a progressive decrease of the rate constant with increase in ionic strength (NaCl), with the constant dropping from 0.113 with no NaCl present (initial NaOH 0.01 molal) to 0.102 L./mole-sec. at 0.51 *M* NaCl. Bell and Waind (11), however, found that increasing concentrations of NaCl up to 0.5 *M* had no significant effect on the rate constant; they reported values in the range 0.099–0.101 L./mole-sec. The value of 0.103 L./mole-sec. obtained in the present study, therefore, lies close to many reliable literature values, though it may be slightly lower than the best value.

Methyl acetate was also studied by the absorbance-stat method. The mean $(k_{OH})^c$ for this ester at 25° was 0.186 L./mole-sec., with a standard deviation of 0.0075 L./mole-sec. [Gooch and Terry (12) give 0.195 L./mole-sec. for this constant.] The same data yielded, for $(k_{OH})^a$, the mean value 0.226 L./mole-sec. (standard deviation 0.067).

Guggenheim's method (13) of plotting first-order rate data was also applied to all experiments, and the rate constants obtained from these plots agreed closely with those from the conventional graphs. The Guggenheim method obviates the necessity of obtaining a value for V_∞, which may be subject to small errors caused by indicator instability, carbon dioxide absorption, or spectrophotometer base line drift.

Other indicators could doubtless be used, especially in the less alkaline pH ranges. Attempts to locate suitable indicators for use above pH 12 were unsuccessful, the substances studied being too unstable for practical use.

The absorbance-stat method for studying reaction rates appears, on the basis of the analysis and results reported in this paper, to be a useful technique for the precise and accurate determination of rate constants in suitable systems. The procedure could easily be placed on an automatic basis by substituting a photometer for the pH meter as the detector in a conventional pH-stat. The absorbance-stat is potentially more sensitive than is the pH-stat. These two techniques will probably complement each other, for the absorbance-stat method will not be successful in heterogeneous systems where the pH-stat might perform satisfactorily, while the absorbance-stat will function in systems (such as low dielectric constant solutions) that might vitiate measurements with the glass electrode; moreover, the absorbance-stat is not limited to reactions involving solvent ions.

REFERENCES

(1) Jacobsen, C. F., Leonis, J., Linderstrøm-Lang, K., and Otterson, M., "Methods of Biochemical Analysis," Glick, D., ed., Interscience Publishers, Inc., New York, N. Y., 1957, vol. IV, p. 171.
(2) Knaffl-Lenz, E., *Medd. Vetenskaps. Nobelinst.*, **6**, 1(1922); through *Chem. Abstr.*, **17**, 1483(1923).
(3) Breuer, M. M., and Jenkins, A. D., *Trans. Faraday Soc.*, **59**, 1310(1963).
(4) Rehm, C., Bodin, J. I., Connors, K. A., and Higuchi, T., *Anal. Chem.*, **31**, 483(1959).
(5) Terry, E. M., and Stieglitz, J., *J. Am. Chem. Soc.*, **49**, 2216(1927).
(6) Salmi, E. J., and Kantola, K. K., *Ann. Acad. Sci. Fennicae*, **A46**, 18(1936); through *Chem. Abstr.*, **31**, 1685(1937).
(7) Shrivastava, H., *J. Indian Chem. Soc.*, **17**, 387(1940).
(8) Myers, R. T., Collett, A. R., and Lazzell, C. L., *J. Phys. Chem.*, **56**, 461(1952).
(9) Flom, D. G., and Elving, P. J., *Anal. Chem.*, **25**, 541(1953).
(10) Wilson, S. D., and Terry, E. M., *J. Am. Chem. Soc.*, **50**, 1250(1928).
(11) Bell, R. P., and Waind, G. M., *J. Chem. Soc.*, **1950**, 1979.
(12) Gooch, W. T., and Terry, E. M., *J. Am. Chem. Soc.*, **51**, 1959(1929).
(13) Guggenheim, E. A., *Phil. Mag.*, **2**, 538(1926).

Colorimetric Analysis of Organic Hydroxyl Groups

By GERALD R. UMBREIT* and RONALD L. HOUTMAN

The reaction of 3,5-dinitrobenzoyl chloride with organic hydroxyl groups has been adapted to provide a colorimetric method of analysis. The ester formed by this reaction is subjected to conditions of controlled basicity after hydrolysis of the excess reagent. A red color is formed by the ester which is stable after 30 min. for an additional 20 min. or more, and is measured at 555 mμ *versus* a reagent blank. Tertiary alcohols react too slowly to interfere, and tertiary amines do not react. Phenols, primary and secondary amines, and certain thiols react similarly to hydroxyl groups and can be determined by this method. The procedure is rapid, simple, and accurate.

THE PRODUCTION of color on the addition of base to polynitroaromatics is well known. It has been used as a means of qualitative and quantitative determination of nitroaromatics by Porter (1). Berezin (2) and Robinson, Cundiff, and Markunas (3) have described the use of 3,5-dinitrobenzoyl chloride for the determination of alcohols in which the excess reagent is hydrolyzed and titrated with base. The end point of the titration is indicated by color formation due to the 3,5-dinitrobenzoate ester of the alcohol and the 3,5-dinitrobenzoate anion. Robinson, Cundiff, and Markunas (3) also pointed out that the ester could be determined directly by potentiometric titration since the end point potential is sufficiently removed from that for the anion to allow a differentiating titration.

A number of other excellent methods are available for the titrimetric analysis of hydroxyl compounds on a milligram or larger scale (5, 10–12). At the sensitivity level of colorimetric analyses several methods are available for specific hydroxyl types. As examples, those of Dal Nogare and Mitchell (4), Schreiber and Eschenmoser (5), and Critchfield and Hutchinson (6) may be cited.

However, few generally applicable colorimetric hydroxyl methods are available. The method of Baggett, Engel, and Fielding (7) may be adapted to general use for all hydroxyl compounds which are subject to acetylation. Acetylation is also the basis of a method proposed by Gutnikov and Schenk (8) which utilizes the ferric hydroxamate reaction after acid-catalyzed esterification of the hydroxyl functions. The sequence of reactions involved necessarily makes this a complex procedure, though it has the compensating advantage of allowing analyses of tertiary alcohols and avoids interference by amines. The method of Johnson and Critchfield (9) utilizes the 3,5-D reaction. It also has the advantage of eliminating amine interferences because of an extraction step, but has several disadvantages when compared to the method described here. Satisfactory response was not obtained by these investigators with diols. Larger volumes and more complex treatment are required, and the color produced is not stable for more than 4 min. The method proposed here is markedly superior on each of these points.

The information reported by Robinson, Cundiff, and Markunas (3) indicated the possibility that the 3,5-dinitrobenzoate esters become colored in a system of lower basicity than that required to produce color with 3,5-dinitrobenzoic acid. The problem of capitalizing on this differential color development was then to devise a system wherein the ester was primarily in the colored state, and the acid was not, and to maintain this condition long enough for convenient measurement. The method described here presents an approach based on this concept.

The method involves formation of the 3,5-dinitrobenzoate ester by reaction of the hydroxyl compound under study with a large excess of 3,5-dinitrobenzoyl chloride in pyridine–dimethylformamide. The excess reagent is hydrolyzed, the resulting mixture buffered with a saturated solution of ammonium acetate in acetone, and concentrated ammonia is added. Final dilution is made with acetone. Under these circumstances only faint color is developed with a reagent blank, and is probably due to impurities in the solvents used. The measurement is made at 555 mμ with a suitable spectrophotometer or colorimeter.

EXPERIMENTAL

Reagents and Apparatus—3,5-Dinitrobenzoyl chloride (Eastman White Label reagent), 540 mg., is dissolved in 2 ml. of dry dimethylformamide. This solution is stable for at least 72 hr. A saturated solution of ammonium acetate is prepared in dry acetone at 25° to 29°. The Cary model 11, Beckman DU, and Beckman B spectrophotometers were used in this study.

Procedure—One milliliter of the sample solution (25–300 mcg./ml. in dry pyridine) is transferred to a 10-ml. volumetric flask. One-tenth milliliter of the

Received October 12, 1966, from the Research Laboratories, The Upjohn Co., Kalamazoo, MI 49002
Accepted for publication December 20, 1966.
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3,5-dinitrobenzoyl chloride solution is added, and the mixture is allowed to stand for 15 min. at room temperature. Approximately 0.25 ml. (5 drops) of water is then added, and hydrolysis of the excess reagent is carried out by heating on a steam bath for 5 min. When the sample has cooled (5 to 15 min.), 2 ml. of the buffer solution and 0.5 ml. of concentrated (15 *N*) ammonium hydroxide reagent are added. After 30 min. the mixture is diluted to 10 ml. with acetone and the absorbance measured at 555 mμ *versus* a similarly prepared reagent blank. The color is stable for at least an additional 20 min.

DISCUSSION

Table I summarizes analytical data obtained for a variety of compound types by this method. In every case, except the tertiary hydroxyl compound (*tert*-butyl alcohol), the response is linear over the normally useful absorbance range. The absorption spectrum as shown in Fig. 1 for ethanol is essentially identical for all compounds listed in the table.

All the listed compounds were determined using the procedure exactly as described above. It is possible that increased reaction time or temperature for the esterification may result in a satisfactory response for tertiary hydroxyls. Careful limitation of reaction time will permit the determination of primary or secondary alcohols in the presence of approximately equivalent amounts of tertiary alcohols (9).

The failure of 2-mercaptobenzothiazole to react may be due to a possible opening of the lactone ring resulting in a nonreactive structure. Mercaptoacetic acid yielded color immediately upon addition of the dinitrobenzoyl chloride reagent. However, this color remained stable throughout the balance of the analytical procedure and presented no difficulties.

The problem of buffering at an appropriate level of basicity to provide reasonable sensitivity without excessive color in the blank solution was most critical. This was dealt with in the following manner.

Table I—Application of the Method

Compd.	Taken, mcg.	Found, mcg.	Error, mcg.
Ethanol	200	203	3
1-Propanol	200	193	7
1-Butanol	200	189	11
2-Butanol	200	204	4
1,3-Propanediol	100	97	3
Testosterone	200	196	4
11α-Hydroxytestosterone	100	101	1
11α-Hydroxyprogesterone	200	202	2
6α-Methyl dienediol[a]	300	294	6
Phenol	278	275	3
n-Butylamine	206	204	2
Aniline	145	143	2
Piperidine	307	309	2
Mercaptoacetic acid	205	210	5
Mercaptoethanol	207	203	4
Mercaptoethyl amine	220	223	3
2-Mercaptobenzothiazole		No reaction	
tert-Butyl alcohol		Slow reaction	

[a] 11β,21-Dihydroxy-6α-methylpregna-4,17(*cis*)-dien-3-one.

With each solvent system and base tested during development of the method, a titration similar to that of Robinson, Cundiff, and Markunas (3) was carried out with appropriate quantities of ethanol

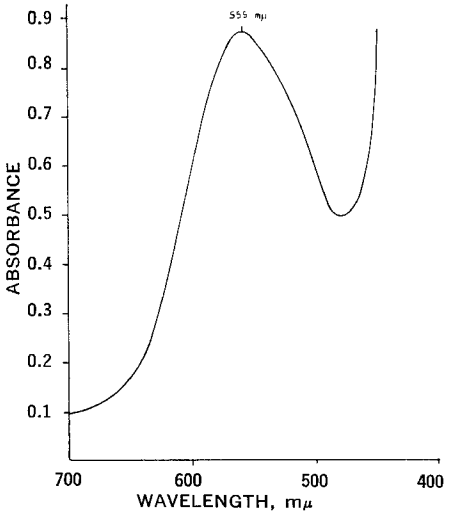

Fig. 1—Absorption spectrum of the 3,5-dinitrobenzoate ester of ethanol (201 mcg. of ethanol in 10 ml. solution vs. reagent blank).

Table II—Analysis of Mixtures

Mixture, Wt. % Each	Sample Taken, mcg. of Ethanol	Sample Recovery, mcg.	Error, mcg.
Ethanol–acetic acid			
0.1–99.9	196	183	13
1.0–99.0	196	193	3
10.0–90.0	196	197	1
Ethanol–acetone			
0.1–99.9	207	214	7
1.0–99.0	207	210	3
10.0–90.0	207	222	15
Ethanol–acetaldehyde			
0.1–99.9	195	207	12
1.0–99.0	195	198	3
10.0–90.0	195	192	3
Ethanol–ethyl acetate			
0.1–99.9	221	237	16
1.0–99.0	221	226	5
10.0–90.0	221	220	1
Ethanol–water			
15.0–85.0	300	305	5
10.0–90.0	300	310	10
7.0–93.0	300	290	10
4.8–95.2	300	270	30
3.6–96.4	300	255	45
3.0–97.0	300	245	55
2.5–97.5	300	235	65
2.1–97.9	300	225	75
Ethanol–concentrated HCl			
15.0–85.0	300	283	17
10.0–90.0	300	287	13
7.0–93.0	300	260	40
4.8–95.2	300	215	85
3.6–96.4	300	167	133

and 3,5-dinitrobenzoyl chloride reagent. The titration was carried out potentiometrically beyond the second break (titration of the benzoate ester) using a glass-saturated calomel electrode system and one of several different bases as titrant. Following this, solid sodium or ammonium acetate was added to determine whether the potential of the solution could be reversed partially and made to stabilize at the beginning of the second break. At this point, color development due to the ester is near a maximum, while color due to the 3,5-dinitrobenzoic acid is negligible. In this manner solvent systems involving pyridine, acetonitrile, dimethylformamide, acetone and chloroform alone, and some selected mixtures, were tested to arrive at the solvent mixture used in the procedure. Tetrabutylammonium hydroxide (1 *M* in methanol) and KOH (1 *M* in water) were tested as bases before turning to concentrated ammonia.

It is obvious that any of the reactive compound types would constitute a positive interference in the analysis of any other. Similarly, reactive acyl functions (anhydrides or halides) will tend to show negative interference depending on the efficiency with which they compete with the 3,5-D reagent for the available hydroxyl groups. The fact that both hydrochloric and 3,5-dinitrobenzoic acids are formed during the esterification reaction indicates that these types of compounds would not constitute interferences if present in small amounts. Water will react preferentially with 3,5-D, but will not interfere seriously unless present in sufficient quantity to consume a large proportion of the available reagent. Additional compounds which have been tested and shown not to interfere seriously in the determination of hydroxyl groups are summarized in the data of Table II. Note also in Table I that the multifunctional steroids imply a lack of interference by unsaturated moieties and by simple and conjugated ketones.

SUMMARY

A method has been presented and described for the colorimetric determination of organic alcohol, amine, and thiol groups. The method is free of interference from most common solvents and other functional groups. The procedure is rapid and the resulting products are adequately stable to provide ease of measurement.

REFERENCES

(1) Porter, C. C., *Anal. Chem.*, **27**, 805(1955).
(2) Berezin, J. V., *Doklady Akad. Nauk, S.S.S.R.*, **99**, 563(1954); through *Chem. Abstr.*, **50**, 859g(1956).
(3) Robinson, W. F., Jr., Cundiff, R. H., and Markunas, P. C., *Anal. Chem.*, **33**, 1030(1961).
(4) Dal Nogare, S., and Mitchell, J., Jr., *ibid.*, **25**, 1376 (1953).
(5) Schreiber, J., and Eschenmoser, A., *Helv. Chim. Acta*, **38**, 1529(1955).
(6) Critchfield, F. E., and Hutchinson, J. A., *Anal. Chem.*, **32**, 862(1960).
(7) Baggett, B., Engel, L. L., and Fielding, L. L., *J. Biol. Chem.*, **213**, 87(1955).
(8) Gutnikov, G., and Schenk, G. H., *Anal. Chem.*, **34**, 1316(1962).
(9) Johnson, D. P., and Critchfield, F. E., *ibid.*, **32**, 865(1960).
(10) Fritz, J. S., and Schenk, G. H., *ibid.*, **31**, 1808(1959).
(11) Schenk, G. H., and Santiago, M., *Microchem. J.*, **6**, 77(1962).
(12) Sully, B. D., *Analyst*, **87**, 940(1962).

Behavior of Erythrocytes in Various Solvent Systems III

Water–Polyethylene Glycols

By B. LESTINA SMITH* and DONALD E. CADWALLADER

Hemolytic behavior of human and rabbit erythrocytes in aqueous solutions of polyethylene glycol (PEG) 200, 300, 400, and 600 was investigated. Complete hemolysis occurred in all PEG 200 and 300 solutions, with discoloration occurring in $\geq$ 25 per cent PEG 200 and $\geq$ 15 per cent PEG 300 solutions. Sodium chloride was effective in preventing hemolysis in $\leq$ 25 per cent PEG 200 or $\leq$ 40 per cent PEG 300 solutions. When possible, *i* values were calculated for sodium chloride in the various water–PEG 200 and 300 solutions. PEG 400 and 600 protected blood cells from damage in > 10 per cent to < 40 per cent solutions, and *i* values were calculated for these PEG's. Solutions containing $\geq$ 40 per cent PEG 400 or 600 (with and without NaCl) were damaging to red cells. The ability of liquid PEG's to penetrate rabbit and human erythrocytes appeared to be 200 > 300 > 400 > 600.

PREVIOUS PAPERS in this series have reported the behavior of erythrocytes in various water–glycerin and water–propylene glycol systems (1, 2). Among other nonaqueous solvents that might be used in the preparation of parenterals would be the liquid polyethylene glycols.

Polyethylene glycols (PEG's) are products possessing a very low order of toxicity. Spiegel and Noseworthy (3) have reviewed the physical properties, toxicities, and parenteral applications of these liquids. Skin penetration studies on

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Accepted for publication December 15, 1966.
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and 3,5-dinitrobenzoyl chloride reagent. The titration was carried out potentiometrically beyond the second break (titration of the benzoate ester) using a glass-saturated calomel electrode system and one of several different bases as titrant. Following this, solid sodium or ammonium acetate was added to determine whether the potential of the solution could be reversed partially and made to stabilize at the beginning of the second break. At this point, color development due to the ester is near a maximum, while color due to the 3,5-dinitrobenzoic acid is negligible. In this manner solvent systems involving pyridine, acetonitrile, dimethylformamide, acetone and chloroform alone, and some selected mixtures, were tested to arrive at the solvent mixture used in the procedure. Tetrabutylammonium hydroxide (1 *M* in methanol) and KOH (1 *M* in water) were tested as bases before turning to concentrated ammonia.

It is obvious that any of the reactive compound types would constitute a positive interference in the analysis of any other. Similarly, reactive acyl functions (anhydrides or halides) will tend to show negative interference depending on the efficiency with which they compete with the 3,5-D reagent for the available hydroxyl groups. The fact that both hydrochloric and 3,5-dinitrobenzoic acids are formed during the esterification reaction indicates that these types of compounds would not constitute interferences if present in small amounts. Water will react preferentially with 3,5-D, but will not interfere seriously unless present in sufficient quantity to consume a large proportion of the available reagent. Additional compounds which have been tested and shown not to interfere seriously in the determination of hydroxyl groups are summarized in the data of Table II. Note also in Table I that the multifunctional steroids imply a lack of interference by unsaturated moieties and by simple and conjugated ketones.

SUMMARY

A method has been presented and described for the colorimetric determination of organic alcohol, amine, and thiol groups. The method is free of interference from most common solvents and other functional groups. The procedure is rapid and the resulting products are adequately stable to provide ease of measurement.

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many of these products show that they do not penetrate the skin in harmful amounts (4). Meyer and Sturmer (5) reported low oral and subcutaneous toxicities of PEG 200 and 600 in mice. Swanson and co-workers (6) found that sodium amobarbital in 60% PEG 200 and sodium secobarbital in 50% PEG 200 had approximately the same potency and toxicity as aqueous solutions of these barbiturates. Lee and Anderson (7) determined the toxicity of vancomycin in 50% PEG 200 and of PEG 200 alone. Their results indicated that PEG 200 produced no apparent toxic effects when given to dogs at 1.0 ml./Kg./day for 80 days intramuscularly, or 0.5, 1.0, 2.5, and 5.0 ml./Kg. as single intravenous doses.

The purpose of this investigation was to conduct experiments to study the behavior of red blood cells in aqueous polyethylene glycol solutions. Experiments were designed to determine the effect that aqueous solutions of PEG's 200, 300, 400, and 600 have in preventing hemolysis alone, and in the presence of sodium chloride.

EXPERIMENTAL

Materials—Polyethylene glycols 200, 300, 400, and 600, supplied by Union Carbide Chemical Corp., were used without further purification. The sodium chloride used was reagent grade.

Collection of Blood—Approximately 10 ml. of blood was obtained from rabbits by heart puncture. An 18-gauge, 2-in. needle attached to a 10-ml. syringe was used to make entrance into the heart. The blood was placed in a 50-ml. round-bottom flask which contained 10–15 glass beads. After gently rotating the flask for approximately 5 min., the defibrinated blood was decanted into a 50-ml. conical flask. The blood was aerated by gently swirling the flask for about 5 min.

The human blood used was obtained from the forearm veins of several 20–25-year-old healthy male Caucasians. The blood was treated in the same manner as the rabbit blood. Fresh blood samples were used in all experiments.

Preparation of Solutions—All of the polyethylene glycol and sodium chloride solutions were weight-in-volume percentage preparations.

Quantitative Determination of Per Cent Hemolysis—The method used to determine the degree of hemolysis in this investigation was dependent upon the fact that the amount of oxyhemoglobin liberated from the red corpuscles in hypotonic solutions is a direct function of the number of cells hemolyzed. A quantitative determination of partial hemolysis in any mixed solvent system was made by centrifuging solutions containing unhemolyzed cells and determining the oxyhemoglobin in the supernatant solutions by means of a photoelectric colorimeter.

The general method consisted of transferring 5 ml. of standard sodium chloride solutions (0.34, 0.36, . . ., 0.44, 0.46%) into each of two test tubes. Identical amounts of the mixed solvent systems were also transferred into each of two test tubes. Then 0.05 ml. of blood was added to each test tube and the tubes inverted several times to obtain complete mixing. After 45 min. at 37°, the blood mixtures were centrifuged. Because of the viscosity of the PEG solutions, especially in high concentrations, it was necessary to use centrifuge speeds of 2000–3000 r.p.m. to bring about complete settling of intact cells. After centrifuging, the light absorbance of the supernatant liquid was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter. These absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100 to obtain per cent hemolysis occurring in each test solution. A blank, used to cancel any light absorbance inherent to the blood sample, was prepared by placing 0.05 ml. of blood in 0.9% sodium chloride solution, allowing to stand for 45 min., and centrifuging in a like manner.

Water–PEG solutions absorbed a small amount of light, and this absorbance increased with an increase in PEG content. This absorbance was determined for the various concentrations of PEG's used in the experiments, and these blank readings were subtracted from the colorimeter readings obtained at the end of the hemolysis experiments.

A battery-operated model M Beckman pH meter was used in all pH measurements.

Calculation of i Values—The i values (isotonic coefficients) for polyethylene glycols were calculated according to the equation of Grosicki and Husa (8) which was modified as follows.

$$\frac{\left(\begin{matrix}i\text{ value}\\ \text{for NaCl}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml.}\\ \text{soln.}\end{matrix}\right)}{(\text{Gm.-mol. wt. of NaCl})} = \frac{\left(\begin{matrix}i\text{ value for}\\ \text{PEG}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of PEG}\\ \text{in 100 ml.}\\ \text{of soln.}\end{matrix}\right)}{(\text{Gm.-mol. wt. of PEG})} \quad \text{(Eq. 1)}$$

Concentrations of sodium chloride and polyethylene glycol causing the same degree of hemolysis (*e.g.*, 25, 50, and 75%) were used in the above equation to calculate i values for the polyethylene glycols.

Experiments were carried out to obtain data for calculating *apparent* i values for sodium chloride when the salt was present in a water–polyethylene glycol system in which the polyethylene glycol itself exhibited no protection to red blood cells, *e.g.*, sodium chloride in 10% PEG 200. In these calculations, it was necessary to assume that polyethylene glycol did not contribute to the osmotic behavior of the solutions and that sodium chloride was solely responsible for the tonicity of the solutions. Since the calculations were concerned with i values of only sodium chloride in different solvents, the molecular weights in Eq. 1 are identical and the equation becomes

$$\left(\begin{matrix}i\text{ value for}\\ \text{NaCl in water}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml. of}\\ \text{water}\end{matrix}\right) = \left(\begin{matrix}i\text{ value for}\\ \text{NaCl in PEG}\\ \text{soln.}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml.}\\ \text{PEG soln.}\end{matrix}\right) \quad \text{(Eq. 2)}$$

The value of i for sodium chloride was taken as 1.86, which is the accepted value of i for 0.9% sodium chloride in water (8).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–PEG solutions were plotted on rectangular coordinate paper. From these curves the concentrations of sodium chloride in Gm./100 ml. of water and PEG, causing 25, 50, and 75% hemolysis, were determined. These values were inserted into Eq. 2, and the values of i for sodium chloride in a particular water–PEG solution at concentrations giving 25, 50, and 75% hemolysis were determined.

Preparation of Hemolysis Curves—Experiments employing human blood were carried out to determine *apparent* i values for sodium chloride in various PEG 200 and 300 solutions. The average readings of these experiments were used to construct a standard hemolysis curve. In constructing the hemolysis curves of the various PEG solutions the grams of sodium chloride per 100 ml. of solution causing 25, 50, and 75% hemolysis were calculated with reference to the standard hemolysis curve. By utilizing Eq. 2, the grams of sodium chloride per 100 ml. in a polyethylene glycol solution causing 25% hemolysis was calculated as

$$X = \frac{A \times B}{C} \qquad \text{(Eq. 3)}$$

where X = Gm. of sodium chloride in 100 ml. of polyethylene glycol solution causing 25% hemolysis, A = 1.86 as the i value for sodium chloride in water, B = Gm. of sodium chloride in 100 ml. of water causing 25% hemolysis (obtained from standard hemolysis curves), and C = previously calculated i value for sodium chloride in appropriate polyethylene glycol solution. Similar calculations were carried out to obtain i values at 50 and 75% hemolysis. With these three i values at 25, 50, and 75% hemolysis, the hemolysis curves for the various polyethylene glycol solutions were constructed.

RESULTS

Polyethylene Glycol 200 and 300—The hemolysis of rabbit and human erythrocytes after 45 min. at 37° in various water–PEG 200 and 300 solutions are shown in Figs. 1 and 2. All of the PEG 200 and 300 solutions void of sodium chloride caused complete hemolysis of rabbit and human erythrocytes; however, hemolysis of red blood cells in aqueous solutions containing more than 15–25% PEG 200 or 300 resulted in brown-green solutions instead of normal red solutions. The concentrations at which this discoloration occurred are shown in Table I.

The inclusion of 0.9% sodium chloride in aqueous solutions containing 0.0 to 25% PEG 200 or 300 afforded complete protection (no hemolysis) to rabbit and human erythrocytes. However, the inclusion of sodium chloride in solutions containing 25% and more of PEG 200 or 40% and more of PEG 300 did not prevent damage of blood cells. At these critical concentrations (see Table I) the red blood cells were destroyed resulting in brown-green solutions. The addition of 2, 3, or 5% sodium chloride to 25, 30, 40, and 50% PEG 200 and 300 solutions prevented hemolysis and discoloration of blood.

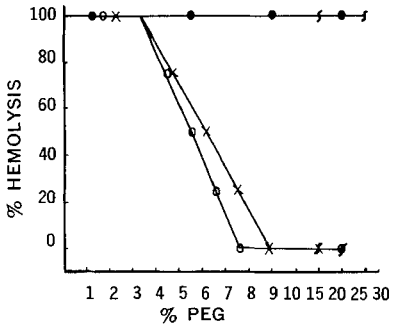

Fig. 1—Hemolysis of rabbit erythrocytes after 45 min. at 37° in various polyethylene glycol–water solutions. Key: ●, PEG 200 and 300; ×, PEG 400; ○, PEG 600; S, discoloration occurred.

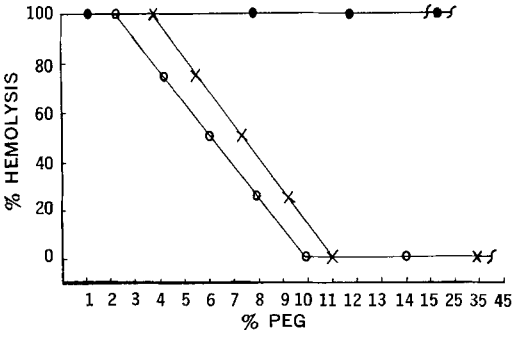

Fig. 2—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol–water solutions. Key: ●, PEG 200 and 300; ×, PEG 400; ○, PEG 600; S, discoloration occurred.

TABLE I—CONCENTRATIONS (Gm./100 ml.) OF PEG IN AQUEOUS SOLUTIONS AT WHICH DISCOLORATION OF BLOOD OCCURRED[a]

PEG	No NaCl		0.9% NaCl	
	Rabbit Blood	Human Blood	Rabbit Blood	Human Blood
200[b]	25	25	30	40
300[b]	15	15	25	25
400[c]	15	40	40	40
600[c]	20	40	40	40

[a] Each value represents an average of at least four blood samples. [b] Discoloration in PEG 200 and 300 solutions resulted in brown-green solutions. [c] Discoloration in PEG 400 and 600 solutions resulted in brown-black precipitates. The solutions were not colored.

The fragility of human erythrocytes in various water–PEG 200 and 300 solutions was modified or corrected by the addition of sodium chloride. It was possible to calculate i values for sodium chloride in various water–PEG 200 and 300 solutions. The average i values for sodium chloride in 5, 10, and 20% PEG 200 and 300 solutions are shown in Table II. The i values for sodium chloride in 10 and 20% PEG 200 solutions were less than 1.86 (the accepted value for 0.9% sodium chloride in water). The i values were greater than 1.86 for PEG 300 solutions.

The pH readings for PEG 200 and 300 solutions

TABLE II—VALUES OF *i* FOR NaCl IN VARIOUS WATER–PEG SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES AT 37°[a]

% w/v	Hemolysis, % 25	50	75	Av.
PEG 200				
5[b]	1.8	2.1	2.2	2.0
10	1.7	1.7	1.9	1.8
20	1.6	1.6	1.7	1.6
PEG 300				
5	4.0	5.2	6.6	5.3
10	2.6	4.4	6.9	4.6
20	2.8	3.1	3.4	3.1

[a] Unless otherwise indicated each *i* value represents an average of at least two blood samples. [b] Average of four blood samples.

before and after the addition of blood were within a range of 3.5 to 4.5.

Hemolysis curves showing the amount of laking that occurred when human blood was added to various water–PEG 200 and PEG 300–sodium chloride solutions are shown in Figs. 3 and 4. These curves were constructed in the manner described under *Experimental* utilizing the data presented in Table II. Unusual data were obtained for experiments using 5% PEG 200 solutions containing various amounts of sodium chloride and the results are shown in Fig. 5. Instead of the continual increase in hemolysis with decreasing sodium chloride concentration, hemolysis in 5% PEG 200 solutions decreased in those solutions containing 0.38 to 0.35% sodium chloride and then increased with further decrease in sodium chloride concentrations.

Polyethylene Glycol 400 and 600—The hemolysis of rabbit and human erythrocytes after 45 min. at 37° in various water–PEG 400 and 600 solutions is shown in Figs. 1 and 2. Hemolysis was prevented in various aqueous PEG 400 and 600 solutions up to critical concentrations where damage of red blood cells occurred resulting in brown-black precipitates (see Table I).

It was possible to calculate *i* values for PEG 400 and PEG 600. Average *i* values are shown in Table III.

The addition of 0.9% sodium chloride to solutions containing less than 40% PEG 400 or 600

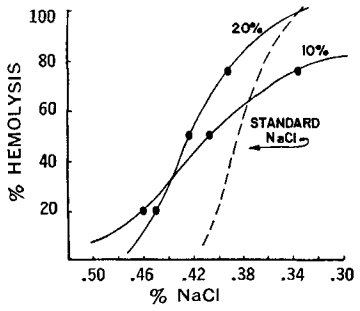

Fig. 3—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol 200–saline solutions.

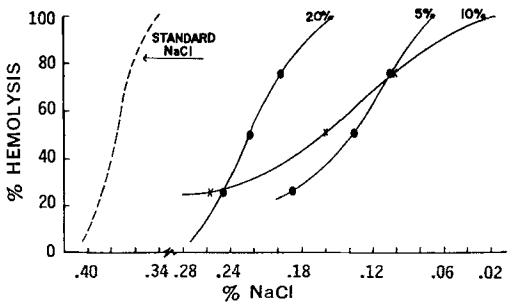

Fig. 4—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol 300–saline solutions.

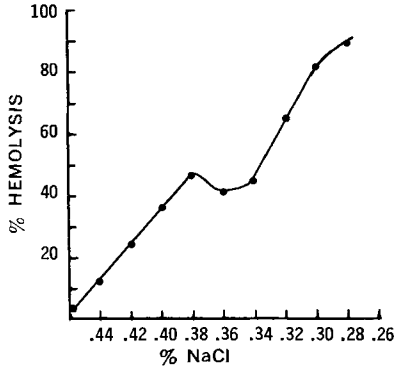

Fig. 5—Hemolysis of human erythrocytes after 45 min. at 37° in 5% polyethylene glycol 200–saline solutions.

TABLE III—VALUES OF *i* FOR PEG 400 AND 600 AT CONCENTRATIONS (Gm./100 ml.) CAUSING 25, 50, and 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES AT 37°[a]

	Hemolysis, % 25	50	75	Av.
	Rabbit Blood			
PEG 400	0.7	0.8	1.0	0.8
PEG 600	1.2	1.4	1.6	1.4
	Human Blood			
PEG 400	0.5	0.6	0.8	0.6
PEG 600	0.9	1.2	1.6	1.2

[a] Each *i* value represents an average of two to seven blood samples.

afforded complete protection (no hemolysis) to rabbit and human erythrocytes; however, at critical concentrations (see Table I) damage of red blood cells occurred as in PEG 400 and 600 solutions void of sodium chloride. This damage was not prevented by the addition of 2, 3, or 5% sodium chloride to 40, 50, and 60% solutions of PEG 400 and 600.

The pH readings of all PEG 400 and 600 test solutions before and after the addition of blood was within a range of 3.5 to 4.5.

DISCUSSION

The concentrations of PEG 200, 300, 400, and 600 in water that are iso-osmotic with 0.9% sodium chloride, according to calculations using the osmotic factor equation (9),

$$\text{osmotic factor} = \frac{\left(\begin{matrix}\text{No. of particles}\\ \text{from 1 molecule}\\ \text{of solute}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of solute}\\ \text{in 100 ml. of}\\ \text{soln.}\end{matrix}\right)}{\text{(Gm.-mol. wt. of solute)}} \quad \text{(Eq. 4)}$$

are 5.8, 8.7, 11.6, and 17.4%, respectively. Regardless of the polyethylene glycol content in aqueous solution, PEG 200 and 300 solutions failed to prevent hemolysis of rabbit and human erythrocytes. These experimental data point out that when water–PEG 200 or water–PEG 300 are used as vehicles for intravenous solutions, the finished product should not be assumed hypertonic with respect to blood, even when there is a high concentration of polyethylene glycol present. In experimental studies, tissue reactions have been observed following parenteral doses of undiluted polyethylene glycols which are severe enough to warrant very thorough study of safety before any parenteral applications are made (10). Hemolysis has resulted from high concentrations injected into the blood stream. Viscosity and lack of diffusion have resulted in discomfort or pain after subcutaneous injections of undiluted material and ischemic necrosis has also been seen after intramuscular injections. In a study of the polyethylene glycols as vehicles for intramuscular and subcutaneous injections by Carpenter and Shaffer (11), tissue reactions at the site of subcutaneous and intramuscular injections of undiluted PEG 300 in dosages 2.5 to 10 times that anticipated for human use caused blanching of the skin and scab formation in 48 hr. In a study by McCabe *et al.* (12), it was found that daily administration of 240 mg. of nitrofurantoin in PEG 300 to 30 patients caused severe metabolic acidosis and nephropathy in seven patients resulting in two deaths. These damaging effects were attributed to polyethylene glycol rather than nitrofurantoin.

Sodium chloride is effective in preventing hemolysis of human erythrocytes in aqueous polyethylene glycol solutions as long as the polyethylene glycol concentrations do not exceed 25 to 40%. At these higher polyethylene glycol concentrations, erythrocytes are not protected from discoloration by the addition of 0.9% sodium chloride. Concentrations of polyethylene glycols causing discoloration of human and rabbit erythrocytes are summarized in Table I. Higher concentrations of sodium chloride prevented discoloration in PEG 200 and 300 solutions but not in PEG 400 and 600 solutions. This damage does not appear to be hemolytic in character, but seems to be a chemical type of destruction. It appears that pH was not a factor since the pH of all concentrations of polyethylene glycol solutions remained within a range of 3.5 to 4.5 before and after the addition of blood.

The van't Hoff factor (i value or isotonic coefficient) is defined as the ratio of the colligative effect produced by a concentration (molal) of electrolyte divided by the effect observed for the same concentration of nonelectrolyte (13). In studying the effect of low concentrations of various substances on erythrocytes, previous workers (1, 2, 8) used molar concentrations in place of molal concentrations in their calculations. The use of molar instead of molal concentration would introduce only a small error at electrolyte concentrations of 0.1 M or less (8). However, in the present study, comparatively high concentrations of liquid polyethylene glycols (4–9%) were involved in calculation of i values. This meant that enough volume of the test solution was occupied by the polyethylene glycol to produce a substantial difference between molar and molal concentrations. Molal concentrations of the test solutions used in this study were calculated by direct proportion (no shrinkage was noticed when polyethylene glycols and water were mixed) and i values calculated using molal concentrations. They were found to be 12 to 15% lower than the i values calculated on a molar basis. However, it was decided to calculate *hemolytic* i values using molar concentrations since pharmaceutical calculations in the area of isotonic solutions are based on this concentration expression.

The i values for sodium chloride in most aqueous PEG 200 solutions were less than 1.86. These low i values indicate that PEG 200 offers no protection to red blood cells against osmotic hemolysis. In fact, there probably is some deleterious effect since more sodium chloride is needed to protect the red blood cells against hemolysis in aqueous PEG 200 solutions than in water. The higher values of i for sodium chloride in aqueous PEG 300 solutions indicate that PEG 300 contributes to the tonicity of aqueous solutions. The fact that i values could be calculated for PEG 400 and 600 shows that these polyethylene glycols have the ability to protect erythrocytes against osmotic hemolysis. Since i values for PEG 400 were less than i values for PEG 600, it can be assumed that PEG 600 gives greater protection to red blood cells than PEG 400. Therefore, the order in which the liquid polyethylene glycols protect rabbit and human erythrocytes against hemolysis is $200 < 300 < 400 < 600$. The ability of the liquid polyethylene glycols to contribute to the tonicity of aqueous solutions is dependent on the molecular weight. A possible explanation for this behavior might be that the lower molecular weight polyethylene glycols are able to penetrate the red blood cell membrane and therefore have little or no effective concentration in the extracellular solutions. It appears that increasing the molecular weight decreases the membrane penetrating properties of liquid polyethylene glycols.

REFERENCES

(1) Cadwallader, D. E., *J. Pharm. Sci.*, **52**, 1175(1963).
(2) Cadwallader, D. E., Wickliffe, B. W., and Smith, B. L., *ibid.*, **53**, 927(1964).
(3) Spiegel, A. J., and Noseworthy, M. M., *ibid.*, **52**, 917(1963).
(4) "Carbowax, Polyethylene Glycols," Union Carbide Chemical Co., New York, N. Y., 1960.
(5) Meyer, G., and Sturmer, E., *Arch. Intern. Pharmacodyn.*, **90**, 193(1952).
(6) Swanson, E. E., Anderson, R. C., Harris, P. N., and Rose, C. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 571(1953).
(7) Lee, C., and Anderson, R. C., *Toxicol. Appl. Pharmacol.*, **4**, 206(1962).
(8) Grosicki, T. S., and Husa, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 632(1954).
(9) "Husa's Pharmaceutical Dispensing," 5th ed., Mack Publishing Co., Easton, Pa., 1959, p. 142.
(10) "Carbowax, Polyethylene Glycols for Pharmaceuticals and Cosmetics," Union Carbide Chemical Co., New York, N. Y., 1959.
(11) Carpenter, C. P., and Shaffer, C. B., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 27(1952).
(12) McCabe, W. R., Jackson, G. G., and Griebel, H. G., *Arch. Internal Med.*, **104**, 710(1959).
(13) Maron, S. H., and Prutton, C. F., "Principles of Physical Chemistry," 3rd ed., Macmillan Co., New York, N. Y., 1958, p. 199.

Metabolite of 4-Amino-4-deoxy-N^{10}-methylpteroylglutamic Acid (Methotrexate)

By DAVID G. JOHNS and TI LI LOO

4-Amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate; MTX) is rapidly metabolized by rabbit liver aldehyde oxidase. The MTX metabolite, on oxidative cleavage with permanganate, yields a simple pteridine having the spectrophotometric and chromatographic properties of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid. Alkaline hydrolysis of the latter at 70° slowly converts it to 2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid. The nonpteridine moiety of the MTX metabolite is identical with the nonpteridine moiety of MTX. The biological properties of the MTX metabolite are compatible with those of a 2,4-diamino derivative of pteroylglutamate. In view of these findings, the structure, 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX), is proposed for the MTX metabolite.

THE FOLIC ACID analog 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate; MTX), a compound of considerable clinical interest in the treatment of neoplastic diseases and of psoriasis, undergoes rapid biological inactivation by a flavin enzyme present in the supernatant fraction of rabbit liver homogenates (1, 2). A similar, but slower conversion occurs in the supernatant fraction of liver homogenates from guinea pig and several other species (3). Johns *et al.* (1) have identified the MTX-inactivating enzyme as aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1) and have shown it to be identical with the enzyme catalyzing the inactivation of 3′,5′-dichloro-MTX and a number of other halogenated 4-amino analogs of pteroylglutamate (4). The structure of the MTX metabolite has not been established, although Redetzki and his co-workers (2), on the basis of elementary analyses, suggested that the compound is 4,7-dihydroxy-MTX (7-hydroxy-N^{10}-methylpteroylglutamic acid). Earlier studies by Loo and Adamson (5) with 3′,5′-dichloro-MTX, however, have shown that, in the latter compound, the 4-amino group remains intact during enzymic inactivation and that the 3′,5′-dichloro-MTX metabolite differs from the parent compound only in the presence of a hydroxyl group at the 7-position. Since the same enzyme system is responsible for the inactivation of both MTX and its 3′,5′-dichloro derivative, it appears unlikely that the 4-amino group of MTX would undergo hydrolysis while the 4-amino group of 3′,5′-dichloro-MTX would not. Furthermore, it is well known that elementary analytical data alone are rarely conclusive in establishing the structure of pteridines (5, 6). The studies described below strongly suggest that, with MTX, as with 3′,5′-dichloro-MTX, enzymic inactivation involves only 7-hydroxylation, and that the 4-amino group remains intact.

Received September 29, 1966, from the Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, and the Department of Developmental Therapeutics, University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, TX 77025

Accepted for publication December 15, 1966.

The authors thank Dr. William C. Alford and associates, Laboratory of Chemistry, National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., for microanalyses.

This work was supported by research grants CA-02817 and CA-08010 from the U. S. Public Health Service, and by contract PH 43-66-1156 with the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Preparation of the MTX Metabolite—Commercial grade MTX[1] was twice precipitated from aqueous solution with glacial acetic acid, washed with ice-cold acetone, and dried over phosphorus pentoxide; of the MTX powder thus obtained, 150 mg. was dissolved in 60 ml. of water with the aid of 1 *N* potassium hydroxide added dropwise. The pH of the solution was adjusted to 7.5 with 2 *N* phosphoric acid.

Frozen mature rabbit liver (600 Gm.; Pel-Freez Biologicals, Inc.) was allowed to thaw at 5° and homogenized in 2 vol. of distilled water. After centrifuging for 10 min. at 27,000 × g, the crude supernatant fraction was collected and immersed in a water bath at 60° for 10 min. Heat-precipitated protein was removed by centrifugation, and the supernatant fractionated with an ammoniacal solution of ammonium sulfate prepared by adding 60 ml. ammonium hydroxide solution (29%) to 940 ml. saturated solution of ammonium sulfate. For each 100 ml. of the supernatant, 59 ml. of the solution of ammoniacal ammonium sulfate was added with stirring, and the precipitate was removed by centrifuging. An additional 23 ml. of ammoniacal solution of ammonium sulfate was then added, and the precipitate was recovered. The precipitate was redissolved in sufficient ammoniacal ammonium sulfate solution of 10% saturation to give a protein concentration of about 30 mg./ml. A typical purification resulted in 28-fold concentration of aldehyde oxidase activity, as compared with that

[1] Kindly provided by Dr. J. M. Ruegsegger, Lederle Laboratories.

of the crude supernatant, and yielded 36 ml. of enzyme solution with a protein concentration of 31 mg./ml. Aldehyde oxidase activity was determined with *N*-methylnicotinamide as substrate (7), and protein concentration was determined by the method of Waddell (8).

To the enzyme solution were added 10,000 units of catalase[2] (Worthington Biochemical Corp.), 6 ml. of 0.4 *M* sodium phosphate buffer, pH 7.8, containing 0.005% iron chelating agent,[3] and 100 ml. of distilled water. The pH was adjusted to 8.4 by the addition of ammoniacal ammonium sulfate, and the MTX solution (prepared as described above) was added dropwise with stirring at room temperature, at a rate of 8 ml./hr. (20 mg. of MTX per hour). The solution was then allowed to stand at 5° overnight, and the yellowish-white flocculent precipitate was collected by centrifuging. The precipitate was suspended in 16 ml. of distilled water, and a 0.2-ml. aliquot was removed for chromatographic examination; the remainder was heated in a boiling water bath for 4 min., centrifuged, and the insoluble protein precipitate discarded. The supernatant fraction was cooled in an ice-bath, 0.8 vol. of glacial acetic acid was added, and the solution was allowed to stand overnight at 5°. The lemon-yellow precipitate was washed twice with cold acetone and dried at room temperature over phosphorus pentoxide. The yield in a typical preparation was 94 mg. Chromatography on DEAE-cellulose, as previously described (9), showed the product to migrate as a single peak, and to be free of MTX. The ultraviolet absorption characteristics of the product are: in 0.1 *N* hydrochloric acid, $\lambda_{max.}$ 218 mμ (log ε 4.48), 300 mμ (log ε 4.26), and 334 mμ (infl.) (log ε 4.07); at pH 7.6 in 0.05 *M* Tris buffer, $\lambda_{max.}$ 224 mμ (infl.) (log ε 4.23), 261 mμ (log ε 4.04), 303 mμ (log ε 4.29), and 340 mμ (infl.) (log ε 4.08); in 0.1 *N* sodium hydroxide solution, $\lambda_{max.}$ 223 mμ (log ε 4.54), 260 mμ (log ε 4.07), 308 mμ (log ε 4.31), and 337 mμ (infl.) (log ε 4.13).

Activity of the MTX Metabolite as an Inhibitor of Dihydrofolate Reductase—The MTX metabolite was tested for ability to inhibit the enzymatic conversion of dihydrofolate to tetrahydrofolate, using a partially purified preparation of dihydrofolate reductase from L1210 mouse leukemia cells (10). Data were plotted by the method of Zakrzewski (11). The MTX metabolite was of intermediate potency as an inhibitor of dihydrofolate reductase; the K_i was 6×10^{-9} *M* at pH 5.5. For comparison, the parent compound, MTX, a strong inhibitor of dihydrofolate reductase, has a K_i of $<3 \times 10^{-11}$ *M* (12), while pteroylglutamate, a weak inhibitor of the enzyme when dihydrofolate is used as substrate, has a K_i of 3×10^{-6} *M* (10).

Oxidative Degradation of the MTX Metabolite—The procedure was similar to that used for 3′,5′-dichloro-MTX (5). The MTX metabolite (50 mg.), dissolved in 10 ml. of 0.1 *N* sodium hydroxide, was oxidized with 1.5 ml. of 5% potassium permanganate added dropwise with vigorous stirring at 90° for 6 min. After all the potassium permanganate had been added, the reaction mixture was kept at 90° for an additional minute. The brown precipitate was removed by centrifugation, washed with water, and centrifuged again. The combined supernatant fractions were acidified with 1 ml. of glacial acetic acid and centrifuged. The weakly acidic supernatant from which the greenish-yellow gelatinous precipitate separated was saved for later investigation (see below). The precipitate was washed with water, redissolved in 6 ml. of 1 *N* sodium hydroxide, warmed with activated charcoal, and filtered. The filtrate was added slowly, dropwise, to hot dilute acetic acid (1 ml. of glacial acetic acid diluted to 20 ml. with water). On cooling, the pale yellow precipitate was collected by centrifugation and washed with water; this treatment was repeated. The product was then washed with acetone and dried in a vacuum desiccator. The pteridine acid (X) so obtained weighed 9 mg.

Anal.—Calcd. for $C_7H_6N_6O_3$ (2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid; the diamino acid): N, 37.84; for $C_7H_5N_5O_4$ (2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid; the dihydroxy acid): N, 31.39. Found: N, 38.15; 38.26.

The pteridine acid (X) showed ultraviolet absorption spectra identical to those of an authentic sample of the diamino acid (13) in 14.7 *N* sulfuric acid, in 0.1 *N* sodium hydroxide solution, and in 0.05 *M* Tris buffer, pH 8.0. Except in alkaline solution, the spectra of acid X bear little resemblance to those of the dihydroxy acid. The spectra of the three acids in 0.05 *M* Tris buffer, pH 8.0, are shown in Fig. 1.

When chromatographed on Whatman No. 1 paper, and developed with 0.5% sodium carbonate solution, ascending flow, the R_f values were 0.46–0.49 for pteridine acid (X), 0.46–0.49 for the diamino acid, and 0.71–0.73 for the dihydroxy acid. With 3% ammonium chloride solution as solvent, the R_f values were 0.19–0.20 for pteridine acid (X), 0.20–0.21 for the diamino acid, and 0.40–0.42 for the dihydroxy acid. All paper chromatograms were viewed under ultraviolet light of 254 mμ.

Alkaline Hydrolysis of Pteridine Acid (X)—Pteridine acid (X) was hydrolyzed at 70° in 2 *N* sodium hydroxide solution. Aliquots were removed at the times indicated in Table I, and ultraviolet absorption spectra determined in 0.05 *M* Tris buffer, pH 8.0. The maximum at 262 mμ displayed a gradual decrease in intensity, with a concomitant increase in intensity at 285 mμ, a change compatible with the conversion of the diamino acid to the dihydroxy acid (Table I).

Nonpteridine Moiety Derived from Permanganate Oxidation of the MTX Metabolite—The weakly acidic supernatant from which the pteridine acid (X) had been removed was further acidified with 3 ml. of concentrated hydrochloric acid, saturated with sodium chloride, and extracted first with 15 ml. of ethyl acetate, and then twice with 10 ml. of the same solvent. The combined extract was washed with 25 ml. of water and dried over anhydrous sodium sulfate. The ethyl acetate extract was then concentrated at 100° in an atmosphere of nitrogen. The reddish oil thus obtained defied all attempts at crystallization; however, its ultraviolet absorption spectra closely resembled that of *p*-methylaminobenzoylglutamic acid (14). When compared with the product obtained from a similar oxidative degradation of MTX, it showed identical *Rf* values on Whatman No. 1 paper, developed by

[2] The addition of catalase is necessary in order to prevent inactivation of the enzyme by hydrogen peroxide formed during the reaction.

[3] Marketed as Versene Fe-3 by the Dow Chemical Co., Midland, Mich.

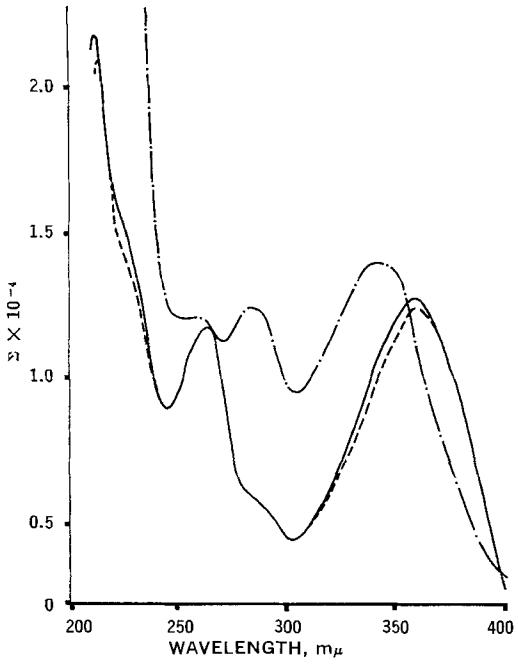

Fig. 1—Ultraviolet absorption spectra of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid (————), 2-amino - 4,7 - dihydroxy - 6 - pteridinecarboxylic acid (- · - · - · -), and pteridine acid (X) derived from permanganate oxidation of the MTX metabolite (- - - - - - - -). Solvent: 0.05 M Tris buffer, pH 8.0.

TABLE I—CHANGES IN ABSORBANCE OF PTERIDINE ACID (X) ON ALKALINE HYDROLYSIS[a]

Time, hr.	$A_{263m\mu}/A_{358m\mu}$	$A_{285m\mu}/A_{358m\mu}$
0	0.808	0.306
2.5	0.749	0.309
13.5	0.746	0.359
21.5	0.696	0.437

[a] Hydrolysis was carried out at 70° in 2 *N* sodium hydroxide solution. Ultraviolet absorption spectra were determined in 0.05 *M* Tris buffer, pH 8.0.

ascending flow, in three solvent systems, as indicated in Table II. All paper chromatograms were viewed under ultraviolet light of 366 mμ.

RESULTS AND DISCUSSION

The demonstration by ultraviolet spectroscopy, by chromatography, and by elementary analysis, that the MTX metabolite gives rise to 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid on permanganate oxidation is compatible with the structure 7-hydroxy-MTX for the parent compound. It would appear, therefore, that the enzyme-catalyzed hydroxylation involves only position-7 of MTX, and that the 4-amino group is unaltered. While 7-hydroxylation is compatible with the known catalytic properties of rabbit liver aldehyde oxidase, this enzyme possesses no known deaminase activity. For the 2-amino-4,7-dihydroxy structure, therefore, one would have to postulate the presence of an MTX-4-deaminase in the reaction mixture, a remote possibility, since the reaction proceeds equally well

TABLE II—R_f VALUES OF THE NONPTERIDINE MOIETY OF MTX (*A*) AND OF MTX METABOLITE (*B*)

Chromatographic System	R_f Value *A*	*B*	Mixture of *A* + *B*
0.1 *M* acetate buffer, pH 4.4	0.83	0.82	0.82
0.5% sodium carbonate soln.	0.92	0.89	0.91
1:1 mixture of 5% ammonium sulfate soln. and isopropanol	0.59	0.63	0.58

with highly purified aldehyde oxidase. Alternatively, nonenzymic hydrolytic deamination might occur at the 4-position. This appears equally unlikely, since lengthy heating at 70° in 2 *N* sodium hydroxide solution is required to bring about the conversion of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid to its 2-amino-4,7-dihydroxy analog.

Supporting evidence for the 2,4-diamino structure is the moderate antifolate activity of the MTX metabolite when tested in an *in vivo* system (2), or *in vitro*, against partially purified dihydrofolate reductase from L1210 mouse leukemia cells; barring alteration of the *p*-methylaminobenzoylglutamic acid moiety, such activity is compatible with a 2,4-diamino structure but not with a 2-amino-4-hydroxy structure.

The suggestion by other workers that the MTX metabolite is 2-amino-4,7-dihydroxy-MTX was based largely on elementary analyses. The difficulties encountered in elementary analysis, however, and particularly nitrogen analysis of pteridines (6), make additional criteria obligatory before identity can be established. The additional data presented here would favor the 7-hydroxy-MTX structure over the 4,7-dihydroxy-MTX structure.

The rapid hydroxylation of MTX and other 4-amino analogs of pteroylglutamate by the hepatic aldehyde oxidase of either rabbit or guinea pig probably accounts for the very high tolerance of these species for these drugs. The resistance of the hamster to the toxicity of MTX recently demonstrated by Freireich and his co-workers (15) would suggest that the latter species also belongs to this group. On the other hand, the extremely low level of MTX-oxidizing activity of the rat and mouse hepatic aldehyde oxidases (3) correlates well with the sensitivity of the two latter species to the antifolates. The human enzyme has not been studied; however, chromatographic studies of human urine after the administration of MTX show that most of the drug is excreted unchanged (16–18);[4] in addition, clinical data suggest that man is relatively sensitive to both MTX and aminopterin. These observations support the contention that the MTX-hydroxylating properties of human hepatic aldehyde oxidase must be rather low.

SUMMARY

1. Methotrexate (MTX) is readily metabolized by rabbit liver aldehyde oxidase.

[4] Chromatographic examination of human urine 2 weeks after the administration of tritium-labeled MTX has shown the presence of a small amount of non-MTX radioactivity (17); the latter migrated more rapidly on DEAE-cellulose than did MTX, however, and thus cannot be attributed to 7-hydroxy-MTX.

2. Permanganate oxidation of the metabolite of MTX yields a simple pteridine with the properties of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid.

3. Heating of the latter compound in 2 *N* sodium hydroxide solution at 70° leads to slow hydrolysis of the 4-amino group.

4. The nonpteridine moiety of the metabolite of MTX is identical with the nonpteridine moiety of MTX.

5. The structure 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX) is proposed for the MTX metabolite.

REFERENCES

(1) Johns, D. G., *et al.*, *Biochim. Biophys. Acta*, **105**, 380(1965).
(2) Redetzki, H. M., Redetzki, J. E., and Elias, A. L., *Biochem. Pharmacol.*, **15**, 425(1966).
(3) Johns, D. G., *et al.*, *ibid.*, **15**, 555(1966).
(4) Loo, T. L., and Adamson, R. H., *ibid.*, **11**, 170(1962).
(5) Loo, T. L., and Adamson, R. H., *J. Med. Chem.*, **8**, 513(1965).
(6) Albert, A., *Quart. Rev.*, **6**, 197(1952).
(7) Rajagopalan, K. V., Fridovich, I., and Handler, P., *J. Biol. Chem.*, **237**, 922(1962).
(8) Waddell, W. J., *J. Lab. Clin. Med.*, **48**, 311(1956).
(9) Johns, D. G., *et al.*, *Life Sci.*, **3**, 1383(1964).
(10) Bertino, J. R., *et al.*, *J. Biol. Chem.*, **239**, 479(1964).
(11) Zakrzewski, S. F., *ibid.*, **238**, 1485(1963).
(12) Werkheiser, W. C., *ibid.*, **236**, 888(1961).
(13) Elion, G. B., Hitchings, G. H., and Russell, P. B., *J. Am. Chem. Soc.*, **72**, 78(1950).
(14) Fu, S.-C. J., Reiner, M., and Loo, T. L., *J. Org. Chem.*, **30**, 1278(1965).
(15) Freireich, E. J., *et al.*, *Cancer Chemotherapy Rept.*, **50**, 219(1966).
(16) Freeman, M. V., *J. Pharmacol. Exptl. Therap.*, **122**, 154(1958).
(17) Johns, D. G., *et al.*, *J. Clin. Invest.*, **43**, 621(1964).
(18) Henderson, E. S., Adamson, R. H., and Oliverio, V. T., *Cancer Res.*, **25**, 1018(1965).

Statistical Analysis of Log-Dose Response Bioassay Experiments with Unequal Dose Ratios for the Standard and Unknown Preparations

By C. PHILIP COX

Well-known statistical analyses are available for the analysis of log-dose response assays when successive doses are in the same ratio for both the standard and unknown preparations. It is, however, sometimes convenient and advantageous in practice to use unequal dose ratios. Appropriate analyses are offered for such cases, analyses which reduce to the usual ones when the ratios are equal. It is seen that the operational flexibility thus permitted is obtained in return for only slightly increased computation. Four and six-point assays are discussed in detail together with a numerical example of the former. An improved method for calculating confidence interval estimates in log-dose response assays is also given.

STATISTICAL ANALYSES for log-dose response parallel line assays in which the ratios of the successively higher doses are the same for both the standard and unknown preparations are well known. (For example, see *Reference 1.*) The case to be considered here is that in which the ratios of successive doses are constant within each preparation but the constant ratio differs from one preparation to the other. Such a relaxation of the usual single constant ratio condition is occasionally desirable in practice (2) and, for example, may permit the linear response log-dose range of the standard preparation to be exploited in the presence of more uncertainty about the linear range for the unknown preparation.

It will be shown that such flexibility can be achieved with only small changes from the single ratio analyses and, in fact, the latter can be regarded as special cases of the more general analysis proposed here. This analysis has some computational advantages over the alternative based on a single log-dose transformation which gives integral dose-metameter values for one preparation but nonintegral values for the other.

SPECIFICATION AND DOSE TRANSFORMATIONS

It will be supposed that, in an $(h + k)$-point assay, the h and k doses (concentrations) of the standard and unknown preparations, respectively, are chosen so that,

$$\left.\begin{aligned} \frac{z_{Si}}{z_{Si-1}} &= D_S,\ i = 1, 2, \ldots, h \\ \text{and} \\ \frac{z_{Uj}}{z_{Uj-1}} &= D_U,\ j = 1, 2, \ldots, k \end{aligned}\right\} \quad \text{(Eq. 1)}$$

where z_{Si} and z_{Uj} are, respectively, the ith dose of the standard and the jth dose of the unknown preparation.

As usual, it will be assumed that the observed

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Accepted for publication December 6, 1966.
This research was supported in part by a graduate training program from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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responses are subject to residual variability components which are normally and independently distributed about population responses and that these are linearly related to logarithms of the doses over, at least, the dose ranges used. Then, writing

$$d_S = \log D_S \text{ and } d_U = \log D_U$$

dose transformations are defined as,

$$\left.\begin{aligned} x_S &= \frac{1}{d_S}\log(z_S/z_{S1}) \\ \text{and} \\ x_U &= \frac{1}{d_U}\log(z_U/z_{U1}) \end{aligned}\right\} \quad \text{(Eq. 2)}$$

where z_{S1} and z_{U1} are the two lowest doses. Hence, because for the ith dose, from Eq. 1,

$$z_{Si} = D_S z_{Si-1} = \ldots = D_S^{i-1} z_{S1}$$

so that,

$$x_{Si} = \frac{1}{d_S}\log(D_S^{i-1} z_{S1}/z_{S1}) = i - 1$$

it is apparent that the integral x_S values 0, 1, 2, . . . , $(h - 1)$, correspond to the z_S values z_{S1}, z_{S2}, . . . z_{Sh}, and similarly, for the unknown preparation, the second of Eq. 2 transforms the z_U values into the integral x_U values 0, 1, 2, . . ., $k - 1$.

EQUIVALENT DILUTION CONDITION AND RELATIVE POTENCY FORMULA

A dilution assay is one in which the unknown preparation acts simply as a dilution (concentration) of the standard preparation in an inert diluent. In this case if z_{ES} is a dose of the standard preparation which is equipotent to, that is, produces the same response value as a dose z_{EU} of the unknown preparation, any two such equipotent doses are connected by the equivalent dilution relationship

$$z_{ES} = \rho_p z_{EU} \quad \text{(Eq. 3)}$$

where ρ_p is the relative potency.

It is well known that, in the single dose-ratio case, this expression leads to a pair of parallel lines for the relationship between responses and log-dose values. The corresponding implication from the equivalent dilution condition based on the transformations in Eq. 2 is readily obtained as follows.

Let x_{ES} and x_{EU} denote equipotent log-dose-values so that, from Eqs. 2 and 3,

$$d_S x_{ES} - d_U x_{EU} = \log\left(\frac{z_{ES}}{z_{EU}}\right)\left(\frac{z_{U1}}{z_{S1}}\right) = \log(\rho_p z_{U1}/z_{S1}) \quad \text{(Eq. 4)}$$

and it may be noted that, if $d_S = d_U$, Eq. 4 reduces to $x_{ES} - x_{EU}$ = a constant, that is to the usual parallelism condition.

The next step is to find the relations between the parameters of the two response lines when these are such that the condition in Eq. 4 obtains. These relations will further lead to the expression from which the relative potency may be estimated.

For these suppose that population values η_S and η_U of standard and unknown preparation responses are linearly related to their corresponding x_S and x_U values by the equations:

$$\left.\begin{aligned} \eta_S &= \alpha_S + \beta_S x_S \\ \eta_U &= \alpha_U + \beta_U x_U \end{aligned}\right\} \quad \text{(Eq. 5)}$$

α_S and α_U being the intercepts, while β_S and β_U are the slopes of the lines. Then, since $\eta_S = \eta_U$ for equipotent doses,

$$\alpha_S + \beta_S x_{ES} = \alpha_U + \beta_U x_{EU}$$

and, multiplying both sides by d_S,

$$d_S\alpha_S + \beta_S d_S x_{ES} = d_S\alpha_U + \beta_U d_S x_{EU}$$

Hence, substituting for $d_S x_{ES}$ from Eq. 4 into the lefthand side,

$$d_S\alpha_S + \beta_S\{d_U x_{EU} + \log(\rho_p z_{U1}/z_{S1})\} = d_S\alpha_U + \beta_U d_S x_{EU}$$

that is,

$$d_S(\alpha_S - \alpha_U) + \beta_S\log(\rho_p z_{U1}/z_{S1}) + (\beta_S d_U - \beta_U d_S)x_{EU} = 0$$

But, for this relation to hold for all x_{EU} in the linear range, it must be an identity and hence,

$$d_S(\alpha_S - \alpha_U) + \beta_S\log(\rho_P z_{U1}/z_{S1}) = 0 \quad \text{(Eq. 6)}$$

and

$$\beta_S d_U - \beta_U d_S = 0 \quad \text{(Eq. 7)}$$

The relation in Eq. 7 is the new statement of the equivalent dilution condition and which reduces to the parallelism condition, $\beta_S = \beta_U$, when $d_S = d_U$. For the relative potency itself it is first convenient to write the condition in Eq. 7 as:

$$\frac{\beta_S}{d_S} = \frac{\beta_U}{d_U} = \beta \quad \text{(Eq. 8)}$$

Then, from Eq. 6,

$$\log(\rho_p z_{U1}/z_{S1}) = -(\alpha_S - \alpha_U)/\beta \quad \text{(Eq. 9)}$$

so that the population value of the relative potency is given by

$$\rho_p = \frac{z_{S1}}{z_{U1}}\text{ antilog}\left\{-(\alpha_S - \alpha_U)/\beta\right\}$$

and the related estimation procedure is next considered.

RELATIVE POTENCY ESTIMATE

Writing Eq. 5 in the equivalent form,

$$\eta_S = \mu_S + \beta_S(x_S - \bar{x}_S) = \mu_S + \beta d_S(x_S - \bar{x}_S)$$

$$\eta_U = \mu_U + \beta_U(x_U - \bar{x}_U) = \mu_U + \beta d_U(x_U - \bar{x}_U)$$

it follows that

$$(\alpha_S - \alpha_U) = (\mu_S - \mu_U) - \beta(d_S\bar{x}_S - d_U\bar{x}_U)$$

and hence, from Eq. 9, that

$$\log(\rho_p z_{U1}/z_{S1}) = d_S\bar{x}_S - d_U\bar{x}_U - \frac{(\mu_S - \mu_U)}{\beta} \quad \text{(Eq. 10)}$$

Conventional statistical methodology then leads to estimates of μ_S, μ_U, and β as $\bar{y}_S$, $\bar{y}_U$, and b, respectively, where

$$b = \frac{d_S\Sigma_S'xy + d_U\Sigma_U'xy}{d_S^2\Sigma_S'xx + d_U^2\Sigma_U'xx} \quad \text{(Eq. 11)}$$

in which quantities such as,

$$\Sigma_S'xy = \Sigma_S(x_S - \bar{x}_S)(y_S - \bar{y}_S) = \Sigma_S(x_S - \bar{x}_S)y_S$$

can be calculated by the usual computation formula, suffixes S and U denoting summations over the standard and test preparation values, respectively.

From Eq. 10 the relative potency estimate R is then given by

$$R = \frac{z_{S1}}{z_{U1}} \text{antilog } [d_S\bar{x}_S - d_U\bar{x}_U - (\bar{y}_S - \bar{y}_U)/b] \quad \text{(Eq. 12)}$$

Pursuance of the general case beyond this point is straightforward but likely to be of limited practical appeal; more usefully the commonly occurring cases of four and six-point assays will now be examined in detail.

FOUR-POINT LOG-DOSE RESPONSE ASSAY

Suppose that a four-point assay is carried out in a completely randomized design and that n responses are observed at each of the four preparation-dose combinations. Let S_1 and S_2 be the response totals for the lower and higher doses of the standard preparation, U_1 and U_2 being the corresponding totals for the unknown preparation. Then, as in the usual case when $d_S = d_U$, it is convenient for the analysis of variance (ANOV) and the estimation of relative potency to obtain three orthogonal contrasts between the four treatment totals.

The difference between the means for the two preparations $(\bar{y}_S - \bar{y}_U)$ is

$$\bar{y}_S - \bar{y}_U = \frac{1}{2n}(S_1 + S_2 - U_1 - U_2) = -L_p/2n \quad \text{(Eq. 13)}$$

where

$$L_p = -S_1 - S_2 + U_1 + U_2 \quad \text{(Eq. 14)}$$

is the first of the contrasts required.

The second contrast is one proportional to the regression coefficient b. In the four-point assay case the transformations in Eq. 2 lead to x-values of 0 and 1 for the upper and lower doses, respectively. Then:

$$\Sigma_S'xy = \Sigma_S(x_S - \bar{x}_S)y_S = \frac{1}{2}(-S_1 + S_2)$$

and

$$\Sigma_S'xx = \Sigma_S(x_S - \bar{x}_S)^2 = n/2$$

and similarly for unknown preparation summations. Hence, from Eq. 11,

$$b = \frac{d_S(-S_1 + S_2) + d_U(-U_1 + U_2)}{n(d_S^2 + d_U^2)} = \frac{L_r}{n(d_S^2 + d_U^2)} \quad \text{(Eq. 15)}$$

so that

$$L_r = -d_SS_1 + d_SS_2 - d_UU_1 + d_UU_2 \quad \text{(Eq. 16)}$$

will be taken as the second contrast.

The third contrast is one for testing the relevance of the model, in particular, being sensitive to departure from the equivalent dilution condition as now expressed by Eq. 8. This contrast can therefore be taken as proportional to the quantity $(d_Ub_S - d_Sb_U)$ where b_S and b_U are regression coefficients for the standard and unknown preparations taken separately. With

$$b_S = \Sigma_S'xy/\Sigma_S'xx = -(S_1 - S_2)/n$$

and

$$b_U = -(U_1 - U_2)/n$$

it follows that

$$d_Ub_S - d_Sb_U = -\frac{1}{n}(d_US_1 - d_US_2 - d_SU_1 + d_SU_2)$$

The third contrast will accordingly be taken as

$$L_d = d_US_1 - d_US_2 - d_SU_1 + d_SU_2 \quad \text{(Eq. 17)}$$

where the suffix d here denotes divergence from the condition in Eq. 8.

The statistical analysis is based on the usual assumptions that the responses are independent and normally distributed with constant variance. Accordingly, because each contrast is a linear combination of independent observations, the appropriate divisors can be found by conventional statistical procedures and it is also readily checked that the three contrasts are orthogonal. From Eqs. 14, 16, and 17 the complete scheme of contrasts together with the ANOV entries, may then be set out as in Table IA.

Defining the correction term as

$$C = (S_1 + S_2 + U_1 + U_2)^2/4n$$

the ANOV is therefore as shown in Table IB.

Model relevance may be tested by comparing the ratio of the mean squares for divergence and for within preparations and levels against the tabulated critical value, $F_c = F(1, 4n - 4, 0.05)$ say. Assuming satisfactory relevance, the point estimate of relative potency is next obtained. For this, values of $(\bar{y}_S - \bar{y}_U)$ and b can be substituted, from Eqs. 13 and 15 into Eq. 12, to give with $\bar{x}_S = \bar{x}_U = \frac{1}{2}$,

$$R = \frac{z_{S1}}{z_{U1}} \text{antilog } \frac{1}{2}\left[d_S - d_U + (d_S^2 + d_U^2)\frac{L_p}{L_r}\right] \quad \text{(Eq. 18)}$$

With the usual assumptions the only quantities subject to statistical variability are the contrasts L_p and L_r. Accordingly, an interval estimate of the relative potency can be obtained from an interval estimate for the population mean of

$$M = (d_S^2 + d_U^2)L_p/L_r \quad \text{(Eq. 19)}$$

The result due to Fieller (3, 4) for interval estimation of a ratio can be applied in this case using the method of Cox and Ruhl (5). The authors showed that r_L and r_H, the lower and upper limits, respectively, of the 95% confidence interval (for example) for the population mean of a ratio $r = L_p/L_r$ can be expressed as

$$r_L, r_H = \frac{1}{(F_r - F_c)} \times \left[rF_r \mp \sqrt{\frac{a_pF_c}{a_r}(F_p + F_r - F_c)}\right] \quad \text{(Eq. 20)}$$

where

TABLE IA—ORTHOGONAL CONTRASTS FOR AN UNEQUAL DOSE RATIO FOUR-POINT ASSAY (n RESPONSES PER DOSE)

Contrast	Response Total S_1	S_2	U_1	U_2	ANOV Sum of Sq.
Prepn.: L_p	-1	-1	1	1	$L_p^2/4n$
Regression: L_r	$-d_S$	d_S	$-d_U$	d_U	$L_r^2/2n(d_S^2 + d_U^2)$
Divergence: L_d	d_U	$-d_U$	$-d_S$	d_S	$L_d^2/2n(d_S^2 + d_U^2)$

TABLE IB—ANOV

Variation Source	d.f.	s.s.	m.s.
Prepn.	1	$L_p^2/4n$	
Regression	1	$L_r^2/2n(d_S^2 + d_U^2)$	
Divergence	1	$L_d^2/2n(d_S^2 + d_U^2)$	
Within prepn. and levels	$4n - 4$	By subtraction	$s^2 = \text{s.s.}/4n - 4$
Total	$4n - 1$	$\Sigma\,(\text{response})^2 - C$ all	

$$F_c = F(1, 4n - 4, 0.05)$$

is, as previously noted, the critical F-value read from standard statistical tables, and

$$\left.\begin{aligned} F_p &= \text{the } F\text{-ratio for preparations in the ANOV} = L_p^2/a_p s^2 \\ F_r &= \text{the } F\text{-ratio for regression in the ANOV} = L_r^2/a_r s^2 \end{aligned}\right\} \quad \text{(Eq. 21)}$$

An improved method can, however, be derived as follows. From Eq. 21,

$$\frac{a_p}{a_r} = \frac{r^2 F_r}{F_p}$$

and substituting this in Eq. 20 gives

$$\begin{aligned} r_L, r_H &= \frac{1}{(F_r - F_c)} \\ &\times \left[rF_r \mp \sqrt{\frac{r^2 F_r F_c}{F_p}(F_p + F_r - F_c)} \right] \\ &= \frac{rF_r}{F_r - F_c} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_p F_r}(F_p + F_r - F_c)} \right] \\ &= \frac{r}{\left(1 - \frac{F_c}{F_r}\right)} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_r} + \frac{F_c}{F_p}\left(1 - \frac{F_c}{F_r}\right)} \right] \end{aligned} \quad \text{(Eq. 22)}$$

Applied in the present case the lower and upper limits, M_L and M_H, with M as defined in Eq. 19 are then, from Eq. 22,

$$\begin{aligned} M_L, M_H &= \frac{M}{\left(1 - \frac{F_c}{F_r}\right)} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_r} + \frac{F_c}{F_p}\left(1 - \frac{F_c}{F_r}\right)} \right] \end{aligned} \quad \text{(Eq. 23)}$$

The final assay results may therefore be cited as:

$$R = \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M) \quad \text{(Eq. 24)}$$

where the 95% confidence interval is defined by

$$\begin{aligned} R_L &= \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M_L), \\ R_H &= \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M_H) \end{aligned} \quad \text{(Eq. 25)}$$

Numerical Example—To illustrate the above method, responses from an assay (6) are used, unequal dose ratios being obtained by assuming that the upper dose of the unknown preparation was 0.9 mg. instead of 1 mg. as used in the actual experiment. The totals, each of 5 individual responses, and the doses taken for present purposes are shown in Table II.

Here then,

$$D_S = 4,\ D_U = 3.6$$

so that,

$$d_S = \log 4 = 0.6021,\ d_U = 0.5563$$

giving,

$$d_S - d_U = 0.0458,\ d_S^2 + d_U^2 = 0.6720$$

The contracts L_p, L_r, and L_d are then calculated in accordance with the scheme in Table IA as,

$$L_p = -350.1 - 213.9 + 335.8 + 240.7 = 12.5$$

$$\begin{aligned} L_r &= -d_S(S_1 - S_2) - d_U(U_1 - U_2) \\ &= (-0.6021)(136.2) - (0.5563)(95.1) \\ &= -134.9102 \end{aligned}$$

$$\begin{aligned} L_d &= d_U(S_1 - S_2) - d_S(U_1 - U_2) \\ &= (0.5563)(136.2) - (0.6021)(95.1) = 18.5083 \end{aligned}$$

With $n = 5$ the corresponding ANOV entries are then,

$$L_p^2/20 = 7.8125$$

$$L_r^2/10(d_S^2 + d_U^2) = (134.9102)^2/6.72 = 2708.4467$$

$$L_d^2/6.72 = (18.5083)^2/6.72 = 50.9758$$

TABLE II—DOSES AND RESPONSE TOTALS

	Std. Prepn., mcg./LH		Unknown Prepn., mg. Tissue	
Dose	0.6	2.4	0.25	0.9
Response total	350.1	213.9	335.8	240.7

From the original ANOV, the total corrected sum of squares was 3692.1775 so that for the present case the ANOV (in which the ratios F_p and F_r are also indicated) is as shown in Table III.

TABLE III—ANOV

Variation Source	d.f.	s.s.	m.s.	
Prepn.	1	7.8125		$F_p = 0.1351$
Regression	1	2708.4467		$F_r = 46.8517$
Divergence	1	50.9758		
Residual	16	924.9425	57.8089	
Total	19	3692.1775		

Since the mean square for divergence is less than the residual mean square relevance may be considered satisfactory. Proceeding to the estimation of relative potency, from Eq. 19,

$$\begin{aligned} M &= (d_S^2 + d_U^2)L_p/L_r \\ &= (0.6720)(12.5)/-134.9102 \\ &= -0.0623 \end{aligned}$$

so that, from Eq. 24,

$$R = \frac{0.6}{0.25} \text{ antilog } \frac{1}{2}(0.0458 - 0.0623)$$

$$= 2.36 \text{ mcg./mg.}$$

For the interval estimate F_c, from statistical tables, is $F_c = F(1, 16, 0.05) = 4.49$ or, if desired, F_c can be obtained from the t-table for 16 degrees of freedom as

$$F_c = t^2(16, 0.05) = 2.12^2 = 4.49$$

Then, with F_p and F_r from the ANOV,

$$\frac{F_c}{F_p} = \frac{4.49}{0.1351} = 33.2346$$

$$\frac{F_c}{F_r} = \frac{4.49}{46.8517} = 0.0958$$

so that, from Eq. 23,

$$\begin{aligned} M_L, M_H &= \frac{-0.0623}{0.9042} \\ &\quad \times [1 \pm \sqrt{0.0958 + (33.2346)(0.9042)}] \\ &= -0.4472, 0.3094 \end{aligned}$$

and, finally, from Eq. 25,

$$R_L, R_H = 2.4 \text{ antilog } \frac{1}{2}(0.0458 - 0.4472),$$

$$2.4 \text{ antilog } \frac{1}{2}(0.0458 + 0.3094)$$

$$= 1.51, 3.61 \text{ mcg./mg.}$$

SIX-POINT LOG-DOSE RESPONSE ASSAY

Suppose that a six-point assay is carried out using a completely randomized design and that n responses are observed at each of doses z_{S1}, $D_S z_{S1}$, $D_S^2 z_{S1}$ for the standard preparation and at doses z_{U1}, $D_U z_{U1}$, $D_U^2 z_{U1}$ for the unknown preparation. Let the respective response totals be denoted in the usual way as S_1, S_2, S_3, and U_1, U_2, U_3. Five orthogonal contrasts together with their ANOV entries can then be defined as shown in Table IV, where $d_S = \log D_S$ and $d_U = \log D_U$.

The first three contrasts are analogous to the three defined for the four-point assay while the contrasts Q_S and Q_U are sensitive to quadratic curvature in the log-dose response lines for the standard and the unknown preparations, respectively. An alternative formulation for the latter two contrasts would be to replace them by contrasts for combined and opposed curvatures Q_1 and Q_2 say, where,

$$Q_1 = Q_S + Q_U = S_1 - 2S_2 + S_3 + U_1 - 2U_2 + U_3$$

$$Q_2 = Q_S - Q_U = S_1 - 2S_2 + S_3 - U_1 + 2U_2 - U_3$$

for which the ANOV entries would be $Q_1^2/12n$ and $Q_2^2/12n$, respectively. The formulation in Table IV, however, is slightly easier to compute and provides for the relevance tests required because appreciable curvature in either response line will render the present analysis inapplicable. The ANOV is then as shown in Table V.

On the assumption that the relevance tests for divergence, curvature(S) and curvature(U) are satisfactory, the relative potency estimate is now obtained by finding appropriate quantities to substitute into Eq. 12. For the 6-point assay the transformations in Eq. 2 lead to x-values of 0, 1, and 2 for both preparations, so that $\bar{x}_S = \bar{x}_U = 1$ and

$$d_S\bar{x}_S - d_U\bar{x}_U = d_S - d_U$$

Next

$$\bar{y}_S - \bar{y}_U = \frac{1}{3n}(S_1 + S_2 + S_3 - U_1 - U_2 - U_3)$$

$$= -L_p/3n \quad \text{(Eq. 26)}$$

with L_p as defined in Table IV.

For the regression coefficient the quantities required are

$$\Sigma_S' xy = -S_1 + 0S_2 + S_3 = -S_1 + S_3$$

$$\Sigma_S' xx = 2n$$

and similarly for the unknown preparation. Hence, from Eq. 11

$$b = \frac{d_S(-S_1 + S_3) + d_U(-U_1 + U_3)}{2n(d_S^2 + d_U^2)}$$

$$= L_r/2n(d_S^2 + d_U^2) \quad \text{(Eq. 27)}$$

From Eqs. 26, 27, and 12 the relative potency estimate is then

$$R = \frac{z_{S1}}{z_{U1}} \text{ antilog } \left[d_S - d_U + \frac{2}{3}(d_S^2 + d_U^2)\frac{L_p}{L_r}\right]$$

$$= \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M] \quad \text{(Eq. 28)}$$

where

$$M = \frac{2}{3}(d_S^2 + d_U^2)\frac{L_p}{L_r} \quad \text{(Eq. 29)}$$

For the interval estimate M_L and M_H are calculated, using the now appropriate values, from Eq. 23 so that, for the relative potency itself the interval estimate is defined by

TABLE IV—ORTHOGONAL CONTRASTS FOR AN UNEQUAL DOSE RATIO SIX-POINT ASSAY
(*n* RESPONSES PER DOSE)

Contrast		Response Total						ANOV Sum of Sq.
		S_1	S_2	S_3	U_1	U_2	U_3	
Prepn.:	L_p	-1	-1	-1	1	1	1	$L_p^2/6n$
Regression:	L_r	$-d_S$	0	d_S	$-d_U$	0	d_U	$L_r^2/2n(d_S^2 + d_U^2)$
Divergence:	L_d	d_U	0	$-d_U$	$-d_S$	0	d_S	$L_d^2/2n(d_S^2 + d_U^2)$
Curvature (S):	Q_S	1	-2	1	0	0	0	$Q_S^2/6n$
Curvature (U):	Q_U	0	0	0	1	-2	1	$Q_U^2/6n$

$$R_L, R_H = \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M_L], \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M_H] \quad \text{(Eq. 30)}$$

CONCLUSION

For a given assay response it has been established in bioassay literature that two highly important requirements for the precision of relative potency estimations are: (*a*) the range, from the lowest to the highest dose investigated, should be as large as possible subject to linearity of the relationship between the response and the log-dose and to the requirement that responses should be normally distributed with constant variance and (*b*) the difference between the mean response of the standard and unknown preparations should be as small as possible.

TABLE V—ANOV

Variation Source	d.f.	s.s.	m.s.
Prepn.	1	$L_p^2/6n$	
Regression	1	$L_r^2/2n(d_S^2 + d_U^2)$	
Divergence	1	$L_d^2/2n(d_S^2 + d_U^2)$	
Curvature (S)	1	$Q_S^2/6n$	
Curvature (U)	1	$Q_U^2/6n$	
Residual	$6n - 6$	By subtraction	s.s./$6n-6$
Total	$6n - 1$		

In planning an assay, therefore, a basic problem is just that of using what is known about the unknown preparation to best meet these requirements. And in early stages, following perhaps some pilot investigations of log-dose response relationships, it may not be desirable to use equal spacing of log-doses for either the standard or the unknown preparations. The analyses of such general assays can be carried through quite straightforwardly using the actual log-doses instead of being based on log-dose transformations giving integral values for at least one of the two sets of log-doses. The advantages of such transformations are primarily computational so that such transformations are commonly used in relatively well-established assays. In this context the present proposals may be regarded as providing a convenient procedure for intermediate cases. It gives simple computations and allows greater flexibility in the choice of doses than is permitted by the completely canonical form which requires the same dose ratio to be used for both preparations.

REFERENCES

(1) Bliss, C. I., "The Statistics of Bioassay," Academic Press Inc., New York, N. Y., 1952.
(2) Folley, S. J., personal communication, 1956.
(3) Fieller, E. C., *Quart. J. Pharm. Pharmacol.*, **17**, 117 (1944).
(4) Fieller, E. C., *J. R. Statist. Soc.*, **B16**, 175(1954).
(5) Cox, C. P., and Ruhl, D. J., *J. Pharm. Sci.*, **55**, 368 (1966).
(6) Schultz, J., personal communication, 1965.

Comparative Study in Man and Dog of the Absorption and Excretion of Dextroamphetamine-^{14} C Sulfate in Sustained-Release and Nonsustained-Release Dosage Forms

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The absorption and excretion of dextroamphetamine-^{14}C sulfate in both sustained-release and nonsustained-release dosage forms were evaluated in identical studies in man and dog. A direct comparison of the results of these studies showed that the various dosage forms performed in a similar way in both man and dog. These results provided information on the design and evaluation of *in vivo* studies intended to indicate which preliminary sustained-release formulations merit objective human testing.

IN THE ABSENCE of an accurately measurable clinical effect, an objective way to evaluate the performance of sustained-release dosage forms in humans is to compare how a drug is absorbed and excreted from this dosage form and from the regimen it is intended to replace. There are many problems involved in conducting such studies: the need for a substantial number of humans; the need for specialized analytical techniques to measure the drug levels in body tissues; the need for knowledge not only of the statistical considerations in experimental design, but also of individual drug kinetics; and the need for professional surveillance to insure faithful adherence to dosage regimens, food and liquid intake, and a set schedule of sample collections.

Because these problems exist, only the most promising experimental formulations merit such objective human testing. So there is a need for *in vivo* animal studies that will provide data comparable to those obtained in human studies and thus indicate which preliminary formulations merit such testing.

The authors recently conducted an absorption and excretion study in humans comparing a dextroamphetamine-^{14} C sulfate pelleted sustained-release product with similarly labeled nonsustained-release regimens (1). It was necessary to tag the drug with the radioactive carbon atom because ordinary biochemical methods are not adequate to measure the low concentration of dextroamphetamine sulfate that appears in blood.

It was decided to carry out an identical study in dogs to obtain directly comparable data, reasoning that a comparison of the data would serve as a useful reference for the design and evaluation of future formulation screening studies in the dog.

This study is a report of the data on the absorption and excretion of various dosage forms of dextroamphetamine-^{14} C sulfate in both man and dog, as well as an analysis of how well these data compare with each other.

EXPERIMENTAL

Preparation of Dosage Forms—Based on human safety considerations, the highest dose of dextroamphetamine sulfate to be administered (15 mg.) was tagged with a maximum single radiation dose of 45 μc. To obviate the problems involved in uniformly mixing labeled with unlabeled drug, we chose to synthesize the labeled drug with a specific activity of approximately 3 μc./mg. The method of preparation described by Blackburn and Burghard was used (2).

The pelleted sustained-release dosage form of the labeled drug was prepared by a method similar to that reported previously by Rosen and Swintosky (3). One kilogram of medicated nonsustained-release pellets was prepared in a 12-in. coating pan located in a disposable glove-box inside a walk-in fume hood fitted with special filters. A portion (135 Gm.) of these nonsustained-release pellets was set aside and the remaining pellets were coated with a wax-fat coating. Six separate groups with approximately 7, 9, 11, 13, 15, and 17% wax-fat coating were prepared. This was done to insure that there would be sufficient spread in patterns to facilitate blending for the *in vitro* pattern of the commercial product.

The *in vitro* release patterns of the six experimental groups are shown in Table I; they were determined by the method of Souder and Ellenbogen (4).

As reported (4), the average *in vitro* pattern of 15 consecutive commercial lots of the specific 15 mg. dextroamphetamine sulfate sustained-release product we chose to simulate was 39, 62, 80, and 90% at the 0.5, 2, 4.5, and 7 hr. time intervals,

Received October 3, 1966, from the Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101, and the † Section of Clinical Pharmacology, Research Institute and Department of Medicine, Presbyterian-University of Pennsylvania Medical Center, Philadelphia, PA 19104

Accepted for publication December 2, 1966.

The authors are grateful to Mr. Louis Gutzait for his technical assistance.

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respectively. In the six experimental groups it was found that the blend consisting of 25% noncoated pellets, 55% W-F3, and 20% W-F2 pellets gave a calculated pattern of 37, 57, 81, and 97% at the same time intervals.

The *in vitro* patterns of this blend before and after encapsulation are shown in Table II.

These data indicated that the *in vitro* release pattern of the experimental ^{14}C-labeled capsule closely approximated that of the commercial product.

The 5-mg. and 15-mg. nonsustained-release capsules which were used in the *in vivo* studies were prepared by filling capsules with nonsustained-release pellets of dextroamphetamine-^{14}C sulfate.

In Vivo Protocols—The following four dosage regimens of labeled drug in man and dog were compared: *A*, 15-mg. sustained-release dosage form given at 0 hr.; *B*, 15-mg. nonsustained-release dosage form given at 0 hr.; *C*, 5-mg. nonsustained-release dosage form given at 0 hr.; *D*, 5-mg. nonsustained-release dosage form given t.i.d., at 0, 4, and 8 hr.

These particular regimens were chosen because the authors wanted to determine five performance criteria: whether the sustained-release formulation provided a prompt initial dose; whether it was similar to t.i.d. administration; whether it was dissimilar to an equivalent nonsustained-release dose; whether equal doses in different dosage forms were equally effective in making the drug available for absorption; and whether there was any significant variability among subjects receiving the various regimens.

The earlier study in humans utilized 16 subjects in a crossover sequence; the study plan is shown in Table III.

Radiation safety considerations precluded the giving of each subject all four dosage regimens. Instead, each subject received two regimens, one week apart. Because four subjects were studied at one time, all four regimens were given on each day of the study. All possible combinations and permutations were covered in the first three groups, so the authors utilized the last group to repeat the most significant direct comparisons—that of *A* with *D*, and *B* with *C*.

The plan for the dog study is shown in Table IV. Eight female purebred beagle dogs ranging in weight from 9 to 11 Kg. were used in this study. They were given no food after 4:30 p.m. the day prior to dosing and the prescribed dosage regimen was administered, with no fluid, starting at 10 a.m. the next morning. The animals were then fed and watered 3.5 hr. after receiving the drug. Each animal received all four dosage regimens.

Blood Sampling—Human blood specimens were collected at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 32, and 48 hr. To accomplish this with a minimum of discomfort to the subject, the first seven specimens were collected through an indwelling anticoagulated venous needle system, as illustrated in Fig. 1. Before each collection, 5 ml. of blood was aspirated into a syringe; this flushed the system with fresh venous blood. Then the syringe was isolated from the system and the required 10-ml. specimen was collected through a heparin vacuum tube inserted in the stopcock. When the specimen had been withdrawn, the system was again flushed with the anticoagulant-blood mixture by returning 5 ml. to the subject. The plasma was removed from each blood sample and frozen until assayed.

TABLE I—*In Vitro* RELEASE PATTERNS OF DEXTROAMPHETAMINE-^{14}C SULFATE WAX-FAT-COATED GROUPS

Group	% Wax-Fat	% *In Vitro* Release at Time Interval 0.5 hr.	2 hr.	4.5 hr.	7 hr.
WF-1	7	46	100	100	100
WF-2	9	34	69	100	100
WF-3	11	20	50	84	100
WF-4	13	4	18	51	87
WF-5	15	2	3	21	70
WF-6	17	2	3	7	33

TABLE II—BLEND AND SUSTAINED-RELEASE CAPSULE *In Vitro* RELEASE PATTERNS (DEXTROAMPHETAMINE-^{14}C SULFATE)

	% *In Vitro* Release at Time Interval 0.5 hr.	2 hr.	4.5 hr.	7 hr.
Blend	34	56	79	92
15-mg. sustained-release capsule	33	57	80	91

TABLE III—HUMAN STUDY PLAN OF VARIOUS DOSAGE REGIMENS[a]

Subject	Initial	1 Wk. Later
1	*A*	*B*
2	*B*	*C*
3	*C*	*D*
4	*D*	*A*
5	*A*	*C*
6	*B*	*D*
7	*C*	*A*
8	*D*	*B*
9	*A*	*D*
10	*B*	*A*
11	*C*	*B*
12	*D*	*C*
13	*A*	*D*
14	*B*	*C*
15	*C*	*B*
16	*D*	*A*

[a] *A*, 15-mg. sustained-release dosage form given at 0 hr.; *B*, 15-mg. nonsustained-release dosage form given at 0 hr.; *C*, 5-mg. nonsustained-release dosage form given at 0 hr.; *D*, 5-mg. nonsustained-release dosage form given t.i.d., at 0, 4, and 8 hr.

TABLE IV—DOG STUDY PLAN OF VARIOUS DOSAGE REGIMENS[a]

Dog	Wk. of Study 1	2	3	4	5	6	7	8
101	*A*		*D*		*C*		*B*	
102	*B*		*C*		*A*		*D*	
103	*C*		*B*		*D*		*A*	
104	*D*		*A*		*B*		*C*	
201		*A*		*D*		*C*		*B*
202		*B*		*C*		*A*		*D*
203		*C*		*B*		*D*		*A*
204		*D*		*A*		*B*		*C*

[a] *A*, 15-mg. sustained-release dosage form given at 0 hr.; *B*, 15-mg. nonsustained-release dosage form given at 0 hr.; *C*, 5-mg. nonsustained-release dosage form given at 0 hr.; *D*, 5-mg. nonsustained-release dosage form given t.i.d., at 0, 4, and 8 hr.

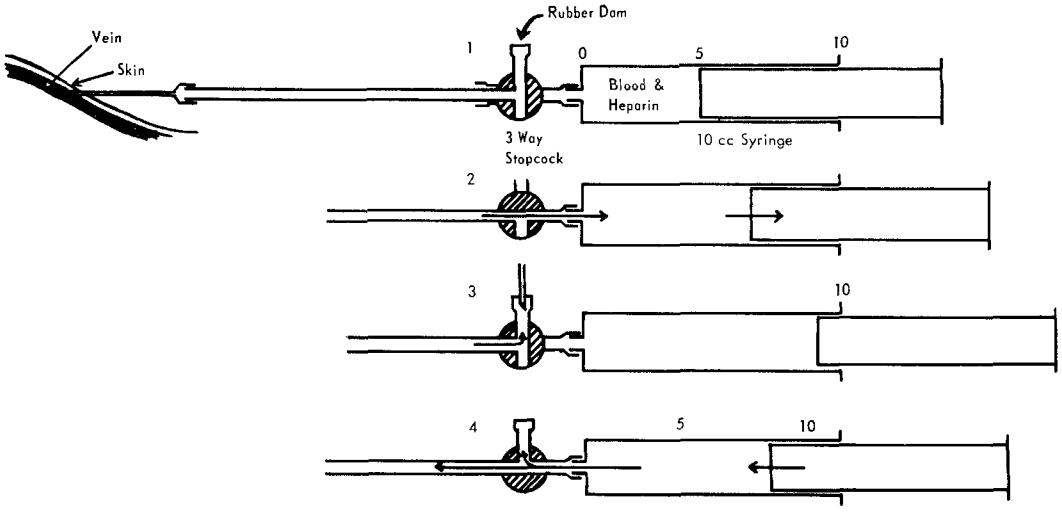


Fig. 1—Technique of blood collection. Key: 1, *needle is inserted in vein;* 2, *specimen collection is begun by aspirating 5 ml. of blood to flush system with fresh blood;* 3, *specimen is collected* via *needle puncture of rubber dam after isolation from syringe;* 4, *following collection, system is flushed with heparin solution to prevent clotting. Stopcock is then closed to await next collection.*

Blood samples from the dog were taken using a 5-ml. heparinized vacuum tube, and the time periods were the same as those in the human study. These plasmas were also separated and frozen until assayed.

Urine Sampling—Urine was collected from all humans for 48 hr. following ingestion of the drug. The collection intervals were 0–3, 3–6, 6–9, 9–12, 12–24, 24–32, and 32–48 hr. Aliquots of these samples were frozen until assayed.

Urine was collected from the dogs by catheterization at the same time intervals as those in the human study, and these samples were also stored frozen until assayed.

Analysis of the Specimens—Samples of plasma and urine ranging from 0.2 to 0.5 ml. were placed in a liquid scintillation counting solution with the following formula: dimethyl POPOP [2,2-*p*-phenylenebis(5-phenyloxazole)], 50 mg.; PPO (2,5-diphenyloxazole), 7-Gm.; naphthalene, 80 Gm.; toluene, 400 ml.; *p*-dioxane, 400 ml.; diluted to a total of 1000 ml. with absolute alcohol.

The samples were counted using a Tri-Carb[1] liquid scintillation spectrometer, and all measurements were corrected for background, quench, and dilution. The efficiency of the counter was approximately 50%.

RESULTS AND DISCUSSION

The human data have been reported previously (1), but because they are an integral part of this comparative study, they are repeated here. In this regard, note that two minor errors were found after reporting these data and, although they in no way alter the original interpretation, they have been corrected in Fig. 2 (ordinate scale) and Table V (*0–48 hr. data column*).

Plasma Data—The radioactivity appearing in the plasma of the humans following the administration of the four dosage regimens is shown in Fig. 2.

[1] Model 314-DC, Packard Instrument Co., Inc., La-Grange, Ill.

Because of the low order of radioactivity, the average plasma data are reported as counts per minute per milliliter. Of course, the authors were measuring the levels of radioactivity not only from the parent compound but also from its metabolites.

The 15-mg. sustained-release dosage form and the 5-mg. capsule given at 0, 4, and 8 hr. produced roughly similar curves, but the sustained-release preparation showed a higher level during most of the first 8 to 10 hr. after administration, with none of the "staircase" effect or 12-hr. peaking seen with the t.i.d. regimen. The 15-mg. nonsustained-release dosage form gave different results. It showed a high peak at 2 hr. that was maintained for at least 8 hr.; this plot paralleled the one for the 5-mg. capsule given once, but at a proportionately higher level.

The radioactivity appearing in the plasma of dogs following the administration of the four regimens is shown in Fig. 3.

Although the plots for the 15-mg. sustained-release dosage form were very similar in man and dog, the plots for the 5-mg. capsule given t.i.d. differed. In the dogs, this plot showed a rapid rise after the first two doses but, unexpectedly, there was no rise after the third dose. And there was no peak at the 12th hr., causing a plateau from the 6th to the 12th hr.

The 15-mg. nonsustained-release dose showed a peak at 2 hr. that was maintained until the 4th hr.; then the level started to drop. As in the human study, this plot paralleled that of the 5-mg. capsule given once, but at a proportionately higher level.

Urine Data—The urinary excretion plots for the humans are shown in Fig. 4; those for the dogs are shown in Fig. 5. Although the excretion values are expressed as milligrams of drug to which the ^{14}C is equivalent, as mentioned before, the radioactivity reflects not only the parent compound but also its metabolites.

The urinary findings in man and dog were consistent with the plasma findings. The 15-mg.

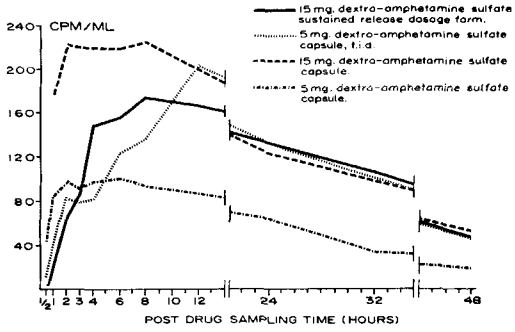


Fig. 2—Adjusted average human plasma levels. Each line represents eight subjects per regimen in a balanced incomplete block crossover design.

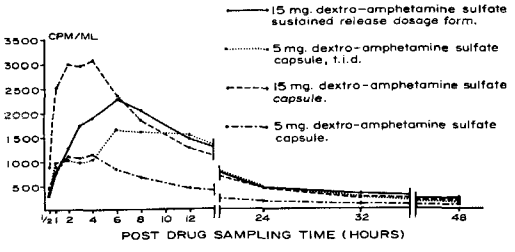


Fig. 3—Adjusted average dog plasma levels. Each line represents eight dogs per regimen.

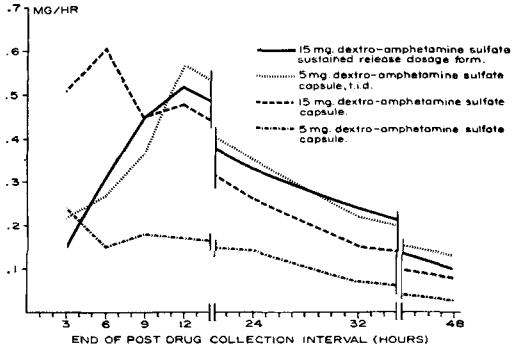


Fig. 4—Adjusted average human urinary excretion rates. Radioactive counts are expressed as average milligrams of dextroamphetamine sulfate per collection interval divided by the number of hours in each interval

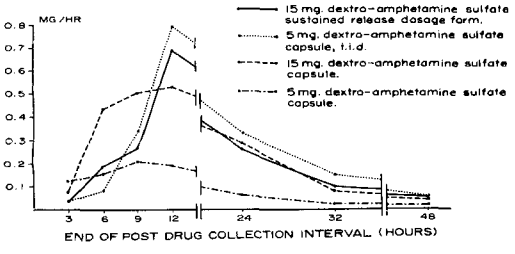


Fig. 5—Adjusted average dog urinary excretion rates. Radioactive counts are expressed as average milligrams of dextroamphetamine sulfate per collection interval divided by the number of hours in each interval.

sustained-release dosage form gave a curve similar to that of the 5-mg. capsule given three times a day. And, just as the plasma data for the 3–10-hr. period showed more drug being absorbed from the sustained-release dosage form, so the urinary data showed that during this same period the excretion was greater from the sustained-release form than from the t.i.d. regimen. The plots of the 15 and 5-mg. plain capsules again paralleled each other at proportionate levels.

The cumulative urinary excretion data for the humans are shown in Table V. These data indicated that in the first 12 hr. following administration of the drug, the sustained-release and t.i.d. regimens made approximately the same amount of drug available for absorption. On the other hand, the 5-mg. capsule given once made significantly less of the drug available and the 15-mg. capsule given once made significantly more of the drug available. Cumulative urinary excretion data for the 24th hr. through to the 48th hr. showed that the three 15-mg. regimens made the same amount of drug available for absorption. Almost proportionately equivalent amounts were made available from the 5-mg. single dose.

TABLE V—ADJUSTED AVERAGE[a] URINE RECOVERIES IN HUMANS, mg. OF DEXTROAMPHETAMINE SULFATE EQUIVALENT

Dosage Form	Collection Interval, hr. 0–12	0–24	0–48
5-mg. capsule	2.18	3.90	4.89
15-mg. sustained-release capsule	4.30	8.24	11.78
5-mg. capsule, three times each day	4.32	8.48	12.30
15-mg. capsule	6.14	9.31	11.86

[a] Each figure is the average for eight humans. [b] No figure included in a brace is significantly different from any other figure included in that brace ($P < 0.05$).

The cumulative urinary excretion data for the dogs are shown in Table VI. In the 0–12 hr. interval, the three highest and three lowest values were not significantly different from each other; only the extreme values showed a significant difference. And, as in the human study, the data in this interval for the sustained-release and t.i.d. regimens showed excellent agreement. It should be noted that although both humans and dogs excreted similar amounts after the 5-mg. acute dose, the dogs excreted less than the humans after the three 15-mg. regimens. It is assumed that this finding is related to the lower total absorption seen in the dogs. All three 15-mg. regimens again made the same amount of drug available by the 24th hr., and through to the 48th hr., with significantly less drug excreted from the 5-mg. single dose.

Although the most valid way to compare drug availability from various dosage forms is to compare cumulative urinary recovery data, plotting plasma data is also informative. When the authors plotted plasma level *versus* time, the area under the 15-mg. acute dose plot was significantly greater than the area under the 15-mg. sustained-release plot or the 5-mg. t.i.d. plot, but the urinary recoveries for all three regimens were similar. And this was true for both man and dog. To explain this apparent paradox,

we assume that the 15-mg. acute dose did not achieve distribution equilibrium during the early time periods.

TABLE VI—ADJUSTED AVERAGE[a] URINE RECOVERIES IN DOGS, mg. OF DEXTROAMPHETAMINE SULFATE EQUIVALENT

Dosage Form	Collection Interval, hr. 0–12	0–24	0–48
5-mg. capsule	2.02[b]	2.74	3.23
15-mg. sustained-release capsule	3.50[b]	6.62[b]	8.28[b]
5-mg. capsule, three times each day	3.77[b]	7.71[b]	9.76[b]
15-mg. capsule	4.62	9.76	9.33

[a] Each figure is the average for eight dogs. [b] No figure included in a brace is significantly different from any other figure in that brace ($P < 0.05$).

Coefficient of Variation Data—Table VII shows the coefficient of variation for the first 8 blood specimens and for the cumulative urine collections in the human study; Table VIII shows these data for the animal study.

In both humans and animals, these data indicated that there was a relationship between the variation at any particular time and the average recovery result at that time, with low intrinsic values showing the highest degree of variation. At most data points, the three 15-mg. regimens showed equivalent variability; in other words, the variability was related to the average ^{14}C recovered at each sampling time or collection interval, and not to the dosage forms studied.

The design of the experiments provided internal checks of its validity by including dose response relationships. In man and dog, the 15 and 5-mg. plain capsules gave plasma and urine plots that paralleled each other at proportionate levels; this fact validated our methodology.

SUMMARY

The study in man and dog was undertaken to determine certain performance criteria for four dosage forms of dextroamphetamine-^{14}C sulfate. The experimental data indicated the following conclusions.

1. In man and dog, the initial 3-hr. portions of the plasma plots for the sustained-release dosage form, the t.i.d. regimen, and the 5-mg. single dose were similar. Therefore, the sustained-release formulation did provide a prompt initial dose.

2. In man and dog, the plasma and urine plots for the sustained-release dosage form and the t.i.d. regimen were similar.

3. In man and dog, the plasma and urine plots for the sustained-release dosage form and the 15-mg. plain capsule were dissimilar.

4. In man and dog, the sustained-release dosage form made as much drug available for absorption as the other two 15-mg. regimens.

5. In man and dog, the variation in plasma and urine data is not greater with the sustained-release dosage form than with the t.i.d. regimen.

Because the preceding five statements can be applied to both man and dog, this example study serves to promote confidence in the selection of sustained-release formulations worthy of objective human testing based on comparative dosage form performance criteria obtained in the dog.

TABLE VII—COEFFICIENTS OF VARIATION[a] FOR HUMAN PLASMA AND URINE DATA

Study Time, hr.	15-mg. Sustained-Release Capsule	15-mg. Capsule	5-mg. Capsule	5-mg. Capsule Given Three Times a Day
	Plasma Results			
0.5	48	46	82	88
1	53	39	58	60
2	44	35	38	20
3	34	30	33	22
4	34	20	33	17
6	22	25	22	22
8	11	23	26	19
12	18	22	23	15
Collection Interval, hr.	Urine Results			
0–12	25	20	20	34
0–24	17	18	12	18
0–48	9	16	9	9

[a] The coefficient of variation is the standard deviation of a set of data divided by the average of the same set, expressed as a percentage.

TABLE VIII—COEFFICIENTS OF VARIATION[a] FOR DOG PLASMA AND URINE DATA

Study Time, hr.	15-mg. Sustained-Release Capsule	15-mg. Capsule	5-mg. Capsule	5-mg. Capsule Given Three Times a Day
	Plasma Results			
0.5	72	108	80	91
1	31	52	24	30
2	27	33	28	16
3	23	34	26	17
4	25	15	58	51
6	28	19	39	26
8	19	24	43	34
12	25	35	51	39
Collection Interval, hr.	Urine Results			
0–12	50	63	48	65
12–24	30	20	35	18
24–48	19	14	27	15

[a] The coefficient of variation is the standard deviation of a set of data divided by the average of the same set, expressed as a percentage.

REFERENCES

(1) Rosen, E., Tannenbaum, P., Ellison, T., Free, S. M., and Crosley, A., *J. Am. Med. Assoc.*, **194**, 1203(1965).

(2) Blackburn, D., and Burghard, G., *J. Pharm. Sci.*, **54**, 1586(1965).

(3) Rosen, E., and Swintosky, J. V., *J. Pharm. Pharmacol.*, **12**, 237(1960).

(4) Souder, J. C., and Ellenbogen, W. C., *Drug Std.*, **26**, 77(1958).

Effect of Urea on Solubility

Role of Water Structure

By STUART FELDMAN and MILO GIBALDI

The solubilities of benzoic and salicylic acid in aqueous urea, methylurea, and 1,3-dimethylurea solutions were studied. From a thermodynamic transfer model, the free energy, enthalpy, and entropy of solution were determined. By virtue of the fact that the solubilization process was nearly athermal and by the magnitude of the free energy and entropy values obtained, it was concluded that the enhanced solubilization of benzoic and salicylic acid in urea and alkylurea solutions did not involve complexation. The effect of urea on water structure and hydrophobic bonding was considered. By examination of the literature and analysis of the data obtained from this investigation it was proposed that urea, methylurea, and 1,3-dimethylurea solubilized benzoic and salicylic acids by "breaking up" water clusters surrounding the nonpolar molecule, increasing the entropy of the system, and producing a "driving" force for the solubilization. It was found that the alkyl substituted ureas, methyl- and 1,3-dimethylurea, produced a greater increase in solubility than urea, with 1,3-dimethylurea having the greater effect upon the solubility of the aromatic acids. It was noted that the urea and the alkylureas had the greatest solubilization effect on the more hydrophobic molecule, salicylic acid. A discussion of the solubilizing effect of urea on the aromatic acids is presented.

IT HAS BEEN reported that the presence of urea increases the water solubility of benzoic and the hydroxybenzoic acids (1, 2). Bolton (1) and Altwein *et al.* (2) studied the effect of urea on the solubilization of benzoic and salicylic acids. Altwein and co-workers (2) concluded that the interaction of urea with salicylic acid was a complex interaction but was definitely a "complexation" even though "urea possesses low complexing ability."

Bolton (1) termed the "interaction" of urea with salicylic and benzoic acids a complexation and calculated approximate stability constants (K_s), on the basis of a 1:1 interaction, of 0.2 for the urea–benzoic acid system and 0.3 for the urea–salicylic acid system.

These values are considerably lower than stability constant values of complexations which have been reported in the literature (3). Literature values have ranged from about $K_{1:1} = 2.0$ for salicylic acid and substituted amides to $K_{1:1} = 44$ for the complexation between salicylic acid and caffeine (3). Benzoic acid has yielded corresponding values for similar interactions (3).

The unusual thermodynamic data reported for urea–benzoic acid and urea–salicylic acid raises some doubt as to the mechanism of this solubilization. It was therefore of interest to consider the phenomenon in terms of the general properties of urea solutions. To appreciate fully the complex properties of aqueous solutions of urea, a discussion of water structure is in order.

Received July 13, 1966, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication December 5, 1966.

Based on a thesis submitted by Stuart Feldman to the College of Pharmaceutical Sciences, Columbia University, New York, N. Y., in partial fulfillment of Master of Science degree requirements.

THEORETICAL ASPECTS

Water Structure and the Effect of Urea—Water, as ice, exists in an open structure consisting of tetrahedral hydrogen bonds between water molecules in three dimensions (4). When ice melts there still remains a high degree of hydrogen bonding in the resulting liquid. The hydrogen bonded water molecules take the form of "clusters" which are nearly spherical in shape (4). Therefore, there exist in liquid water two types of water molecules: (*a*) the free or unbonded water molecule; and (*b*) the clusters of hydrogen bonded water.

When nonpolar solutes dissolve in water, they increase the ordering of water molecules around them (5). It was from this model that the concept of "icebergs" or water molecule clusters, forming about nonpolar solutes, was evolved. Essentially, the nonpolar solute becomes associated with the tetrahedral water molecules and becomes its fifth neighbor through van der Waals interactions. The water cluster forms a partial cage around the hydrocarbon. Since there is greater stability in this penta-coordinated state, more molecules will occupy lower energy levels, promoting more hydrogen bonding and an ordering effect on the solvent (4). This process is known as *hydrophobic hydration* and is accompanied by a small decrease in enthalpy, a large decrease in entropy, and a resulting large positive free energy of the system.

An opposite effect is achieved when nonpolar moieties or residues associate in the presence of water. This phenomenon is termed *hydrophobic bonding*. The formation of the hydrophobic bond will involve a decrease in the ordering of the system since the "ice-like" regions between the nonpolar residues become partially melted as the residues come together. Therefore, there will be an increase in the entropy of the system, a small positive enthalpy, and a free energy of from -0.2 to -1.5 Kcal./mole. Hydrophobic bonding, from a mechanistic point of view, actually places a limitation on the solubility of nonpolar compounds in water. The hydrophobic interaction, which is "driven" by entropic contributions, would tend to "pull"

hydrocarbons out of solution, and is responsible for the low water solubility manifested by nonpolar compounds.

It is widely held that urea has the ability to reduce hydrophobic bonding (5–7). This effect has been ascribed to the unusual capacity of urea for "breaking up" the icebergs in liquid water. Urea becomes associated with the structured water by hydrogen bonding and takes an active part in the formation of a more open "lattice" structure (8). The increase in entropy gained by "disruption" of water structure by urea makes the association of nonpolar molecules by hydrophobic bonding thermodynamically less favorable, and there is an increase in water solubility of the nonpolar compound.

Mukerjee and Ghosh (6) have shown that urea effectively reduces the degree of self-association of methylene blue in aqueous solution, thereby increasing its water solubility. Katayama *et al.* (9) showed the effect of urea on the aqueous solubility of a series of azo-dyes. They noted that urea had a more pronounced effect on the solubility of those compounds having the larger "hydrophobic surface."

Abu-Hamdiyyah (8) proposed that urea participated in the formation of "mixed" clusters in aqueous solution. The "mixed" clusters were found to be less thermally stable than pure water clusters, indicating a higher enthalpy in the aqueous urea solutions and an entropic driving force for the spontaneous transfer of nonpolar solutes from pure water to aqueous urea solutions (10). The increase in entropy was attributed to the "increase in the number of available cavities or interstices for the solute molecules in aqueous urea solutions."

Abu-Hamdiyyah (8) also predicted, on the basis of the above conclusions, the effect of alkyl-substituted ureas on the strength of the hydrophobic bond. He stated that (*a*) an alkylurea derivative would have a decreased ability to hydrogen bond with water compared to the ability of urea itself, thereby decreasing the ability of the urea derivative to participate in cluster formation, and (*b*) the alkyl groups themselves could only be accommodated in the cavities produced in the aqueous solution, resulting in a decrease in the number of cavities available for the nonpolar compound. He concluded that alkyl substituted ureas would have a minimal effect on hydrophobic bonding as compared to urea.

Thermodynamic Considerations—When considering the process where a compound is transferred from water to urea solution, the following equation may be written (5, 9):

compd. in water (mole fraction N_0) $\rightarrow$
compd. in urea solution (mole fraction N) (Eq. 1)

The ratio of the mole fractions N/N_0, may be considered as a partitioning ratio since it involves the distribution of the compound between two phases, the water phase and the urea solution phase.

In this case the free energy of partitioning (ΔF) may be determined from the following expression (9):

$$\Delta F = -2.3\,RT \log (N/N_0) \qquad \text{(Eq. 2)}$$

The enthalpy of partitioning (ΔH) can be determined from the following relationship, which represents a modification of the van't Hoff equation (9, 11):

$$\frac{d \ln (N/N_0)}{dT} = \frac{\Delta H}{RT^2} \qquad \text{(Eq. 3)}$$

Rearranging to solve for ΔH;

$$\Delta H = -2.3R\,[d \log (N/N_0)/d(1/T)] \qquad \text{(Eq. 4)}$$

From this equation it can be seen that a plot of log (N/N_0) *versus* $1/T$ for each urea concentration would result in a straight line with a slope equal to $-\Delta H/2.3R$, providing ΔH remains reasonably constant over the temperature range studied. The change in entropy (ΔS) associated with this process can be determined by use of the following equation (9):

$$\Delta S = \frac{\Delta H - \Delta F}{T} \qquad \text{(Eq. 5)}$$

EXPERIMENTAL

Materials—The benzoic acid (m.p. 122°), salicylic acid (m.p. 159°), and urea (m.p. 132°) used in this study were Fisher certified reagent grade. Methylurea (m.p. 101°) and 1,3-dimethylurea (m.p. 107°) were obtained from Eastman Kodak. The purity of each drug was determined by melting point analysis and determination of ultraviolet spectra.

Assay Procedures—Beer's law plots were obtained for benzoic and salicylic acid by diluting aliquot portions of standard solutions of each drug with 0.1 *N* HCl–anhydrous methanol (1:10) solution.

Absorbance was determined by means of a Beckman DB recording spectrophotometer at the 272 mμ peak for benzoic acid and the 304 mμ peak for salicylic acid. Absorbance *versus* concentration plots were then constructed, and the slope was determined by the method of least squares.

Possible interference with the assay procedure was determined by adding various quantities of 3 *M* urea or urea derivative to known concentrations of each drug. No shifts in absorbance maxima and no alterations in absorbance values were noted.

Solubility Determinations—Initial solubility studies were conducted according to the methods of Altwein *et al.* (2). After encountering significant experimental difficulties, which are discussed in a subsequent section of this report, the following methodology was adopted.

The quantity of urea, methylurea, or 1,3-dimethylurea needed to produce a given volume of 3 *M* solution was carefully weighed out on a Mettler balance (model H15). The urea was then dissolved in a minimum amount of water. The pH of the solution was adjusted to pH 1 with concentrated HCl to suppress ionization of the acid species. All pH measurements were recorded by means of a Beckman Zeromatic pH meter. The solution was then transferred to a suitable volumetric flask and brought to volume with 0.1 *N* HCl.

Solutions of urea or urea derivative ranging from 0 to 3 *M*, at half-molar intervals, were prepared in 25-mm. screw cap culture tubes, by adding an appropriate amount of 3 *M* urea solution and sufficient 0.1 *N* HCl to bring the total volume to 10 ml.

A sufficient amount of drug (benzoic acid, 0.5 Gm.; salicylic acid, 0.6 Gm.) was added to each tube to insure an excess at equilibrium. The

mouth of each tube was covered with aluminum foil and capped to prevent evaporation.

The samples were equilibrated in a Metabolyte water bath shaker[1] at temperatures of 30°, 33°, 40°, and 45° and in a Gyrotory incubator shaker model G-25[1] at 37° for periods not less than 12 or more than 18 hr. Equilibrium was determined by repetitive sampling.

Before the tubes were to be sampled, the shaker was turned off to allow the excess solid material to settle. The liquid was then filtered through a Millipore[2] filter (0.45 μ pore size) to insure that no solid particles were present in the sample. To eliminate any variation due to a temperature differential, the filtration and sampling equipment were maintained at the same temperature as that of the equilibrium study.

One milliliter of sample was withdrawn from the filtrate and was diluted to 10 ml. with acidified methanol (1 ml. 0.1 *N* HCl to 100 ml. anhydrous methanol). All subsequent dilutions were made with 0.1 *N* HCl–acidified methanol (1:10). The absorbance of each solution was determined and the drug concentration was calculated from the absorbance values by use of the Beer's law plots.

RESULTS AND DISCUSSION

The initial solubility studies, using the method of Altwein *et al.* (2), involved equilibrating the drug in stock solutions of urea prepared in 0.1 *N* HCl. However, this approach led to a number of unusual findings. The pH values of 1 to 5 *M* urea solutions prepared in this manner were then checked to determine whether a substantial difference in pH existed between samples. Values ranged from pH 1.45 in 1 *M* urea to pH 2.40 at a 5 *M* urea concentration. The increase in pH of 0.1 *N* HCl solution as a function of urea concentration is apparently due to the presence of impurities in the urea, since urea itself is too weak a base to produce these effects. Bull *et al.* (12) have reported that even recrystallized urea contains small amounts of ammonium cyanate present in sufficient quantity to elevate the pH of HCl solutions. The effect of increasing pH on the solubility of benzoic and salicylic acids is quite significant in view of the fact that the greater the degree of ionization, the greater the solubility.

The procedures described under *Experimental* were adopted to overcome these difficulties. At each urea concentration, sufficient HCl was added to insure that essentially all the benzoic or salicylic acid in solution existed in the undissociated form. Under these experimental conditions little degradation of urea will occur (13). In addition, the ionic strength of these solutions was sufficiently high so that the small ionic contamination in the urea sample should have little effect on the solubility data.

Each drug was equilibrated with urea and methylurea at 30°, 37°, and 45° and with 1,3-dimethylurea at 30°. The N/N_0 ratios were determined from the base solubility of each drug (N_0) and the corresponding solubility in urea (N) solution. The solubility curves in mg./ml. of drug *versus* molarity of urea for each drug and urea derivative at the temperatures studied are represented by Figs. 1–4. Solubility data are also tabulated in Tables I and II. It can be seen from the graphs that the solubility of benzoic and salicylic acid is a linear function of urea concentration. The plots of solubility of drug *versus* moles of urea derivative for methylurea show curvature. Similar plots were observed for 1,3-dimethylurea at 30°. In each case the solubility of both drugs is increased significantly

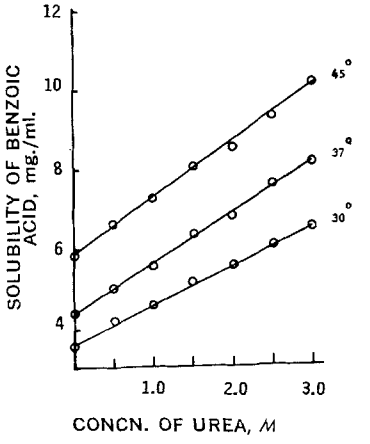


Fig. 1—Solubility of benzoic acid as a function of urea concentration.

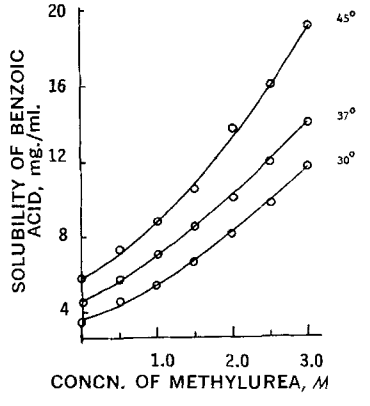


Fig 2—Solubility of benzoic acid as a function of methylurea concentration.

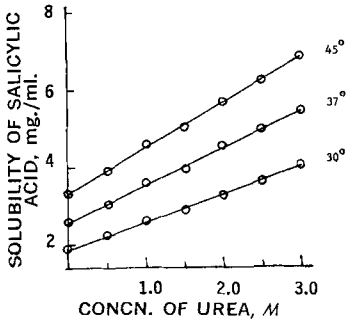


Fig. 3—Solubility of salicylic acid as a function of urea concentration.

[1] New Brunswick Scientific Corp., New Brunswick, N. J.
[2] Millipore Filter Corp., Bedford, Mass.

by the addition of urea or urea derivative to the aqueous solutions. For each drug the order of effectiveness of the urea derivatives as solubilizers decreased in the following manner: 1,3-dimethylurea > methylurea > urea. Comparative solubility plots of benzoic and salicylic acid at 30° as a function of urea derivative are presented in Figs. 5 and 6.

To determine the enthalpic contribution to the dissolution process, log N/N_0 values of salicylic and benzoic acid for each concentration of urea and methylurea between 0.5 and 3.0 M were plotted *versus* $1/T$. The slope of each plot was determined by the method of least squares and the ΔH calculated. The change in enthalpy was found to be exceedingly small, in the order of ±100 cal. The random nature of the results, however, prevented a definitive statement as to whether the process was endothermic, exothermic, or athermal. The variation in ΔH from concentration to concentration showed no trend and appeared to involve a random error.

In an attempt to refine the data, solubility determinations of each drug were carried out in 2 M solutions of urea and methylurea at 33° and 41°,

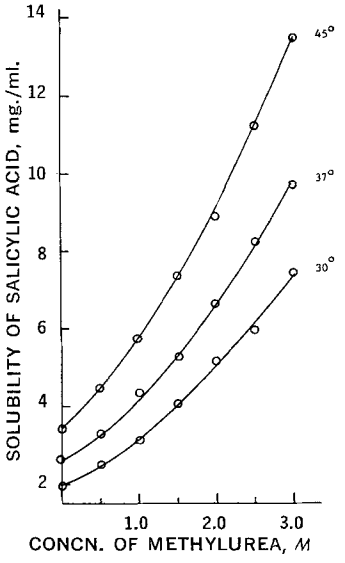


Fig. 4—Solubility of salicylic acid as a function of methylurea concentration.

TABLE I—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF UREA CONCENTRATION AND TEMPERATURE

	Solubility of Benzoic Acid, mg./ml.		
	30°	37°	45°
Urea, *M*			
0.0	3.56	4.41	5.83
0.5	4.23	5.10	6.66
1.0	4.61	5.63	7.31
1.5	5.25	6.45	8.16
2.0	5.67	6.93	8.63
2.5	6.22	7.75	9.46
3.0	6.60	8.35	10.35
Methylurea, *M*			
0.0	3.56	4.41	5.83
0.5	4.51	5.74	7.34
1.0	5.50	7.11	8.81
1.5	6.83	8.47	10.50
2.0	8.20	10.21	13.66
2.5	9.80	12.02	16.19
3.0	11.85	14.35	19.26
1,3-Dimethylurea, *M*			
0.0	3.56	...	...
0.5	4.95	...	...
1.0	7.37	...	...
1.5	9.72	...	...
2.0	12.32	...	...
2.5	15.56	...	...
3.0	19.01	...	...

TABLE II—SOLUBILITY OF SALICYLIC ACID AS A FUNCTION OF UREA CONCENTRATION AND TEMPERATURE

	Solubility of Salicylic Acid, mg./ml.		
	30°	37°	45°
Urea, *M*			
0.0	1.97	2.62	3.40
0.5	2.27	3.05	3.87
1.0	2.61	3.60	4.51
1.5	2.86	3.96	5.03
2.0	3.32	4.46	5.74
2.5	3.68	4.94	6.35
3.0	4.03	5.57	6.92
Methylurea, *M*			
0.0	1.97	2.62	3.40
0.5	2.52	3.35	4.46
1.0	3.14	4.39	5.75
1.5	4.03	5.25	7.39
2.0	5.17	6.64	8.92
2.5	5.96	8.24	11.21
3.0	7.42	9.74	13.46
1,3-Dimethylurea, *M*			
0.0	1.97	...	...
0.5	2.97	...	...
1.0	4.04	...	...
1.5	5.61	...	...
2.0	7.33	...	...
2.5	9.71	...	...
3.0	12.53	...	..

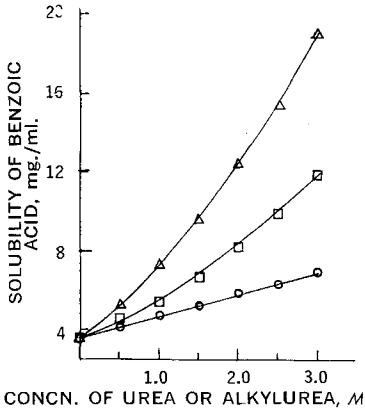


Fig. 5—Effect of urea, methylurea, and 1,3-dimethylurea on solubility of benzoic acid at 30°. Key: Δ,1,3-dimethylurea; □, methylurea; ○, urea.

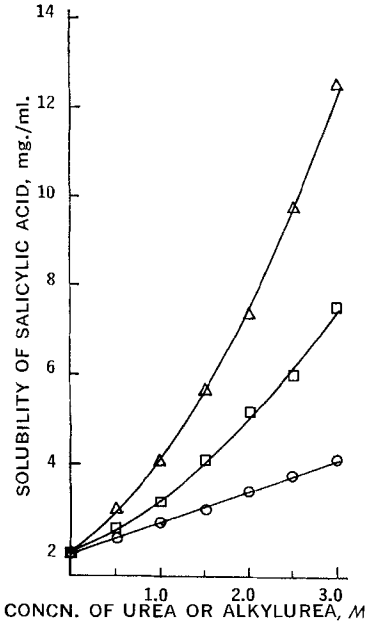


Fig. 6—Effect of urea, methylurea, and 1,3-dimethylurea on solubility of salicylic acid at 30°. Key: Δ, 1,3-dimethylurea; □, methylurea; O, urea.

and in 2 *M* 1,3-dimethylurea at 33°, 37°, 41°, and 45°. It was anticipated that a total of five temperature data points for each drug in each urea solution would clarify the extent of enthalpic contribution. Unfortunately, this was not the case. The ΔH values closely approximated zero, but the degree of inherent error prevented assignment of a positive or negative sign to the enthalpic component.

Further analysis of the data indicated that the method used for determining ΔH values was considerably more sensitive than the experimental procedures. The following illustration may serve to clarify the problem. Assume that the N/N_0 ratios for a particular experimental condition at 30° and 45° were 1.50 and 1.53, respectively. Further assuming that the N/N_0 ratio is really constant, these data would be in error to the extent of 1.5%. However, when the N/N_0 ratios are converted to logarithms the error is increased to approximately 4.5%. The 1.5% deviation (which is a reasonable approximation of experimental error) and the corresponding 4.5% deviation in the log of N/N_0 yields a ΔH of approximately −255 cal./mole, rather than zero.

The failure to refine the data by no means negates the value of determining the thermodynamic parameter, albeit an approximation. The methods employed were sufficiently sensitive to permit the conclusion that the enthalpic component was indeed small, certainly no greater than ±100 cal., and to a first approximation considered as zero.

The type of complexation most frequently described in the pharmaceutical literature involves the formation of a hydrogen bond (11). The enthalpy for the formation of a hydrogen bond is between 3 and 5 Kcal./mole (14), well above the small ΔH's noted in this investigation. Furthermore, it has been reported (15) that the strength of the hydrogen bond formed by urea molecules is of the same order of magnitude as those bonds formed by water. Therefore, even if urea formed hydrogen bonds with the benzoic or salicylic acid molecule, the strength of the bond would be about the same as the hydrogen bond formed between water and the drug molecule, and there should be no significant increase in solubility of the drug in aqueous urea solutions.

The free energies (ΔF) for the solubilization process have also been computed and are reported in Tables III and IV. They are small negative values similar to values reported by Katayama (9) and others (10, 15), who have solubilized nonpolar solutes in urea solutions. The negative sign is indicative of the spontaneity of the process.

Bolton (1), relying on complexation theory to explain the interaction of urea and salicylic acid, calculated apparent stability constants for the process. The magnitude of these constants, however, would yield a positive ΔF indicating a nonspontaneous process, which is clearly not the case. Moreover, the free energy values obtained in this investigation also tend to rule out a complexation mechanism in that the ΔF values of complexation are usually in the 2–5 Kcal./mole range (3, 16).

Since salicylic acid and benzoic acid do not appear to be solubilized by urea *via* a complexation mechanism, the possibility remains that urea is in some way "disrupting" water structure and thereby bringing about an increase in the solubility of the aromatic acids.

The solubility of benzoic and salicylic acid in water is due largely to the presence of polar groups on the aromatic ring. Water can solvate the benzoic or salicylic acid molecule through dipole interaction forces, particularly hydrogen bond formation. Thus, benzoic acid has a higher water solubility than salicylic acid. The latter, by forming intramolecular hydrogen bonds, presents a more "hydrophobic surface" to the solvent. When benzoic or salicylic acid is placed in water two interactions are possible.

The first possibility is that there is an interaction between the polar group on the aromatic nucleus and the water clusters. This hydrogen bonding between the polar group on the acid molecule and the water clusters would tend to break the clusters of hydrogen bonded water molecules and replace them partially with hydrogen bonds between the polar portion of the aromatic acid and the free water molecules. This interaction would tend to increase the solubility of the drug molecule through the negative ΔH values produced by hydrogen bond formation and the positive ΔS values brought about by the break up of water clusters.

The second possible point of interaction is between the nonpolar or hydrophobic portion of the drug molecule and the water clusters. The interaction comes about through van der Waal's forces and results in a hydrophobic hydration of the nonpolar molecule. An interaction of this type would tend to be a limiting factor in the solubility of the compound because of the large negative entropies and positive free energies which result. As noted previously, extensive hydrophobic hydration produces a situation where hydrophobic bonding between drug molecules and the resulting limitation on solubility would be energetically favored.

Therefore, the degree of solubility of benzoic or

TABLE III—THERMODYNAMIC PARAMETERS FOR SALICYLIC ACID IN UREA SOLUTIONS

	ΔF, cal./mole 30°	37°	45°	ΔH, cal./mole	ΔS (e.u.)
Urea, *M*					
0.5	− 85	− 76	− 83		+0.3
1.0	− 173	−195	−181		+0.6
1.5	− 225	−255	−249	≅ 0	+0.8
2.0	− 318	−329	−333		+1.1
2.5	− 385	−394	−391		+1.3
3.0	− 435	−460	−453		+1.4
Methylurea, *M*					
0.5	− 149	−153	−172		+0.5
1.0	− 281	−318	−333		+1.0
1.5	− 435	−429	−492	≅ 0	+1.4
2.0	− 583	−575	−612		+1.9
2.5	− 671	−711	−759		+2.3
3.0	− 803	−814	−876		+2.7
1,3-Dimethylurea, *M*					
0.5	− 249	...	...		+0.8
1.0	− 436	...	...		+1.4
1.5	− 634	...	...	≅ 0	+2.1
2.0	− 795	...	...		+2.6
2.5	− 966	...	...		+3.2
3.0	−1120	...	...		+3.7

TABLE IV—THERMODYNAMIC PARAMETERS FOR BENZOIC ACID IN UREA SOLUTIONS

	ΔF, cal./mole 30°	37°	45°	ΔH, cal./mole	ΔS (e.u.)
Urea, *M*					
0.5	− 105	− 92	− 83		+0.4
1.0	− 159	−133	−126		+0.5
1.5	− 237	−234	−214	≅ 0	+0.8
2.0	− 281	−279	−249		+0.9
2.5	− 339	−350	−307		+1.1
3.0	− 372	−394	−366		+1.2
Methylurea, *M*					
0.5	− 145	−162	−147		+0.5
1.0	− 265	−295	−262		+0.9
1.5	− 395	−404	−405	≅ 0	+1.3
2.0	− 504	−521	−540		+1.7
2.5	− 612	−622	−650		+2.0
3.0	− 728	−730	−759		+2.4
1,3-Dimethylurea, *M*					
0.5	− 199	...	...		+0.7
1.0	− 440	...	...		+1.5
1.5	− 608	...	...	≅ 0	+2.0
2.0	− 751	...	...		+2.5
2.5	− 893	...	...		+3.0
3.0	−1014	...	...		+3.4

salicylic acid in pure water would depend upon the relative magnitudes of the hydrophobic surface of each molecule. Since salicylic acid, by virtue of its intramolecular bonding, has the larger hydrophobic surface and can, therefore, demonstrate self-interaction to a greater extent by hydrophobic bonding, it should (based on the above consideration) have a lower water solubility than benzoic acid. This is, of course, the case.

Since urea effectively breaks up the clusters of hydrogen bonded water molecules in aqueous solution resulting in an increase in the entropy of the system, there would be less entropy to be gained by the hydrophobic bonding between the relatively nonpolar benzoic or salicylic acid molecules. Since formation of the hydrophobic bond has been decreased, there is a resulting increase in water solubility of the drug molecule.

If this mechanism is correct, then urea should have a greater effect upon the more hydrophobic molecule. A comparison of N/N_0 values reveals that salicylic acid, the more hydrophobic molecule, is solubilized by urea to a greater extent than the less hydrophobic benzoic acid molecule.

The effect of the alkyl substituted ureas, methylurea and 1,3-dimethylurea, on the solubility of benzoic and salicylic acid raises a number of interesting considerations. Based on Abu-Hamdiyyah's theory (8), the substituted ureas should have less of an effect on hydrophobic bonding than unsubstituted urea. This, in turn, would result in less solubilization of drug molecules by the alkylurea derivatives. The results of this study clearly show the opposite effect. Methylurea and 1,3-dimethylurea were more effective in solubilizing benzoic and salicyclic acid than urea, with 1,3-dimethylurea having the greatest effect. The values of log N/N_0 for 2 *M* 1,3-dimethylurea with both aromatic acids at various temperatures showed that ΔH was extremely small but slightly positive, and ΔF was negative (approximately −700 to −900 cal./mole). These values indicate the solubility increase was entropic in origin as was the case with unsubstituted urea.

These findings, however, should not be construed as a criticism of Abu-Hamdiyyah's model because the present experimental system is different from the system considered in the model. Considering the basicity of urea, and particularly the alkyl ureas, it is reasonable to conclude that at pH 1, there is present significant amounts of the conjugate acids of the ureas. While there exists a likelihood that these solutions have the same structure as aqueous solutions of nonionic ureas, this fact is yet to be experimentally demonstrated.

REFERENCES

(1) Bolton, S., *J. Pharm. Sci.*, **52**, 1071(1963).
(2) Altwein, D. M., Delgado, J. N., and Cosgrove, F. P., *ibid.*, **54**, 603(1965).
(3) Higuchi, T., and Connors, K. A., *Advan. Anal. Chem. Instr.*, **4**, 117(1965).
(4) Scheraga, H. A., and Nemethy, G., *J. Chem. Phys.*, **36**, 3382(1962).
(5) Kauzmann, W., *Advan. Protein Chem.*, **14**, 1(1959).
(6) Mukerjee, P., and Ghosh, A. K., *J. Phys. Chem.*, **67**, 193(1963).
(7) Bruning, W., and Holtzer, A., *J. Am. Chem. Soc.*, **83**, 4865(1961).
(8) Abu-Hamdiyyah, M., *J. Phys. Chem.*, **69**, 2720(1965).
(9) Katayama, A., Matsuura, T., Konishi, K., and Kuroki, N., *Kolloid-Z.*, **202**, 157(1965).
(10) Wetlaufer, D. B., Malik, S. K., Stoller, L., and Coffin, R. L., *J. Am. Chem. Soc.*, **86**, 508(1964).
(11) Martin, A. N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, p. 668.
(12) Bull, H. B., Bresse, K., Ferguson, G. L., and Swenson, C. A., *Arch. Biochem. Biophys.*, **104**, 297(1964).
(13) Shaw, W. H. R., and Bordeaux, J. J., *J. Am. Chem. Soc.*, **77**, 4729(1955).
(14) Pimentel, G., and McClellan, A. L., "The Hydrogen Bond," W. H. Freeman and Co., San Francisco, Calif., 1960, pp. 206–225.
(15) Nozaki, Y., and Tanford, C., *J. Biol. Chem.*, **238**, 4074(1963).
(16) Higuchi, T., and Zuck, D. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 138(1953).

Photochemical Formation of Hydrogen Peroxide in White Lotion U.S.P.

By EARL P. GUTH and Z. MANSOUR

White lotion U.S.P. produces hydrogen peroxide when it is irradiated with ultraviolet radiation or with natural sunlight. The amount of hydrogen peroxide formed during irradiation is sufficient to oxidize the sulfide ion to sulfate. When cholesterol is added and the product irradiated, the β-hydroxyl group on the cholesterol molecule is oxidized.

WHITE LOTION is a widely used external preparation in which insoluble compounds of zinc are present. It has been used for the topical treatment of a number of dermatologic diseases. The lotion was proposed for the American Recipe Book in 1912 (1). It became official in the U.S.P. XV and U.S.P. XVI with the following formula:

Zinc sulfate	40 Gm.
Sulfurated potash	40 Gm.
Purified water, a sufficient quantity to make	1000 ml.

"Dissolve the zinc sulfate and sulfurated potash separately, each in 450 ml. of purified water, and filter each solution. Add slowly the sulfurated solution with constant stirring. Then add the required amount of purified water, and mix.

"Note: Prepare the Lotion freshly and shake thoroughly before dispensing."

The mode of action of white lotion is attributed to be astringent and protective (2). The principal activity is associated with the sulfur content (3), and at one time the presence of residual hydrogen sulfide in the lotion was considered to be the important reason for preparing the lotion freshly.

DISCUSSION

A number of papers on the catalytic production of hydrogen peroxide by irradiation of zinc oxide in aqueous medium have appeared (4–13) since 1944. All these studies showed that zinc oxide catalyzed the production of hydrogen peroxide when exposed to light in the presence of water and air, the rate of production being influenced by the kind of light and temperature. Since this is a catalytic process, the production of peroxide would continue indefinitely as long as all components of the system were present. The source of ultraviolet light used in this study was a Dazor floating fixture ultraviolet lamp model No. U-58, equipped with a General Electric UA-3, 360-w. quartz photochemical lamp. The radiant energy is greatest at 2537 Å., 3131 Å., and 3564 Å. Irradiations were also done by exposing samples directly to sunlight.

Received April 26, 1966, from the College of Pharmacy, Ohio State University, Columbus, OH 43210

Accepted for publication December 29, 1966.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

Samples for irradiation were placed in 600-ml. beakers. These were placed in a water bath situated on a six-plate magnetic stirrer. The U.V. lamp was suspended 30 cm. over the surface of the sample, and the entire assembly was enclosed in an aluminum box. Irradiation time was 30 min. in all cases unless otherwise noted.

Since sulfurated potash is a complex and variable mixture of potassium polysulfides and potassium thiosulfate depending on the particular batch and age of the product, and since zinc sulfate undergoes dissociation in water to give hydronium, sulfate, and zinc ions, a number of chemical reactions are possible. The extent of any one of these reactions can be affected by concentration and temperature of the system.

Possible reactions:

$$\text{sulfurated potash in water} \rightleftharpoons K^+, S^{=}, S_x^{=}, S_2O_3^{=}, OH^-$$

$$S_2O_3^{=} + 2H^+ \rightleftharpoons SO_2 + H_2O + S$$

$$SO_2 + 2H_2S \rightleftharpoons 3S + 2H_2O$$

$$S^{=} + Zn^{++} \rightleftharpoons ZnS$$

$$xS + Zn^{++} \rightleftharpoons ZnS_x \text{ (where } x \text{ is mostly 2 or 3)}$$

$$SO_4^{=} + 2K^+ \rightleftharpoons K_2SO_4$$

$$S_2O_3^{=} + Zn^{++} \rightleftharpoons ZnS_2O_3$$

The formation of zinc hydroxide in an acid medium of zinc sulfate solution occurs as follows:

$$[Zn(OH_2)_6]^{++} + H_2O \rightleftharpoons [Zn(OH_2)_5(OH)]^+ + H_3O^+$$

hexa-aquo zinc (II)

$$[Zn(OH_2)_5(OH)]^+ + H_2O \rightleftharpoons [Zn(OH_2)_4(OH)_2]^0 + H_3O^+$$

(zinc hydroxide)

The OH^- ions in the sulfated potash shift both reactions to the right forming zinc hydroxide.

In view of these considerations, white lotion was restudied to evaluate the following: (*a*) composition of the soluble phase of the lotion, (*b*) composition of the insoluble phase of the lotion, (*c*) the effect of order of mixing on the composition of the two phases, (*d*) the effect of temperature on the composition, (*e*) the production of hydrogen peroxide by catalytic action of zinc hydroxide, (*f*) the effect of hydrogen peroxide on the components of the lotion, (*g*) the effect of aging lotion, (*h*) the effect of hydrogen peroxide on additives such as cholesterol.

EXPERIMENTAL

Qualitative Analysis of the Fresh Lotion

Soluble Phase—*Potassium*—This was determined by cobaltinitrite solution: a finely divided yellow to orange-yellow precipitate was obtained.

Thiosulfate, Sulfate, Sulfite, and Zinc—The U.S.P. tests for these ions were applied. The presence of sulfate and thiosulfate ions was confirmed. No sulfite ion was detected.

Hydrogen Sulfide—Van Itallie reagent (14) (freshly prepared solution of *p*-diazobenezensulfonic acid) was added to the filtrate of the freshly prepared lotion (pH 7.3). No coloration occurred (reaction negative).

Insoluble Phase—*Sulfides*—This test depends on the ability of sulfides to facilitate the reaction between equal quantities of 0.2 *N* aqueous solution of iodine and 0.2 *N* sodium azide solution. A violent evolution of nitrogen occurs in this reaction. The washed precipitate obtained by filtration of white lotion gave a positive test for sulfides.

Zinc Hydroxide—The gelatinous precipitate of zinc hydroxide is amphoteric and dissolves in alkalies to form the complex ion $[Zn(OH)_4]^=$. The identification of zinc hydroxide was done by treating the insoluble phase with dilute ammonium hydroxide solution. The sample is filtered and the filtrate neutralized with acid. A white precipitate indicated zinc hydroxide (reaction positive).

Quantitative Analysis—A quantitative analysis was run on the washed and dried precipitate obtained from the lotion. The dried precipitate was extracted with carbon disulfide, filtered, and the solvent evaporated. The free sulfur was determined by a gravimetric or a volumetric procedure depending on the amount of the extracted sulfur obtained (15–17).

Sulfides were determined by Kolthoff's method (18) for insoluble sulfides as modified for polysulfides (19). Zinc was determined by the U.S.P. XVI method (20).

Table I gives percentage composition of dried precipitate from three differently prepared lotions. Each entry is the average of seven determinations.

Ultraviolet and sunlight irradiations were performed on suspensions made by resuspending the washed precipitate of the lotion in 40 ml. of water. The amounts of precipitate represented 25 and 5 ml. of lotion, respectively. After irradiation, both the filtrate and the precipitate were analyzed. Results are shown in Table II.

According to Goudah (9), increasing the zinc oxide irradiated in the aqueous system increases the peroxide formed but not in a linear relationship. In this work, irradiations were performed in identical containers, for the same period of time, stirred at the same speed, and placed in the same circulating water bath. The samples representing 25 ml. of lotion showed sulfite and zinc ions, free sulfur, zinc oxide, and no free hydrogen peroxide. The samples representing 5 ml. of lotion show the presence of sulfate and no zinc sulfides. It is concluded from these results that in the 25-ml. sample, the amount of hydrogen peroxide produced in the 30 min. of irradiation was insufficient to oxidize the larger amount of sulfides present in the 25-ml. samples. The 5-ml. sample, however, did produce sufficient peroxide in the 30 min. of irradiation to completely oxidize the sulfides to sulfates. This is probably due to the fact that in the irradiation of the sample under the conditions used, the exposure of zinc oxide to light in the 25-ml. samples was not proportionally greater than the zinc oxide in the 5-ml. samples. This is a reasonable conclusion since the area of the exposure was identical in all samples.

Table III lists the amount of peroxide present after irradiation of different amounts of washed precipitate resuspended in 40 ml. of water. The amount of residual peroxide detected showed an increase with the decrease of the amount of precipitate irradiation.

The iodometric method of Kolthoff (21), as modified by Chari and Qureshi (12), was utilized for the quantitative determination of hydrogen peroxide.

To the aqueous suspension made from the washed precipitate of 5 ml. of lotion, 200 mg. of cholesterol dissolved in 5 ml. of ethanol was added. The mixture was irradiated for 4 hr. in the sunlight, or

TABLE I—PERCENTAGE COMPOSITION (W/W) OF DRIED PRECIPITATE FROM THREE DIFFERENTLY PREPARED LOTIONS

Lotion[a]	Contents, % w/w in the Dry Precipitate: Free Sulfur	Zinc Oxide	Zinc Sulfides[b]	Sulfides' Sulfur
A	4.93	45.54	49.52	36.45
B	4.32	48.1	47.5	35.2
C	4.36	46.1	49.54	35.4

[a] *A*, freshly prepared U.S.P. lotion; *B*, lotion prepared by reverse order of mixing; *C*, lotion prepared according to the U.S.P. but using heated solutions at 80°. [b] Zinc sulfides are calculated values by subtracting free sulfur and zinc hydroxide content from 100% dry precipitate.

TABLE II—ANALYSIS OF SAMPLES AFTER 30 min. ULTRAVIOLET IRRADIATION

	Filtrate: SO_4^-	SO_3^-	H_2O_2	Zn^{++}	Precipitate: S	ZnO	ZnS
25-ml. lotion	−	+	−	+	+	+	+
5-ml. lotion	+	−	+	+	+	+	−

TABLE III—AMOUNT OF PEROXIDE PRESENT AFTER IRRADIATION OF WASHED PRECIPITATE RESUSPENDED IN 40 ml. WATER

Amt. of Precipitate	H_2O_2, mg.	Presence of ZnS_x
Ultraviolet Irradiation, 30 min.		
2 ml. original lotion	0.24	−
3 ml. original lotion	0.23	−
4 ml. original lotion	0.25	−
5 ml. original lotion	0.17	−
6 ml. original lotion	0.10	−
7 ml. original lotion	0.08	−
10 ml. original lotion	0.03	−
11 ml. original lotion	−	+
12 ml. original lotion	−	+
25 ml. original lotion	−	+
Sunlight Irradiation, 4 hr.		
2 ml. lotion	0.23	−
3 ml. lotion	0.19	−
5 ml. lotion	0.13	−
25 ml. lotion	−	+

30 min. under the ultraviolet light, with continuous stirring. The samples were then extracted with chloroform, the extract condensed under vacuum to 10 ml., dried in a desiccator, and analyzed by infrared spectroscopy. A sample of cholesterol, ethanol, and water was treated in the same manner. In addition, the precipitate suspension with cholesterol was placed in the dark. The entire process was repeated using samples made of 5 ml. of the entire lotion.

Infrared spectra are shown in Figs. 1, 2, 3, 4, and 5. Finally, the effect of aging white lotion was determined by storing the lotion in clean glass bottles at room temperature, and samples analyzed at intervals. Results are shown in Table IV.

RESULTS

The results of this study show that white lotion U.S.P. XVI is a mixture of several insoluble and soluble materials and that the percentage composition of each of these materials is not significantly affected by the order of mixing or alteration of the temperature of the reactants. The composition of the precipitate of freshly prepared lotion appears to contain approximately 4.5% of free sulfur, 45–48% of zinc oxide, and 47–49% of zinc sulfides. The zinc sulfides represent sulfur to the extent of 35 to 36% of the precipitate.

When the precipitate is separated and washed with distilled water, then resuspended in distilled water and subjected to irradiation by ultraviolet light, considerable alteration occurs. When 25 ml. of lotion is used the filtrate shows, after irradiation, sulfite and zinc ions. When 5 ml. of lotion is irradiated for the same time, the filtrate shows the presence of sulfate and zinc ions, and hydrogen peroxide, while the precipitate shows the absence of sulfide ions.

These changes show that the peroxide oxidizes the sulfide to sulfite and finally to sulfate and only after this reaction is completed is it possible to show the presence of hydrogen peroxide. Table III shows the results of peroxide formation from increasing amounts of resuspended precipitate when irradiated by ultraviolet light for 30 min., and by sunlight for 4 hr. These values show that the amounts of peroxide produced by the two light sources are approximately the same for the time periods used. This also indicates that these reactions may occur when white lotion is applied to the skin.

The existence of cholesterol on the skin, and the probability of its being a precursor of 7-dehydro-

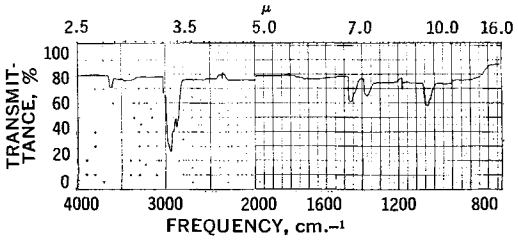


Fig. 1—Infrared spectrum of cholesterol.

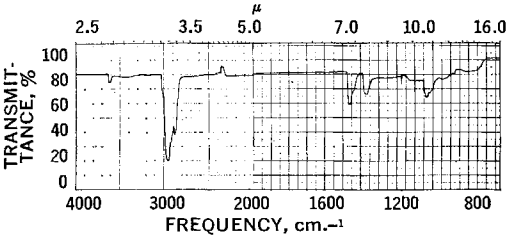


Fig. 2—Infrared spectrum of cholesterol extracted from precipitate (no irradiation).

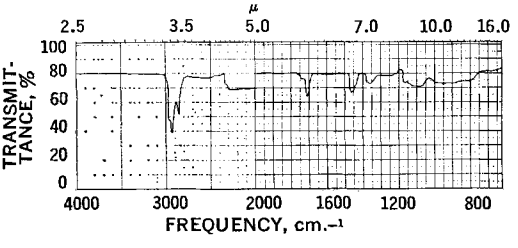


Fig. 3—Infrared spectrum of cholesterol extracted from precipitate after ultraviolet irradiation (30 min.).

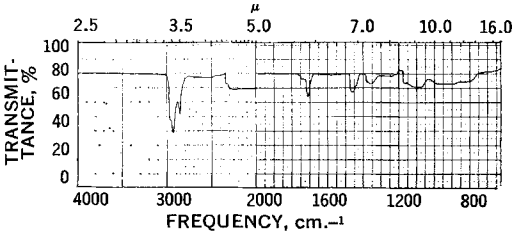


Fig. 4—Infrared spectrum of cholesterol extracted from precipitate after sunlight irradiation (4 hr.).

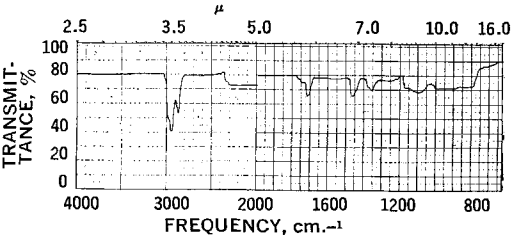


Fig. 5—Infrared spectrum of cholesterol extracted from white lotion after ultraviolet irradiation (30 min.).

TABLE IV—COMPOSITION OF WHITE LOTION STORED AT ROOM TEMPERATURE

	% w/w in the Dry Precipitate			
Time, Days	Free Sulfur	Zinc Oxide	Polysulfides	Sulfides' Sulfur
0	5	45.5	49.50	36.46
10	15.40	45.6	39.00	26.10
20	23.20	45.3	31.50	18.43
30	27.56	45.6	26.84	14.10
40	28.95	45.2	25.90	12.80
50	29.50	45.2	25.30	12.50
60	29.60	45.2	25.20	12.30

cholesterol (provitamin D_3) suggested the determination of the effect of irradiation of cholesterol in the presence of the white lotion. The results represented in the infrared spectra of cholesterol before and after irradiation show oxidation of cholesterol when irradiated in the presence of both the resuspended washed precipitate and in the entire white lotion.

The characteristic absorption band of the hydroxyl group of cholesterol is seen at 3600^{-1} or 2.75 μ wavelength in the infrared spectra of cholesterol and in that of cholesterol extracted from a nonirradiated sample. A band is noted at 1075 cm.$^{-1}$ due to the presence of a secondary alcohol (Figs. 2 and 3). These respective characteristic absorption bands disappear in the I.R. spectra of cholesterol extracted from the precipitate and from entire lotion samples after ultraviolet irradiation (30 min.) or sunlight irradiation (4 hr.) (Figs. 3, 4, and 5). A new absorption band appears in these spectra in the region of 6 μ or 1750 cm.$^{-1}$. This band is ascribed to the vibrations of carbonyl function C=O, and is found in about the same region as in ketones and aldehydes. The irradiation of cholesterol alone under the above condition does not produce any changes in the absorption peaks of the I. R. spectra. The results obtained are in agreement with previous work by Guth and Goudah (9).

The findings of this study suggest further investigation of the effect of the peroxide produced by white lotion on the sterols in the skin.

The results on the effect of aging on the white lotion showed an increase in the free sulfur content and a decrease in sulfides' sulfur as shown in Fig. 6. The physical stability of white lotion is affected only slightly on aging. Colloidal sulfur particles seem to aggregate and the components of the precipitate do not remain uniformly distributed, but since no caking or flocculation is noted, the lotion is readily redispersed into a uniform mixture when the container is shaken.

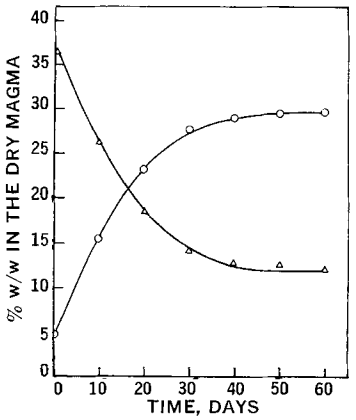


Fig. 6—Effect of aging on white lotion. Key: O, free sulfur; △, sulfides' sulfur.

It seems that the freshly prepared lotion was reported beneficial in acne and other dermatologic diseases due to its high content of insoluble sulfides. It appears from this study that there may be merit in requiring a freshly prepared lotion, since the effectiveness of white lotion could in part be associated with the gradual release of colloidal sulfur.

SUMMARY

1. White lotion U.S.P. contains zinc mono and polysulfides, zinc hydroxide, and free sulfur all suspended in an aqueous medium containing sulfate, thiosulfate, and potassium ions.

2. Zinc oxide catalyzed hydrogen peroxide is formed in white lotion when irradiated by ultraviolet and sunlight. The peroxide so formed is very active in oxidizing sulfides to sulfites and then to sulfates.

3. When cholesterol is present, β-hydroxyl group is oxidized.

4. On aging, there is a marked increase in free sulfur in the lotion.

REFERENCES

(1) Raubenheimer, O., *J. Am. Pharm. Assoc.*, **7**, 761 (1912).
(2) Martin, E. W., "Remington's Practice of Pharmacy," 13th ed., Mack Publishing Co., Easton, Pa., 1965, p. 805.
(3) Olso, A., Ferrar, E. F., and Pratt, R., "United States Dispensatory," vol. 102, J. B. Lippincott Publishing Co., Philadelphia, Pa., 1960, p. 1508.
(4) Lozada, H. A., and Guth, E. P., *Drug Std.*, **28**, 736 (1960).
(5) Reese, D. R., and Guth, E. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 491(1954).
(6) Mathias, M., and Guth, E. P., *Drug Std.*, **22**, 185 (1954).
(7) Minardi, F. C., and Guth, E. P., *ibid.*, **22**, 192(1954).
(8) Blubaugh, F. C., and Guth, E. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 696(1956).
(9) Goudah, M. W., and Guth, E. P., *J. Pharm. Sci.*, **52**, 799(1963).
(10) Winter, G., *Nature*, **163**, 326(1949).
(11) Stephens, R. E., Ke, B., and Trivich, D., *J. Phys. Chem.*, **59**, 966(1955).
(12) Chari, N., and Qureshi, M., *J. Indian Chem. Soc.*, **21**, 297(1944).
(13) Garn, F. D., "The Catalytic Activity of Zinc Oxide in the Photochemical Formation of Hydrogen Peroxide," Ph.D. Thesis, Ohio State University, Columbus, Ohio, 1952.
(14) "The Merck Index," 5th ed., Merck & Co., Inc., Rahway, N. J., 1940, tests and reagents.
(15) Kolthoff, I. M., and Elving, P. J., "Treatise on Analytical Chemistry II," vol. 7, Interscience Publishers, New York—London, 1961, p. 101.
(16) Beckett, A. H., and Stenlake, J. B., "Practical Pharmaceutical Chemistry, Quantitative Analysis," Oxford University Press, Inc., New York, N. Y., 1962, p. 173.
(17) "Standard Methods for the Examination of Water, Sewage and Industrial Wastes," 10th ed., American Public Health Association, New York, N. Y., 1955, pp. 196–200.
(18) Kolthoff, L. M., and Belcher, R., "Volumetric Analysis III," Interscience Publishers, New York–London, 1957, p. 291.
(19) *Ibid.*, pp. 307–308.
(20) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 807.
(21) Kolthoff, I. M., *Z. Anal. Chem.*, **60**, 400(1921).

Irreversible Enzyme Inhibitors LXIX

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase III. Bromoacyl Derivatives of 5-(*p*-Aminophenoxypropyl)-2,4,6-triaminopyrimidines

By B. R. BAKER and DANIEL V. SANTI

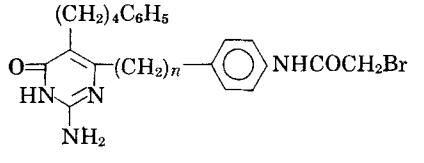
5-(*p*-Aminophenoxypropyl)-2,4,6-triaminopyrimidine (XIV*d*) was synthesized by alkylation of malononitrile by 3-bromopropyl *p*-nitrophenyl ether (X*b*), followed by ring closure with guanidine and catalytic reduction of the nitro group. Selective haloacylation of the aromatic amino group of XIV*b* was accomplished by protonation of the triaminopyrimidine moiety of XIV*d* with acetic acid. Treatment with the anhydrides of bromoacetic acid, *p*-bromoacetamidophenylbutyric acid, and *N*-bromoacetyl-β-alanine gave the pure bromoacyl derivatives, XIV*e*, *i*, and *j*. These three compounds were good reversible inhibitors of dihydrofolic reductase, but failed to show irreversible inhibition; these failures are attributed to the phenoxypropyl group of the inhibitors (XIV) being complexed with a hydrophobic region on the enzyme—a region not apt to have groups that could form a covalent bond.

PRIOR TO THE discovery of a strong hydrophobic bonding region on dihydrofolic reductase (1) with its conformational requirements (2–6) and the possible pyrimidine rotomers that could bind to the enzyme (5–9), more than 30 candidate compounds were synthesized and evaluated as active-site-directed irreversible inhibitors (5, 10) of this enzyme with negative results. By proper utilization of the hydrophobic region, successful active-site-directed irreversible inhibitors (I–III) were found (11, 12). By complexing the hydrophobic region by a 5-phenylbutyl group—as in the pyrimidines, I and II (11)—or by 6-phenyl as in III (12), the remaining aralkyl group then projected into a hydrophilic region on the enzyme and could covalently link a nucleophilic group on the enzyme.

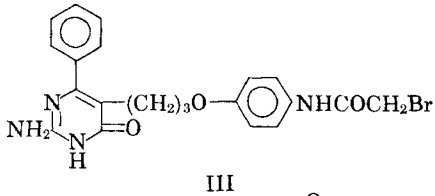
DISCUSSION

The first candidate active-site-directed irreversible inhibitor successfully synthesized in this laboratory was IV; the latter was a good reversible inhibitor of dihydrofolic reductase, but gave no inactivation of the enzyme. Subsequent work on hydrophobic bonding to dihydrofolic reductase (1–9) allowed a suitable rationalization for this failure; the 5-side chain of IV was most probably complexed in a hydrophobic region and, therefore, was not likely to be juxtaposed to a nucleophilic region on the enzyme. The synthesis of inhibitors such as IV was accomplished only after a number of false starts; this successful synthesis of IV paved the way for the ultimate synthesis and enzymic evaluation of the successful candidate inhibitors (I–III) (11, 12).

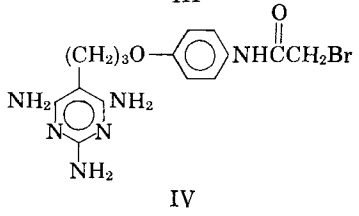
Since the anilinopropyl pyrimidinol (VII*a*) was a reasonably good inhibitor of dihydrofolic reductase (13, 14), the related *p*-bromoacetamidoanilinopropyl pyrimidinol (VII*e*) was selected as a candidate active-site-directed irreversible inhibitor (5, 10) for dihydrofolic reductase, and its synthesis was undertaken. The various reactions following are outlined in Scheme I. The first general route developed to structures of type VII was the reductive condensation of an arylamine (VI) with the pyrimidine-5-propionaldehyde (V) (13, 15). The condensation failed to give VII*b* with the more weakly basic *p*-nitroaniline (VI*b*). The required intermediate (VII*b*) was synthesized by the second general route starting the *N*-(*p*-nitrophenyl)-*p*-toluenesulfonamide *via* VIII*b* and IX*b* (16). Re-



I, $n=2$
II, $n=4$

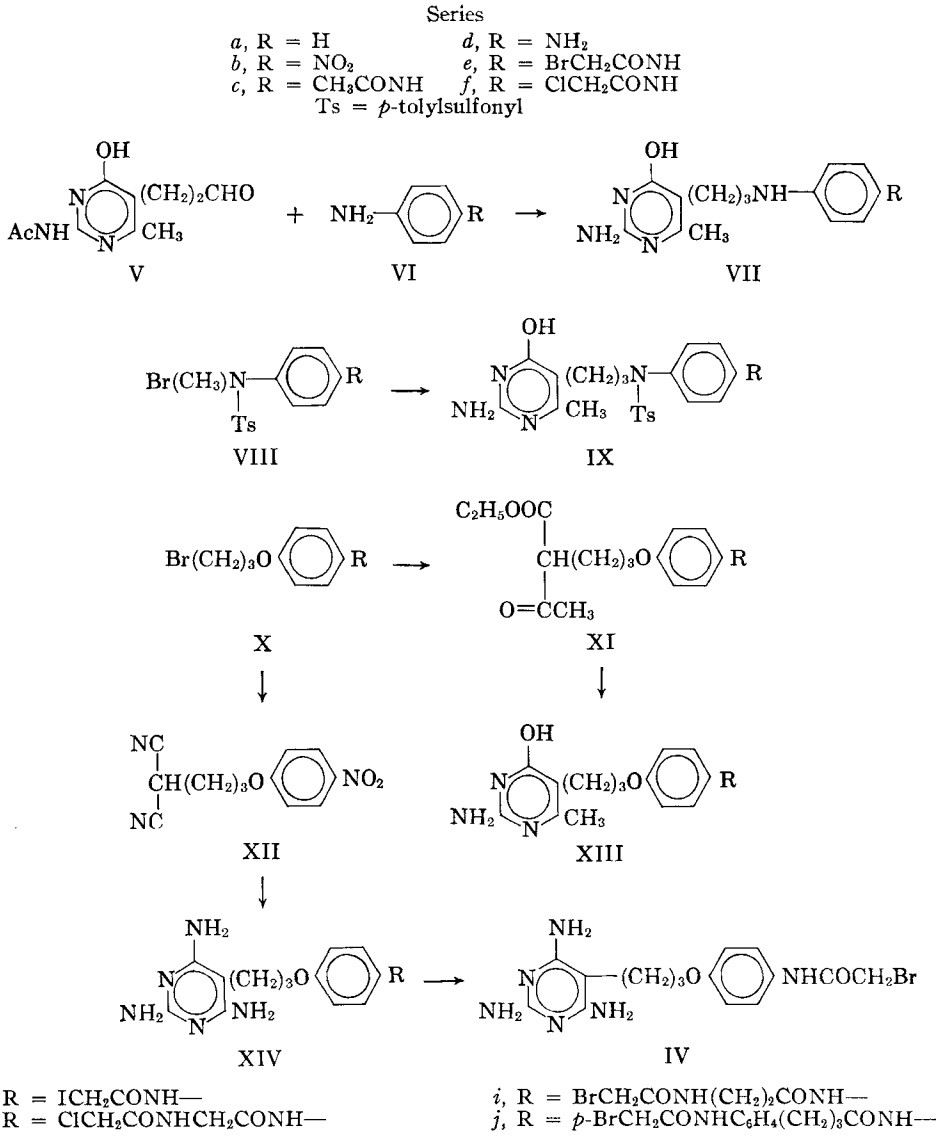


III



IV

Received August 5, 1966, from the Department of Chemistry, University of California, Santa Barbara, CA 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication September 21, 1966.
This work was generously supported by grants CA-05867, CA-06624, and CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Previous paper of this series see *Reference 12*.



Scheme I

duction of the nitro group of VII*b* to the substituted *p*-phenylenediamine (VII*d*) or preparation of VII*d* from the *p*-acetamidoanilinopropyl pyrimidinol (VII*c*) in this and the corresponding 6-phenyl series (17) appeared to be intractable due to the instability of the *p*-phenylenediamine moiety.

Since the anilinopropyl group of VII*a* can be replaced with phenoxypropyl (XIII*a*) with no change in binding to the enzyme (18) and since the *p*-anisidine is much more stable than *p*-phenylenediamine, the synthesis of XIII*b* was investigated. The latter was readily synthesized from *p*-nitrophenyl 3-bromopropyl ether (X*b*) by alkylation of ethyl acetoacetate to XI*b* followed by ring closure with guanidine to XIII*b*. Such a synthesis has considerable latitude in variation of the 4- and 6-substituents of the pyrimidine ring. Therefore, the 2,4,6-triaminopyrimidine ring system bearing a 5-(*p*-nitrophenoxypropyl) group (XIV*b*) was selected for study with the extra consideration that 2,4,6-triaminopyrimidines are better reversible inhibitors of dihydrofolic reductase than the corresponding 2-amino-6-methyl-4-pyrimidinols (1, 5, 19).

Alkylation of malononitrile with the bromopropyl phenyl ether (X*b*) gave a crude product (XII) which was condensed with guanidine in ethanol to give the triaminopyrimidine (XIV*b*) in 51% over-all yield; this product contained some minor impurities (by TLC) that could not be removed. Since XIV*b* was rather intractable, it was reduced catalytically to XIV*d* with a Raney nickel catalyst; the anisidine derivative (XIV*d*) was readily purified in 56% yield.

Methods were then developed for selective halo-

acylation of the polyfunctional XIV*d* to give derivatives of type XIV*e–j* where only the aromatic amino group has become acylated. The first studies in this laboratory on selective acylation of aminophenyl groups attached to an aminopyrimidine were performed on XIV*d*. Although the methods developed for selective bromoacylation of these aminopyrimidines have been collected in one paper (20) to indicate the scope and limitations of the reaction, the background work was performed on XIV*d* and will be reported here.

It was reasoned that the greatest selectivity in acylation of the aromatic amino group of XIV*d* would occur if the more strongly basic triaminopyrimidine moiety could be protonated throughout the experiment. Such a procedure would be more difficult with an acid chloride than with an anhydride, since the evolved hydrogen chloride would be more difficult to keep neutralized at acid pH than the organic acid evolved from an anhydride. The first acylation was performed with chloroacetic anhydride on XIV*d* in acetone containing excess aqueous 5% acetic acid; within 10 min. precipitation occurred. By use of excess chloroacetic anhydride to overcome partial aqueous hydrolysis of the anhydride, the reaction was carried to completion. The salt that separated appeared to be a mixture of monoacetate and monochloroacetate. Upon warming the mixture, the salt redissolved; the solution was added to excess aqueous potassium carbonate, then the isolated free base of XIV*f* was converted to the acetate salt; when dried without heating, this material gave combustion values for a diacetate salt. Extended drying at 100° gave varying amounts of acetate in the salt. Attempts to convert XIV*f* to the iodo derivative (XIVg) with sodium iodide in acetone containing glacial acetic acid were unsuccessful, the products seeming to be unstable.

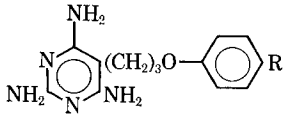
Similarly, the anhydride of chloroacetylglycine could be used to form XIV*h*—isolated as an acetate salt. However, with bromoacetic anhydride, the product (XIV*e* = IV) could not be treated with aqueous potassium carbonate since some of the bromoacetyl group hydrolyzed to hydroxyacetyl. Therefore, a different set of criteria and a different isolation had to be devised, namely (*a*) the Bratton-Marshall test (13, 15, 21) for aromatic amines was negative; (*b*) thin-layer chromatography showed the presence of a single spot that gave a positive *p*-nitrobenzylpyridine test for active halogen (22, 23); (*c*) ultraviolet spectra were essentially unchanged showing that an amino group on the pyrimidine had not become acylated (24).

Treatment of XIV*d* in 2:1 acetone–10% acetic acid at 0° with 2–3 equivalents of bromoacetic anhydride resulted in a salt of the product (XIV*e* = IV) separating from solution. Since this salt was probably a mixture of acetate salt and bromoacetate salt and since IV could not be converted to the free base without decomposition, the acetate salt of IV was directly converted to the salt of a stronger acid such as hydrobromic or sulfuric acid; either a monohydrobromide or dihydrobromide of IV could be prepared, depending upon the conditions (20).

Similarly, the use of the anhydrides of *N*-bromoacetyl-β-alanine and *p*-bromoacetamidophenylbutyric acid (20) gave XIV*i* and *j*, respectively, isolated as their acetate and sulfate salts, respectively.

Enzymatic Evaluation—Table I lists the results on reversible and irreversible inhibition of dihydrofolic reductase with the bromoacyl derivatives of structure IV or XIV. All three compounds are better reversible inhibitors than the 4-pyrimidinol (II). However, when incubated at a concentration sufficient to convert 50% of the enzyme to the reversible complex (E · · · I), the bromoacyl derivatives of structure IV or XIV failed to show any inactivation in 1 hr. at 37°. In contrast, the 5-phenylbutyl-6-(*p*-bromoacetamidophenylbutyl)-4-pyrimidinol (II) showed 50% inactivation in 18 min. (11). These results are believed to be due to the 5-phenoxypropyl group of XIV being complexed to a hydrophobic region on the enzyme where there are not apt to be any polar nucleophilic groups.

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Reversible[a] μM Concn. for 50% Inhibition	Reversible[a] Estimated $Ki \times 10^6$ M[c]	Irreversible[b] μM Concn.	Irreversible[b] % EI[d]	Irreversible[b] Time, min.	Irreversible[b] % Inactivation
XIV*d*	NH_2	57	9				
IV	$BrCH_2CONH$	28	5	5	50	60	0
XIV*i*	$BrCH_2CONH(CH_2)_2CONH$	100	16	16	50	60	0
XIV*j*	p-$BrCH_2CONHC_6H_4(CH_2)_3CONH$	13	2	2	50	120	0
II[e]		240	40	40	50	18	50
				10	20	25	43

[a] The dihydrofolic reductase was a 45–90% ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μ*M* dihydrofolate and 12 μ*M* TPNH in 0.05 *M* Tris buffer (pH 7.4) containing 10 m*M* mercaptoethanol, as previously described (14). [b] Dihydrofolic reductase was incubated with the inhibitor at 37° in 0.05 *M* Tris buffer (pH 7.4) containing *no* mercaptoethanol and *no* TPNH, but diluted with 10% of its volume with *N*,*N*-dimethylformamide in which the inhibitor had been dissolved; in each case an enzyme control was run that showed 0–4% inactivation (22). [c] Estimated from $Ki = I \times Km/S$ where I = inhibitor concentration giving 50% inhibition (5, 25); this equation is valid since $S = 6\ Km > 4\ Km$. [d] Calculated from $[EI] = [Et]/[1 + Ki/I]$, where $[Et]$ = the concentration of total active enzyme and $[EI]$ = fraction of Et reversibly complexed by I (5, 26). [e] Data from *Reference 11*.

EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B recording spectrophotometer. Ultraviolet spectra were taken in water, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF, and spots were detected by visual examination under ultraviolet light.

3-Bromopropyl p-Nitrophenyl Ether (Xb)—To a well stirred solution of 120 Gm. (0.60 mole) of 1,3-dibromopropane in 75 ml. of dimethylsulfoxide was added 29.4 Gm. (0.15 mole) of sodium *p*-nitrophenolate dihydrate. After 1 hr. the mixture had formed a jell; it was heated on a steam bath for 30 min., then spin-evaporated *in vacuo* at 100°. The residue was partitioned between 750 ml. of benzene and 500 ml. of water. The organic layer was washed successively with five 500-ml. portions of water, two 500-ml. portions of 0.5 *N* aqueous sodium hydroxide, then once more with water. The benzene layer was spin-evaporated *in vacuo;* the last traces of higher boiling materials were removed at 100° at 1 mm. The oily residue was crystallized from ethanol. The product was collected on a filter and washed with petroleum ether (b.p. 30–60°) until the washings were colorless; yield, 26.0 Gm. (67%), m.p. 52–54°; this product was suitable for further transformations even though TLC with petroleum ether (b.p. 60–110°)–benzene (1:1) showed it was only about 90% pure. Recrystallization from petroleum ether (b.p. 60–110°) gave yellow crystals, m.p. 57–58°. [Lit. m.p. 58–59° (27).]

2-Amino-6-methyl-5-(p-nitrophenoxypropyl)-4-pyrimidinol (XIIIb)—A mixture of 70 ml. of *tert*-butyl alcohol, 3.90 Gm. (0.03 mole) of ethyl acetoacetate, and 0.72 Gm. (0.03 mole) of sodium hydride as a 56.5% suspension in mineral oil was magnetically stirred until hydrogen evolution was complete. After the addition of 5.2 Gm. (0.02 mole) of X*b*, the mixture was gently refluxed for 24 hr. protected from moisture. Acidified with glacial acetic acid, the mixture was spin-evaporated *in vacuo* finally at about 1 mm. to remove ethyl acetoacetate. The oily residue was dissolved in about 50 ml. of chloroform and washed with water. After being dried with magnesium sulfate, the solution was evaporated *in vacuo* to give crude XI*b* as an oil; yield, 5.7 Gm. of an oily residue containing some mineral oil.

A part (3.12 Gm., 10 mmoles) of the residual XI*b* and 0.99 Gm. (5 mmoles) of guanidine carbonate in 20 ml. of *tert*-butyl alcohol was gently refluxed with magnetic stirring for 48 hr. The cooled mixture was filtered and the product washed with 25 ml. of hot ethanol. The crude product was dissolved in about 15 ml. of hot 1 *N* aqueous sodium hydroxide. The hot solution was clarified by filtration, then acidified to about pH 5 with 3 *N* aqueous hydrochloric acid. After being cooled to 0°, the mixture was filtered and the product was recrystallized from 2-methoxyethanol–water; yield, 1.26 Gm. (42% based on X*b*) of light yellow crystals, m.p. 260–261° dec. $\nu_{max.}$ 3400–3100 (broad, NH); 1640, 1600, 1590 (NH, C=O, C=C, C=N); 1525, 1320 (NO_2); 1255 (C—O—C); 750, 720, 690 cm.$^{-1}$ (p-C_6H_4).

Anal.—Calcd. for $C_{14}H_{16}N_4O_4$: C, 55.3; H, 5.30; N, 18.4. Found: C, 55.1; H, 5.43; N, 18.6.

5-(p-Nitrophenoxypropyl)-2,4,6-triaminopyrimidine (XIVb)—To a magnetically stirred mixture of 5 ml. of dimethylsulfoxide and 0.24 Gm. (10 mmoles) of sodium hydride as a 56.5% dispersion in mineral oil was added 0.72 Gm. (11 mmoles) of malononitrile in 2 ml. of dimethylsulfoxide over a period of about 5 min. with cooling in a water bath. When hydrogen evolution was complete, a solution of 2.6 Gm. (10 mmoles) of X*b* in 4 ml. of dimethylsulfoxide was added. After 10 min. at ambient temperature, the mixture was heated at 75° for 30 min., then poured into 50 ml. of benzene. The mixture was washed with three 50-ml. portions of water, then spin-evaporated *in vacuo*. To the residual XII*b* was added a filtered solution of 1.05 Gm. (11 mmoles) of guanidine hydrochloride and 0.595 Gm. (11 mmoles) of sodium methoxide in 10 ml. of absolute ethanol. After being refluxed with magnetic stirring for 3 hr. protected from moisture, the mixture was cooled. The amorphous product was collected on a filter and washed with 100 ml. of hot ethanol in portions; yield, 1.57 Gm. (51%), m.p. >250°. $\nu_{max.}$ 3450, 3390, 3200 (NH); 1660, 1620–1575 (broad) (NH, C=C, C=N, NO_2); 1335 (NO_2); 757, 720, 690 cm.$^{-1}$ (p-C_6H_4). $\lambda_{max.}$ (pH 1): 284, 320 mμ; (pH 13): 278, 320 mμ.

TLC in ethanol showed one major spot and several minor spots; no suitable purification method could be found, but the reduction product (XIV*d*) could be purified.

5-(p-Aminophenoxypropyl)-2,4,6-triaminopyrimidine (XIVd)—A mixture of 7.6 Gm. (0.025 mole) of crude XIV*b*, 200 ml. of 70% aqueous ethanol, and about 15 Gm. of Raney nickel was shaken with hydrogen at 2–3 Atm. until reduction was complete (4 hr.). The mixture was filtered through a pad of diatomaceous earth[1] then spin-evaporated *in vacuo*. The oily residue readily crystallized on trituration with ethanol. Two recrystallizations from ethanol afforded 3.9 Gm. (56%) of buff-colored crystals, m.p. 179–180°; the compound moved as a single spot on TLC in ethanol. $\lambda_{max.}$ (pH 1): 283 mμ (ϵ 18,400); (H_2O): 279 mμ (ϵ 12,600); (pH 13): 277 mμ (ϵ 12,400). $\nu_{max.}$ 3500, 3400, 3150 (NH); 1650–1575 (broad) (NH, C=C, C=N); 1240 (C—O—C); no nitro band near 1340 cm.$^{-1}$.

Anal.—Calcd. for $C_{13}H_{18}N_6O$: C, 56.9; H, 6.62; N, 30.7. Found: C, 56.7; H, 6.62; N, 30.9.

5-(p-Chloroacetamidophenoxypropyl)-2,4,6-triaminopyrimidine (XIVf)—To a magnetically stirred solution of 822 mg. (3 mmoles) of XIV*d* in 12 ml. of acetone and 12 ml. of 5% aqueous acetic acid cooled in an ice bath was added a solution of 1.026 Gm. (6 mmoles) of chloroacetic anhydride in 6 ml. of acetone. The mixture was stirred at ambient temperature for 2 hr., then the separated solid was redissolved by warming. The solution was poured into 60 ml. of 5% potassium carbonate previously cooled to 0°. The product was collected on a filter and washed with 25 ml. of cold water, then dried. The dry XIV*f* free base was dissolved in a small volume of glacial acetic acid, then slowly precipitated by dropwise addition of ethyl acetate.

[1] Marketed as Celite by Johns-Manville Corp., New York, N. Y.

The *diacetate* salt was collected, washed with ethyl acetate and ether, then dried at room temperature *in vacuo;* yield, 1004 mg. (71%), m.p. 144–146° dec. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500, 3400, 3300–3150 (NH); 1670 (amide I); 1640–1610 (C=C, C=N); 1570 cm.$^{-1}$ (amide II). TLC in chloroform–ethanol (5:3) showed one spot.

Anal.—Calcd. for $C_{15}H_{19}ClN_6O_2 \cdot 2CH_3COOH$: C, 48.5; H, 5.78; N, 17.8. Found: C, 48.7; H, 5.94; N, 17.6.

When dried at 100° in high vacuum to constant weight, the analysis indicated that most of the diacetate had been converted to the free base; the m.p. had now changed to >200° dec.

Anal.—Calcd. for $C_{15}H_{19}ClN_6O_2$: C, 51.6; H, 5.43; O, 10.8. Found: C, 50.9; H, 5.64; O, 10.8.

N-Bromoacetyl-β-alanine—To a magnetically stirred solution of 4.56 Gm. (0.05 mole) of β-alanine and 2.81 Gm. (0.05 mole) of potassium hydroxide in 15 ml. of water cooled in an ice–salt bath was added, in five portions (5 min. apart), 5.25 ml. (0.06 mole) of bromoacetyl bromide; immediately after each portion of bromoacetyl bromide was added in five portions 25 ml. of 2.8 *N* aqueous potassium carbonate. After being stirred an additional 1 hr. at −5 to 0°, the solution was acidified to about pH 3 with 12 *N* aqueous hydrochloric acid, then extracted with ethyl acetate (3 × 75 ml.). The combined extracts were washed with 75 ml. of water, dried with magnesium sulfate, then spin-evaporated *in vacuo*. The residue was leached with hot petroleum ether (b.p. 60–110°) (2 × 50 ml.), then crystallized from chloroform at −20°; yield, 4.07 Gm. (40%), m.p. 71–77°. Two more recrystallizations from chloroform at −20° gave 2.44 Gm. (23%) of white crystals, m.p. 83–84°. $\nu_{max.}^{Nujol}$ 3300 (amide NH); 1700 (carboxyl C=O); 1640 (amide I); 1550 cm.$^{-1}$ (amide II).

Anal.—Calcd. for $C_5H_8BrNO_3$: C, 28.6; H, 3.82; N, 6.70. Found: C, 28.4; H, 3.84; N, 6.55.

No effort was made to obtain optimum yields.

5-[(p-Chloroacetylglycylamido)phenoxypropyl]-2,-4,6-triaminopyrimidine (XIVh) Acetate—To a magnetically stirred solution of 1.516 Gm. (10 mmoles) of *N*-chloroacetylglycine in 30 ml. of reagent ethyl acetate cooled in an ice bath was added 1.236 Gm. (6 mmoles) of *N,N*-dicyclohexylcarbodiimide. After being stirred 2 hr. at ambient temperature, the mixture was filtered and the dicyclohexylurea was washed with two 10-ml. portions of ethyl acetate. The combined filtrate and washings were spin-evaporated *in vacuo* (bath 25°) to about 10 ml. Addition of about 35 ml. of petroleum ether (b.p. 30–60°) gave 0.896 Gm. (67%) of *N*-chloroacetylglycine anhydride that was not further purified and had $\nu_{max.}$ 3300 (NH); 1825, 1760 (anhydride C=O); 1675 (amide I); 1550 cm.$^{-1}$ (amide II).

Reaction of XIV*d* with an excess of this anhydride as described for the preparation of XIV*f* gave 56% of product after recrystallization from glacial acetic acid–ethyl acetate and drying at 100° *in vacuo* for 24 hr. After being dried at 100° in high vacuum, it showed no definite m.p., but gradually decomposed over 200°. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500–3200 (NH); 1780 (amide I); 1660–1600 (NH, C=C, C=N); 1560 cm.$^{-1}$ (amide II). TLC in chloroform–ethanol (5:3) showed a single spot.

Anal.—Calcd. for $C_{17}H_{22}ClN_7O_3 \cdot {}^1/_2CH_3COOH$: C, 49.3; H, 5.50; N, 22.4; O, 14.6. Found: C, 49.2; H, 5.54; N, 22.4; O, 13.7.

5-[(p-Bromoacetyl-β-alaninamido)phenoxypropyl]-2,4,6-triaminopyrimidine (XIVi) Diacetate—To a magnetically stirred solution of 274 mg. (1 mmole) of XIV*d* in 6 ml. of acetone and 2 ml. of 10% aqueous acetic acid cooled in an ice bath was added 643 mg. (1.6 mmoles) of *N*-bromoacetyl-β-alanine anhydride (prepared as described for the anhydride of chloroacetylglycine in tetrahydrofuran as solvent). After 1 hr. at 0°, during which time a salt of XIV*i* separated, the mixture was filtered, and the product was washed with acetone. The product was recrystallized from glacial acetic acid by addition of ethyl acetate; yield, 485 mg. (83%), m.p. 158–161°. A second recrystallization gave 395 mg. (68%), m.p. 160–162°. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500–3200 (NH); 1680–1600 (amide I, C=C, C=N); 1570 cm.$^{-1}$ (amide II). The compound traveled as a single spot on TLC in chloroform–ethanol (5:3); the spot gave a negative Bratton-Marshall test and a positive *p*-nitrobenzylpyridine test (20). For analysis the compound was dried at room temperature in high vacuum.

Anal.—Calcd. for $C_{18}H_{24}BrN_7O_3 \cdot 2CH_3COOH$: C, 45.1; H, 5.50; Br, 13.6. Found: C, 45.1; H, 5.65; Br, 13.9.

REFERENCES

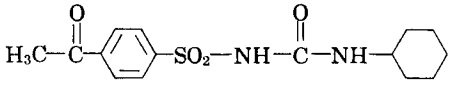
(1) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(2) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(3) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(4) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 470 (1966).
(5) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.
(6) Baker, B. R., and Shapiro, H. S., *J. Pharm. Sci.*, **55**, 308(1966).
(7) Baker, B. R., and Jordaan, J. H., *ibid.*, **54**, 1740 (1965).
(8) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *ibid.*, **55**, 295(1966).
(9) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(10) Baker, B. R., *ibid.*, **53**, 347(1964).
(11) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417 (1966).
(12) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422 (1966).
(13) Baker, B. R., Santi, D. V., Almaula, P. I., and Werkheiser, W. C., *J. Med. Chem.*, **7**, 24(1964).
(14) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(15) Baker, B. R., and Morreal, C. E., *J. Pharm. Sci.*, **52**, 840(1963).
(16) Baker, B. R., Santi, D. V., and Shapiro, H. S., *ibid.*, **53**, 1317(1964).
(17) Baker, B. R., and Shapiro, H. S., *ibid.*, **56**, 33(1967).
(18) Baker, B. R., Ho, B.-T., and Chheda, G. B., *J. Heterocyclic Chem.*, **1**, 88(1964).
(19) Baker, B. R., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1252(1965).
(20) Baker, B. R., Santi., D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(21) Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537(1939).
(22) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(23) Epstein, J., Rosenthal, R. W., and Ess, R. J., *Anal. Chem.*, **27**, 1435(1955).
(24) Baker, B. R., Morreal, C. E., and Ho, B.-T., *J. Med. Chem.*, **6**, 658(1963).
(25) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(26) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(27) Gagnon, P. E., Nadeau, E., and Côté, R., *Can. J. Chem.*, **30**, 592(1952).

Qualitative and Quantitative Tests for Acetohexamide

By EDWARD F. SALIM* and W. W. HILTY†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1 - [(*p* - ACETYLPHENYL)SULFONYL] - 3 - CYCLOHEXYLUREA; $C_{15}H_{20}N_2O_4S$; mol. wt. 324.40. The structural formula of acetohexamide may be represented as:



Physical Properties—Acetohexamide occurs as a practically odorless, white, crystalline powder, m.p 184–189° (U.S.P. class Ia). It is practically insoluble in water and in ether, and slightly soluble in alcohol and in chloroform. It is soluble in pyridine and in dilute solutions of alkali hydroxides.

Identity Tests—A 1 in 100,000 solution of acetohexamide in 0.01 *N* sodium hydroxide exhibits an ultraviolet absorbance maximum at about 249 mμ [absorptivity (*a*) about 43]. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of acetohexamide in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Dissolve about 100 mg. of 2,4-dinitrophenylhydrazine in 1 ml. of sulfuric acid and cautiously add 5 ml. of alcohol. Cool, filter if necessary to obtain a clear solution, add 5 ml. of a solution of acetohexamide in hot alcohol (1 in 200), and warm on a steam bath: an orangish yellow precipitate is formed.

Purity Tests—Dry about 1 Gm. of acetohexamide, accurately weighed, at 105° for 3 hr.: it loses not more than 1% of its weight.

Char about 1 Gm. of acetohexamide, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the heavy metals content of acetohexamide by the U.S.P. heavy metals test, method II: the heavy metals limit for acetohexamide is 20 p.p.m.

Assay—Transfer about 300 mg. of acetohexamide, accurately weighed, to a 125-ml. conical flask, and dissolve in 40 ml. of dimethylformamide. Add 5 drops of thymol blue T.S., and titrate with 0.1 *N* sodium methoxide, using a magnetic stirrer and

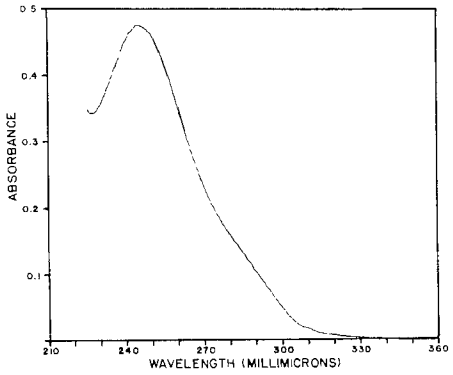


Fig. 1—Ultraviolet absorption spectrum of acetohexamide in 0.01 N sodium hydroxide (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

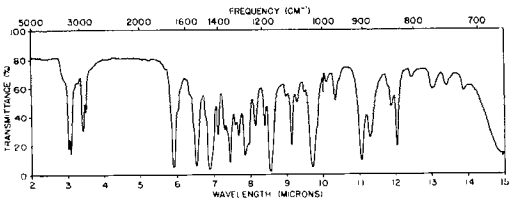


Fig. 2—Infrared spectrum of acetohexamide in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Received September 2, 1966, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication November 30, 1966.

† Eli Lilly and Co., Indianapolis, IN 46206. Eli Lilly and Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The authors express their appreciation to J. M. Woodside and E. G. Spinks, Eli Lilly and Co., and Carolyn Damon, Drug Standards Laboratory, for valuable contributions to the monograph.

taking precautions against absorption of atmospheric carbon dioxide. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 32.44 mg. of $C_{15}H_{20}N_2O_4S$. The amount of acetohexamide found is not less than 97% and not more than 101%, calculated on the dried basis.

DOSAGE FORMS OF ACETOHEXAMIDE

Acetohexamide Tablets

Identity Tests—The final solution prepared from the tablet sample in the *Assay* exhibits an absorbance maximum and minimum at the same wavelengths as the acetohexamide standard solution.

Assay—Weigh and finely powder not less than 20 acetohexamide tablets. Weigh accurately a portion of the powder, equivalent to about 500 mg. of acetohexamide, and transfer to a 100-ml. volumetric flask. Add about 60 ml. of 0.1 *N* sodium hydroxide and shake for 30 min. Dilute to volume with water, and mix. Filter the solution, discarding the first portion of filtrate, and transfer 20.0 ml. into a 125-ml. separator. Add 2 ml. of diluted hydrochloric acid and extract with four 40-ml. portions of chloroform, filtering each portion through chloroform-washed paper into a 200-ml. volumetric flask. Make to volume with chloroform, and mix. Pipet 20.0 ml. of this solution into a suitable beaker and evaporate to dryness on a steam bath. Transfer the residue quantitatively, with the aid of 0.1 *N* sodium hydroxide, into a 100-ml. volumetric flask, add alkaline solution to volume, and mix. Dilute 10.0 ml. of this solution to 100.0 ml. with water, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of acetohexamide, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 249 mμ, with a suitable spectrophotometer, using 0.01 *N* sodium hydroxide as the blank. Calculate the quantity, in milligrams, of $C_{15}H_{20}N_2O_4S$ in the portion of tablets taken by the formula $50C \times (A_u/A_s)$, in which C is the exact concentration of the standard solution, in micrograms per milliliter, A_u is the absorbance of the sample solution, and A_s is the absorbance of the acetohexamide standard solution. The amount of acetohexamide found is not less than 90.0% and not more than 110.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Acetohexamide[1] is an oral hypoglycemic agent effective in the treatment and management of selected patients with stable diabetes mellitus. The reduction of blood sugar levels by sulfonylurea drugs is achieved primarily by stimulating the release of endogenous insulin and secondarily by inhibition of glucose formation from liver glycogen.

Identity Tests—The qualitative tests included for acetohexamide provide suitable differentiation from official sulfonylurea compounds. The ultraviolet absorption maximum of acetohexamide in alkaline solution is exhibited at about 249 mμ as compared to 230 and 228 mμ for chlorpropamide and tolbutamide, respectively, in the same medium. The formation of the hydrazone derivative with acetohexamide which does not occur with chlorpropamide nor tolbutamide constitutes a rapid distinctive test.

Quantitative Tests—The nonaqueous titration of acetohexamide with sodium methoxide using thymol blue T.S. gave an average value of 100.2 ± 0.5%.[2] Satisfactory recoveries can be obtained by dissolving acetohexamide in a known excess of 0.1 *N* sodium hydroxide and back titration of the excess base with 0.1 *N* hydrochloric acid using phenolphthalein T.S. Neutralized alcohol is added prior to the back titration to retard precipitation of acetohexamide at the end point. Spectrophotometric analyses of commercial tablets labeled to contain 250 and 500 mg. of acetohexamide gave average values of 102.2 ± 0.7%[2] and 100.6 ± 0.4%[2] of the labeled amounts, respectively.

[1] Marketed as Dymelor by Eli Lilly and Co., Indianapolis, Ind.

[2] Maximum deviation from the mean value.

Assay of Methotrexate in the Presence of Its Decomposition Products and Other Folic Acid Analogs

By JOSEPH F. GALLELLI and GLENN YOKOYAMA

Extensive studies with the folic acid antagonist methotrexate in cancer chemotherapy prompted the development of an assay for methotrexate in the presence of its decomposition products and other folic acid analogs. This was accomplished by quantitative chromatographic separation and spectrophotometric analysis of intact methotrexate and its decomposition products. Methotrexate U.S.P. samples were assayed and found to be at best approximately 90 per cent pure.

THE PRESENT U.S.P. method of assay for methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) is based solely on the characteristic ultraviolet absorption of the compound. However, spectrophotometric studies of this type are subject to several limitations. Methotrexate is known to be contaminated with its decomposition products and other closely related folic acid analogs, often differing by as little as one functional group. The similarity of the ultraviolet absorption characteristics of methotrexate, its decomposition products, and other folic acid analogs requires that some type of quantitative separation and purification technique be utilized prior to a spectrophotometric assay of the intact methotrexate in the ultraviolet region.

Although paper chromatography (1) has been used for the separation and identification of closely related derivatives of pteroylglutamic acid, it is not suitable for purification and resolution of folic acid analogs on the preparative scale of milligram or gram quantities. Furthermore, compounds in the folic acid series have been reported (2, 3) to be degraded when subjected to variations of paper chromatography due to photolytic breakdown and transformation due to extreme pH changes.

Column chromatography of proteins on a cellulose anion exchange adsorbent has been described by Sober and Peterson (4). Mixtures of proteins were separated using gradients of pH and salt concentrations for elution. Heinrich *et al.* (5) have employed a strongly basic anion-exchange resin[1] for the resolution of mixtures of certain folic acid analogs.

Oliverio (6) applied diethylaminoethyl (DEAE) cellulose and linear gradient elution by means of phosphate buffers to separate folic acid analogs in milligram quantities. The present study involved an adaptation of this method. A specific method of assay was developed for methotrexate in the presence of its decomposition products and other folic acid analogs, utilizing in part modified techniques and methods of separation and purification.

EXPERIMENTAL

Reagents—Methotrexate[2] U.S.P., m.p. 182–189°, and sodium methotrexate for injection,[3] 50 mg. methotrexate per vial. One of the decomposition products of methotrexate, N^{10}-methylpteroylglutamic acid, had m.p. 224–228°. Stock solutions of 0.1 *M* and 0.4 *M* ammonium bicarbonate were prepared and adjusted to pH 8.3 with ammonium hydroxide U.S.P. Diethylaminoethyl (DEAE) cellulose,[4] 0.75 meq./Gm., was passed through a 100-mesh screen and prepared batch-wise by successive washings with 1 *N* sodium hydroxide, water, 1 *N* hydrochloric acid, water, until pH 5.6 to 6.5. The cellulose was finally washed with 0.1 *M* ammonia–ammonium bicarbonate buffer until pH was approximately 8.3 and then stored in the refrigerator. All other chemicals used were of reagent grade.

Apparatus—A Gilson Medical Electronics (GME) volumetric fractionator model V 15-square[5] was used for fractionations. The eluent was monitored by a GME ultraviolet absorption meter, model UV-265 1F[5] and transmittance recorded on a Texas Rectilinear recording milliammeter.[6] A Sigmamotor pump[7] was employed to monitor rates of approximately 2 ml. per minute. Borosilicate glass containers, 1-cm. inner diameter and 25-cm. in length, held the cellulose adsorbent. A Beckman DU spectrophotometer was used for spectrophotometric determinations.

Chromatographic Separation Procedure—A dilute suspension of the washed DEAE cellulose in 0.1 *M* ammonia–ammonium bicarbonate buffer was added to a column and packed by gradually increasing air

Received October 4, 1966, from the Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, U. S. Public Health Service, Bethesda, MD 20014

Accepted for publication November 10, 1966.

The authors express their appreciation to Mrs. Altice Frazier for her technical assistance.

[1] Marketed as Dowex 1 (chloride form) by the Dow Chemical Co., Midland, Mich.

[2] Obtained from Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y.

[3] Marketed as Methotrexate Sodium Parenteral by Lederle Laboratories.

[4] Marketed as Cellex-D by California Corporation for Biochemical Research, Los Angeles, Calif.

[5] Gilson Medical Electronics, Middleton, Wis.

[6] Texas Instruments, Inc., Houston, Tex.

[7] Sigmamotor, Inc., Middleport, N. Y.

pressure from 2 to 10 p.s.i. Five milliliters of a sample solution representing theoretically 5 mg. of methotrexate in 0.1 *M* ammonia–ammonium bicarbonate buffer was allowed to run slowly into the prepared column of washed DEAE cellulose. The methotrexate is separated from its decomposition products and other folic acid analogs by a linear gradient with pH 8.3 ammonia–ammonium bicarbonate buffer of increasing molarity. The mixing chamber contained 500 ml. of 0.1 *M* ammonia–ammonium bicarbonate buffer, and the reservoir contained 500 ml. of 0.4 *M* ammonia–ammonium bicarbonate buffer. The column was attached *via* polyethylene tubing to the automatic fraction collector. The transmittance of the eluent was continually monitored by a 265 mμ ultraviolet light source and per cent transmittance plotted on a rectilinear recorder. Five-milliliter fractions were collected at the rate of 2 ml. per minute. All chromatographic operations were carried out at room temperature in subdued light to protect the sample from any possible photochemical effects. The peak tubes, as indicated by the per cent transmittance on the chromatogram, were then pooled and the ultraviolet absorption spectrum (230 to 340 mμ) of each peak was measured on a Beckman DU spectrophotometer.

Determination of the Purity of Methotrexate—The sample solution of 1 mg. methotrexate per ml. before fractionation was diluted to 1 mg. per 100 ml. with ammonia–ammonium bicarbonate buffer, pH 8.3, and its absorbance at a wavelength of 302 mμ was recorded on a Beckman DU spectrophotometer. The sample solution was then fractionated as described under *Chromatographic Separation Procedure*. The major peak (methotrexate) tubes were pooled, and a dilution to a theoretical 1 mg./100 ml. solution was prepared and its absorbance recorded at a wavelength of 302 mμ on a spectrophotometer. The purity of methotrexate was determined by calculating the per cent of its absorbance at a wavelength of 302 mμ recovered following chromatographic separation. Further confirmation of the purity of the methotrexate peak was obtained by taking the pooled fractionated solution from the major peak (methotrexate) and refractionating it by first concentrating the methotrexate fractions *via* freeze-drying, followed by reloading on a fresh column utilizing the chromatographic separation procedure previously described.

Identification of the Decomposition Products—In addition to the major peak (methotrexate) on the chromatogram (Fig. 1), three other peaks were obtained. The tubes of each peak were pooled and the ultraviolet absorption spectra (230 to 340 mμ) were recorded on a Beckman DU spectrophotometer. (See Fig. 2.) The identities of the single peaks were then obtained by comparing the ultraviolet curve with that of the authentic sample.

RESULTS AND DISCUSSION

Chromatographic separation of methotrexate U.S.P. is shown in Fig. 1, in which the per cent transmittance has been plotted against the volume of ammonia–ammonium bicarbonate buffer (eluent). Figure 1 shows, in addition to the major peak (methotrexate), the transmittance of three other peaks. These indicated that contaminants or hydrolysis products were present in the methotrexate U.S.P. sample. The hydrolysis products appearing as peaks 1, 2, and 4 on the chromatogram all absorbed at the wavelength of maximum absorbance, about 302 mμ, for methotrexate (see Fig. 2) and accounted for approximately 10% of the methotrexate sample. When the methotrexate fraction was collected, freeze-dried, reconstituted, and refractionated, none of the possible decomposition products showed up in the chromatogram seen in Fig. 3.

The spectrum of the major decomposition product of methotrexate U.S.P. was identical to an authentic sample of N^{10}-methylpteroylglutamic acid. This hydrolysis product appearing as the last peak (No. 4) on the chromatogram accounts for approximately one-half of the total impurities. The molar ab-

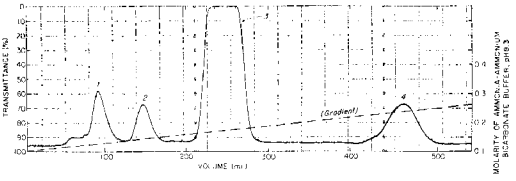


Fig. 1—Chromatogram of methotrexate U.S.P. from DEAE ion-exchange column by linear gradient elution from 0.1 M *to 0.4* M *ammonia–ammonium bicarbonate buffer, pH 8.3. Gradient is represented by dashed line.*

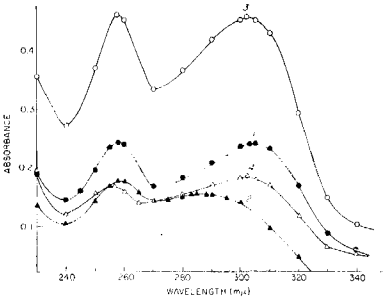


Fig. 2—Ultraviolet spectra of peaks 1, 2, 3, and 4 obtained from chromatographed methotrexate U.S.P. in ammonia–ammonium bicarbonate buffer, pH 8.3. Key: ○, peak 3 (methotrexate); ●, peak 1; △, peak 4 (N^{10}-methylpteroylglutamic acid); ▲, peak 2.

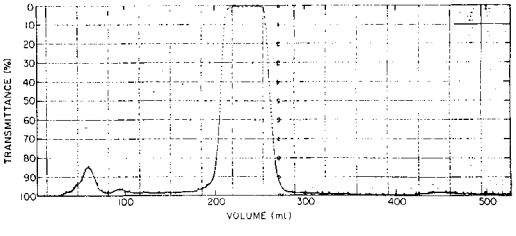


Fig. 3—Chromatogram of refractionated methotrexate U.S.P. from DEAE ion-exchange column by linear gradient elution with ammonia–ammonium bicarbonate buffer, pH 8.3.

sorptivity of N^{10}-methylpteroylglutamic acid was determined, at a wavelength of 302 mμ, and found to be the same as that of the parent methotrexate compound. The molar absorptivities of the unidentified first and second peaks were assumed because of their spectral characteristics of pteroyls to be essentially the same as that of the parent methotrexate compound. It should be noted that molar absorptivity values have been reported in the literature (7, 8) for analogs of pteroyl and pteroylglutamic compounds and found to be approximately the same as that for methotrexate at a wavelength of 302 mμ in alkaline media.

Specificity of Assay for Intact Methotrexate—The present U.S.P. XVII method of assay for methotrexate consists of determining and comparing the absorbance of both the solution of an unknown sample and a standard solution of a U.S.P. methotrexate reference substance (theoretically 100% methotrexate) at the wavelength of maximum absorbance at about 306 mμ with a suitable spectrophotometer using 0.1 *N* hydrochloric acid as the blank. This method is unsatisfactory for distinguishing intact drug from decomposition products, because all decomposition products isolated were shown (see Fig. 2) to absorb at the wavelength of maximum absorbance at about 302 mμ for methotrexate.

The quantitative chromatographic separation of a solution of a methotrexate sample followed by the determination of the absorbance of the methotrexate fraction at a wavelength of 302 mμ on a suitable spectrophotometer does provide a specific method of assay for the intact drug in the presence of its decomposition products and other folic acid analogs. Also, the assay method is such that it might not require the use of a U.S.P. methotrexate reference substance as employed in the U.S.P.

Calculation of Intact Methotrexate—The following formula would permit calculation of per cent intact methotrexate (% IM) on an anhydrous basis at a wavelength of 302 mμ in a 0.1 *M* ammonia–ammonium bicarbonate buffer, pH 8.3:

$$\% \text{ IM} = (A_u/A_s)\,(100 - \% \text{ water})$$

where A_u is the absorbance of the methotrexate solution after chromatographic separation, and A_s is the absorbance of the methotrexate solution before chromatographic separation. The absorptivity (a) of pure or intact methotrexate at a wavelength of 302 mμ in 0.1 *M* ammonia–ammonium bicarbonate buffer, pH 8.3, was calculated in terms of anhydrous methotrexate and found to be 53.9. The above formula can be further expressed as

$$\% \text{ IM} = \frac{(A_u)\,(10)\,(d)}{53.9\,(W_s)}$$

where A_u = absorbance of the chromatographed methotrexate solution whose fractions made up to 100 ml. are diluted to a theoretical 1 mg. methotrexate per 100 ml. solution, d = dilution factor required to dilute the methotrexate fractions made up to 100 ml. to a theoretical 1 mg. methotrexate per 100 ml. solution, and W_s = weight, in grams, of methotrexate sample put on the column.

SUMMARY

A chromatographic separation followed by a spectrophotometric method of analysis is reported for the determination of methotrexate in the presence of its products of hydrolytic decomposition and other folic acid analogs.

All methotrexate samples assayed were found to be at best approximately 90% pure methotrexate.

Recommendation is made for revision of the present U.S.P. method of assay for methotrexate to the one discussed in this paper.

REFERENCES

(1) Kwienty, H., and Bergmann, F., *J. Chromatog.*, **2**, 162(1959).
(2) Albert, A., Brown, D. J., and Cheesemen, G., *J. Chem. Soc.*, **1951**, 474.
(3) Jukes, T. H., *Science*, **120**, 324(1954).
(4) Sober, H. A., and Peterson, E. A., *Federation Proc.*, **17**, 1116(1958).
(5) Heinrich, M. R., Dewey, V. C., and Kidder, G. W., *J. Chromatog.*, **2**, 296(1959).
(6) Oliverio, V. T., *Anal. Chem.*, **33**, 263(1961).
(7) Cosulich, D. B., and Smith, J. M., *J. Am. Chem. Soc.*, **70**, 1924(1948).
(8) Seeger, D. R., Cosulich, D. B., Smith,, J. M., Jr. and Hultquist, M. E., *ibid.*, **71**, 1755(1949).

Improved Method of Assay of Pyrvinium Pamoate and Its Official Preparations

By H. D. BECKSTEAD and S. J. SMITH

A simple, rapid, and reliable assay procedure for pyrvinium pamoate and its official preparations using 80 per cent acetic acid solutions and absorption measurement at 508 mμ is presented. Results with this method are found to be more precise and can be obtained with greater facility than with the U.S.P. method. In addition a thin-layer chromatography system using Silica Gel G and dioxane–water (3:1), for the identification of pyrvinium base is described.

THE ASSAY procedure described in the "United States Pharmacopeia" XVII (1) for pyrvinium pamoate {6-dimethylamino-2-[2-(2,5-dimethyl-1-phenyl-3-pyrrolyl)vinyl]-1-methylquinolinium 4,4′-methylenebis[3-hydroxy-2-naphthoate]} specifies the use of low actinic glassware and minimal exposure to light of the sample being analyzed. In the revised method of assay of the bulk drug (2) the use of sodium hydroxide in methoxyethanol has been deleted since this was found to depress the absorbance of solutions.

Analysis of oral suspensions and tablets containing pyrvinium pamoate requires (in addition to protecting the sample from unnecessary exposure to bright light) the use of a rather involved partition chromatography system [both phases of chloroform–hexane–water–methoxyethanol–acetic acid (30:20:10:10:1) on acid washed siliceous earth].

In this laboratory, results from analyses of official preparations of pyrvinium pamoate were found to be closely coupled to the rate of column elution. A maximum elution rate of 4 ml./min., as specified in the U.S.P., gave results with very low values and broad analytical ranges (Table I). When the elution rate was held at a maximum of 3 ml./min. (elution time: 30 to 35 min.) reasonable results were obtained. However, when duplicate samples of U.S.P. reference standard pyrvinium pamoate were eluted through columns on each of 3 consecutive days, a broad analytical range was obtained while standard deviations between days varied from 1.53 to 2.45.

This apparent sensitivity of the official method to variation in elution rates (and perhaps variation in column density), together with the resulting imprecision and inaccuracy, led to the search for a more satisfactory assay procedure.

The only other method of assay of pyrvinium pamoate apparent in the literature is that described by Alves, who used absorption measurement in dimethylformamide (DMF) and acetic acid solutions (3). In this paper a simple, rapid, and reliable spectrophotometric assay procedure for pyrvinium pamoate and its preparations employing 80% acetic acid solutions is reported.

Received June 9, 1966, from the Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, Canada.

Accepted for publication November 14, 1966.

The authors thank Dr. L. Levi and Dr. W. N. French for their comments and Dr. D. F. Bray and Mr. J. M. Airth for their statistical evaluations.

EXPERIMENTAL

Light absorption measurements in stability studies were made using a Beckman model DK2 and in assays a Beckman model DU spectrophotometer. Both were calibrated previously by means of a mercury source.

Solvents—Reagent grade DMF, acetic acid, dimethylsulfoxide, and methoxyethanol were used as supplied. Technical grade formamide was distilled (b.p. 97–99°/14 mm. Hg) before use.

Stability Studies—Ten micrograms per milliliter solutions of pyrvinium pamoate (U.S.P. reference standard) in the various solvents were prepared and their absorbances recorded at suitable time intervals.

Assay Procedure—*U.S.P.*—Pyrvinium pamoate: assay according to the official revised procedure (2). Oral suspension and tablets: assay as described in the U.S.P. (1) observing either the maximum elution rate of 4 ml./min. or 3 ml./min.

Proposed Method—Standard solution: accurately weigh 10 mg. of U.S.P. reference standard pyrvinium pamoate and transfer to a 10-ml. volumetric flask. Add approximately 5 ml. of 80% acetic acid, shake mechanically for 5 min., make up to volume, and mix. Dilute 1.0 ml. of this solution to 100 ml. in a volumetric flask with 80% acetic acid.

Pyrvinium pamoate: prepare as described for the standard solution.

Oral suspension: into a 100-ml. volumetric flask weigh by difference 0.7 Gm. oral suspension. Add 50 ml. 80% acetic acid, shake mechanically for 5 min., make up to volume, and mix. Dilute 10 ml. of this solution to 100 ml. with 80% acetic acid in a volumetric flask.

Tablets: determine the average weight of 20 tablets. Powder 5 tablets and transfer an aliquot equivalent to about 50 mg. pyrvinium base to a 100-ml. volumetric flask. Add 50 ml. 80% acetic acid, shake mechanically for 5 min., make up to volume, mix, and filter through Whatman No. 1 paper, or equivalent. Pipet 1 ml. of the filtrate into a 100-ml. volumetric flask and dilute to volume with 80% acetic acid.

Measure the absorbance of all solutions at 508 mμ on a suitable instrument using 80% acetic acid as a blank.

TABLE I.—ANALYSIS OF PYRVINIUM PAMOATE

Method →	U.S.P. XVII Method, 4 ml./min. Max. Elution			U.S.P. XVII Method, 3 ml./min. Max. Elution			80% Acetic Acid				80% Acetic Acid				N,N-Dimethylformamide				N,N-Dimethylformamide				Dimethylsulfoxide[e]	
Glassware →	Low Actinic			Low Actinic			Low Actinic				Normal				Low Actinic				Normal				Low Actinic	
Preparation	Av.,[a] %	Range, %	S.D.	Av.,[a] %	Range, %	S.D.	Av.,[a] %	Range, %	S.D.		Av.,[a] %	Range, %	S.D.		Av.,[a] %	Range, %	S.D.		Av.,[a] %	Range, %	S.D.		Av.,[b] %	Range, %	S.D.
Powder	95.72	5.05	1.82[d]	98.08	3.15	1.15[e]	99.49	0.64	0.25		105.51	7.55	2.80		99.50	0.32	0.13		97.08	3.42	1.25		106.19	20.97	3.29
Oral suspension No. 1	85.64	12.42	4.30	94.01	6.29	2.20	92.73	0.60	0.26		93.19	2.93	1.06		92.53	0.60	0.26		88.81	3.42	1.19		102.39	1.07	0.57
Oral suspension No. 2	89.30	5.75	2.00	...	...	...	91.93	0.82	0.28		92.45	2.05	0.72		92.03	0.70	0.30		90.69	3.54	1.36		102.81	0.84	0.47
Oral suspension No. 3	80.63	8.43	3.41	97.02	5.13	2.00	103.88	0.59	0.23		101.08	0.62	0.24		104.48	0.58	0.20		101.15	1.97	0.82		101.95	0.49	2.58
Oral suspension No. 4	79.93	15.63	5.73	92.73	4.66	1.27	91.63	1.10	0.43		90.87	1.01	0.38		91.80	0.60	0.23		88.96	0.77	0.30		105.05	5.06	0.29
Tablets No. 1	87.39	13.37	5.10	89.99	4.20	1.64	97.30	0.60	0.22		99.21	1.52	0.64		98.68	0.90	0.37		95.97	2.75	1.05		99.39	19.94	11.10
Tablets No. 2	82.81	9.54	3.69	84.27	3.44	1.18	93.65	0.72	0.35		94.06	7.14	2.29		94.41	0.84	0.30		93.21	3.18	1.06		110.41	17.36	9.40
Simulated suspension[f]	...	...	...	...	...	...	99.51	0.97	0.29		...	...	...		...	...	...		...	...	...		...	...	...
Simulated tablet[f]	...	...	...	...	...	...	100.14	0.70			...	...	...		...	...	...		...	...	...		...	...	...

[a] Average of six assays. [b] Average of three assays. [c] Formamide was also tried; results for powder were 108.12%; 106.56%; 102.64%. Mean: 106.56%; standard deviation: 3.28. [d] Superceded procedure for powder. [e] Revised procedure for powder. [f] Corresponding in composition to a commercial preparation.

Calculations—For pyrvinium pamoate:

$$\frac{A \times \text{wt. of ref.}}{B \times \text{wt. of sample taken}} \times 100 = \% \text{ (w/w) pyrvinium pamoate} \quad \text{(Eq. 1)}$$

For the oral suspension:

$$\frac{A \times SG \times 0.6644}{B \times \text{wt. of sample taken}} \times 100 = \% \text{ (w/v) pyrvinium base} \quad \text{(Eq. 2)}$$

For tablets:

$$\frac{A \times 100 \times \text{av. wt./tablet (Gm.)} \times 0.6644}{B \times \text{wt. of sample taken (Gm.)} \times \text{labeled wt. (mg.)}} \times 100 = \% \text{ (w/w) pyrvinium base/tablet} \quad \text{(Eq. 3)}$$

where

A = absorbance of sample solution,
B = absorbance of standard solution,
SG = specific gravity of the suspension,
0.6644 = ratio of the formula weight or pyrvinium base to one-half of the molecular weight of pyrvinium pamoate (the pamoate moiety being dibasic).

Thin-Layer Chromatography

Plates—Prepare plates with standard equipment for thin-layer chromatography (*cf. Reference 4*). To obtain five plates 20 × 20 cm., mix 25 Gm. of Silica Gel G (Merck) with 50 ml. of 0.5 *N* NaOH. Apply the slurry to plates (layer thickness about 250 μ), air dry, and activate within 1 hr. of use by heating at 110° for 20 min.

Solvent System—1,4-Dioxane–water (3:1 v/v).

Spray Reagents—(*a*) Mix 20 ml. of 10% w/v aqueous $FeCl_3$ and 10 ml. 5% w/v aqueous $K_3Fe(CN)_6$ with 70 ml. water. (*b*) Prepare 2 *N* HCl.

Preparation of Samples—Dissolve sufficient bulk drug, suspension, or powdered tablet in methoxyethanol to give a concentration of about 1 mg./ml. pyrvinium pamoate. If the solution is turbid, filter or centrifuge it before spotting.

Chromatographic Procedure—Equilibrate a suitable jar with the chromatographic solvent. Spot 5 μl. of solutions being examined next to a similar amount of U.S.P. reference pyrvinium pamoate. Insert the plate in the chromatographic jar and allow the solvent to rise to a height of 15 cm. (about 2 hr.). Air dry plates and view under shortwave U.V. light or treat with spray (*a*) followed by spray (*b*).

RESULTS AND DISCUSSION

The revised assay of pyrvinium pamoate has deleted the use of sodium hydroxide in the methoxyethanol. Figures 1 and 2 clearly indicate the deleterious effects of this base. Furthermore, the figures indicate that the degradative effects of the base are greatly in excess of breakdown due to actinic activity, since the sample curves obtained for solutions stored in both low actinic and normal glassware are superimposable (Figs. 1 and 2). The deleterious activity of sodium hydroxide is further demonstrated when the lower analytical results from the now-superseded procedure for pyrvinium pamoate powder (1) are compared with data obtained from the revised method (2) (Table I).

The use of heat recommended to aid solution of

pyrvinium pamoate may reduce the accuracy of the official method of assay due to possible breakdown of the drug. Furthermore, analyses of oral suspensions and tablets require that aliquots of material subjected to assay be adsorbed on chromatographic support prior to transfer to the column. Each of these factors increases the exposure of the sample to light and introduces the possibility of error. A major factor causing variability in assay results employing the chromatographic column was found to be variation in the rate of column elution. Elution of the column at a maximum rate of 4 ml./min. as specified in the U.S.P. gave results which were, in all cases, below U.S.P. limits and exhibited broad analytical ranges (Table I). In all analyses pink material was noted to remain on the column. However, when the elution rate was kept at a maximum of only 3 ml./min. (elution time: 30–35 min.), results and standard deviations were found to be reasonable (Table I).

Relatively minor variations in elution rates, and perhaps also in the density of the column packing, may have given rise to relatively broad ranges in the U.S.P. assay results. This can be inferred from the data obtained when duplicate samples of U.S.P. reference material were chromatographed in a uniform manner on 3 consecutive days. Standard deviations between pairs of results were 1.82, 1.53, and 2.45. The six results examined *in toto* had a standard deviation of 3.49. The variation in absorbance of the eluates was the equivalent of an analytical range of 9.29%. Thus in addition to variation within the sample itself inconsistencies are even obtained with the reference standard using the official method.

To find a suitable medium for the assay of pyrvinium pamoate, the stability of the drug in 80% acetic acid, DMF, dimethylsulfoxide, and methoxyethanol in low actinic and normal glassware was determined (Figs. 1 and 2). Solutions kept in low actinic glassware were found to be quite stable, in all cases exhibiting at least 97% of the original absorption at 508 mμ after 60 min. Solutions stored in normal glassware were somewhat less stable (Fig. 2), showing a rapid initial decrease in absorption and thereafter their absorbances remaining fairly constant. No satisfactory absorption curve could be obtained with formamide solutions of the drug, although the trend of readings appeared to indicate that the order of stability was very similar to that in solvents listed above.

Concomitant with these studies, assays of pyrvinium pamoate and its preparations were carried out in both low actinic and normal glassware employing the five solvent systems listed in Table I. Results obtained are shown therein. As anticipated, low actinic glassware always afforded more reliable results than normal glassware. With formamide and also with dimethylsulfoxide solutions, satisfactory results could not be obtained. Assays were calculated as shown under *Experimental*, and for consistency all figures in Table I have been quoted as per cent of labeled claim. To conform with the U.S.P. procedure, values quoted for the suspension (Eq. 2; Table I) should be divided by 100 to read grams per 100 ml.

The data in Table I indicate that results from analyses carried out in dimethylsulfoxide and formamide (powder only) gave the highest values.

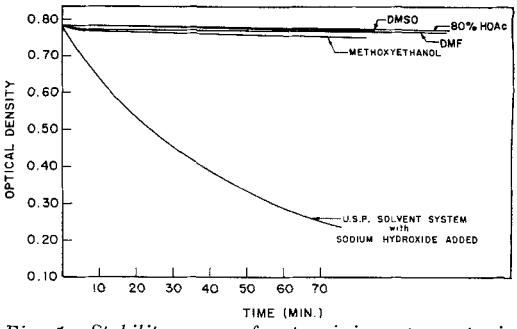


Fig. 1—Stability curves for pyrvinium pamoate in low actinic glassware.

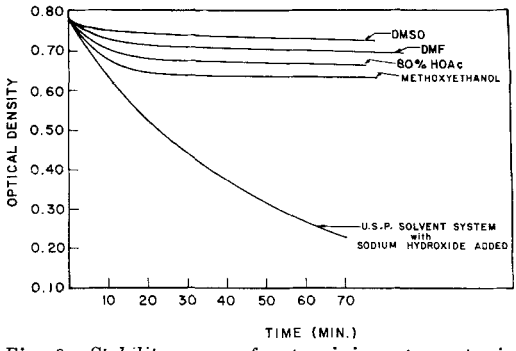


Fig. 2—Stability curves for pyrvinium pamoate in normal glassware.

In addition, results from both of these methods exhibited, in some instances, large standard deviations. Analyses based on the use of both 80% acetic acid and DMF in low actinic glassware showed consistently smaller experimental ranges and smaller standard deviations (probability <0.05) than analyses based on the other systems. These ranges were also smaller than those obtained from assays using 80% acetic acid and DMF in normal glassware. There is little difference in the variability between 80% acetic acid and DMF solvent systems when low actinic glassware (aluminum foil wrapped around the volumetric flask suffices) is employed. However, the greater stability of pyrvinium pamoate in acetic acid solution compared to DMF solution (Fig. 1), coupled with the more general availability, lower toxicity, and lower cost of acetic acid, leads to the proposal of the acetic acid solvent system as the medium of choice for the assay of pyrvinium pamoate and its preparations.

Most of the preparations of pyrvinium pamoate examined did not completely dissolve in methoxyethanol. In addition, when some brands were brought into contact with the solvent, a copious precipitate was formed which appeared to adsorb pyrvinium pamoate, thus making it difficult to obtain homogeneous aliquots for subsequent chromatography. This source of error was eliminated by the use of 80% acetic acid in which all excipients were found to be completely soluble.

To confirm the accuracy of the proposed method a simulated tablet as well as a suspension was prepared and assayed according to the new method.

The values shown in Table I are per cent recovery. These results appear to confirm that the proposed method is both accurate and precise. In addition to its greater precision, it is also much faster than the official method; about 8 min. compared with near 40 min. in the latter.

Thin-layer chromatographic examination of official dosage forms of pyrvinium pamoate and a U.S.P. reference standard indicated that the drug consists of a number of components. Pyrvinium pamoate contained in official preparations separated into two major red spots at R_f 0.33 and 0.44 and a minor red spot at R_f 0.50. Upon spraying, these spots stained intense blue, while pamoic acid, previously visible only by fluorescence at R_f 0.50, stained light blue. U.S.P. reference standard pyrvinium pamoate was found to contain all of the above components as well as another minor red constituent, staining intense blue, at R_f 0.15. Pamoic acid could be separated from the red material also appearing at R_f 0.50 by using 0.1 *N* NaOH instead of 0.5 *N* NaOH in preparing the chromatographic plates (new R_f of pamoic acid: 0.69).

The occurrence of a greater number of red spots than expected, on the basis of two isomeric configurations of the compound, should not be attributed to the degradative effect of sodium hydroxide in the aqueous system since, for example, chromatography with chloroform–ethanol (7:3) on neutral Silica Gel G (Merck), although giving very poor separation, clearly indicated three red constituents in commercial and four such components in U.S.P. reference standard pyrvinium pamoate. These compositional characteristics of the drug will be examined further.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 548–551.
(2) "First Interim Revision Announcement," U.S.P. XVII, August 1965, p. 3.
(3) Alves, M. A. P., *Rev. Port. Farm.*, **14**, 13(1964); through *Chem. Abstr.*, **61**, 9357*c*(1964).
(4) Stahl, E., "Thin-Layer Chromatography," Academic Press Inc., New York, N.Y., 1965, p. 5 *ff*.

Analysis of Combinations Containing Phenylephrine in Liquid Dosage Forms

By K. O. MONTGOMERY, P. V. JENNINGS, and M. H. WEINSWIG

A method is presented for the isolation, separation, and determination of phenylephrine, an antitussive, and an antihistamine in commercial products containing the combination. All three compounds are extracted with strong cation exchange resin AG 50W-X4. The phenylephrine is eluted from the resin bed with 8.0 *N* phosphoric acid. The antitussive is then eluted with 1.0 *N* hydrochloric acid in 60 per cent methanol in water. Finally the antihistamine is eluted with 3.5 *N* hydrochloric acid in 40 per cent methanol in water. The compounds are determined by subjecting the eluates to ultraviolet spectrophotometry. The method is used successfully on several commercial products.

THE ASSAY of complex formulations containing several active ingredients is usually a difficult and time-consuming problem. This is especially true if the active ingredients are similar in their chemical and physical properties. Ion-exchange chromatography was selected as the method of choice for this assay because of its ability to isolate completely the cationic active ingredients from the inactive ingredients, and separate these active ingredients by selective elution. Each ingredient was then quantitatively eluted in a form suitable for determination by ultraviolet spectrophotometry.

Ion-exchange chromatography has been used extensively to accomplish difficult separations of inorganic ions. Hofer (1) used an ion-exchange resin[1] to absorb calcium and magnesium from tissue extracts. Magnesium was then eluted with 2 *N* hydrochloric acid and calcium was eluted with 3 *N* hydrochloric acid. Grasselly (2) used anion-exchange resins to separate iron, aluminum, manganese, calcium, and magnesium. Pollard (3) separated magnesium, strontium, barium, and calcium on the ion-exchange resin[1] utilizing a gradient elution technique. Pitstick and associates (4) separated magnesium, cobalt, zinc, copper, and iron with anion-exchange resins.

Most of the references to organic separations utilize a simple adsorption–elution technique to separate anions or cations from neutral components. Wang and Hunter (5) assayed 11 different alkaloids separately by adsorbing them on an ion-exchange resin,[2] eluting with glacial acetic acid, and titrating the eluate with perchloric acid.

Morphine has been separated from the nonphenolic alkaloids of opium utilizing ion exchange (6). Blake and associates (7, 8) applied tech-

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Accepted for publication November 29, 1966.

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The values shown in Table I are per cent recovery. These results appear to confirm that the proposed method is both accurate and precise. In addition to its greater precision, it is also much faster than the official method; about 8 min. compared with near 40 min. in the latter.

Thin-layer chromatographic examination of official dosage forms of pyrvinium pamoate and a U.S.P. reference standard indicated that the drug consists of a number of components. Pyrvinium pamoate contained in official preparations separated into two major red spots at R_f 0.33 and 0.44 and a minor red spot at R_f 0.50. Upon spraying, these spots stained intense blue, while pamoic acid, previously visible only by fluorescence at R_f 0.50, stained light blue. U.S.P. reference standard pyrvinium pamoate was found to contain all of the above components as well as another minor red constituent, staining intense blue, at R_f 0.15. Pamoic acid could be separated from the red material also appearing at R_f 0.50 by using 0.1 *N* NaOH instead of 0.5 *N* NaOH in preparing the chromatographic plates (new R_f of pamoic acid: 0.69).

The occurrence of a greater number of red spots than expected, on the basis of two isomeric configurations of the compound, should not be attributed to the degradative effect of sodium hydroxide in the aqueous system since, for example, chromatography with chloroform–ethanol (7:3) on neutral Silica Gel G (Merck), although giving very poor separation, clearly indicated three red constituents in commercial and four such components in U.S.P. reference standard pyrvinium pamoate. These compositional characteristics of the drug will be examined further.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 548–551.
(2) "First Interim Revision Announcement," U.S.P. XVII, August 1965, p. 3.
(3) Alves, M. A. P., *Rev. Port. Farm.*, **14**, 13(1964); through *Chem. Abstr.*, **61**, 9357*c*(1964).
(4) Stahl, E., "Thin-Layer Chromatography," Academic Press Inc., New York, N.Y., 1965, p. 5 *ff*.

Analysis of Combinations Containing Phenylephrine in Liquid Dosage Forms

By K. O. MONTGOMERY, P. V. JENNINGS, and M. H. WEINSWIG

A method is presented for the isolation, separation, and determination of phenylephrine, an antitussive, and an antihistamine in commercial products containing the combination. All three compounds are extracted with strong cation exchange resin AG 50W-X4. The phenylephrine is eluted from the resin bed with 8.0 *N* phosphoric acid. The antitussive is then eluted with 1.0 *N* hydrochloric acid in 60 per cent methanol in water. Finally the antihistamine is eluted with 3.5 *N* hydrochloric acid in 40 per cent methanol in water. The compounds are determined by subjecting the eluates to ultraviolet spectrophotometry. The method is used successfully on several commercial products.

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niques similar to those used in inorganic separation to determine active ingredients in pharmaceuticals.

The combinations of compounds considered in this paper have been studied by several groups. Schriftman and Shultz (9) used paper electrophoresis for their separation and measured spot size on a densitometer to determine the active ingredients. Thin-layer chromatography has also been used with spot size measurement (10). Hyatt (11, 12) has proposed the use of partition columns to separate the active ingredients. Three columns are used and the separated compounds determined spectrophotometrically.

EXPERIMENTAL

Apparatus—Glass column 20 cm. × 1 cm. with a stopcock made of Teflon and containing a built-in needle valve for control of flow rate; the column is also fitted with a reservoir with a capacity of 250 ml.

A suitable recording ultraviolet spectrophotometer such as a Beckman DK-2A or Spectronic 505 which records in absorbance units.

Reagents—Cationic exchange resin AG 50W-X4, 100–200 mesh, hydrogen form, available from Bio-Rad Laboratories, Richmond, Calif. Enough resin, about 3 Gm., is added in the form of an aqueous slurry to the glass column; the resin bed is rinsed with water.

Phosphoric acid, 8.0 *N* in water; hydrochloric acid, 0.05 *N* in 50% methanol in water; hydrochloric acid, 1.0 *N* in 60% methanol in water; hydrochloric acid, 3.5 *N* in 40% methanol in water.

Standard Solutions—Prepare the following standard solutions using U.S.P., N.F., or other suitable reference standards. (*a*) Phenylephrine hydrochloride 10 mg./250 ml.; 8.0 *N* phosphoric acid in water. (*b*) Phenylephrine hydrochloride, 20 mg./250 ml.; 8.0 *N* phosphoric acid in water. (*c*) Codeine phosphate, 20 mg./200 ml.; 1.0 *N* hydrochloric acid in 60% methanol in water. (*d*) Dextromethorphan hydrobromide, 30 mg. in 200 ml.; 1.0 *N* hydrochloric acid in 60% methanol in water. (*e*) Chlorpheniramine maleate, 4 mg./200 ml.; 3.5 *N* hydrochloric acid in 40% methanol in water. (*f*) Promethazine hydrochloride, 10 mg./200 ml.; 3.5 *N* hydrochloric acid in 40% methanol in water.

Sample Treatment—Use 10-ml. samples of preparations *A*,[3] *B*,[4] and *C*.[5] For preparations *D*,[6] *E*,[7] and *F*[8] use 20-ml. samples. Pipet the sample into the reservoir, rinse the pipet with distilled water, and add to the reservoir.

Add distilled water to the sample to make the volume approximately 100 ml. and mix well. Allow this sample solution to flow through the resin bed at the rate of 2–3 ml./min. Wash the column by adding 100 ml. of distilled water to the reservoir and allow it to flow through the resin at the rate of 5 ml./min.

Traces of aromatic amines from flavors or coloring agents are removed by allowing 50 ml. of 0.05 *N* hydrochloric acid in 50% methanol in water to flow through the column at 5 ml./min.

Position a 250-ml. volumetric flask under the column and add 240 ml. of 8.0 *N* phosphoric acid to the reservoir. Allow this to flow through the column at the rate of 2 ml./min. The eluate volume is adjusted with 8.0 *N* phosphoric acid. This eluate contains the phenylephrine.

Position a 200-ml. volumetric flask under the column and add 190 ml. of 1.0 *N* hydrochloric acid in 60% methanol in water to the reservoir. Allow this to flow through the resin at 3 ml./min. Adjust the eluate volume with 1.0 *N* hydrochloric acid in 60% methanol in water. This eluate contains the antitussive.

Position another 200-ml. volumetric flask under the column and add 190 ml. 3.5 *N* hydrochloric acid in 50% methanol in water to the reservoir. Allow this to flow through the resin at the rate of 4 ml./min. Adjust the eluate volume with 3.5 *N* hydrochloric acid in 40% methanol in water. This eluate contains the antihistamine.

For re-use the column is washed with 100 ml. of distilled water.

Determinations—Record the ultraviolet spectra of the sample eluates and the standard solutions using a suitable spectrophotometer. Absorbance units should be used. Using the base line technique, determine the absorbances for each sample and standard at the particular maximum. Calculate the amounts of each compound present in each sample from the values obtained for the standard solutions.

DISCUSSION AND RESULTS

Standard solutions, when subjected to the above procedures, gave the results shown in Table I. These results are derived from ten determinations for each compound. These data indicate that the method is accurate to within 1% of the true values and is reproducible to ±1.5% at 95% confidence limits. The data for the analyses of marketed products are shown in Table II.

A slightly raised base line was noted with some products where a small portion of the coloring agent was held and eluted with the sample. This did not cause any problem since the base line technique was used in the calculations.

When the maleate salt of a compound is used for a standard, there is a small difference in the base line caused by ultraviolet absorbance of the maleic acid radical. This also is overcome for the most part by the use of the base line technique.

One deviation from the described procedures was necessary for preparation *E*. Since terpin hydrate is precipitated on dilution with water, the sample dilution prior to column application and the washing of the column were performed using 50% methanol in water instead of distilled water.

In products containing acetaminophen, a small amount of *p*-aminophenol is usually present which could interfere with the analysis of phenylephrine. This is effectively removed during the rinse using

[3] Marketed as Phenergan VC Expectorant with Codeine by Wyeth Laboratories, Philadelphia, Pa.
[4] Marketed as Tussquellin by Circle Pharmaceuticals.
[5] Marketed as Novahistine Expectorant by Pitman-Moore Division of the Dow Chemical Co., Indianapolis, Ind.
[6] Marketed as Pediacof by Winthrop Laboratories, New York, N. Y.
[7] Marketed as Adulton by Bristol-Myers Co., Syracuse, N. Y.
[8] Marketed as Coryban D by J. B. Roerig and Co., New York, N. Y.

TABLE I—ACCURACY, REPRODUCIBILITY, AND PRECISION ANALYSIS DATA

Std. Material	Concn., mg./10 ml.	Means ($\bar{x}$) of Results of 10 Determinations in % of Theoretical	S.D. of 10 Determinations in % of Theoretical
Phenylephrine HCl	20	99.42	±0.61
Codeine phosphate	20	99.94	±0.64
Chlorpheniramine maleate	4	100.38	±0.44
Dextromethorphan hydrobromide	15	99.04	±0.52

TABLE II—ANALYSIS OF PRODUCTS CONTAINING THREE AMINE COMPONENTS

Prepn.	Ingredients	Label Claim, mg./5 ml.	Found % of Label Claim
D	Phenylephrine HCl	2.5	99.9
	Codeine phosphate	5	100.2
	Chlorpheniramine maleate	0.75	99.6
A	Phenylephrine HCl	5	98.4
	Codeine phosphate	10	97.9
	Promethazine HCl	5	99.0
E	Phenylephrine HCl	5	101.1
	Dextromethorphan HBr	15	100.3
	Chlorpheniramine maleate	1	98.6
F	Phenylephrine HCl	5	102.8
	Dextromethorphan HBr	7.5	99.5
	Chlorpheniramine maleate	1	100.2
B	Phenylephrine HCl	5	98.8
	Dextromethorphan HBr	15	98.1
	Chlorpheniramine maleate	2	100.2
C	Phenylephrine HCl	10	100.85
	Codeine phosphate	10	100.82
	Chlorpheniramine maleate	2	101.65

0.05 *N* hydrochloric acid in 50% methanol in water.

One product, preparation *B*, actually contained four amines as active ingredients: phenylephrine, dextromethorphan, chlorpheniramine, and phenylpropanolamine. The last compound does not have a significant ultraviolet absorbance and therefore was outside the scope of this study. However, because of its lack of ultraviolet absorbance character, it did not interfere with the assay for the other active ingredients.

To demonstrate the characteristics of this method of assay, a mixture of phenylephrine, codeine, and chlorpheniramine standards was sorbed onto a column and eluted according to the previously described scheme. The eluate from the column was monitored utilizing a flow-through cell in an ultraviolet spectrophotometer. A strip chart recorder was connected to the spectrophotometer to record absorbance *versus* volume of eluate. (Fig. 1.) This chart shows a complete and concise separation of the three compounds.

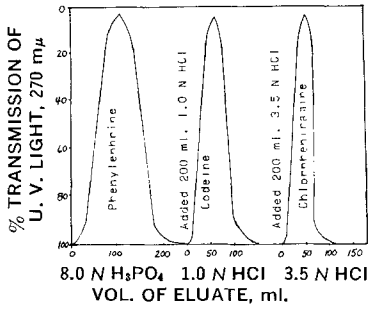

Fig. 1—Graph of per cent transmission of column eluate vs. *volume.*

SUMMARY

A method of assay for liquid dosage forms containing the combination of phenylephrine, an antitussive, and an antihistamine in a single product has been presented. The procedures have been used successfully on commonly available products with accurate and reproducible results.

REFERENCES

(1) Hofer, M., *Experientia*, **19** (7), 367(1963).
(2) Grasselly, G., *Acta Univ. Szeged., Acta Mineral.-Petrog.*, **15**, 7(1962); through *Chem. Abstr.*, **59**, 3309*h*(1963).
(3) Pollard, F. H., Nickless, G., and Spencer, D., *J. Chromatog.*, **11** (4), 542(1963).
(4) Pitstick, G., Sweet, T., and Morie, G., *Anal. Chem.*, **35**, 995(1963).
(5) Wang, S. M., and Hunter, R. W., *J. Pharm. Sci.*, **50**, 265(1961).
(6) Brochmann-Hanssen, E., and Svendsen, A., *ibid.*, **52**, 1134(1963).
(7) Blake, M. I., and Nona, D. A., *ibid.*, **52**, 945(1963).
(8) Blake, M. I., and Siegel, F. P., *ibid.*, **51**, 944(1962).
(9) Schriftman, H., and Shultz, R. C., *ibid.*, **50**, 332 (1961).
(10) Morrison, J. C., and Chatten, L. G., *ibid.*, **53**, 1205 (1964).
(11) Hyatt, R., *J. Assoc. Offic. Agr. Chemists*, **47**, 475 (1964).
(12) *Ibid.*, **48**, 594(1965).

Effects of 5-Fluorotryptophan on the Biosynthesis of Ergot Alkaloids by *Claviceps purpurea*

By W. A. SKINNER, J. J. MORRIS, and J. V. STEVENSON

The possibility of incorporation of 5-fluorotryptophan into ergot alkaloids by *Claviceps purpurea* PRL 1565 was explored utilizing randomly tritiated material. Very little radioactivity was found in the alkaloid fraction, most of it residing either in the mycelium or in the base-soluble fraction which would contain unchanged 5-fluorotryptophan. Thin-layer chromatography allowed the isolation of an alkaloid containing radioactivity, but fluoride analysis using neutron activation indicated the absence of fluorine in the molecule. It was concluded that 5-fluorotryptophan was not incorporated into the ergot alkaloids produced by *C. purpurea* PRL 1565.

THE BIOSYNTHESIS of ergot alkaloids has been the subject of many studies (1–5), primarily with regard to the elucidation of the pathway followed and identification of precursors. Tryptophan is known to be actively incorporated into the ergot or clavine alkaloids by certain strains of *Claviceps*. However, except for two studies, ring-substituted tryptophans have not been investigated as precursors of the ergot nucleus. Arcamone *et al.* (6) investigated the effect of 4-,5- or 6-methyltryptophans on the ergot alkaloid production by *Claviceps paspali* Stevens and Hall in submerged culture. They concluded on the basis of comparison of chromatography of the products isolated from the media containing the 5- or 6-methyltryptophans with that containing tryptophan that no incorporation of the methyltryptophans had occurred. Further evidence cited for this was comparison of the infrared spectra of hydrazides of the *dl*-isolysergic acid obtained from the culture containing the 5- or 6-methyltryptophans with that of an authentic sample of the unsubstituted derivative. However, one would expect only small differences between the chromatographic behavior and infrared spectra of ring methyl substituted isolysergic acid amides and hydrazides and the nonring substituted analogs. The added 4-methyltryptophan inhibited alkaloid production, while the 5- and 6-methyl substituted tryptophans allowed production of alkaloids greater than the control without the addition of the tryptophans. This increase in alkaloid production could be due either to incorporation or enzyme induction.

The other study of the effect of substituted tryptophans in *Claviceps* on alkaloid production was conducted by Floss and Mothes (7) using a saprophytic culture of *Claviceps*-Stammes SD 58. These workers investigated the effects of 4-, 5-, 6-, and 7-methyltryptophans and 5-fluorotryptophan on alkaloid yield. The methyl ring-substituted tryptophans were all found to increase alkaloid production somewhat with the 5- and 6-isomers giving the greatest increase. A marked reduction in alkaloid yield was obtained with the addition of 5-fluorotryptophan but no incorporation data were obtained. The incorporation into elymoclavine of tritium marked 5- and 7-methyltryptophans was studied. No appreciable incorporation into elymoclavine was obtained, and the authors concluded that the increases in alkaloid production obtained with these ring methyl derivatives were due to enzyme induction.

In these laboratories, the authors have also been interested in whether ring-substituted tryptophans could be incorporated into ergot alkaloids as a possible biosynthetic method for the preparation of new ring-substituted ergot derivatives. The results of studies using randomly tritium-labeled 5-fluorotryptophan in submerged cultures of *Claviceps purpurea* PRL 1565 are reported.

METHODS

Two strains of *C. purpurea*, initially obtained from Dr. W. A. Taber, were used for experiments. Strain 1565 was found to give the highest yields of alkaloids when grown on a modified Abe medium plus yeast extract and glucose and strain 1578 when grown on Abe's medium plus ammonium succinate and a 19:1 mixture of galactose and glucose. The extraction procedure used was that of Vining and Taber (8). By using ergometrine as a control, the efficiency was determined to be satisfactory. Alkaloid content was ascertained by the van Urk method using ergometrine as a standard. The maximum alkaloid production was attained in 20–50 days. Addition of L-tryptophan, either just before the time of peak alkaloid production or earlier in the growth period, resulted in increased alkaloid production.

Investigation of various silica gel GF thin-layer chromatographic systems using authentic ergot and clavine alkaloids indicated that the two solvent systems, benzene–ethanol (2:1) and ethyl acetate–dimethylformamide–95% ethanol (10:1:1), were sufficient to separate most of the alkaloids of interest. Alkaloids could be detected under ultraviolet light or by spraying with *p*-dimethylaminobenzaldehyde reagent. In both solvent systems 5-fluorotryptophan and 5-fluorotryptamine stayed at the origin. In butanol–acetic acid–water (10:1:1), 5-fluorotryptophan has an R_f of 0.4.

Received October 7, 1966, from Life Science Research Stanford Research Institute, Menlo Park, CA 94025
Accepted for publication November 21, 1966.
Presented to the Fourth International Symposium on the Chemistry of Natural Products, Stockholm, Sweden, July 1966.
This investigation was supported by grant 5 S01-FR-5522-03 from the Division of Research Facilities and Resources, U. S. Public Health Service, Bethesda, Md.
The authors thank Mr. D. Brown and Mr. M. Leaffer for technical assistance. The counting was conducted under the direction of Dr. C. Mitoma.

TABLE I—ALKALOID YIELDS PRODUCED BY *C. purpurea* PRL 1565

Expt.	Sample	Yields of Alkaloid, mcg./ml. 10 Days	15 Days	18 Days	20 Days	28 Days	35 Days
33	Control	8.5	...	...	8.5	...	9.0
	5-FT (labeled)[a]	2.5	...	...	2.5	...	19
	5-FT (labeled)[b]	6.5	...	...	12	...	15
36	Control	...	10	...	...	10	10
	5-FT (labeled)[c]	...	3.5	...	...	8.5	5.0
	5-FT (labeled)[b]	...	3.5	...	...	4.5	8.5
39	Control	...	9.5	10	10	10	...
	5-FT (labeled)[d]	...	...	2.0	3.5	5.0	
	5-FT (labeled)[b]	...	18	...	15	17	...

[a] Added on day 24 (1.5 mg. with specific activity of 2.46×10^7 d.p.m./mg.). [b] In addition to labeled 5-FT added on day 24, L-tryptophan added at time of inoculation to yield final concentration of 0.1%. [c] Added on day 6 (1.1 mg. with specific activity of 2.46×10^7 d.p.m./mg.). [d] Added on day 4 (1.3 mg. with specific activity of 2.34×10^7 d.p.m./mg.).

RESULTS

5-Fluorotryptophan (K and K Chemical Co.) was identified by its melting point and infrared absorption spectrum and randomly tritiated at the New England Nuclear Co. by the Wilzbach technique of exposure of 375 mg. at 250 mm. pressure to 3 c. of tritium at 27° for 2 weeks. The labile tritium was removed by dissolving the material in 95% ethanol and by removal of the solvent *in vacuo*. This material was purified by crystallization from isopropanol–water to yield material whose infrared absorption spectrum and thin-layer behavior in butanol–acetic acid–water (10:1:1) and in *n*-propanol–concentrated ammonium hydroxide (70:30), R_f 0.5, agreed with that of authentic material. This purified material, which also showed no change in specific activity upon recrystallization, was added to submerged cultures of *C. purpurea* PRL 1565 and alkaloidal yields were determined colorimetrically by using van Urk reagent (Table I). In some experiments L-tryptophan was added at the time of inoculation and the labeled 5-fluorotryptophan was added later.

TABLE II—DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS FROM *C. purpurea* PRL 1565

Expt.	% of Counts Basic Extract	Neutral Extract	Acidic Extract	Mycelium
33	20	8.2	5.0	66.8
36	33.6	0.8	3.0	62.6
39	10.6	7.5	7.7	74.2

TABLE III—THIN-LAYER CHROMATOGRAPHY OF ALKALOIDS FROM *C. purpurea* PRL 1565 AND STANDARD ALKALOIDS

Alkaloid Extract	Ethyl Acetate–Dimethylformamide–95% Ethanol (10:1:1) Orig., 0.55, 0.90	Benzene–Ethanol (2:1) Orig., 0.60, 0.90
Chanoclavine	0.05	0.06
Ergonovine	0.17	0.20
Agroclavine	0.40	0.44
Ergosine	0.54	0.48
Ergotamine	0.55	0.65
Ergocristine	0.58	0.70
Ergocryptine	0.60	0.75
Ergocornine	0.68	0.70
Ergocristinine	0.75	0.70
Ergotaminine	0.90	0.85

Distribution of the radioactivity among extracts from the various experiments is shown in Table II. Each per cent represents the average of results from four separate flasks.

Investigation of the thin-layer behavior (silica gel GF) of the acidic extracts after basification with ammonium hydroxide and extraction of the alkaloids with $CHCl_3$ and ether gave results as seen in Table III when compared to authentic samples of ergot and clavine alkaloids. These R_f values are averages of a large number of determinations.

The spots were all detected by their fluorescence and all but the origin spots were van Urk positive. The alkaloid at 0.55 in ethyl acetate–dimethylformamide–95% ethanol and 0.60 in benzene–ethanol corresponds most closely to ergotamine. The alkaloid at 0.90 corresponds most closely to ergotaminine.

The alkaloid mixture isolated from experiment 39 (Table II) was put on a thick-layer silica gel GF plate and developed with ethyl acetate–dimethylformamide–95% ethanol (10:1:1). The plate was sectioned to separate the fluorescent spots at the origin, 0.55 and 0.90 and the silica gel was extracted, first with methanol, then with chloroform. The combined extracts were dried *in vacuo* and counted in a scintillation counter. The products had the following counts: origin (8,040 c.p.m.), 0.55 (92,300 c.p.m.), and 0.90 (6,390 c.p.m.). Nonfluorescent sections between these zones had only background counts.

The van Urk positive radioactive material at 0.55 was isolated from a silica gel H plate by removal of the zone of ultraviolet absorption and extraction with methanol. The methanol was removed *in vacuo* and the sample submitted along with a control sample to General Atomic Division of General Dynamics, San Diego, Calif., for fluoride analysis by neutron activation. The results were 1.1 ± 0.2 mcg. F for the blank and 0.7 ± 0.3 mcg. F for the alkaloid extract.

DISCUSSION

5-Fluorotryptophan was found to somewhat inhibit production of ergot alkaloids from *C. purpurea* PR 1565 when added early after inoculation, agreeing with the results that Floss and Mothes found on clavine alkaloid yields using *Claviceps*-Stammes SD 58. However, when the 5-fluorotryptophan was added on the day 24 after inoculation, the alkaloid yields were increased.

The distribution of radioactivity after addition of

randomly tritiated 5-fluorotryptophan to *C. purpurea* PRL 1565 was found to be largely in the mycelium and the basic extract which would contain unincorporated labeled 5-fluorotryptophan. In the alkaloid containing extract, the majority of the radioactivity was found to be concentrated in the thin-layer chromatographic spot at R_f 0.55 when developed with ethyl acetate–dimethylformamide–95% ethanol (10:1:1). The low yields of alkaloids produced by the organism prevented conventional fluoride analyses on them. The sensitivity of neutron activation analysis for fluoride was useful in indicating the absence of fluorine in the radioactive, van Urk positive material. The conclusion is that 5-fluorotryptophan was not incorporated into the ergot alkaloids by *C. purpurea* PRL 1565. Two possible explanations could account for the radioactivity of the alkaloid at R_f 0.55: tritium exchange between the tritiated 5-fluorotryptophan and tryptophan or enzymatic reduction of the 5-fluoro group followed by incorporation of the tryptophan. Doubly labeled 5-fluorotryptophan with a better alkaloid producing organism would be required to distinguish between these explanations.

REFERENCES

(1) Weygand, F., Floss, H. G., Mothes, U., Gröger, D., and Mothes, K., *Z. Naturforsch.*, **19**, 202(1964); Weygand, F., "Abstracts of the Sixth International Congress of Biochemistry," New York, N. Y., July 26–August 1, 1964.

(2) Taber, W. A., and Vining, L. C., *Chem. Ind.*, **1959**, 1218.

(3) Baxter, R. M., Kandel, S. I., Okany, A., and Tom, K. L., *J. Am. Chem. Soc.*, **84**, 4350(1962).

(4) Gröger, D., Mothes, K., Simon, H., Floss, H. G., and Weygand, F., *Z. Naturforsch.*, **15**, 141(1960).

(5) Plieninger, H., Hobel, M., and Liede, V., *Chem. Ber.*, **96**, 1618(1963).

(6) Arcamone, F., Chain, E. B., Ferretti, A., Minghetti, A., Pennella, P., and Tonolo, A., *Biochim. Biophys. Acta*, **57**, 174(1962).

(7) Floss, H. G., and Mothes, U., *Arch. Mikrobiol.*, **48**, 213(1964).

(8) Vining, L. C., and Taber, W. A., *Can. J. Microbiol.*, **5**, 444(1959).

Gastrointestinal Irritant Effect of Glycerin as Compared with Sorbitol and Propylene Glycol in Rats and Dogs

By R. STAPLES, A. MISHER, and J. WARDELL, JR.

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GLYCERIN is a fairly common component of liquid pharmaceutical vehicles. However, there is little definitive information in the literature on its gastrointestinal effects. Johnson *et al.* (1) reported that ingestion of 30 ml. (approximately 0.6 ml./Kg.) of 95% glycerin with orange juice three times daily for 50 days by 14 normal humans produced no consistent changes in body weight or temperature and no overt pharmacological effects. The daily number and consistency of stools was unaffected by the quantities of glycerin fed. Subjectively, four subjects (29%) reported an occasional "sensation of warmth" in the stomach after ingestion, especially in the rare instance when glycerin was taken on an empty stomach. No enterogastric examinations were done, however, to assess gastrointestinal effects directly. These same investigators noted "no ill effects" in 11 fasted rats which received single oral doses of glycerin ranging from 7 to 18 Gm./Kg. (5.6 to 14.3 ml./Kg.). Arnschnik (2) cited "no illness" in dogs after single oral doses of glycerin up to 8 Gm./Kg. (6.3 ml./Kg.). However, at 11 Gm./Kg. orally (8.7 ml./Kg.), the same investigator reported "intestinal disturbances" and vomiting. Kobert (3) and Sollmann (4) stated that repeated ingestion of large doses of glycerin lead to a chronic inflammation of the gastrointestinal tract in man. Deichmann's review of the pharmacology of glycerin (5, 6) cited Kobert's report (3) of the accidental ingestion of 300 Gm. (about 238 ml.) of glycerin by a 2-year-old child who lost consciousness but recovered following stomach lavage. Drill (7) noted that oral administration of 100 to 300 ml. of undiluted glycerin has caused "severe symptoms" in humans, whereas the same amount diluted or mixed with food produces "little or no effect." Sroka (8), in a patient given "more than 100 grams orally" while being treated for kidney stones, observed dizziness, drowsiness, violent headache, bloody diarrhea, and kidney pains (differing from the orginal kidney pains).

The purpose of this study was twofold: (*a*) to compare the gastrointestinal irritation liability of glycerin with that of sorbitol (in aqueous solution) and propylene glycol, two other commonly used constituents of oral liquid pharmaceutical preparations; and (*b*) to determine the effect of water dilution on the gastrointestinal irritant potential of these three compounds.

METHODS

Female Charles River rats, weighing 150 to 210 Gm., and adult mongrel dogs of either sex, weighing 8.9 to 16.0 Kg., were used. The rats were housed five per cage, and the dogs caged individually, in rooms maintained at 74° F. The compounds studied (glycerin, sorbitol, and propylene glycol) were administered by stomach tube, three times

Received December 5, 1966, from the Pharmacology Section, General Therapeutics Department, Smith Kline & French Laboratories, Philadelphia, PA 19130

Accepted for publication January 5, 1967.

randomly tritiated 5-fluorotryptophan to *C. purpurea* PRL 1565 was found to be largely in the mycelium and the basic extract which would contain unincorporated labeled 5-fluorotryptophan. In the alkaloid containing extract, the majority of the radioactivity was found to be concentrated in the thin-layer chromatographic spot at R_f 0.55 when developed with ethyl acetate–dimethylformamide–95% ethanol (10:1:1). The low yields of alkaloids produced by the organism prevented conventional fluoride analyses on them. The sensitivity of neutron activation analysis for fluoride was useful in indicating the absence of fluorine in the radioactive, van Urk positive material. The conclusion is that 5-fluorotryptophan was not incorporated into the ergot alkaloids by *C. purpurea* PRL 1565. Two possible explanations could account for the radioactivity of the alkaloid at R_f 0.55: tritium exchange between the tritiated 5-fluorotryptophan and tryptophan or enzymatic reduction of the 5-fluoro group followed by incorporation of the tryptophan. Doubly labeled 5-fluorotryptophan with a better alkaloid producing organism would be required to distinguish between these explanations.

REFERENCES

(1) Weygand, F., Floss, H. G., Mothes, U., Gröger, D., and Mothes, K., *Z. Naturforsch.*, **19**, 202(1964); Weygand, F., "Abstracts of the Sixth International Congress of Biochemistry," New York, N. Y., July 26–August 1, 1964.

(2) Taber, W. A., and Vining, L. C., *Chem. Ind.*, **1959**, 1218.

(3) Baxter, R. M., Kandel, S. I., Okany, A., and Tom, K. L., *J. Am. Chem. Soc.*, **84**, 4350(1962).

(4) Gröger, D., Mothes, K., Simon, H., Floss, H. G., and Weygand, F., *Z. Naturforsch.*, **15**, 141(1960).

(5) Plieninger, H., Hobel, M., and Liede, V., *Chem. Ber.*, **96**, 1618(1963).

(6) Arcamone, F., Chain, E. B., Ferretti, A., Minghetti, A., Pennella, P., and Tonolo, A., *Biochim. Biophys. Acta*, **57**, 174(1962).

(7) Floss, H. G., and Mothes, U., *Arch. Mikrobiol.*, **48**, 213(1964).

(8) Vining, L. C., and Taber, W. A., *Can. J. Microbiol.*, **5**, 444(1959).

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METHODS

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TABLE I—GASTROINTESTINAL IRRITATING EFFECTS OF GLYCERIN, SORBITOL, AND PROPYLENE GLYCOL AFTER ORAL ADMINISTRATION t.i.d. FOR 8 DOSES TO RATS

Compd.	Dose	Concn., %	Dose, Vol., ml./Kg.	No. Animals Showing Effect/No. Tested: No Irritation	Slight Hyperemia	Severe Hyperemia	Petechial Hemorrhage	Erosions
Glycerin	0.75 ml./Kg.	100 v/v	0.75	6/10	3/10	0/10	1/10	
	1.50	100	1.50	3/10	2/10	3/10	1/10	1/10
	3.00	100	3.00	0/10	3/10	1/10	4/10	2/10
	3.00	80	3.75	2/10	0/10	2/10	5/10	1/10
	3.00	60	5.00	3/10	5/10	1/10	1/10	
	3.00	40	7.50	4/10	4/10	1/10	1/10	
	3.00	20	15.00	9/10	1/10			
Sorbitol	0.675 Gm./Kg.	90 w/v[a]	0.75	8/10	2/10			
	1.35	90	1.50	7/10	0/10	1/10	1/10	1/10
	2.70	90	3.00	5/10	0/10	2/10	3/10	
	3.00	45	6.67	8/10	2/10			
	3.00	25	12.00	9/10	1/10			
Propylene glycol	0.75 ml./Kg.	100 v/v	0.75	9/10	1/10			
	1.50	100	1.50	9/10	0/10	1/10		
	3.00	100	3.00	8/10	2/10			
	3.00	75	4.00	10/10				
	3.00	50	6.00	10/10				
Distilled water–controls	3.00 ml./Kg.	100	3.00	20/20				

[a] Sorbitol, 90% w/v in distilled water is sorbitol solution U.S.P.

daily (at 10 a.m., 1 p.m., and 4 p.m.), for 3 days. Food and water were withheld during the dosing period (from 8:30 a.m. to 4:30 p.m.) on each day but were available *ad libitum* at all other times.[1] All animals were observed for overt effects following each dose. The animals were sacrificed about 1 hr. after the second dose on the third day (after having received 8 doses). Rats were killed by a blow on the head; dogs received a lethal intravenous dose of a veterinary euthanasia agent.[2] Following sacrifice, the stomach and attached portion of the duodenum (about one-third the entire duodenal segment) of each animal were removed carefully and rinsed in tepid water. The stomach was cut along its greater curvature from cardiac sphincter through the pylorus, and the duodenum was cut longitudinally by extending the incision. The mucosal surface was examined grossly and irritated areas were observed under a binocular microscope.

The degree of irritation was graded according to the following descriptions, arranged progressively from least to most severe: *normal*, appearance of mucosa same as in water-treated controls; *slight hyperemia*, slight reddening of mucosa; *severe hyperemia*, deep reddening of mucosa; *petechial hemorrhage*, hyperemia and pinpoint submucosal hemorrhages; *erosion*, mucosal erosion with associated severe hyperemia and hemorrhage.

RESULTS AND DISCUSSION

The results of this study in rats (Table I) and dogs (Table II) clearly indicate that at equivalent undiluted oral doses glycerin was more irritating to the gastrointestinal tract than either sorbitol or propylene glycol. Chronic dosing (t.i.d. for 8 doses) in both species with glycerin at doses up to 3.00 ml./Kg. produced hyperemia, petechial hemorrhages, and erosions. These effects may be due to the hygroscopicity of the compound which, in high concentration, is somewhat dehydrating and irritating to exposed tissue (9). In contrast, undiluted sorbitol solution U.S.P. was far less irritating than equivalent doses of glycerin, and propylene glycol was relatively innocuous. Gastrointestinal irritation following oral administration of undiluted glycerin has been cited in the literature. The doses used, however, were in the toxic range. Deichmann (6) reported an acute irritation of the entire gastrointestinal tract in rats receiving lethal oral doses of undiluted glycerin. The previously cited "severe symptoms" in humans (3, 7, 8) were produced by doses in the lethal range since Dreisbach (10) estimated the fatal oral undiluted dose in humans to be 100 Gm. of glycerin (79.4 ml.). The highest dose used in the present study, 3.00 ml./Kg. of undiluted glycerin, was sublethal, and was about one-seventh of the reported oral LD_{50} in rats (11).

Thus, the irritating properties of undiluted glycerin have been observed in two species at doses below that required to produce overt toxicity.

According to Drill (7), the gastrointestinal irritating potential of glycerin in humans was reduced by dilution of the dose. Johnson, Carlson, and Johnson (1) reported a similar observation in rats and dogs. Results of the present study in rats confirm these earlier investigations. Rather severe irritation was produced by 3.00 ml./Kg. of undiluted glycerin; but when the same dose was given as 80%, 60%, 40%, or 20% dilutions in distilled water, the irritation was reduced as the concentration was decreased. Similar results were obtained with both sorbitol and propylene glycol, although the initial irritation produced by either compound undiluted was less than that produced by glycerin. It appeared that irritation was reduced to minimal

[1] Rats received Purina rat chow. Dogs received 1 lb. of Red Heart dog food daily.

[2] Marketed as Euthanasia Solution by Arnold Laboratories, New Castle, Ind. Each milliliter of the solution contains 100 mg. each of sodium pentobarbital, sodium secobarbital, and mephenesin.

TABLE II—GASTROINTESTINAL IRRITATING EFFECTS OF GLYCERIN, SORBITOL, AND PROPYLENE GLYCOL AFTER ORAL ADMINISTRATION t.i.d. FOR 8 DOSES TO DOGS

Compd.	Dose, ml./Kg.	Dog No.	Description of Stomach and Duodenum
Glycerin	0.75	1	Appeared "normal."
	1.50	2	All mucosa severely hyperemic with widespread petechial hemorrhages.
	3.00	3	All stomach mucosa slightly to severely hyperemic with several localized areas of petechial hemorrhages; duodenal mucosa mostly "normal" with scattered hyperemic areas.
		4	Several localized severely hyperemic areas containing several small erosions; duodenum appeared "normal."
Sorbitol[a] soln.	0.75	5	Appeared "normal."
	1.50	6	Most of stomach slightly to severely hyperemic; duodenum appeared "normal."
	3.00	7	Appeared "normal."
Propylene glycol	0.75	8	Appeared "normal."
	1.50	9	All stomach mucosa slightly hyperemic; duodenum "normal" except for several small, localized slightly hyperemic areas.
	3.00	10	Appeared "normal."
Distilled water—controls	3.00	11	Appeared "normal."

[a] 0.75 ml./Kg. of sorbitol solution U.S.P. = 0.675 Gm./Kg., as sorbitol. 1.50 ml./Kg. of sorbitol solution U.S.P. = 1.35 Gm./Kg., as sorbitol. 3.00 ml./Kg. of sorbitol solution U.S.P. = 2.70 Gm./Kg., as sorbitol.

levels: (*a*) when 3.00 ml./Kg. of glycerin was administered in a concentration of under 40% v/v; and (*b*) when 3.00 Gm./Kg. of sorbitol was administered at concentrations of 45% w/v, or less. Propylene glycol, even undiluted, produced only minimal irritation.

These studies in rats and dogs have shown that repeated oral administrations of high concentrations of glycerin are irritating to the gastrointestinal mucosa. This effect might be a factor for consideration in the development of vehicles for liquid pharmaceutical preparations intended for repeated administration to humans. The results suggest that additional investigations in man would be desirable to establish the possible clinical significance of our observations in animals.

SUMMARY

In this study, undiluted glycerin, administered orally to rats and dogs, produced gastrointestinal irritation to a much greater degree than did either sorbitol or propylene glycol. The degree of severity of the irritant effect of each compound was dose-dependent in both species and was reduced in rats by dilution of the dose.

REFERENCES

(1) Johnson, V., Carlson, A. J., and Johnson, A., *Am. J. Physiol.*, **103**, 517(1933).

(2) Arnschnik, L., *Z. Biol.*, **23**, 413(1887).

(3) Kobert, R., "Lehrbuch der Intoxikationen," 2nd ed., F. Emke, Stuttgart, Germany, 1906.

(4) Sollmann, T., "A Manual of Pharmacology," 8th ed., W. B. Saunders Co., Philadelphia, Pa., 1957, p. 127.

(5) Deichmann, W., *Ind. Med.*, **9**, 60(1940).

(6) *Ibid.*, **10**, 5(1941).

(7) Dipalma, J., "Drill's Pharmacology in Medicine," 3rd ed., McGraw-Hill Book Co., Inc., New York, N. Y. 1965, p. 228.

(8) Sroka, K., *Muench. Med. Wochschr.*, **94**, 1417(1952).

(9) Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics," 2nd ed., The Macmillan Co., New York, N. Y., 1960, pp. 124, 128.

(10) Dreisbach, R., "Handbook of Poisoning: Diagnosis and Treatment," 3rd ed., Lange Medical Publications, Los Altos, Calif., 1961, p. 129.

(11) Deichmann, W., and LeBlanc, T., *J. Ind. Hyg. Toxicol.*, **25**, 415(1943).

Structure Elucidation and Chemistry of *Catharanthus* Alkaloids II. Isolation and Partial Structure of Catharine, a Dimeric Indole Alkaloid from *C. lanceus* and *C. roseus*

By DONALD J. ABRAHAM, NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, and ROBERT E. RHODES

A continuing study of *Catharanthus lanceus* alkaloid fractions, in a search for new antineoplastic alkaloids, has led to the isolation of catharine, a dimeric indole alkaloid found previously only in the related *C. roseus*. High resolution mass spectrometric measurements have shown that catharine has the formula $C_{46}H_{54}N_4O_{10}$ and is made up of a vindoline moiety and an alkaloid moiety with a molecular formula of $C_{21}H_{23}N_2O_4$.

PREVIOUS STUDIES in these laboratories on *Catharanthus lanceus* have led to the isolation of the monomeric alkaloids lanceine (1), tetrahydroalstonine (2), ajmalicine (3), and yohimbine (4), which were previously reported from this plant by Janot *et al.* (5–7). In addition, monomeric alkaloids previously unreported from this plant, but reported present in related genera or species, were found to be vinosidine (1), perivine (4), perimivine (3), pericalline (tabernoschizine, apparicine, gomezine) (3), vindoline (4), and lochnerinine (2). Three new and hitherto unreported monomeric alkaloids, catha-lanceine (3), pericyclivine (8), and periformyline (2, 9), were similarly isolated in the authors' studies. On the other hand, only one dimeric alkaloid, leurosine (4), had been encountered in this investigation of *C. lanceus* alkaloids. This alkaloid was previously isolated from *C. roseus* by Svoboda *et al.* (10), and has been subsequently shown to be highly active against the P-1534 leukemia in DBA/2 mice (11–13).

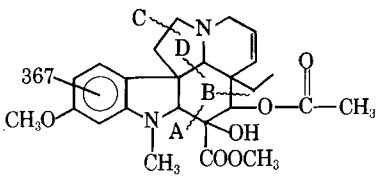
A continued investigation of alkaloid fractions obtained from *C. lanceus* has resulted in the isolation of a second dimeric alkaloid, catharine, from this plant. The isolation, characterization, and partial structure elucidation of this alkaloid is reported here.

EXPERIMENTAL

Isolation of Catharine.—In a previous paper the authors investigated the leaf alkaloid (A_1) fraction from *C. lanceus* and isolated the alkaloids tetrahydroalstonine, lochnerinine, and a new alkaloid periformyline (2). The structure for periformyline was subsequently shown by us to be $N_{(b)}$-formyl perivine, the first example of an $N_{(b)}$-substituted formyl iodole alkaloid to be found in nature (9). Additional studies on this leaf (A_1) fraction have resulted in the isolation of catharine, previously isolated only by Svoboda *et al.* from *C. roseus* (14).

Work-up of the chloroform eluted fractions 175–181 from the column chromatographic separation of the (A_1) alkaloids (100 Gm.), as previously described (Table I), resulted in the formation of 0.730 Gm. of crystals from benzene after several weeks of refrigeration. Recrystallization of these fine crystals from methanol–anhydrous ether afforded an analytical sample of catharine, m.p. 257–258° dec. A mixed melting point with reference to catharine showed no depression,[1] and an infrared absorption spectrum (Fig. 1) was superimposible with that of a reference sample, as was that of a comparison ultraviolet absorption spectrum. The molecular weight of catharine, as determined by mass spectrometry, was found to be 822.

Partial Structure Elucidation of Catharine.—The mass spectrum[2] of catharine showed major peaks at *m/e* 822, 763, 735, 733, 622, 555, 554, 158, 144, 136, 135, 130, 122, 121, 108, and 107. From the above data it became apparent that vindoline made up one-half of this dimeric alkaloid. If one considers the partial structure (I) for catharine, the mass spectral data can be interpreted reasonably. The 367 in I



I

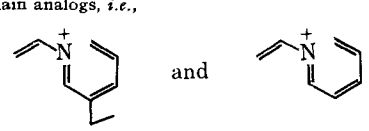
represents the molecular weight of the other half of the dimeric alkaloid. The *m/e* 763 peak represents a loss of 59 mass units, or a carbomethoxy group. Previous mass spectral degradations of vindoline (16, 17) are also evident here. The *m/e* 822 to *m/e* 662 (II) transition (Scheme I), with loss of 160 mass units, indicates ABD cleavage to give the fragment II*a*. A strong metastable ion for this transition is observed at *m*/e* 533.1. The *m/e* 554 peak can possibly be represented as the following ion (III) (Scheme II), from cleavage at E in II, which is similar to that basic ion formed by vindoline at *m/e* 188. The *m/e* 282 (IV) to *m/e* 222 (V) transition has a weak metastable ion at *m/e** 174.8 and is pictured as similar to that particular degradation in vindoline (Scheme III).

Other characteristic vindoline peaks (17) are at *m/e* 135 (VI) and *m/e* 107 (VII)[3] with a metastable

[1] Although the literature (11, 14) states 271–275° as the m.p. for catharine, an authentic sample, found to be homogeneous when examined by thin-layer chromatography using three different solvent systems (15), and supplied by G. H. Svoboda, gave m.p. 257–258° dec.

[2] The AEI MS 9 high resolution mass spectrometer was used in this work.

[3] These structures are designated in *Reference 16* as the open chain analogs, *i.e.*,



Received August 1, 1966, from the Departments of Pharmacognosy and Pharmaceutical Chemistry, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213
Accepted for publication September 26, 1966.
This investigation was supported by research grants CA-08228, CA-08509, and FR-05455 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
Previous paper: Abraham, D. J., Farnsworth, N. R., Blomster, R. N., and Sharkey, A. J., Jr., *Tetrahedron Letters*, 1965, 317.

TABLE 1.—COLUMN CHROMATOGRAPHIC SEPARATION OF *C. lanceus* LEAF (A_1) FRACTION, 100 GM.

Eluent	Fraction[a]	Fraction Wt., Gm.[b]	Alkaloid Isolated	Wt., Gm.
Benzene (fractions 1–87)	1–2	0.29	...	...
	3	2.10	...	...
Benzene–chloroform (9:1)	4	1.33	...	...
(fractions 88–97)	5–6	8.55	...	...
Benzene–chloroform (3:1)	7–15	10.67	Lochnerinine	1.950
(fractions 98–118)	16–28	10.57	Tetrahydroalstonine	7.415
Benzene–chloroform (2:1)	29–40	1.69	Tetrahydroalstonine	0.760
(fractions 119–160)	41–52	0.29	...	...
Benzene–chloroform (1:1)	53–89	0.58	...	...
(fractions 161–173)	90–112	0.54	...	...
Chloroform	113–120	5.79	...	...
(fractions 174–243)	121–125	1.17	...	...
Chloroform–methanol (99:1)	126–133	1.29	...	...
(fractions 244–280)	134–140	0.86	...	...
Chloroform–methanol (4:1)	141–149	1.12	...	...
(fractions 281–418)	150–162	1.65	Periformyline	0.220
Chloroform–methanol (2:1)	163–174	1.31	...	...
(fractions 419–439)	175–181	6.56	Catharine	0.730
Chloroform–methanol (1:1)	182–186	0.55	...	...
(fractions 440–453)	187–214	1.61	...	...
Methanol	215–247	0.65	...	...
(fractions 454–481)	248–357	3.38	...	...
	358–438	1.28	...	...
	439–454	0.57	...	...
	455–474	7.40	...	...
	475–481	...	...	...

[a] All fractions collected were 1000 ml. [b] All fractions collected were alkaloidal.

peak at *m/e** 84.8, and at *m/e* 122 (VIII), resulting from B,C,D cleavage (Scheme IV).

High resolution mass spectrometry measurements on several of these peaks showed them to be in agreement with the postulated formulas (Table II). However, the site of the attachment of the 367 group to vindoline has not as yet been determined.

Some possible molecular formulas for catharine are listed in Table III and it can be seen from high resolution data that the formula for catharine is in agreement with $C_{46}H_{54}N_4O_{10}$.

If $C_{46}H_{54}N_4O_{10}$ is the correct molecular formula for catharine, it follows that with the vindoline moiety having a molecular formula of $C_{25}H_{31}N_2O_6$, then the other half of the dimer has the formula $C_{21}H_{23}N_2O_4$. The authors are at present investigating the nature of this second half of the dimer.

Preliminary results on the acid cleavage of catharine show desacetylvindoline, by thin-layer chromatography, to be one of the products. This would be expected, and attempts are in progress to isolate the cleavage products and rigorously establish their structures.

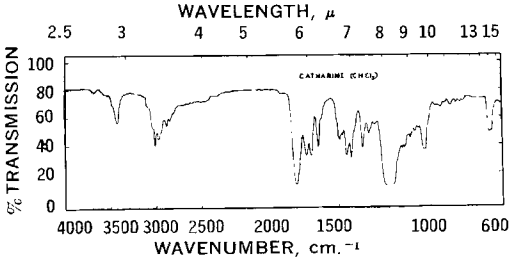
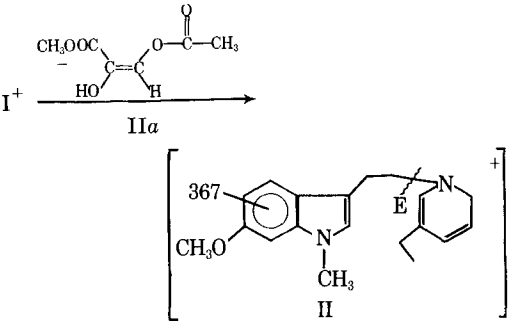
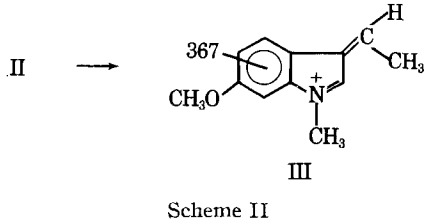


Fig. 1.—Infrared spectrum of catharine.

It was previously reported (14) by other workers, that the formula of catharine was $C_{46}H_{52}N_4O_9 \cdot CH_3OH$; however, they only reported an elemental analysis as their proof for the proposed structure. Their first elemental analysis of catharine is also in agreement with our proposed formula of $C_{46}H_{54}N_4O_{10}$.[4]



Scheme I

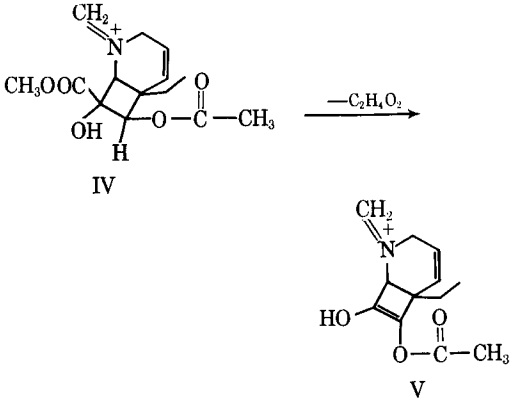


Scheme II

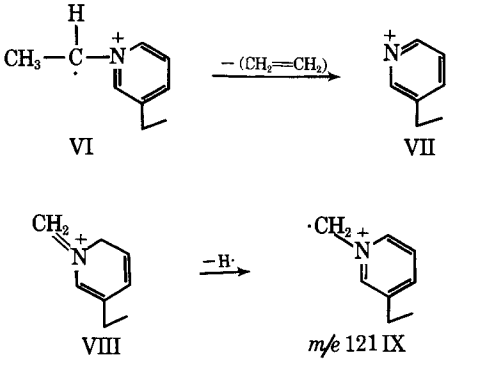
[4] *Anal.*—Calcd. for $C_{46}H_{54}N_4O_{10}$: C, 67.15; H, 6.57; N, 6.81; O, 19.46; 4-OCH_3, 15.08; OAc, 7.18. Found (14): C, 67.45; H, 6.95; N, 6.97; O, 19.68; 4-OCH_3, 15.34; OAc, 7.81.

TABLE II.—HIGH RESOLUTION DATA OF FRAGMENTED IONS FROM CATHARINE

Ion	Formula	Calcd. Wt. *m/e*	Observed Wt. *m/e*	Difference in Mass Units
I-($C_2H_3O_2$)	$C_{44}H_{51}N_4O_8$	763.3707	763.3691	−0.0016
II	$C_{40}H_{46}N_4O_5$	662.3468	662.3433	−0.0035
III	$C_{33}H_{36}N_3O_5$	554.2655	554.2635	−0.0020
V	$C_{12}H_{16}NO_3$	222.1130	222.1123	−0.0007
VI	$C_9H_{13}N$	135.1048	135.1047	−0.0001
VII	C_7H_9N	107.0735	107.0734	−0.0001
VIII	$C_8H_{12}N$	122.0970	122.0962	−0.0008
IX	$C_8H_{11}N$	121.0892	121.0882	−0.0010



Scheme III



Scheme IV

TABLE III.—HIGH RESOLUTION MOLECULAR WEIGHT OF CATHARINE

Proposed Formulas	Calcd. Wt. *m/e*	Observed Wt. *m/e*	Difference
$C_{45}H_{50}N_4O_{11}$	822.3476	822.3793	+0.0317
$C_{46}H_{54}N_4O_{10}$	822.3840	822.3793	−0.0047
$C_{47}H_{58}N_4O_9$	822.4203	822.3793	−0.0410
$C_{48}H_{62}N_4O_8$	822.4567	822.3793	−0.0774

REFERENCES

(1) Blomster, R. N., Farnsworth, N. R., and Abraham, D. J., *J. Pharm. Sci.*, **56**, 284(1967).
(2) Maloney, E. M., Farnsworth, N. R., Blomster, R. N., Abraham, D. J., and Sharkey, A. G., Jr., *ibid.*, **54**, 1166 (1965).
(3) Blomster, R. N., Martello, R. E., Farnsworth, N. R., and Draus, F. J., *Lloydia*, **27**, 480(1964).
(4) Loub, W. D., Farnsworth, N. R., Blomster, R. N., and Brown, W. W., *ibid.*, **27**, 470(1964).
(5) Janot, M.-M., and LeMen, J., *Compt. Rend.*, **239**, 1311(1954).
(6) Janot, M.-M., LeMen, J., and Gabbai, Y., *Ann. Pharm. Franc.*, **15**, 474(1957).
(7) Janot, M.-M., LeMen, J., and Hammouda, Y., *ibid.*, **14**, 341(1956).
(8) Farnsworth, N. R., Loub, W. D., Blomster, R. N., and Gorman, M., *J. Pharm. Sci.*, **53**, 1558(1964).
(9) Abraham, D. J., Farnsworth, N. R., Blomster, R. N., and Sharkey, A. G., Jr., *Tetrahedron Letters*, **1965**, 317.
(10) Svoboda, G. H., Neuss, N., and Gorman, M., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 659(1959).
(11) Svoboda, G. H., Johnson, I. S., Gorman, M., and Neuss, N., *J. Pharm. Sci.*, **51**, 707(1962).
(12) Johnson, I. S., Armstrong, J. G., Gorman, M., and Burnett, J. P., Jr., *Cancer Res.*, **20**, 1016(1960).
(13) Farnsworth, N. R., Blomster, R. N., and Buckley, J. P., *J. Pharm. Sci.*, **56**, 23(1967).
(14) Svoboda, G. H., Gorman, M., Neuss, N., and Barnes, A. J., Jr., *ibid.*, **50**, 409(1961).
(15) Farnsworth, N. R., Blomster, R. N., Damratoski, D., Meer, W. A., and Cammarato, L. V., *Lloydia*, **27**, 302(1964).
(16) Moza, B. K., Trojanek, J., Hanus, V., and Dolejs, L., *Coll. Czech. Chem. Commun.*, **29**, 1913(1964).
(17) Gorman, M., Neuss, N., and Biemann, K., *J. Am. Chem. Soc.*, **84**, 1058(1962).

Simultaneous Determination of Ethanol and Chloroform in Liquid Cough Preparations by Gas Chromatography

By HORACE P. WARRINGTON, JR., and JOHN M. LENNON

A gas chromatographic procedure using a styrene-divinylbenzene polymer as column-packing material is described. The use of this column allows for the complete separation and simultaneous determination of ethanol and chloroform contained in liquid cough preparations.

THE WIDESPREAD current use of gas chromatography for the quantitative measurement of ethanol (1) and of chloroform (2) suggests that this approach would be desirable in the analytical examination of formulations containing both substances. In practice, however, important difficulties arise. The volume ratio of ethanol to chloroform is always large (*e.g.*, 20 to 1). Response of a flame ionization detector is approximately 10 times greater to ethanol than it is to chloroform. With column materials available until recently, separation characteristics were such that chloroform showed up on the chromatogram as a very small peak on the tail of the ethanol peak. The contribution of ethanol peak tail area to the area under the chloroform peak was large enough to result in poor precision for the chloroform estimation. Recently, Hollis (3) introduced the use of a styrene-divinylbenzene polymer as a column-packing material. This material gives excellent separations of low molecular weight compounds with a minimum of tailing and permits column temperature programming with very low column bleed. A column packed with a styrene-divinylbenzene polymer[1] shows a complete separation of ethanol and chloroform. It has been found possible to measure 0.5% chloroform and 10% ethanol accurately and simultaneously in a cough syrup which also contains dextromethorphan hydrobromide, chlorpheniramine maleate, phenylephrine hydrochloride, glyceryl guaiacolate, glucose, and common preservative, sweetening, coloring, and flavoring agents.

EXPERIMENTAL

An F and M model 609 instrument was set up as described in Fig. 1 with a glass wool plug inserted in the injection port. The column was a 6-mm. o.d. glass tube 4 ft. long packed with 80 to 120-mesh styrene-divinylbenzene polymer.

An ethanol–chloroform standard solution was prepared by accurately weighing 10.5 ml. of absolute ethanol and 0.55 ml. of chloroform into about 20 ml. of distilled water contained in a 100-ml. volumetric flask. The mixture was made to volume with distilled water and thoroughly mixed. An isopropanol–*n*-butanol internal standard solution was prepared by accurately weighing 11 ml. of isopropanol and 0.1 ml. of *n*-butanol into about 20 ml. of distilled water in a 100-ml. volumetric flask. This mixture was made to volume with distilled water and thoroughly mixed.

Detector response calibration factors were determined from a chromatogram obtained from a 3-μl. injection of a solution prepared by mixing 10.0 ml. of the isopropanol–*n*-butanol internal standard solution and 10.0 ml. of the ethanol–chloroform standard solution. The ethanol peak area was compared to the isopropanol peak area, and the chloroform peak area was compared to the *n*-butanol peak area. In the cough preparation already described, the amounts of chloroform and ethanol were determined from a chromatogram of a 3-μl. aliquot of a mixture of 10.0 ml. of the cough syrup and 10.0 ml. of the isopropanol–*n*-butanol internal standard solution.

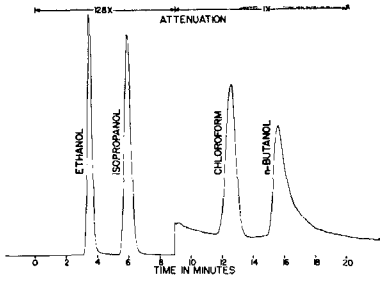


Fig. 1—Chromatogram of detector response calibration solution. Column temperature: 125° isothermally for 3 min., then programmed to 150° at a rate of 2.3° per minute. Injection port temperature = 125°. Helium carrier gas flow rate = 40 ml. per minute. Range = 10. Detector = flame ionization.

DISCUSSION

The glass wool plug in the injection port retains sugars and other high boiling components. At injection port temperatures greater than 125°, spurious peaks are observed which presumably originate from the thermal decomposition of components. Results using this assay for determining the amounts of chloroform and ethanol in various cough medicines are shown in Table I.

TABLE I—RESULTS OF ASSAY

Cough Medicine Lot No.	Chloroform		Ethanol	
	% Theory	% Found	% Theory	% Found
Pn-N-7a	0.55	0.46	10.0	9.99
Pn-N-7b	0.55	0.54	10.0	9.94
Pn-N-33	0.56	0.49	10.0	10.22
Pn-N-48	0.56	0.55	10.0	9.85
Drm-D-159	0.56	0.55	10.0	10.0

Received October 13, 1966, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144

Accepted for publication January 5, 1967.

[1] Polypak-2. F and M Scientific Division of Hewlett Packard.

REFERENCES

(1) Wesselman, H. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 320(1960).

(2) Brealey, L., Elvidge, D. A., and Proctor, K. A., *Analyst*, **84**, 221(1959).

(3) Hollis, O. L., *Anal. Chem.*, **38**, 309(1966).

Effects of Sex Hormones on the Duration of Drug Action in Mice

By T. GESSNER, M. ACARA, J. A. BAKER, and L. L. EDELMAN

An altered drug response is induced in mice following pretreatment not only with repeated doses, but even after a single dose, of a sex hormone. Testosterone pretreatment produces a biphasic effect on the duration of action of hexobarbital, prolonging the action initially and shortening the action in 4–8 days after the pretreatment. The early action of testosterone appears to be associated with an effect on the hypnotic property of a drug, since both hexobarbital and barbital sleep times are prolonged while the duration of action of the muscle relaxant chlorzoxazone remains unaffected. The long-term pretreatment with testosterone leads to a shorter duration of action of drugs that are deactivated by detoxification, notably hexobarbital and chlorzoxazone, but has no effect on the duration of hypnosis produced by barbital, a drug which is predominantly eliminated unchanged.

THERE IS increasing evidence that steroids are endogenous substrates of drug-metabolizing enzymes in mammals (1–8). Such a relationship between the drug and steroid metabolism could be of great consequence for drug therapy in man. In animals the evidence for the above interrelationship consists of two kinds of data. On one hand, compounds which have been known for some time to stimulate drug-metabolizing enzymes (2, 3) have been found more recently to be effective in stimulating steroid metabolism *via* hydroxylation (1, 4–7). On the other hand, sex hormones exert a marked effect on drug-metabolizing enzymes in the rat (8, 9). This latter phenomenon is seen both as an inherent sex difference in the susceptibility to drugs (female rats sleep four times longer than male rats after the same dose of hexobarbital), and as a virtual obliteration of such a sex difference through pretreatment of male rats with estrogens or of female rats with androgens (1, 8, 9). Similar experiments with sex hormones in mice and guinea pigs have been unsuccessful (8, 9).

It seemed important to the authors that if the relationship between drug- and steroid hormone-metabolizing enzymes is applicable to mammalian species in general, sex hormones should have an effect on the duration of drug action in species other than rats, though such effects might be more subtle, and hence less readily discernible. The authors re-examined the effect in mice.

EXPERIMENTAL

Animal Treatment—Swiss Webster mice of ICR strain from Blue Spruce Farms were used for the experiments. The mice weighed 18–26 Gm. on arrival and were maintained on commercial chow diet and water *ad libitum*. Each animal was used only once. Animals were randomly assigned to either a test or the corresponding control group. Both groups were treated similarly in all respects, except that the control mice received injections of the vehicle (corn oil) in place of the hormone solution.

In the study of the effects of chronic sex hormone pretreatment the hormone in corn oil solution was administered subcutaneously every other day for a period of 1 week or longer, as stated. Female mice were thus treated either with 4 doses of 10 mg./Kg. of ethinyl estradiol in an 8-day period, or with 8 doses of 20 mg./Kg. of testosterone acetate in a 16-day period. Male mice were treated with 7 doses of 25 mg./Kg. of diethylstilbestrol in a 14-day period. Chlorzoxazone and hexobarbital assays were performed 24 hr. after the last injection of the hormone.

In the study of the development of an altered drug response due to pretreatment with only a single dose of a sex hormone, assays were carried out using chlorzoxazone,[1] hexobarbital, and barbital at 0 hr., 24 hr., 96 hr. (4 days), and 192 hr. (8 days) after the hormone pretreatment. In the special case of the 0-hr. pretreatment the hormone in corn oil was injected intraperitoneally immediately before the assay drug; in all other cases the injections were subcutaneous. In all experiments corn oil was administered to control mice on a schedule identical with that used for the corresponding test animals.

Drug Assays—The duration of drug action was assayed by means of the following drugs at the given dosages: chlorzoxazone[2] (150 mg./Kg.), hexobarbital[3] (150 mg./Kg. unless otherwise stated), barbital[4] (250 mg./Kg.). The compounds were administered as sodium salts in isotonic saline. The duration of drug effect (the chlorzoxazone prostration time and the hexobarbital or barbital sleep time) was determined by noting the time interval during which the righting reflex was lost.

Where direct comparisons were made between assays with different agents (hexobarbital, chlorzoxazone, or barbital), control and test mice were randomly selected for assay with either agent and the assays were performed contemporaneously under identical conditions. The statistical significance of the observed differences (the P value) was determined by use of the tailed t test except where the F test showed the variance ratio itself to be significant. In the latter event Wilcoxon's two-tailed Rank test was employed (10).[5]

RESULTS AND DISCUSSION

The results (Table I) show that the duration of drug action is significantly changed after chronic pretreatment of mice with an estrogen (diethylstilbestrol or ethinyl estradiol) or with an androgen (testosterone acetate). Estrogen treatment leads to the prolongation of drug action in both male and female mice; conversely, androgen treatment leads to a shorter duration of drug action.

Received September 15, 1966, from the School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication December 8, 1966.

This investigation was supported in part by the undergraduate research participation program grant GY-212 to J. A. Baker and L. L. Edelman from the National Science Foundation.

[1] The gift of chlorzoxazone from McNeil Laboratories is gratefully acknowledged.

[2] 5-Chlorobenzoxazolidone.

[3] 5-(1-Cyclohexen-1-yl)-1,5-dimethylbarbituric acid.

[4] 5,5-Diethylbarbituric acid.

[5] The authors thank Dr. P. K. Gessner for the statistical computations.

TABLE I—EFFECT OF MULTIPLE DOSE PRETREATMENT WITH SEX HORMONES ON THE DURATION OF DRUG ACTION

Sex	N[a]	Pretreatment[c] Sex Hormone	Dose, mg./Kg.	Length, Days	Duration of Drug Effect Assay Drug	Time, min.	P
F	10	Ethinyl Estradiol	10	8	Hexobarbital	60.2 ± 4.9	<0.01
F	10	None[b]			Hexobarbital	43.4 ± 3.2	
F	8	Testosterone acetate	25	16	Chlorzoxazone	50.7 ± 4.7	<0.05[d]
F	5	None[b]			Chlorzoxazone	65.7 ± 7.2	
M	5	Diethylstilbestrol	25	14	Chlorzoxazone	52.2 ± 5.0	<0.01
M	5	None[b]			Chlorzoxazone	31.6 ± 2.0	
M	7	Diethylstilbestrol	25	14	Hexobarbital	97.2 ± 13.8	<0.05
M	7	None[b]			Hexobarbital	56.5 ± 7.1	

[a] Number of animals. [b] All control animals received injections of the vehicle (corn oil) on a schedule identical to that used for the corresponding tests. [c] During the pretreatment period the indicated dose of the sex hormone was administered every 48 hr. [d] Single-tailed *t* test.

It is interesting that female mice do not appear to have an inherently greater susceptibility to hexobarbital than male mice (Table I, hexobarbital control groups). In fact, the trend appears reversed but the observed difference in the sleep times is not statistically significant ($P < 0.06$). However, a highly significant sex difference ($P < 0.001$) is apparent in the duration of action of chlorzoxazone; female mice exhibit chlorzoxazone prostration times twice as long as those of the males.

The results of the sex hormone pretreatment are amenable to two interpretations. First, it could be argued that a period of chronic hormone pretreatment is necessary for the sought effect on drug action to become manifest. Second, the possibility exists that a single administration of hormone is sufficient, but that a certain length of time has to elapse before the process initiated by this hormone injection (in effect, the first injection of a chronic pretreatment regimen) results in an observable effect. In order to distinguish between these two possibilities, the authors injected female mice with a single high dose of testosterone acetate and carried out the duration of drug effect assays after various time intervals. As shown in Table II, the authors found a significant decrease in the duration of action of chlorzoxazone and of hexobarbital 192 hr. after a single injection of a dose of either 140 mg./Kg. or 46 mg./Kg. of testosterone.

Many compounds which stimulate drug metabolism when long-term pretreatment is employed are found to be inhibitors of drug-metabolizing enzymes both *in vivo* and *in vitro* (2, 11, 12). Accordingly, such compounds are seen to have a biphasic effect on the duration of action of drugs which are subject to inactivation through rapid metabolism. In order to test whether testosterone pretreatment can elicit a biphasic effect on the duration of drug action the authors carried out a series of duration of drug effect assays immediately after the hormone administration (0 hr. pretreatment, Table II). The results show that indeed such a short-term pretreatment with either testosterone or testosterone acetate produces a prolongation of the hypnotic action of barbiturates, but that this occurs irrespective of whether the barbiturate is one which is extensively metabolized, *i.e.*, hexobarbital (13), or one which is hardly metabolized at all, *i.e.*, barbital (14). Moreover, the identical pretreatment has no effect on the duration of the paralysis produced by chlorzoxazone, a drug which is inactivated mainly by metabolism (15). These results therefore suggest that the observed longer duration of action of barbiturates is mainly unrelated to an effect of testosterone on drug-metabolizing enzymes, but rather is associated with a potentiating effect on the hypnotic action. In this latter respect, the report of Selye (16) on the anesthetic property of testosterone in partially hepatectomized rats is noteworthy.

Novick *et al.* (17) have reported that daily pretreatment of mice with testosterone for 4–5 days resulted in a prolongation of hexobarbital sleep time in these animals, whereas we found that long-term testosterone pretreatment shortens the duration of drug action (Tables I and II). In the experiments of Novick *et al.*, high levels of circulating testosterone would be expected to be present at the time of the sleep-time assay, since, in contrast to our experiments, these workers administered the last injection of the hormone only 1-hr. before the hexobarbital assay. In such circumstances testosterone could extend the duration of hexobarbital action, not only by any possible inhibition of the drug-metabolizing enzyme (Novick *et al.* report somewhat decreased microsomal metabolism in the livers of testosterone pretreated mice) but also by a direct potentiation of the hypnotic action of the barbiturate.

The authors conclude that testosterone exerts a biphasic effect on the hypnotic action of hexobarbital. The prolongation of hexobarbital sleep time in the initial period following pretreatment with the hormone appears to be mainly unrelated to an effect on drug metabolism, but instead seems to involve potentiation or synergism of the hypnotic property of the drug, as is evidenced by the fact that hypnosis due to barbital is affected similarly. On the other hand, the shorter duration of drug action after long-term pretreatment with testosterone is probably associated with a stimulation of drug-metabolizing enzymes. This is indicated by the fact that an 8-day (192 hr.) pretreatment with testosterone has no effect on the barbital sleep time, but foreshortens the duration of action of the two drugs that are extensively metabolized, hexobarbital and chlorzoxazone.

TABLE II—TIME COURSE OF DEVELOPMENT OF AN ALTERED DRUG RESPONSE IN FEMALE MICE PRETREATED WITH A SINGLE DOSE OF AN ANDROGEN

	Pretreatment			Duration of Drug Effect		
N^a	Hormone	Dose, mg./Kg.	Length, hr.	Assay Drug	Time, min.	P
9	Testrosterone acetate	140	192	Chlorzoxazone	38.4 ± 2.2	<.02
10	None[b]			Chlorzoxazone	47.4 ± 2.5	
7	Testosterone acetate	140	192	Hexobarbital	86.4 ± 8.6	<.01
9	None[b]			Hexobarbital	125.1 ± 9.4	
10	Testosterone acetate	140	192	Barbital	107.4 ± 12.7	N.S.
10	None[b]			Barbital	112.2 ± 12.1	
10	Testosterone acetate	46	192	Hexobarbital	95.2 ± 7.6	<.01
10	None[b]			Hexobarbital	138.3 ± 11.7	
10	Testosterone acetate	46	96	Chlorzoxazone	47.6 ± 3.2	N.S.
10	None[b]			Chlorzoxazone	49.4 ± 3.6	
9	Testosterone acetate	46	96	Hexobarbital	146.3 ± 7.6	N.S.
10	None[b]			Hexobarbital	162.5 ± 10.4	
10	Testosterone acetate	46	24	Chlorzoxazone	25.4 ± 3.1	N.S.
10	None[b]			Chlorzoxazone	27.4 ± 1.9	N.S.
9	Testosterone acetate	140	24	Chlorzoxazone	27.5 ± 2.4	
10	Testosterone acetate	46	24	Hexobarbital	66.2 ± 8.5[c]	N.S.
10	None[b]			Hexobarbital	69.0 ± 4.6[c]	N.S.
10	Testosterone acetate	140	24	Hexobarbital	61.2 ± 6.5[c]	
9	Testosterone acetate	140	0	Chlorzoxazone	29.5 ± 2.0	N.S.
10	None[b]			Chlorzoxazone	26.8 ± 2.3	
10	Testosterone acetate	140	0	Barbital	237.2 ± 6.9	<.01
8	None[b]			Barbital	176.5 ± 15.9	
10	Testosterone	120	0	Chlorzoxazone	27.3 ± 2.0	N.S.
10	None[b]			Chlorzoxazone	26.8 ± 2.3	
9	Testosterone	120	0	Barbital	245.8 ± 13.4	<.005
8	None[b]			Barbital	176.5 ± 15.9	
10	Testosterone acetate	280	0	Hexobarbital	76.2 ± 4.1[c]	<.005
10	None[b]			Hexobarbital	53.6 ± 4.8[c]	
10	Testosterone	240	0	Hexobarbital	94.9 ± 10.1[c]	<.01
10	None[b]			Hexobarbital	53.6 ± 4.8[c]	

[a] Number of animals. [b] All control animals received injections of the vehicle (corn oil) on a schedule identical to that used for the corresponding tests. [c] Hexobarbital was injected at a dose level of 100 mg./Kg.

REFERENCES

(1) Conney, A. H., Schneidman, K., Jacobson, M., and Kuntzman, R., *Ann. N. Y. Acad. Sci.*, **123**, 98(1965).

(2) Remmer, H., *Proc. Intern. Pharmacol. Meeting, 1st., Stockholm*, **6**, 235(1962).

(3) Burns, J. J., Conney, A. H., and Koster, R., *Ann. N. Y. Acad. Sci.*, **104**, 881(1963).

(4) Jellinck, P. H., *Proc. Am. Assoc. Cancer Res.*, **5**, 123 (1964).

(5) Kuntzman, R., Jacobson, M., Schneidman, K., and Conney, A. H., *J. Pharmacol. Exptl. Therap.*, **146**, 281(1964).

(6) Kuntzman, R., and Jacobson, M., *Federation Proc.*, **24**, 133(1965).

(7) Conney, A. H., and Schneidman, K., *ibid.*, **24**, 152 (1965).

(8) Quinn, G. P., Axelrod, J., and Brodie, B. B., *Biochem. Pharmacol.*, **1**, 152(1958).

(9) Booth, J., and Gillette, J. R., *J. Pharmacol. Exptl. Therap.*, **137**, 374(1962).

(10) Goldstein, A., "Biostatistics," The Macmillan Co., New York, N. Y., 1964, pp. 51–57.

(11) Serrone, D. M., and Fujimoto, J. M., *J. Pharmacol. Exptl. Therap.*, **133**, 12(1961).

(12) Kato, R., Chiesara, E., and Vassanelli, P., *Biochem. Pharmacol.*, **13**, 69(1964).

(13) Bush, M. T., Butler, T. C., and Dickinson, H. L., *J. Pharmacol. Exptl. Therap.*, **108**, 104(1953).

(14) Ebert, A. G., Yim, G. K. W., and Miya, T. S., *Biochem. Pharmacol.*, **13**, 1267(1964).

(15) Conney, A. H., and Burns, J. J., *Ann. N. Y. Acad. Sci.*, **86**, 167(1960).

(16) Selye, H., *Endocrinology*, **30**, 437(1942).

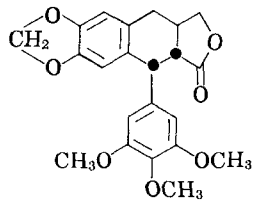
(17) Novick, W. J., Stohler, C. M., and Swagzdis, J., *J. Pharmacol. Exptl. Therap.*, **151**, 139(1966).

Tumor Inhibitors XIX. Desoxypodophyllotoxin, the Cytotoxic Principle of *Libocedrus decurrens*

By S. MORRIS KUPCHAN, RICHARD J. HEMINGWAY, and JANE C. HEMINGWAY

An alcoholic extract of *Libocedrus decurrens* Torr. was found to show significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB). Systematic fractionation of the extract led to the isolation and characterization of desoxypodophyllotoxin as the active principle.

In the course of continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Libocedrus decurrens* Torr.[1] (*Pinaceae*) was found to have significant inhibitory activity against cells derived from human carcinoma of the nasopharynx in tissue culture (KB).[2] Consequently, a systematic study aimed at isolation of the KB-inhibitory principle of *L. decurrens* Torr. was undertaken. The preliminary fractionation of the alcohol extract is summarized in Scheme I. Fraction H material, dissolved in chloroform, was subjected to chromatography on silicic acid, whereupon the total activity was eluted in the first yellow band using chloroform as eluant. Further careful rechromatography on silicic acid, collecting fractions which were analyzed by thin-layer chromatography, led to the isolation of desoxypodophyllotoxin (I) from the fraction eluted with 10% chloroform in benzene. The assay data are reported in Table I. Evaluation of the tissue culture assay results by the CCNSC is such that a material is considered active if the ED_{50} (dose inhibiting growth to 50% of control growth) equals or is less than 1 mcg./ml. The fractions containing desoxypodophyllotoxin were found to show the highest cytotoxicity. The results indicate that desoxypodophyllotoxin is the major cytotoxic principle of the extract of *L. decurrens* Torr.[3]



I

EXPERIMENTAL

Melting points were determined on a Hoover Uni-Melt capillary melting point apparatus. Infrared spectra were determined in chloroform solution on a Beckman IR5A infrared spectrophotometer. The petroleum ether used was Skellysolve B, b.p. 60–68°. Evaporations were carried out at temperatures less than 40°.

Extraction and Preliminary Fractionation of L. decurrens—Coarsely ground leaves and stems of *L. decurrens* (500 Gm.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump pressure to a thick dark syrup (A, 142 Gm.). A portion of the extract (96 Gm.) was partitioned between water (1.5 L.) and chloroform (2.5 L.), and the two solutions were evaporated under reduced pressure (B, 59 Gm., and D, 27 Gm., respectively) and the insolubles (C, 8.1 Gm.) collected separately. The chloroform solubles, except a small assay sample (50 mg.), were partitioned between petroleum ether (1 L.) and 10% aqueous methanol (750 ml.) and the two solutions were evaporated under reduced pressure (E, 9.0 Gm., and F, 15.0 Gm., respectively). The 10% aqueous methanol solubles were dissolved in methanol (250 ml.) and treated with a saturated methanol solution of neutral lead acetate until no more precipitate formed. The precipitate was removed by centrifuging and washed with methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered after regeneration had been completed and the filtrate was evaporated to a gummy residue (G, 1.94 Gm.). The supernatant liquid from lead acetate precipitation was also treated with hydrogen sulfide, and the lead sulfide was filtered off and the solvent evaporated (H, 11.9 Gm.).

Isolation of Desoxypodophyllotoxin—A larger batch of fraction H (151 Gm.) was prepared from the crude extract (1.5 Kg.) and a portion (57.9 Gm.) was dissolved in chloroform and added to a column of silicic acid (2.6 Kg.). The column was eluted with chloroform until the first orange-yellow band was collected (I, 16.445 Gm.) and the remaining material was stripped with methanol (J, 36.0 Gm.).

A portion of the active fraction I (5.6 Gm.), was dissolved in benzene, added to a column of silicic acid (80 Gm.) packed in benzene, and chromatographed using 10% chloroform in benzene as solvent. Fractions were collected and examined by thin-layer chromatography on silica gel using chloroform as solvent and ceric sulfate (3% in 3 *N* sulfuric acid) spray reagent. The fractions richest in desoxypodophyllotoxin were combined, evaporated to dryness (533 mg.), and crystallized from ethanol. The colorless crystalline product (K, 195 mg.) was characterized as desoxypodophyl-

Received October 20, 1966, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison, WI 53706

Accepted for publication November 29, 1966.

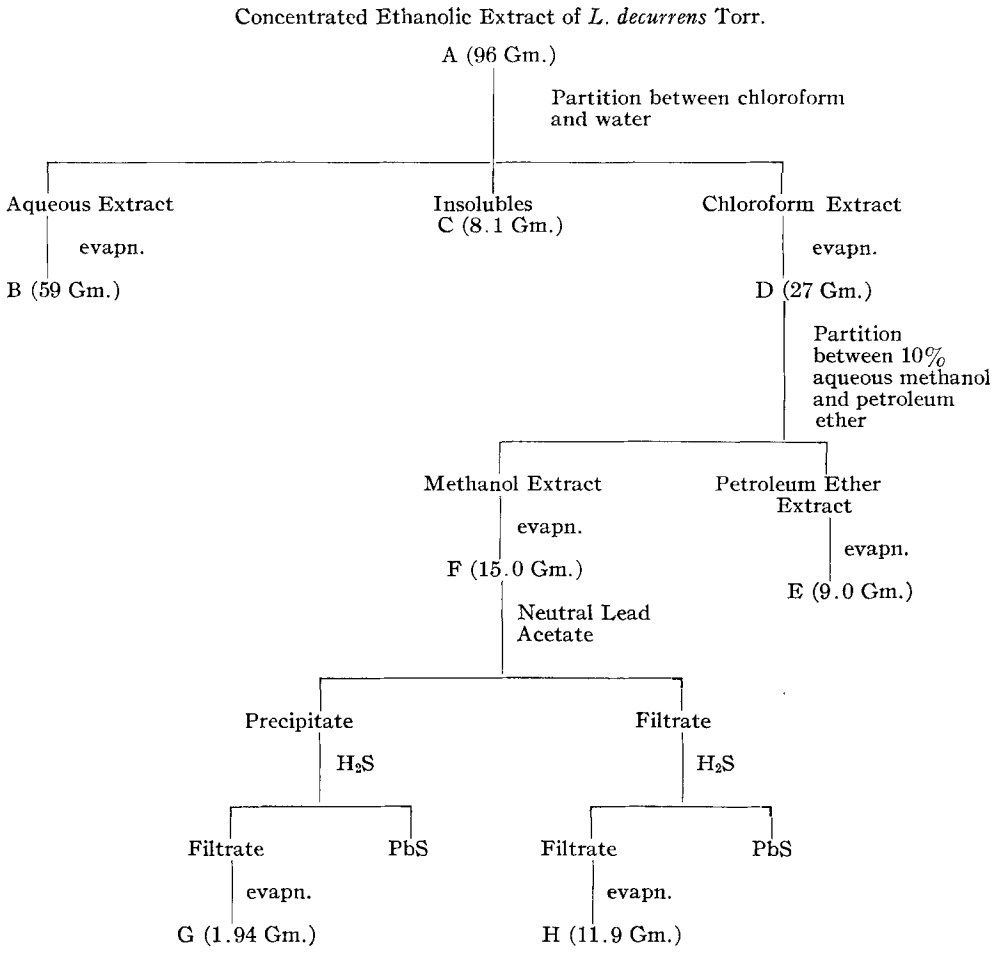
This investigation was supported by grant CA-04500 from the National Cancer Institute, grant T-275 from the American Cancer Society, and contract PH 43-64-551 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Previous paper: Abbott, B. J., Hartwell, J. L., Iltis, H. H., Kupchan, S. M., Leiter, J., Perdue, R. E., Jr., and Schepartz, S. A., *Cancer Res. (Suppl.)*, **26**, 1131(1966).

[1] Leaves and stems gathered in California in September 1964. The authors acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center.

[2] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[3] It has been reported previously (1) that desoxypodophyllotoxin occurs in *Libocedrus chilensis* and is active against sarcoma 37. The same authors, however, found that their sample of *L. decurrens* was inactive against sarcoma 37.



Flow Sheet for Fractionation of Cytotoxic Extract from *L. decurrens* Torr.

Scheme I

TABLE I—CYTOTOXICITY OF FRACTIONS FROM *L. decurrens* TORR.

Fraction	ED_{50}, mcg./ml.
A	0.26
B	>100
C	27
D	0.063
E	3.0
F	0.024
G	2.6
H	0.23
I	<0.001
J	2.5
K	1.6×10^{-9}

lotoxin by mixed melting point, mixed thin-layer chromatography, and infrared spectral comparison with an authentic sample.[4]

Other chromatographic fractions with contained desoxypodophyllotoxin also showed cytotoxic activity. The desoxypodophyllotoxin-free fractions were found to be essentially inactive in the KB assay.

REFERENCE

(1) Fitzgerald, D. B., Hartwell, J. L., and Leiter, J., *J. Natl. Cancer Inst.*, **18**, 83(1957).

[4] The authors thank Dr. J. L. Hartwell, CCNSC, National Cancer Institute, for the authentic sample of desoxypodophyllotoxin.

Molecular Weight of the Antifungal Antibiotic Saramycetin

By JOEL KIRSCHBAUM and A. ASZALOS

The molecular weight of the antibiotic saramycetin was determined as 2100 by two ultracentrifugal methods: (*a*) approach to sedimentation equilibrium and (*b*) combination of the zero concentration diffusion constant ($D_{20} = 34 \times 10^{-7}$ cm.^{2} sec.$^{-1}$) with the zero concentration sedimentation constant ($S_{20}, w = 1.2 \times 10^{-13}$ cm. sec.$^{-1}$). At concentrations above 5 mg./ml. saramycetin associates, forming aggregates with a molecular weight of 55,000.

THE MOLECULAR weight of the antifungal agent saramycetin had been previously reported by Baudet and Cherbuliez (1) to be 14,000 daltons on the basis of sedimentation and diffusion constant measurements. Evidence will be presented in this paper that saramycetin is capable of molecular association and that the apparent molecular weight is dependent on polarity of solvent and solute concentration.

EXPERIMENTAL

Molecular weights were measured by the Archibald (2) approach to sedimentation equilibrium method. One to 0.1% solutions, in 12-mm. interference cells, were centrifuged in an An-D rotor at 22°. Concentration gradients were formed at speeds ranging from 8766 to 42,040 r.p.m. using the schlieren optical system with a phase bar angle of 75°. The concentration gradient was evaluated using Engelberg's modification (3), at the meniscus and cell bottom from photographs recorded on Kodak metallographic plates magnified tenfold on a Nikon magnifier. Total solute concentration was measured with a synthetic boundary centerpiece in an interference cell centrifuged at 29,500 r.p.m.

Sedimentation coefficients for saramycetin solutions with concentrations from 10 mg./ml. to 1.25 mg./ml. were measured in a 4°, 12-mm. cell centrifuged in an An-D rotor at 42,040 r.p.m. For solutions with concentrations of 1.25 mg./ml. to 0.313 mg./ml., the sedimentation coefficients were evaluated from schlieren peaks produced with the aid of a 12-mm. synthetic boundary centerpiece in an interference cell, rotated at speeds to 42,040 r.p.m.

Diffusion constants were evaluated in the analytical ultracentrifuge (4), at 22°, using a synthetic boundary centerpiece and the schlieren optical system. Solute concentrations were from 10 mg./ml. to 0.313 mg./ml. The first image was recorded at two-thirds of the final speed of 42,040 r.p.m. or 29,500 r.p.m. This first image was considered to be recorded at zero time for the diffusion constant calculations.

Partial specific volume was determined at 20° in a 1-ml. pycnometer.

RESULTS AND DISCUSSION

As the concentration of saramycetin is diminished from a concentration of 10 mg./ml. to 0.313 mg./ml. in 0.2 *M* NaCl–0.02 *M* phosphate buffer, pH 6.85, the sedimentation coefficient (*s*) first increases and then decreases (Fig. 1). Two *S* values at infinite dilution can be calculated by extrapolating to zero solute concentration both the positively sloping and negatively sloping regions of the curve. At the higher concentrations of saramycetin (10 mg./ml. to 5 mg./ml.) $S_{20}, w = 5.28 \times 10^{-13}$ cm. sec.$^{-1}$ and at concentrations of saramycetin below 2.5 mg./ml., $S_{20}, w = 1.2 \times 10^{-13}$ cm. sec.$^{-1}$. The *S* value found for saramycetin depends then on the solute concentration in 0.2 *M* NaCl–0.02 *M* phosphate buffer.

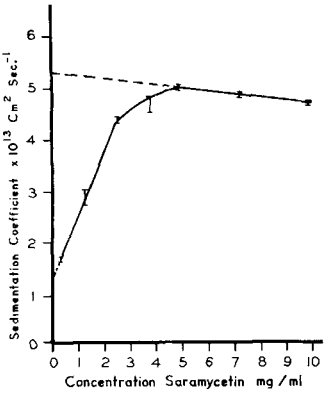


Fig. 1—Dependence of sedimentation coefficient of saramycetin on the concentration of saramycetin in 0.2 M *NaCl–0.02* M *phosphate buffer, pH 6.85.*

Measurements of the diffusion constant also give two values dependent on saramycetin concentration. At the higher saramycetin concentrations, $D_{20} = 6.0 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ and at the lower concentrations, $D_{20} = 34 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, extrapolating to zero concentration.

The partial specific volume, $\bar{V}$, was found to be 0.58, and agrees with the value of 0.60 ± 0.05 published by Baudet and Cherbuliez.

By combining the *S* and *D* data (5) from each concentration range, two molecular weights have been calculated: 55,000 for the 10 to 5 mg./ml. solute concentration and 2100 for the 2.5 to 0.313 mg./ml. concentration range. Acid titration yields an equivalent weight of 2200. This evidence suggests that 2100 represents the true molecular weight. In this solvent system, if it can be assumed that the mass action law is valid, the monomer concentration term in an equilibrium gradient expression would have an exponent of about 25.

Further evidence for aggregate formation is summarized in Table I, which gives the molecular weight of saramycetin in various solvents.

Approach to equilibrium sedimentation (6) yielded a molecular weight of 54,000 ± 2000 daltons at the meniscus and cell bottom for a 1% solution of saramycetin dissolved in any of the following buffers: 1 *M* acetate buffer, pH 4.05; 0.2 *M* NaCl–0.02 *M* phosphate buffer, pH 6.85; 2 *M* NH_4OH–2 *M* NH_4Cl, pH 9.4; or an isotonic solvent containing the inorganic cations and anions found in human serum.

Received October 20, 1966, from the Squibb Institute for Medical Research, New Brunswick, NJ 08903
Accepted for publication November 29, 1966.
This investigation was supported by contract TH 43-65-1056 with the Cancer Chemotherapy National Service Center, National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

TABLE I—MOLECULAR WEIGHT OF 1% SARAMYCETIN SOLUTIONS IN VARIOUS SOLVENTS AS DETERMINED BY APPROACH TO EQUILIBRIUM SEDIMENTATION

Solvent	Mol. Wt.
1 *M* acetate buffer, pH 4.05	55,000 ± 2,000
0.2 *M* NaCl–0.02 *M* phosphate buffer, pH 6.85	56,000 ± 2,000
2 *M* NH_4OH–2 *M* NH_4Cl buffer, pH 9.4	55,000 ± 2,000
Isotonic phosphate buffer containing K^+, Na^+, Ca^{++}, Mg^{++}, Cl^-, and $SO_4^=$	54,000 ± 2,000
0.68 *M* borate buffer, pH 8.6	6,000–20,000
8 *M* urea in NaCl–phosphate buffer	7,000 ± 1,000
90% ethanol (95%)–10% NaCl–phosphate buffer	2,100 ± 100

Sedimentation of 1% saramycetin in 8 *M* urea–NaCl–phosphate buffer diminished the apparent molecular weight to 7000 ± 1000 daltons, indicating a disruption of the aggregate.

Borate buffer, pH 8.6, 0.68 *M*, also affected the aggregate molecular weight with several components found with uncorrected molecular weights of from 6000 to 20,000 daltons.

By diminishing the concentration of the phosphate–NaCl buffer to concentrations of $^1/_{100}$ of the original, a molecular weight of 5500 ± 1000 daltons was found ($S = 1.37 \times 10^{-13}$ cm. sec.$^{-1}$). The physical effect of diluting the buffer is to diminish the apparent molecular weight of the saramycetin aggregate. The *in vitro* microbiological activity of 0.1% saramycetin solutions varied inversely with the ionic strength of the buffer (7). This indicates that the biological effect of using a more dilute buffer is to increase the *in vitro* antifungal activity of saramycetin solutions.

In summary, saramycetin, molecular weight 2100, may form aggregates in solution. The molecular weight of these aggregates may range upward to 55,000 depending on the solvent and on electrolyte ionic strength.

REFERENCES

(1) Baudet, P., and Cherbuliez, E., *Helv. Chim. Acta*, **47**, 661(1964).
(2) Schachman, H. K., "Ultracentrifugation in Biochemistry," Academic Press Inc., New York, N. Y., 1959, p. 182.
(3) Engelberg, J., *Anal. Biochem.*, **6**, 530(1963).
(4) Schachman, H. K., in "Methods in Enzymology," Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1959, vol. 4, p. 59.
(5) Svedberg, T., and Pedersen, K. O., "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940, p. 5.
(6) Archibald, W. J., *J. Phys. Colloid. Chem.*, **51**, 1204 (1947).
(7) Robinson, R., and Davis, S., unpublished data.

Communications

Mathematics of the Three-Phase *In Vitro* Absorption Models

Sir:

Several reports on three-phase models for drug absorption have recently appeared in the literature (1–3). However, it appears that volume terms should be included in the rate equations as well as in the equilibrium equations to obtain more meaningful values for the transfer rates. In these models, the drug (usually weakly basic or weakly acidic) is transferred from an aqueous buffer (*A*) of a pH found in the alimentary tract, through a water immiscible organic phase (*B*) acting as the "membrane," to a buffer of blood pH (*C*). Assuming that only the unionized drug is soluble in the organic phase, then the equilibrium concentrations (C_A, C_B, C_C) can be expressed in the equations derived below in terms of the initial concentration of the drug (a_0), the true distribution coefficient of the drug between the organic layer and water (D), the pH's of the buffers (pH_A and pH_C), the pKa of the drug, and the volumes of the phases (V_A, V_B, and V_C).

For a monobasic acid

$$C_B = \frac{a_0 D}{1 + 10^{pH_A - pK_A} + \frac{V_C}{V_A}(1 + 10^{pH_C - pK_A}) + \frac{V_B D}{V_A}}$$

$$C_A = \frac{C_B}{D}(1 + 10^{pH_A - pK_A})$$

$$C_C = \frac{C_B}{D}(1 + 10^{pH_C - pK_A})$$

For a mono-acidic base

$$C_B = \frac{a_0 D}{1 + 10^{pK_A - pH_A} + \frac{V_C}{V_A}(1 + 10^{pK_A - pH_C}) + \frac{V_B D}{V_A}}$$

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In the kinetics of the three-phase distribution

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$$C_A = \frac{C_B}{D}(1 + 10^{pH_A - pK_A})$$

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For a mono-acidic base

$$C_B = \frac{a_0 D}{1 + 10^{pK_A - pH_A} + \frac{V_C}{V_A}(1 + 10^{pK_A - pH_C}) + \frac{V_B D}{V_A}}$$

$$C_A = \frac{C_B}{D}(1 + 10^{pK_A - pH_A})$$

$$C_C = \frac{C_B}{D}(1 + 10^{pK_A - pH_C})$$

In the kinetics of the three-phase distribution

experiment, volume terms should be included to obtain a mass balance. Consider the transfers

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C$$

then

$$V_A \frac{dA}{dt} = -V_A k_1 A + V_B k_{-1} B$$

$$V_B \frac{dB}{dt} = V_A k_1 A - V_B k_{-1} B - V_B k_2 B + V_C k_{-2} C$$

$$V_C \frac{dC}{dt} = V_B k_2 B - V_C k_{-2} C$$

where A, B, C are the concentrations in the three compartments at any instant. These equations are perfectly general and all transfers in the three-phase model should obey them.

Estimates of the values of the transfer constants k_1, k_{-1}, k_2, and k_{-2} can be obtained with or without the aid of a computer. These transfer constants are dependent on the pH of the solutions, the pKa of the drug, the true distribution coefficient, and the interfacial area, but not on the volumes of the phases. Often several of the terms are negligible (substitution in the equilibrium equations given above will aid in predicting this), so simplifying solution of the equations. The simplest case observed (3) and the one possibly nearest to an *in vivo* absorption situation is the transfer of a weakly acidic drug from a buffer of low pH, in which ionization is negligible, through an organic solvent, in which the drug is much more soluble than the unionized entity is in water, to a buffer of pH 7.4 ("blood"), in which the drug is fully ionized. In this case the back reactions are negligible and if $V_A = V_C$ then on integration we have

$$A = a_0 e^{-k_1 t}$$

$$B = \frac{V_A}{V_B} \frac{a_0 k_1}{k_2 - V_A k_1 / V_B} \left(e^{-V_A k_1 t / V_B} - e^{-k_2 t}\right)$$

$$C = a_0 \left[1 - \frac{1}{k_2 - V_A k_1 / V_B} \left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t} - \frac{V_A}{V_B} k_1 e^{-k_1 t} + k_1 e^{-V_A k_1 t / V_B}\right)\right]$$

Many of the other special cases for this type of transfer have been reported by Doluisio and Swintosky (2); here the authors have a different approach, avoiding the use of volumes in the differential equations. However, if these equations are integrated and/or used in the comparison of theoretical with experimental data, then volume terms must be introduced at some stage.

(1) Doluisio, J., and Swintosky, J. V., *J. Pharm. Sci.*, **53**, 597(1964).
(2) *Ibid.*, **54**, 1594(1965).
(3) Perrin, J., *J. Pharm. Pharmacol.*, to be published.

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Received October 31, 1966.
Accepted for publication January 20, 1967.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JOURNAL OF **Pharmaceutical Sciences**

April 1967 volume 56, number 4

Review Article

Applications of Thin-Layer Chromatography in Pharmaceutical Analyses

By J. P. COMER and I. COMER

THIS REVIEW is a humble effort toward providing a guide to the literature for those pharmaceutical analysts who are not specialists in thin-layer chromatography (TLC), but who are using TLC as one of the many analytical techniques available today. Data on adsorbents, solvent systems, and R_f values are presented so that this review may be used as an intermediate reference between the textbooks and the original journals when they are not conveniently available.

Unfortunately, many good references listed in the abstracting journals were not available to the authors. Because of the repetitive nature of TLC publications, data in the articles omitted will probably appear in future papers. Some classes of compounds, such as amino acids, lipids, and carbohydrates, have been omitted for the sake of brevity. Most of the references reviewed were subsequent to 1959 and for alkaloids, steroids, and vitamins after 1963. Only the first author's name is mentioned in the text but full credit is given to all authors under *References*.

REVIEWS

The popularity of TLC by virtue of its simplicity and economy has produced a large number of general reviews (about 80) since 1960. Most of these were short articles designed for the introduction of the technique. Publication of the texts by Stahl (1), Bobbitt (2), Randerath (3), and Truter (4) consolidated many of the facts and generalizations available from the earlier literature.

Gänshirt's (5) publication summarized some of the earlier work on separation and identification of drug formulations by TLC in his laboratory.

Publications on pharmaceuticals previous to 1960 are included in reviews by Stahl (6,7) in 1961 with sections on pharmaceuticals, steroids, and vitamins. Wollish (8) listed 8 references on pharmaceuticals and showed construction details for an applicator, plate holder, and storage cabinet. Demole (9) gave a general review (French) with 61 references including some alkaloids and vitamins. Teijgeler (10) in 1962 presented a review (Dutch) with 132 references outlining the history, advantages over other forms of chromatography, principles, and reference to six publications on quantitative analyses. The compounds were categorized in terms convenient to the pharmaceutical analysts. Teijgeler (11) brought his review up to date with 64 additional references tabulated according to his original scheme. The book edited by Stahl published in German in 1962 and translated in 1965 (1) is the most convenient starting place for review of the earlier literature, and it is recommended for all analysts active in TLC. It includes chapters by experts in their fields.

A paper by Giacobazzi (12) includes 36 references of pharmaceutical interest. Heft-

Received from the Control Division, Eli Lilly and Co., Indianapolis, IN 46206

mann's comprehensive bibliography (13) included literature from December 1961 through December 1963 on column, paper, and thin-layer chromatography for many classes of compounds and 30 references specifically for pharmaceuticals.

Russel's (14) review (English) is a very good condensed general outline of TLC particularly in reference to types of development. It could be used as a guide for a short course for the introduction of analysts to the techniques of TLC. No attempts were made by Russel for the classification of compounds.

Zarnack (15) published a similar paper (German) designed for the teaching and practice of drug analyses. Solvent systems and examples are given for analgesics, antipeptics, purines, sulfonamides, and alkaloids with 67 references through 1963.

In a section of a book on biochemical analysis, Mangold (16) presented another condensed summary (90 pages) of the techniques of TLC. No attempt to list classes of compounds was made by the authors. A table of common reagents for detection was shown on page 410. An elutropic series was listed on page 422, and a very good section on means of quantitation was included. A table of increasing affinity of functional groups and adsorption power of adsorbents was shown. In other tables, material and solvents were listed for adsorption, ion exchange, partition, and reversed phase partition TLC. The section was concluded with a very convenient list of manufacturers of TLC apparatus and 87 references.

Coombe (17) gave a brief summary (two pages) on the application of quantitative TLC to pharmaceutical analyses.

Heftmann's (18) bibliography in 1966 with 2,329 references covering TLC, paper, and column chromatography (December 1963–December 1965) gave TLC separate discussion on technique and application to classes of compounds. About 90 references were listed for pharmaceuticals and a separate listing made for steroids, vitamins, and antibiotics. Heftmann's review, even though not specific for TLC nor pharmaceutically oriented, is the best starting point for a literature survey of recent publications. Unfortunately, the only way to find data on specific compounds is to read the whole section on that class and then look up the original references cited. Another survey by Teijgeler (19) added 243 references to his earlier paper.

Special bibliographies furnished by suppliers of apparatus and chemicals are available particularly since the introduction of precoated plates and sheets. One of these by Baitsholt (20) includes sections on pharmaceuticals.

EXPLANATION OF TABLES

There has been much written on the merits of and the factors influencing R_f values. In fact, this itself has been the subject of several publications (21–23). Active workers in the field know that observing a fresh TLC plate or suitable reproduction of such tells much more than observing a table in regard to size of spots, shape, tailing, *etc.* A summary of data using reproduction of plates is not practical in a review article such as this. The original R_f values or averages of ranges of values are listed in the tables. The reference for each publication is listed first. The alphabetical designation for solvent system refers to the list of solvents at the bottom of the table.

An "X" was used to show that the R_f value was not reported or was unavailable to the authors when this review was written.

The compounds are generally listed alphabetically, except when other arrangements make interpretation more convenient. Nomenclature presented the usual problems (24) of various trade names in different languages. Classification of the drugs under the general headings was by convenience and according to type of articles in which the work was reported. These lists certainly should not be used as a therapeutic or pharmacological index.

Some duplication, ambiguity, and inaccuracies are undoubtedly present; but workers active in the specific fields will recognize the various names. The adsorbents are designated by a number which refers to the list in Table I.

TABLE I—ADSORBENTS

1, Silica gel (with or without $CaSO_4$)
2, Silica gel with 0.1 *M* NaOH
3, Silica gel with 0.1 *M* KOH
4, Silica gel with 0.1 *M* $KHSO_4$
5, Silica Gel HF254
6, Silica Gel GF
7, Silica gel with rice starch
8, Silica gel with kieselguhr (1:1)
9, Silica gel with $AgNO_3$
10, Silica gel with EDTA
11, Silica gel with NaEDTA
12, Silica gel with $ZnCO_3$ (1:1)
13, Aluminum oxide (with or without $CaSO_4$)
14, Basic aluminum oxide
15, Silica gel + PEG 200
16, Aluminum oxide with $ZnCO_3$ (1:1)
17, Cellulose
18, Cellulose-MN300
19, Kieselguhr
20, Kieselguhr with formamide
21, Carbon
22, Carbon with acid
23, Polyamide powder

TABLE II—TLC OF ERGOT ALKALOIDS

Ref. →	(28)	(28)	(28)	(28)	(27)	(31)	(32)	(32)	(25)	(25)	(25)	(25)	(25)	(25)	(25)
Solvent[a] →	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Adsorbent →	1	1	13	13	20	2	1	1	1	1	1	1	1	13	13	2
Compd.	$R_f \times 100$															
Aci-dihydroergotamine						31										
Dihydroergocornine					38		47	48								
Dihydroergocristine					30		47	48	42	30	03	0	07	15	07	69
Dihydroergocryptine					50		47	48								
Dihydroergotamine					09	45	28	29	21	12	0	0	03	07	0	61
Ergocornine	58	59	10	24	50											
Ergocorninine	83	73	31													
Ergocristine	54	56	09		41				51	38	14	05	13	46	15	70
Ergocristinine	80	74	38						61	57	13	0	20	0	27	70
Ergocryptine	60	55	15	30	61											
Ergocryptinine	85	75	46													
Ergonovine	17	12	0			19										
Ergononovinine						36										
Ergometrine							14	17	14	06	0	0	02	03	0	64
Ergometrinine	44	38	01						42	25	03	0	08	12	10	62
Ergosine	35	31	02		17											
Ergosinine	75	68	12													
Ergotamine	31	31	01		11	51	29	32	24	16	0	0	03	10	05	59
Ergotaminine	68	64	07			76			24	51	0	0	14	42	15	68
LSD						61										
Methylergonovine						23										
Methysergide						35										

[a] *A*, ethyl acetate–*N*,*N*-dimethylformamide–ethanol (130:19:1); *B*, benzene-*N*,*N*-dimethylformamide (13:2); *C*, $CHCl_3$–ethyl ether–water (175:25:50); *D*, $CHCl_3$–ethyl ether–water (3:1:1); *E*, ethyl acetate–*n*-heptane–Et_2NH (250:300:1); *F*, $CHCl_3$–MeOH (9:1); *G*, C_6H_6–acetone–ether–10% NH_3 (4:6:1:0.3); *H*, C_6H_6–acetone–ether–25% NH_3 (4:6:1:0.3); *I*, $CHCl_3$–acetone–Et_2NH (50:40:10); *J*, $CHCl_3$–Et_2NH (90:10); *K*, cyclohexane–$CHCl_3$–Et_2NH (50:40:10); *L*, cyclohexane–Et_2NH (90:10); *M*, C_6H_6–ethyl acetate–Et_2NH (70:20:10); *N*, $CHCl_3$; *O*, cyclohexane–$CHCl_3$ (30:70) + 0.05% diethylamine (3 drops); *P*, CH_3OH.

The descriptions of the means of visualization are included in the text. Usually if good separation is obtained, detection is not a problem since there are many means and reagents available, such as I_2 vapor, H_2SO_4, $KMnO_4$, U.V. and fluorescence, and bioautograph. Analysts usually improvise means of detection suitable for their specific problems.

ALKALOIDS

One of the many papers on the separation and identification of alkaloids was an early report by Waldi (25) in 1961. A total of 54 alkaloids were studied using various solvent systems and silica gel or alumina as the adsorbent. The chapter by Waldi in Stahl's book (*Reference 1*, page 279), the chapter on TLC in Stolman's book (26), and the chapters in the book by Marini-Bettolo (*Reference 22*, pages 144, 149, 155) survey the early literature. This review will mention some pertinent studies reported after 1963.

Hydrogenated ergot alkaloids were separated by Hohmann (27) on cellulose powder impregnated with formamide (Table II). The alkaloids were detected by irradiating with a low pressure Hg burner giving a yellow-green fluorescent zone. The method also separated the nonhydrogenated alkaloids which appeared as blue fluorescent zones.

McLaughlin (28) developed a method for the identification and determination of ergot alkaloids. The solvents shown in Table II were chosen from 111 solvent systems studied. Regression analyses for data obtained by a quantitative determination of the eluted alkaloid by reaction with *p*-dimethylaminobenzaldehyde (PDAB) was tabulated.

A TLC procedure using silica gel and C_6H_6–$CHCl_3$–EtOH (2:4:1) was used by Ziner (29) for the quantitative evaluation of the stability of ergot alkaloids in aqueous preparations with the PDAB reaction.

The separation of ergot alkaloids from pharmaceuticals by Sahli (30) was accomplished on silica gel plates using $CHCl_3$–95% EtOH (9:1) as solvent. The plates were examined under U.V. light and the spots were then determined fluorimetrically using blank zones for comparison.

French (31) used TLC for the determination of identity and purity of ergot alkaloids and other active components present in pharmaceutical dosage forms.

Wasicky (33) used microscope slides as a support for silica gel and a small chromatographic chamber with butanol containing 10% acetic acid and saturated with water as the solvent for opium alkaloids. Spots were detected with PDAB reagent.

Brochmann-Hanssen (34) observed the TLC zones (Table III) under U.V. light, then sprayed

TABLE III—TLC OF OPIUM ALKALOIDS

Ref. →	(43)	(36)	(36)	(36)	(36)	(36)	(36)	(36)	(36)	(44)	(34)	(34)	(35)
Solvent[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	13
Compd.	$R_f \times 100$												
Piminodine ethanesulfonate[b]		97	98	97	93	78							
Anileridine		95	95	93	77	67							
Cotarnine													
Codeine	26	28	42	40	05	05	11	24	21		35	15	77
Cryptopine											48	40	
Diacetylmorphine													
Hydrocodone bitartrate[c]		36	35	42	05	05							
Dihydrocodeine													
Hydrocodone													
Dihydromorphine		09	16	17	01	02							
Hydromorphone													
Hydromorphone HCl[d]		18	19	21	02	01							
Dionin		40	46	47	05	05							
Heroin		74	67	73	19	11							
Hydromorphone													
10-Hydroxycodeine											17	15	
Laudanidine											47	28	
Laudanine											47	28	
Laudanosine											74	36	
Levorphanol tartrate[e]		57	54	44	20	17							
Meperidine		63	70	70	27	28							
Metapon		28	30	34	03	02							
Methadon		79	81	82	58	60							
Monoacetylmorphine		50	55	56	11	10							
Morphine	12	18	20	23	02	02	05	08	07	05	12	08	03
Nalorphine										60			
Narceine											09	03	
Narcotine	74	80	83	85	72	57	80	89	85		97	87	92
Narcotoline											88	71	
Neopine											38	12	
Alphaprodine HCl[f]		83	79	87	25	22							
Normorphine										42			
Oxymorphone HCl[g]		56	56	58	15	14							
Papaverine	59	73	77	77	53	35	61	81	72		97	77	89
Peronin		53	51	52	09	07							
Phenazocine		96	96	95	80	70							
Protopine											46	42	
Thebaine	45	65	73	74	23	15	34	62	54		65	38	

[a] *A*, xylene–methyl ethyl ketone–MeOH–Et_2NH (20:20:3:1); *B*, EtOH–dioxane–benzene–NH_4OH (5:40:50:5); *C*, $CHCl_3$–dioxane–EtOAc–NH_4OH (25:60:10:5); *D*, EtOH–$CHCl_3$–dioxane–30-60° pet. ether–C_6H_6–NH_4OH–EtOAc (5:10:50:15:10:5:5); *E*, EtOAc–benzene–NH_4OH (60:35:5); *F*, EtOAc–dibutyl ether–NH_4OH (60:35:5); *G*, EtOAc–C_6H_6–acetonitrile–NH_4OH (50:30:15:5); *H*, acetonitrile–$CHCl_3$–EtOAc–NH_4OH (40:30:25:5); *I*, acetonitrile–EtOAc–C_6H_6–NH_4OH (40:25:30:5); *J*, $CHCl_3$–isopropanol (1:3); *K*, CH_3OH–$CHCl_3$ (1:9); *L*, C_2H_5OH–C_6H_6 (1:4); *M*, acetone; *N*, chloroform; *O*, benzene–$CHCl_3$–acetone (70:15:15); *P*, benzene–acetone–ether–10% aq. ammonia (4:6:1:0.3); *Q*, benzene–acetone–ether–25% am-

Continued ⟶

the chromatogram with potassium iodoplatinate. Ikram (35) used modified Dragendorff's spray reagent after TLC (Table III) for the identification of opiates obtained in narcotic seizures. Eight solvent systems were studied by Steele (36) for the same purpose on 26 compounds.

A table of reactions of some opium alkaloids and acetylated derivatives with ten reagents and U.V. after separation in MeOH–$CHCl_3$–NH_3 23% (85:15: 0.7) was prepared by Vignoli (37). Continuing a series on TLC without binding agents, Schwarz (38) tabulated R_f values for 19 alkaloids in nine systems including ethylmorphine and codeine. Some of these systems were applied to the separation of mixtures in tablets, extracts, and tinctures.

A method for the separation and the U.V., I.R., and color test identification of acetyl codeine from illicit heroin was described in detail by Nakamura (39). Pfeifer (40) thoroughly studied and tabulated the R_f values of alkaloids of the genus *Papaver* on Silica Gel G with benzene–acetone–methanol (7:2:1) and alumina with heptane–$CHCl_3$–ether (4:5:1). The R_f values of 22 opium alkaloids and 22 rauwolfia alkaloids in $CHCl_3$–acetone–Et_2NH (5:4:1) and chloroform–cyclohexane–Et_2NH (7:2:1) systems along with the fluorescence color and color with iodoplatinate were tested by Kaess (41). Other TLC studies on opium alkaloids are mentioned under *Analgesics and Antipyretics.*

The TLC of cinchona alkaloids (Table IV) was studied intensively by Suszko-Purzycka (45–48) and Steele (36) and also was included in the recent work of Fike (42). The detection of zones was by modified Dragendorff reagent

TABLE III—(*Continued.*)

Ref. → / Solvent[a] → / Adsorbent → / Compd.	(35) N 13	(25) N 13	(35) O 13	(32) P 1	(32) Q 1	(25) R 1	(25) S 1	(25) T 1	(25) U 1	(25) V 1	(25) W 13	(25) X 2	(42) X 2	(42) X 4	(42) Y 2	(42) M 2	(42) Z 4
	$R_f \times 100$																
Piminodine ethanesulfonate[b]																	
Anileridine																	
Cotarnine		0				60	90	43	31	45	25	0					
Codeine	38	12	33	18	24	38	53	16	04	26	27	35	28	24	07	06	10
Cryptopine																	
Diacetylmorphine													39	24	22	20	09
Hydrocodone bitartrate[c]																	
Dihydrocodeine		10				38	54	18	06	28	30	25					
Hydrocodone		48				51	65	21	04	30	43	18	17	11	06	04	03
Dihydromorphine																	
Hydromorphone		05				24	23	08	01	11	08	16	15	13	05	03	05
Hydromorphone HCl[d]																	
Dionin																	
Heroin																	
Hydromorphone				09	13												
10-Hydroxycodeine																	
Laudanidine																	
Laudanine																	
Laudanosine																	
Levorphanol tartrate[e]																	
Meperidine													48	49	55	21	24
Metapon																	
Methadon													37	51	76	43	25
Monoacetylmorphine																	
Morphine	03	03	06	09	09	10	08	0	0	03	0	34	28	23	02	04	10
Nalorphine																	
Narceine		0				03	0	0	0	0	0	0					
Narcotine	77	81	77	78	81	72		51	10	57	79	72					
Narcotoline																	
Neopine																	
Alphaprodine HCl[f]																	
Normorphine																	
Oxymorphone HCl[g]																	
Papaverine	88	85	73	64	69	67		42	03	47	84	70	62	62	11	53	21
Peronin																	
Phenazocine																	
Protopine																	
Thebaine		71		46	51	65		51	16	50	76	40					

monia (4:6:1:0.3); *R*, $CHCl_3$–acetone–Et_2NH (50:40:10); *S*, $CHCl_3$–Et_2NH (90:10); *T*, cyclohexane–$CHCl_3$–Et_2NH (50:40:10); *U*, cyclohexane–Et_2NH (90:10); *V*, benzene–ethyl acetate–Et_2NH (70:20:10); *W*, cyclohexane–$CHCl_3$ (30:70) + 0.05% Et_2NH (3 drops); *X*, CH_3OH; *Y*, cyclohexane–benzene–Et_2NH (75:15:10); *Z*, 95% ethanol. [b] Marketed as Alvodine by Winthrop Laboratories, New York, N. Y. [c] Marketed as Dicodid by Knoll Pharmaceuticals, Orange, N. J. [d] Marketed as Dilaudid by Knoll Pharmaceuticals. [e] Marketed as Levo-Dromoran by Roche Laboratories, Nutley, N. J. [f] Marketed as Nisentil by Roche Laboratories. [g] Marketed as Numorphan by Endo Laboratories, Garden City, N. J.

TABLE IV—TLC OF CINCHONA ALKALOIDS

Ref. → / Solvent[a] → / Adsorbent → / Compd.	(32) A 1	(32) B 1	(47) C 1	(48) C 2	(46) D 2	(45) D 1	(25) E 1	(25) F 1	(25) G 1	(25) H 1	(25) I 1	(25) J 13	(25) K 13	(25) L 2	(42) L 2	(42) L 4	(42) M 2	(42) N 2	(42) O 4
	$R_f \times 100$																		
Cinchonine			30		40	40	38	44	17	07	27	0	22	40	37	22	10	11	09
Cinchonidine			33		40	37													
Dihydrocinchonine			18		30	30													
Dihydrocinchonidine			23		31														
Dihydroquinine			26	37	40	42													
Dihydroquinidine			23	31	38	38													
Quinidine	28	43	37	43	47	46	33	40	15	0	25	12	18	50					
Quinine	23	35	34	47	46	50	19	26	07	0	17	09	18	43	47	37	05	11	14

[a] *A*, benzene–acetone–ether–10% NH_3 (4:6:1:0.3); *B*, benzene–acetone–ether–25% NH_3 (4:6:1:0.3); *C*, $CHCl_3$–MeOH–diethylamine (50:50:1); *D*, $CHCl_3$–MeOH–diethylamine (80:20:1); *E*, $CHCl_3$–acetone–diethylamine (50:40:10); *F*, $CHCl_3$–diethylamine (90:10); *G*, cyclohexane–$CHCl_3$–diethylamine (50:40:10); *H*, cyclohexane–diethylamine (90:10); *I*, benzene–ethyl acetate–diethylamine (70:20:10); *J*, $CHCl_3$; *K*, cyclohexane–$CHCl_3$ (30:70) + 0.05% diethylamine (3 drops); *L*, CH_3OH; *M*, cyclohexane–benzene–diethylamine (75:15:10); *N*, acetone; *O*, 95% EtOH.

and U.V. Rauwolfia alkaloids reported by Ikram (35), Waldi (25), and Zarnack (32) are recorded in Table V. A single tablet assay of reserpine developed by Weaver (49) used Silica Gel G with an acetone–$CHCl_3$ (3:10) solvent. Quantitation was obtained by a comparison of the size

and intensity of the PDAB spots to the standard.

Tropane alkaloids reported by Fike (42), Cuven (50), Zarnack (32), French (31), and Waldi (25) are given in Table VI. Detection was made by modified Dragendorff reagent or iodoplatinate. Kaess (51) reacted a 2% solution of the alkaloid with a few drops of 1 *N* KOH in a sealed capillary tube for 1 hr. before TLC.

Atropine was estimated by Ikram (52) in tropane alkaloids by TLC on alumina with MeOH. The eluted atropine zone in acid was back titrated with base with a relative error of 2%.

Chen (53) separated strychnine and brucine on alumina using as a solvent benzene–ethyl acetate (1:2). The strychnine was eluted from the

TABLE V—TLC OF RAUWOLFIA ALKALOIDS

Ref. →	(35)	(35)	(35)	(32)	(32)	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(25)
Solvent[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
Adsorbent →	13	13	13	1	1	1	1	1	1	1	13	13	2
Compd.	$R_f \times 100$												
Ajmalicine	77												
Ajmaline	24	87	51			47	42	12	03	30	06	13	56
Rauwolscine						55	63	18	04	36	36	15	68
Reserpine	60		89	72	75	72	80	20	0	46	63	35	69
Serpentine	24	75	34			24	15	0	0	04	0	0	0
Serpentinine		86	73			53	56	08	0	10	0	03	12
Yohimbine						63	62	18	03	37	33	15	60

[a] *A*, $CHCl_3$–acetone (85:15); *B*, absolute EtOH; *C*, $CHCl_3$–EtOH–acetone (90:5:5); *D*, benzene–acetone–ether–10% NH_3 (4:6:1:0.3); *E*, benzene–acetone–ether–25% NH_3 (4:6:1:0.3); *F*, $CHCl_3$–acetone–diethylamine (50:40:10); *G*, $CHCl_3$–diethylamine (90:10); *H*, cyclohexane–$CHCl_3$–diethylamine (50:40:10); *I*, cyclohexane–diethylamine (90:10); *J*, benzene–EtOAc–diethylamine (70:20:10); *K*, $CHCl_3$; *L*, cyclohexane–$CHCl_3$ (30:70) + 0.05% diethylamine (3 drops); *M*, CH_3OH.

TABLE VI—TLC OF TROPANE ALKALOIDS

Ref. →	(50)	(31)	(32)	(32)	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(42)	(42)	(42)	(42)	(42)
Solvent[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*L*	*L*	*M*	*N*	*O*
Adsorbent →	13	2	1	1	1	1	1	1	1	13	13	2	2	4	2	2	4
Compd.	$R_f \times 100$																
Atropine	89	05	08	20	38	40	16	05	12	0	10	17	11	31	09	01	14
Apoatropine			19	43	54	67	40	20	26	15	40	16					
Homatropine	87	07			37	45	15	05	23	04	24	15					
Scopolamine		39	37	52	56	60	19	03	34	30	0	52	54	34	09	33	13
Tropine	43																
Tropacocaine					65		56	34	45	58	78	35					
Hyoscyamine		05															

[a] *A*, EtOH–pyridine–H_2O (10:60:40); *B*, $CHCl_3$–MeOH (9:1); *C*, benzene–acetone–ether–10% NH_3 (4:6:1:0.3); *D*, benzene–acetone–ether–25% NH_3 (4:6:1:0.3); *E*, $CHCl_3$–acetone–Et_2NH (50:40:10); *F*, $CHCl_3$–Et_2NH (90:10); *G*, cyclohexane–$CHCl_3$–diethylamine (50:40:10); *H*, cyclohexane–Et_2NH (90:10); *I*, benzene–ethyl acetate–Et_2NH (70:20:10); *J*, $CHCl_3$; *K*, cyclohexane–$CHCl_3$ (30:70) + 0.05% diethylamine (3 drops); *L*, CH_3OH; *M*, cyclohexane–benzene–diethylamine (75:15:10); *N*, acetone; *O*, 95% ethanol.

TABLE VII—TLC OF OTHER ALKALOIDS

Ref. →	(32)	(32)	(55)	(54)	(54)	(56)	(56)	(56)	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(25)
Solvent[a] →	*A*	*B*	*C*	*D*[b]	*E*[b]	*F*	*G*	*H*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*
Adsorbent →	1	1	1			13	1	13	13	1	1	1	1	1	13	2
Compd.	$R_f \times 100$															
Aconitine									36	68		35	03	49	60	65
Brucine	10	25							50	42	63	18	0	19	54	12
Cephaeline									25	56	63	19	02	23	17	37
Colchicine	15	20							11	47	41	04	0	04	0	57
Emetine									38	67		40	06	45	58	50
Hydrocodon	18	25														
Leurocristine			16	51												
Leurosidine			06	23												
Leurosine			45		27	27	35	20								
Oxycodon	64	69														
Pethidine	48	60														
Physostigmine	47	60							59	65		32	04	44	50	46
Pilocarpine	25	37							32	41	52	09	0	13	25	55
Sparteine	11	32							0	70		68	68	55	55	05
Strychnine	20	40							57	53	76	28	05	38	60	22
Vincaleucoblastine				24		36										

[a] *A*, benzene–acetone–ether–10% aq. ammonia (4:6:1:0.3); *B*, benzene–acetone–ether–25% ammonia (4:6:1:0.3); *C*, $CHCl_3$–MeOH (95:5); *D*, 5% EtOH in acetonitrile; *E*, 30% acetonitrile in benzene; *F*, $CHCl_3$–ethyl acetate (1:1); *G*, ethyl acetate–EtOH (3:1); *H*, $CHCl_3$; *I*, $CHCl_3$–acetone–Et_2NH (50:40:10); *J*, $CHCl_3$–Et_2NH (90:10); *K*, cyclohexane–$CHCl_3$–Et_2NH (50:40:10); *L*, cyclohexane–Et_2NH (90:10); *M*, benzene–ethyl acetate–Et_2NH (70:20:10); *N*, cyclohexane–$CHCl_3$ (30:70) + 0.05% Et_2NH (3 drops); *O*, CH_3OH. [b] Silica gel with 0.1 *N* LiOH.

chromatogram and determined by polarography. The differentiation of *Vinca rosea* alkaloids by TLC (Table VII) has been reported by Jakovljevic (54) with ceric ammonium sulfate in phosphoric acid as the reagent. The same reagent was used by Farnsworth (55) for a study on *Catharanthus* alkaloids shown in Table VII. In a study by Cone (56), the R_f values of 26 vinca alkaloids were obtained. Some of the veratrum alkaloids were resolved by cyclohexane–EtOH (17:3) on Silica Gel HF254 by Zeitler (57) and observed under U.V. after treatment with trichloroacetic acid and heat.

ANALGESICS-ANTIPYRETICS

The early work of Gänshirt (5) was the beginning of many articles on the separation of acetylsalicylic acid, phenacetin, and caffeine. These components from extracts of tablets and suppositories were identified by U.V., potassium ferricyanide, and with chlorine, HCl, and then NH_3. His system was later used for a laboratory experiment in teaching pharmaceutical analysis by Conners (58). Simple equipment for coating onto microslides and spray applicators were described. The authors stated: "Despite this simplification the procedures described are entirely practical for serious analytical and research use." This was a very conservative statement, for these "simple" techniques have been used to solve many age-old problems such as the measurement of *p*-chloroacetanilide and acetanilide in phenacetin (59–61) by U.V. and permanganate and for the determination of

TABLE VIII—TLC OF ANALGESICS–ANTIPYRETICS

Ref. →	(5)	(58)	(64)	(65)	(65)	(15)	(15)	(69)	(69)	(70)	(66)	(62)	(71)
Solvents[a] →	*A*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Adsorbent →	1	1	1	1	1	1	1	1	1	5	13	1	1
Compd.	$R_f \times 100$												
Acetylsalicylic acid	X	97		0	0	83	19	0	26		20		
Acetylmethadol													
Acetophenetidin	X	45	X	36	70	57	81	25	57	30			
Amidopyrine			X	42	65	04	75						
Aminopyrine								16	21				
Anileridine													08
Antipyrine								03	09				
Cocaine													39
Caffeine	X	10	X	47	61	25	53	04	13	10			
p-Chloroacetanilide													
Dimethylaminoantipyrine													
Ethoheptazine													12

Ref. →	(71)	(72)	(73)	(67)	(68)	(68)	(68)	(68)	(68)	(68)	(68)	(60)	(31)
Solvents[a] →	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	2
Compd.	$R_f \times 100$												
Acetylsalicylic acid													
Acetylmethadol					64	60	99	40	38	52	62		
Acetophenetidin			70									X	67
Amidopyrine													
Aminopyrine													
Anileridine	37												
Antipyrine			50										
Cocaine	75												
Caffeine			05										70
p-Chloroacetanilide												X	
Dimethylaminoantipyrine			64										
Ethoheptazine	24												

Ref. →	(5)	(58)	(64)	(65)	(65)	(15)	(15)	(69)	(69)	(70)	(66)	(62)	(71)
Solvents[a] →	*A*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Adsorbent →	1	1	1	1	1	1	1	1	1	5	13	1	1
Compd.	$R_f \times 100$												
Isopropylantipyrine								59	72	50			
dl-Methadone													42
Merperidine													14
Dextromethorphan													11
Morphine													0
Normorphine													0
Codeine				24	43	03	38						
Norcodeine													
Heroin													
Nalorphine													
Methyldihydromorphine													
Hydromorphone													

(*Continued on next page.*)

TABLE VIII—(*Continued.*)

Ref. →	(71)	(72)	(73)	(67)	(68)	(68)	(68)	(68)	(68)	(68)	(68)	(60)	(31)
Solvents[a] →	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	2
Compd.	$R_f \times 100$												
Isopropylantipyrine													
dl-Methadone	58				34	59	99	17	17	55	62		
Merperidine	32												
Dextromethorphan	20												
Morphine	0	33			29	27	11	21	07	54	34		
Normorphine	0	15			08	48	04	07		66	62		
Codeine	05				30	29	39	25	08	53	30		
Norcodeine					12	50	13	09	06	63	49		
Heroin					37	35	76	35	15	61	32		
Nalorphine					71	55	35	67	25	59	41		
Methyldihydromorphine					16	24	25	15		45	26		
Hydromorphone					11	21	17	13		41	25		

Ref. →	(5)	(58)	(64)	(65)	(65)	(15)	(15)	(69)	(69)	(70)	(66)	(62)	(71)
Solvents[a] →	*A*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Adsorbent →	1	1	1	1	1	1	1	1	1	5	13	1	1
Compd.	$R_f \times 100$												
Ethylmorphine													
Dihydrohydroxymorphinone													
Dihydromorphine													
Hydrocodone													
Dihydrohydroxycodeinone													
6-Acetylmorphine													
n-Allylmorphine													
Paracetamol						46	67	16	48				
Papaverine				57	64	11	80						
Pethidine													
Phenazone				24	53	21	60						

Ref. →	(71)	(72)	(73)	(67)	(68)	(68)	(68)	(68)	(68)	(68)	(68)	(60)	(31)
Solvents[a] →	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	2
Compd.	$R_f \times 100$												
Ethylmorphine					33	25	46	27	08	53	33		
Dihydrohydroxymorphinone					46	29	34	24	10	45	28		
Dihydromorphine					15	21	10	10		43	29		
Hydrocodone					17	25	41	19		42	23		
Dihydrohydroxycodeinone					46	24	87	29	16	32	34		
6-Acetylmorphine		55			38	40	64	29	19	37	37		
n-Allylmorphine		85											
Paracetamol													
Papaverine													
Pethidine					42	41	97	36	20	46	44		
Phenazone													

Ref. →	(5)	(58)	(64)	(65)	(65)	(15)	(15)	(69)	(69)	(70)	(66)	(62)	(71)
Solvents[a] →	*A*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Adsorbent →	1	1	1	1	1	1	1	1	1	5	13	1	1
Compd.	$R_f \times 100$												
Phenylbutazone						90	40						
Piminodine													
d-Propoxyphene													48
Quinine				28	17	02	55						
Salicylic acid											28	35	
Salicylsalicylic acid											72		
p-Hydroxyisophthalic acid													
Salicylamide						61	59						

Ref. →	(71)	(72)	(73)	(67)	(68)	(68)	(68)	(68)	(68)	(68)	(68)	(60)	(31)
Solvents[a] →	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	2
Compd.	$R_f \times 100$												
Phenylbutazone													
Piminodine					88	73	99	85	76	69	50		
d-Propoxyphene	71				73	68	97	54	56	53	61		
Quinine													
Salicylic acid													
Salicylsalicylic acid													
p-Hydroxyisophthalic acid				31									
Salicylamide													

[a] *A*, MeOH–AcOH–ether–benzene (1:18:60:120); *B*, cyclohexane–acetone (4:5); *C*, benzene + 5% EtOH; *D*, chloroform + 2% *n*-BuOH; *E*, butyl acetate–$CHCl_3$–85% formic acid (6:4:2); *F*, butyl acetate–acetone–*n*-BuOH–10% NH_3 (5:4:3:1); *G*, ether; *H*, ether–ethyl acetate (1:4); *I*, $CHCl_3$–ethyl acetate (1:1); *J*, pet. ether–ethyl acetate–AcOH (85:10:5); *K*, hexane–AcOH–$CHCl_3$ (85:15:10); *L*, benzene–NH_3 atmosphere; *M*, $CHCl_3$–NH_3 atmosphere; *N*, MeOH–*n*-BuOH–benzene–water (60:15:10:15); *O*, $CHCl_3$–acetone–water (2:9:0.5); *P*, benzene–dioxane–AcOH (90:25:4); *Q*, EtOH–pyridine–dioxane–water (50:20:25:5); *R*, EtOH–AcOH–water (60:30:10); *S*, EtOH–dioxane–benzene–NH_4OH (5:40:50:5); *T*, MeOH–*n*-BuOH–benzene–water (60:15:10:15); *U*, *tert*-amyl alcohol–*n*-butyl ether–water (80:7:13); *V*, *n*-BuOH–AcOH–water (4:1:2); *W*, *n*-BuOH–concentrated HCl saturated with water (90:10); *X*, cyclohexane–acetone–isobutylketone–MeOH–H_2O (100:80:30:5:1); *Y*, $CHCl_3$–MeOH (9:1).

salicylic acid in acetylsalicylic acid by reflectance spectroscopy following TLC by Frodyma (62).

Gänshirt (63) also published a table showing reaction of seven common analgesics with reagents already mentioned plus $AgNO_3$–NH_3, Cl_2 + benzidine, I_2, and rhodamine B. Gänshirt (64) reported the accuracy and relative standard deviation results from quantitative TLC for caffeine was 113% ± 12%, amidopyrine as 98.6% ± 5%, and phenacetin as 100% ± 27% from ten replicates. Sarsunova (65) gave solvent systems of choice for the separation of various combinations of acetylsalicylic acid, phenacetin, amidopyrine, caffeine, and quinine. Bailey (66) was able to measure 0.05% or less of salicylsalicylic acid (SSA) in salicylic acid (SA) by U.V. at 310 mμ following TLC. Verification was obtained by the shifting of maxima of SSA to 345 mμ in acid alcohol while SA remained at 310 mμ. Shelly (67) extracted TLC to detect *p*-hydroxyisophthalic acid in SA. Mule (68) applied TLC along with gas chromatography and U.V. spectrophotometry for the identification of analgesics in human urine, plasma, and tissue homogenates. The data on R_f values were organized by Mule according to chemical families and offer a good reference for toxicological studies. Some of these data from the latter authors are presented in Table VIII. Fike (42) gave a comprehensive list of 140 drugs including some analgesics with a separate table for 112 basic drugs and 28 phenothiazines. Five solvent systems were used and a correlation of structure and R_f values discussed. (See Table III.)

ANTIBIOTICS

Nicolaus (74) published one of the early papers on antibiotics using agar containing triphenyltetrazolium for microbiological detection of zones.

TABLE IX—TLC OF ANTIBIOTICS

Ref. →	(84)	(83)	(83)	(85)	(89)	(92)
Solvent[a] →	*Q*	*V*	*W*	*Z*	*AB*	*AF*
Adsorbent →	1	18	18	1	1	1
Compd.	R_f × 100					
Actinomycin C	70					
Actinomycin F	50					
Amidinomycin		53				
Bacitracin A					35	
Bacitracin F					22	
Catenulin		15				
Erythromycin A				X		20
4-Acetylerythromycin				X		
2-4-Diacetylerythromycin				X		
Erythromycin hemiacetal				X		
Fradiomycin		10				
Gentamycin		35				

Ref. →	(74)	(78)	(78)	(80)	(83)	(83)	(92)	(90)
Solvent[a] →	*A*	*P*	*P*	*Q*	*V*	*W*	*AE*	*AC*
Adsorbent →	1	22	21	1	18	18	1	1
Compd.	R_f × 100							
Gramicidin				94				
Kanamycin					17			
Neomycin A		61	54		10			
Neomycin B		21	24					33
Neomycin C		43	45					33
Netropsin						39		

Ref. →	(74)	(78)	(78)	(80)	(83)	(83)	(92)	(90)
Solvent[a] →	*A*	*P*	*P*	*Q*	*V*	*W*	*AE*	*AC*
Adsorbent →	1	22	21	1	18	18	1	1
Compd.	R_f × 100							
Actinomycin C								
Actinomycin F								
Amidinomycin								
Bacitracin A								
Bacitracin F								
Catenulin								
Erythromycin A								
4-Acetylerythromycin								
2-4-Diacetylerythromycin								
Erythromycin hemiacetal								
Fradiomycin								
Gentamycin								

Ref. →	(74)	(78)	(78)	(80)	(83)	(83)	(92)	(90)
Solvent[a] →	*A*	*P*	*P*	*Q*	*V*	*W*	*AE*	*AC*
Adsorbent →	1	22	21	1	18	18	1	1
Compd.	R_f × 100							
Gramicidin								
Kanamycin								
Neomycin A								
Neomycin B								
Neomycin C								
Netropsin								

Ref. →	(74)	(78)	(78)	(80)	(83)	(83)	(92)	(90)
Solvent[a] →	*A*	*P*	*P*	*Q*	*V*	*W*	*AE*	*AC*
Adsorbent →	1	22	21	1	18	18	1	1
Compd.	R_f × 100							
Acetyloleandomycin							20	
Diacetyloleandomycin							50	
Triacetyloleandomycin							70	
Paromomycin					15			
6-Aminopenicillinic acid	09							

Ref. →	(74)	(75)	(75)	(77)	(83)	(83)	(87)	(92)	(92)
Solvent[a] →	*A*	*M*	*N*	*Q*	*V*	*W*	*AA*	*AE*	*AF*
Adsorbent →	1	1	1	7	18	18	1	1	1
Compd.	R_f × 100								
Pen. G K		16		67					
Pen. G NA									75
Pen. V acid	23			71					
Pen. V K		68	64						
Pen. P K		62	58						
Procaine pen.				66					
DBED pen.				68					
Me pen. V K		72	68						
Streptomycin					44	31			
Dihydrostreptomycin					44	31			
Hydroxystreptomycin					32	23			
Dihydrost. phenyl hyd.							92		

Ref. →	(74)	(74)	(74)	(74)	(74)	(74)	(74)	(74)	(74)	(74)	(80)	(81)	(82)	(82)	(82)	(83)	(83)	(94)	(91)
Solvent[a] →	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*Q*	*R*	*S*	*T*	*U*	*V*	*AF*	*AG*	*S*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	19[b]	10	10	10	18	18	17	10
Compd.	R_f × 100																			
Tetracycline	36	38	60	61	50	50	23	50	67	68	64		X	23	26	38				36
Epitetracycline																				36
Anhydrotetracycline	10	45	64	68	50	50	23	53	69	68	64								100	50
4 Epianhydrotet.																			60	40
Chlortetracycline	30	43	64	72	60	58	24	50	69	67	65		X	30	35	49				
Anhydrochlorotet.	10	46	65	75	52	52	19	55	70	70	65									
Oxytetracycline	58	41	62	70	55	60	23	52	68	69	65		X	27	31	46				
Tylosin																		83		
Tyrocidin												35								
Viomycin																	21			
Zyomycin																	15			

[a] *A*, BuOH buffered to 4.6; *B*, citric acid 10%; *C*, BuOH–MeOH–citric acid, 10% (40:10:2); *D*, BuOH–MeOH–citric acid 10% (40:15:20); *E*, BuOH–MeOH–citric acid 10% (40:20:20); *F*, BuOH–MeOH–citric acid 10% (40:30:20); *G*, BuOH–MeOH–citric acid 10% (40:40:20); *H*, citric acid 10% saturated with BuOH + 5% MeOH; *I*, citric acid 10% saturated with BuOH + 10% MeOH; *J*, citric acid 10% saturated with BuOH + 15% MeOH; *K*, citric acid 10% saturated with BuOH + 20% MeOH; *L*, citric acid 10% saturated with BuOH + 25% MeOH; *M*, acetone–MeOH (50:80); *N*, isopropanol–MeOH (30:70); *O*, butyl acetate–*n*-BuOH–AcOH–PO_4 buffer–MeOH (80:5:40:24:5) penicillin hydrol. 0.5 hr. in MeOH–HCl at 50°; *P*, 0.5 *N* H_2SO_4; *Q*, BuOH–AcOH–H_2O (10:1:3); *R*, $CHCl_3$–Me_2CO (1:1); *S*, *n*-BuOH saturated with H_2O circular TLC; *T*, *n*-BuOH–tartaric acid–water (100:6:100) circular TLC; *U*, *n*-BuOH–oxalic acid–water (100:5 Gm.:100) circular TLC; *V*, *n*-PrOH–pyridine–AcOH–H_2O (15:10:3:12); *W*, BuOH + 2% *p*-toluene sulfonic acid; *Z*, methylene chloride–MeOH–benzene–formamide (80:20:20:3); *AA*, BuOH–H_2O–MeOH (40:20:10) + 1 Gm. *p*-toluene sulfonic acid; *AB*, BuOH–AcOH–H_2O (4:1:5); *AE*, $CHCl_3$–MeOH–toluene (80:17:23); *AF*, MeOH–acetone (60:40); *AG*, EDTA 0.1 *M* – 0.1% NH_4Cl pH 4.5, then two-dimensional in $CHCl_3$ saturated with above solution. [b] Adjusted to pH 3.7 with PO_4 buffer.

Experimental details were given for preparation of the TLC plates of Silica Gel G with buffers pH 6.85, 4.5, and 2.2, and the agar inoculation with *Sarcina lutea* for rifomycin, *Bacillus subtilis* for tetracycline, and *Staphylococcus aureus* for penicillin. After cooling, another layer of agar was applied to protect the tetrazolium from the air. Preliminary chilling before incubation allowed the antibiotics to diffuse through the agar. They found that sensitivity of TLC was 10 times greater than paper chromatography, with detection limits of 0.1 mcg. chemically and 0.001 mcg. microbiologically. Fischer (75) used iodine–azide solution for detecting 1–2 mcg. including a white and a yellow spot for some penicillins. Only the single component R_f values are reported in Table IX.

Nussbaumer (76) studied the effect of 40 tablet excipients on the identification of penicillins and found most of them were removed by TLC. A penicillin V spot (77) was removed for measurement at 276 mμ after separation, on silica gel plus rice starch, from phenoxyacetic acid and 20 possible tablet excipients.

Brodasky (78) used acid and neutral carbon black for TLC of neomycin A, B, and C followed by agar diffusion inoculated with *Bacillus pumilus*. Bromophenol blue spray 15 min. after 5% $KMnO_4$ was found by Akita (79) to lower the detection limit for antibiotics over $KMnO_4$ alone. The acid oxidation products produce blue or greenish colors which lasted for 1 month. Tyrocidin and gramicidin (80) were observed on Silica Gel G by U.V. and reaction with *o*-tolidine in AcOH after treatment with Cl_2. Sprays of $SbCl_3$ 50% in AcOH and H_2SO_4, and fluorescence under 350 mμ intensified by NH_4OH or NaOH identified three tetracyclines for Sonanini (81) on regular and circular TLC. Kapadia (82) also used circular TLC for the same compounds. EDTA was incorporated into Silica Gel G to prevent iron from forming complexes with the tetracyclines. A regular TLC plate was inverted over a Petri dish containing the development solvent. A roll of filter paper served as the wick. Ferric chloride and fluorescence were the means of visualization.

Nine basic water-soluble antibiotics were separated on MN cellulose by Ito (83) with oxidized nitroprusside and ninhydrin used for visualization. Actinomycins were separated by Cassani (84) into C and F groups (Table IX). Other systems isolated C_1, C_2, C_3, F, and F_3 portions. Erythromycins were detected on Silica Gel G with phosphomolybdic acid by Anderson (85) and 0.05 mcg. of erythromycin by *Streptococcus lactis* on agar using a reprint from 0.5 mm. Al_2O_3 by Meyers (86). Preliminary treatment of streptomycin and dihydrostreptomycin with phenylhydrazine allowed the separation of these two antibiotics (87). Streptomycin yields 2 spots and dihydrostreptomycin one spot following the reaction and subsequent TLC. The spots of these two antibiotics were removed (88), centrifuged with PO_4 buffer pH 7.9, and the supernatant used for microbiological assay with *B. subtilis*. The procedure was recommended for the control of injectables of streptomycin. The degradation of bacitracin was followed by TLC by Nussbaumer (89) with the spots detected by U.V. and ninhydrin. The microbiological inactivity of the new spot confirmed the technique. Other quantitative TLC work by Foppiano (90) on neomycin was based on its ribose moiety determined with orcinol–ferric chloride–AcOH reagent. Assays after TLC invariably were lower (0.5 to 13%) than before chromatography. The variation of the TLC method was no greater than an official method. Circular TLC with silica gel and disodium EDTA of degradation products in tetracycline using NH_3 and U.V. for detection was reported by Rustici (91).

Dragendorff's reagent detected 2 mcg. spots after TLC of mono-, di-, and triacetyl derivatives of oleandomycin by Gantes (92). Kline (93) used a paint sprayer to coat silica gel and kieselguhr TLC plates with agar inoculated with *B. subtilis* or *S. lutea*. Sensitivities were 0.1 mcg. for streptomycin, 0.25 mcg. for tylosin and erythromycin, and 0.005 units for penicillin.

ANTIHISTAMINICS

Antihistamine compounds have been considered along with tranquilizers by Cochin (95). The phenothiazine derivatives respond to reagents described later under *Psychotropic Drugs*. In a quantititative study, Morrison (96) used ceric sulfate (5%) as a reagent and measured the area of the spot by comparison to a plastic sheet imprinted with circles and ellipses of known area. A formula was used for calculating the concentration of unknown drugs with accuracies for concentrations of 25–50 mcg. of about 3%. Thirty replicates on six different capsule preparations gave recoveries from 94.3–104.5% of label with relative standard deviations 5.8 to 11.4%. Fike (97) used three solvent systems and two spray reagents (Dragendorff and 1% solution ammonium vanadate in H_2SO_4) to identify all but six of 30 antihistamines.

TABLE X—TLC OF ANTIHISTAMINIC AND RELATED COMPOUNDS

Ref. → / Solvent[a] → / Adsorbent → / Compd.	(95) A 1	(95) B 1	(95) C 1	(95) D 13	(96) E 1	(67) F 1	(97) G 2	(97) G 4	(42) H 2	(42) G 2	(42) I 2	(42) G 4	(42) J 4
	$R_f \times 100$												
Antazoline	31	72	40	38		08	11	61					
Bromodiphenylhydramine	86	80	33	63		50	37	48	08	11	06	61	38
Brompheniramine	42	47	16	48									
Buclizine									73	72	84	75	64
Carbinoxamine	33	40	17	63		29	21	05					
Chlorcyclizine	90	68	40	49		49	44	43	49	44	30	43	19
Chlorothen	70	56	29	66		43	37	15	43	37	28	15	02
Chlorpheniramine	40	41	20	52	27	38	19	08	38	19	06	08	01
Clemizole						33	67	47	33	67	61	47	27
Covatin						58	54	52					
Cyclizine						55	46	41	55	46	27	41	16
Diphenhydramine	91	61	25	53	55	52	37	45	52	37	26	45	25
Diphenylpyraline						42	25	44	42	25	11	44	23
Doxylamine	52	38	17	60									
Hydroxyzine	27	70	62	84		08	59	56	08	59	39	56	25
Meclizine						69	71	74	69	71	84	74	60
Methaphenilene						55	46	44	55	46	40	44	24
Methapyrilene	83	52	30	56	38	47	36	14					
Phenindamine	90	67	48	65	35	55	53	41	55	53	35	41	25
Pheniramine	68	38	19	63		40	08	06	40	18	05	06	01
Phenyltoloxamine						46	51	42					
Pyrilamine	82	43	27	56	35	42	33	12	42	33	24	12	01
Pyrrobutamine	84	82	32	78		62	39	59	62	39	34	59	40
Thenyldiamine						47	32	12	47	32	25	12	01
Thenylpyramine									47	36	27	14	02
Thonzylamine	65	57	32	59		41	38	29	41	38	27	29	12
Tripelennamine	92	48	23	62		50	35	12	50	35	27	12	03
Triprolidine	40	48	26	81		41	40	17	41	45	13	18	02

[a] *A*, benzene–dioxane–aq. NH_3 (60:35:5); *B*, EtOH–AcOH–water (50:30:20); *C*, MeOH–BuOH (60:40); *D*, BuOH–butyl ether–AcOH (40:80:10); *E*, AcOH–water (20:80); *F*, cyclohexane–benzene–diethylamine (75:15:10); *G*, MeOH; *H*, cyclohexane–benzene–Et_2NH (75:15:10); *I*, acetone; *J*, 95% ethanol.

TABLE XI—TLC OF BARBITURATES

Ref. → / Solvents[a] → / Adsorbent → / Compd.	(98) A 1	(26) A 1	(105) A 1	(98) B 1	(98) C 1	(102) D 1	(103) E 1	(105) F 1	(105) G 1	(31) H 2	(31) I 2	(107) J 1	(107) K 20	(107) L 20
	$R_f \times 100$													
Allylbarbituric acid		48								54	71			
Amobarbital	60	48	38	42	52	61	74	40	55			58		
Barbital	50	33	24	38	40	48		34	45	40	67	31	13	
Butabarbital	62	42	32	49	52	58		37	50			53		
Cyclobarbital	68		32	55	38	51		36	50			30	46	
Diallylbarbituric acid	55			43	48	62	0					34	24	
Hexobarbital	77	68	46	49	58	61		41	54			77		
Itobarbital	67			48	50									
Mephobarbital	98	79		85	60							64		90
Pentobarbital	57	47	36	40	49	64	40	40	55	61	73	66		50
Phenobarbital	50	33	25	36	26	46	51	27	49	29	64	20		
Secobarbital	64	55	41	46	54	75	29	39	59			63		43

[a] *A*, $CHCl_3$–acetone (9:1); *B*, benzene–AcOH (9:1); *C*, dioxane–benzene–aq. NH_3 (20:75:5); *D*, diisopropyl ether–$CHCl_3$ (1:1); *E*, acetone–*n*-butyl alcohol–NH_3 (9:9:2), preliminary treatment of sample with H_2SO_4; *F*, benzin–dioxane (5:2) DMF stationary phase; *G*, benzol–ether (1:1); *H*, isopropanol–NH_3 25%–$CHCl_3$ (45:10:45); *I*, $CHCl_3$–MeOH (9:1); *J*, ethyl acetate–hexane–NH_4OH (20:9:10); *K*, $CHCl_3$–CCl_4 (2:1); *L*, $CHCl_3$–CCl_4 (1:1).

BARBITURATES

The analyses of the barbiturates and related compounds has received exhaustive and often repetitive attention from many authors. Most of the work was designed for the identification of barbiturates in blood, urine, and tissue samples [Cochin (98), Walker (99), Bogan (100), Eberhardt (101), Shellard (102), Petzold (103)]. Stolman's book (26) is an excellent starting reference for separation on Silica Gel G using the most popular system of $CHCl_3$–acetone (9:1). (Designated *A* in Table XI.) Pretreatment of the barbiturates by H_2SO_4 on the plate by Petzold (103) allowed separation of five common (U.S.) drugs. The same technique was suggested later by Walker (99) following earlier use of this on paper chromatography. Qualitative analyses in pharmaceuticals have received less attention. Phenobarbital was identified in a suppository

containing amidopyrine and quinine by Kraus (104) using benzene–96% ethanol (37:3) on silica gel–$CaSO_4$ (4:1) and other barbiturates by Shellard (102) in galenicals and tablets using diisopropyl ether–$CHCl_3$ (1:1) on Silica Gel G. Sahli (105) was able to separate 15 compounds using three systems, *A*, *F*, *G*, in Table XI. Using a system similar to *H*, Table XI, Kiger (106) differentiated 23 barbiturates on silica gel.

Many detection reagents are available. Mercurous salts gave grey, black, and white colors [Sahli (105), Kraus (104), Stolman (26)]. Cobalt nitrate, then NH_3 vapor, formed a deep blue color (102). Different colors of fluorescence were recorded (101) at 254 and 366 mμ with and without $HgNO_3$. Silver acetate–diphenylcarbazone gave purple colors (103). Permanganate was used to differentiate unsaturated substituents (98) by the yellow colors produced.

CARDIAC GLYCOSIDES AND CARDENOLIDES

The section on cardiac glycosides by Waldi in Stahl's book (1) described the evolution of the work on TLC of the cardiac glycosides. Stahl (108) was able to separate most of these compounds on silica gel. Chloramine–trichloroacetic acid reagent and U.V. were used for visualization.

Mixtures of scillarin and digitoxin were resolved on silica gel and identified by Steinegger and van der Walt (109) with $SbCl_3$. Reichelt (110), using silica gel without binder inactivated with water or acetic acid, observed separation of many of the cardenolides in water saturated by the reagent benzene–alcohol (3:1) reported by Stahl (108).

Duncan (111) was able to postulate possible success of scale up from TLC to column chromatography by the R_f found on material from *Pachycarpus concolor*. Bobbitt (2) discussed in detail Duncan's formula of

$$r = \frac{a}{b + 0.1a}$$

where a and b are the R_f values of the fast and slow moving substance, and pointed out that even though r is greater than 1, good column separation may not be obtained.

Faucennet (112) reported a greater sensitivity on TLC than paper chromatography for cardenolides of digitalis with H_3PO_4–Br_2 reagent. Sonanini (113) chose previously mentioned reagents for observing several compounds separated on kieselguhr impregnated with formamide. Momose (114) introduced 1,3,5-trinitrobenzene 0.1% in DMF–5% aqueous Na_2CO_3 as a reagent to produce orange-red spots with oleandrin, digitoxin, digoxin, and ouabain.

Commercial K-strophanthin analyses by Lukas (115) revealed four components on silica gel with a BuOH–MeOH–formamide (17:2:1) system.

Corona (116) identified five components of K-strophanthin on Silica Gel G and observed another unidentified spot using $CHCl_3$–AcOH–MeOH (85:2:13) solvent.

CENTRAL STIMULANTS

Many reports of other central stimulants such as the sympathomimetic amines are included in review articles previously discussed. Stolman's book (26) shows tables taken from literature for several of these compounds.

Noirfalise's (117) comprehensive paper on central stimulants tabulated responses of many of these compounds to U.V., $KMnO_4$, Dragendorff, iodoplatinate, PDAB, $HClO_4$–KNO_3–$FeCl_3$, and furfural–H_2SO_4. Beckett (118) found multiple spots formed when the amines were treated with trichloroacetic acid in the solvent system used but not with the same system without the acid on cellulose powder (Table XII). Some of the primary amines and their *N*-methyl derivatives are included in Fike's (42) discussion on correlation of structure to R_f. The identification by Troup (119) of the degradation products of phenylephrine in tablet formulations containing aspirin were accomplished by TLC and detection with diazotized *p*-nitroaniline.

Catecholamines and their metabolites were assayed quantitatively following TLC by de Potter (120). Fluorimetric measurement of the eluted spots indicated recoveries of 97–100% for norepinephrine, epinephrine, and metanephrine.

CRUDE DRUGS AND EXTRACTS

The use of TLC in pharmacognosy is a field of its own. Analysts in this work have taken advantage of the simplicity and utility of TLC for the identification of tinctures, fluid extracts, and powdered extracts. It is used routinely in the authors' laboratory for the identification of about 40 such preparations (121). A partial summary of the literature is shown in Table XIII.

DIURETICS

Margasinski (131) applied TLC before and after hydrolysis of benzothiadiazine derivatives to obtain separation on Al_2O_3 with visualization by PDAB. Without the buffer in the ethyl acetate solvent, Adam (132) separated most of these and other compounds of the same series and observed them under U.V. at 250 mμ. (Table XIV.)

TABLE XII—TLC OF CENTRAL STIMULANTS

Ref. →	(118)	(119)	(120)	(117)	(117)	(117)	(42)	(42)	(42)	(42)	(42)
Solvent[a] →	A	B	C	D	E	F	G	H	I	H	J
Adsorbent →	17	1	18	1	1	1	2	2	2	4	4
Compd.	$R_f \times 100$										
Amphetamine	75			38	30	11	34	28	33	59	53
Adrenaline											
Benzphetamine							79	70	85	54	49
Cyclopentamine							52	10	02	52	40
Dextroamphetamine				39	30	11					
Ephedrine	74						08	18	02	54	42
Epinephrine	55						01	07	11	62	40
Hydroxyamphetamine							03	29	36	59	21
Histamine	27										
Iproniazid				46	62	69	04	64	34	34	22
Isocarbazid				74	69	81	28	71	70	65	61
Isophenaline	60										
Methamphetamine							46	18	04	50	39
Metanephrine			X								
Nialamide				21	54	62	0	55	09	35	23
Norepinephrine	47		X				02	21	15	66	50
Normetanephrine			X								
Phenylephrine		X					05	21	33	60	45
Phenylethylamine	72										
Phenylpropanolamine							09	35	50	58	56
Propylhexedrine							54	15	03	54	46

[a] *A*, *n*-BuOH–AcOH–H_2O (4:1:5); *B*, $CHCl_3$–AcOH–H_2O (5:1:1); *C*, *n*-BuOH saturated with 3 *N* HCl; *D*, acetone–NH_3 25% (99:1); *E*, MeOH–NH_3 25% (99:1); *F*, $CHCl_3$–MeOH (50:50); *G*, cyclohexane–benzene–Et_2NH (75:15:10); *H*, MeOH; *I*, acetone; *J*, 95% EtOH.

TABLE XIII—IDENTIFICATION OF CRUDE DRUGS OR ADULTERANTS BY TLC

Recent Lit. Drug	Ref.
Review	(122)
Aloe	(123, 124)
Althaea	(125)
Datura stramonium	(125)
Hyoscyamus niger	(125)
Digitalis lanta	(125)
Cassia angustifolia	(125)
Umbelliferen roots	(126)
Pimpinella soxifraga	(127)
Coptis japonica	(128)
Senna	(129)
Volatile oils	(130)

TABLE XIV—TLC OF DIURETICS

Ref. →	(131)	(131)	(132)	(132)
Solvent[a] →	A	A	B	C
Adsorbent →	13	13[b]	13	13
Compd.	$R_f \times 100$			
Chlorothiazide	10	87	X	
Hydrochlorothiazide	94	90	X	X
Trichloromethiazide			X	X
Benzothiazide	62	54		
Thiabutazide			X	
Polythiazide	100	98	X	
Methylclothiazide			X	
Hydroflumethiazide			X	X
Bendroflumethiazide			X	
Cyclopentathiazide	100	86		

[a] *A*, ethyl acetate, pH 6.7–7.2, with NH_4OH; *B*, ethyl acetate; *C*, ethyl acetate–C_6H_6 (80:20). [b] After hydrolyses.

DYES, EXCIPIENTS, FLAVORS, AND PRESERVATIVES

Although TLC offers a very fast method for separation of dyes, the comprehensive summary of paper chromatography of dyes (133) should be consulted before attacking a complicated dye mixture problem with TLC.

Gasparic (134) listed 87 dyes separated on Al_2O_3 with benzene, and Synodinos (135) separated 7 FD&C dyes on $CaCO_3$ with BuOH–EtOH–water (2:1:1) + 10% NH_3. Stahl's book (1) offers a guide to other systems.

The excipients in pharmaceuticals pose the greatest problems and are the least mentioned in the literature. Those working in stability assays of creams, ointments, and suppositories have experienced many frustrations by the obscuration of good TLC by the smearing action of PEG's, surfactants, flavors, and preservatives, particularly when attempting to observe minor amounts of degradation products. There are a few publications which may offer guidance in these types of problems. Mannitol and sorbitol were separated using isopropanol–0.1 *N* boric acid (85:15) (136). Breithurd (137) dissolved PEG's in methylene chloride. The 2% solutions were applied to silica gel plates and developed with acetone–NH_4OH 20% (85:15).

Vakhtina (138) used alumina with $CHCl_3$–EtOH (98:2) for glycols with molecular weights up to 2000. Thoma (139) found that $CHCl_3$–MeOH–H_2O (3:25:12) would separate the stearates of PEG. Hartsaw (140) studied several excipients with some common systems and general means of visualization (Table XV).

TABLE XV—TLC OF EXCIPIENTS

Ref. →	(140)										Visualization			
Solvent[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*d*	*e*	*f*	*g*
Adsorbent →	1	1	1	1	1	1	1	1	1	1				
Compd.	$R_f \times 100$													
Polyethylene glycol										49	−	+	−	+
Cetyl alcohol	71	70							02		+	+	−	+
Citric acid				04		04					−	+	+	+
Cocoa butter	77	81	82	81	91	93		73	73	80	−	+	+	+
Cream of tartar				04		04				02	−	−	+	+
Ethylcellulose	80	71									−	+	−	+
Ethyl *p*-hydroxybenzoate	61	65	68	25	54	74	76	07	06	66	+	+	+	+
Glucose										14	−	+	+	+
Glycerin	0		07	02	02	25	79	0		33	−	−	+	+
Heavy mineral oil										78	−	−	+	+
Lactose				04		04				05	−	+	+	+
Lt. paraffin oil	77	77									−	+	−	+
Mannitol										18	−	+	+	+
Paraffin hard		79									−	+	−	−
Polyethylene glycol 200	01	07	07	0	14	20		07	14[c]		−	−	−	+
Polyoxyl 40 stearate										54	−	+	+	−
Sorbitan sesquioleate	79	66	75	32	61	89		0	0	77	−	+	+	+
	64[b]	83	82	60	88									
Spermaceti	79	81	82	81	88				73	77	−	+	−	−
Stearic acid		93			63						−	+	−	−
Sucrose				04		04				09	−	−	+	+
White petrolatum	80	80	82	81	88				73	77	−	+	−	−
White wax	80	82	82		88				73	77	−	+	−	−
Magnesium stearate										50	−	−	+	+
Agar[b]											−	+	−	+
$CaSO_4$[b]											−	+	−	−
Cellulose[b]											−	−	−	−
Citrus pectin[b]											−	+	+	+
Gelatin soft[b]											−	+	+	+
Kaolin[b]											−	+	−	−
Acacia powder[b]											−	+	−	+
Methylcellulose[b]											−	+	+	+
Silicon dioxide[b]											−	+	−	−
Polyvinyl pyrrolidine[b]											−	+	+	+
Starch powder[b]											−	+	−	+
Talc											−	−	−	−

[a] *A*, cyclohexane–acetone (40:50); *B*, $CHCl_3$–EtOH (9:1); *C*, $CHCl_3$–EtOH–heptane (1:1:1); *D*, cyclohexane–$CHCl_2$–AcOH (40:50:10); *E*, MeOH–benzene–AcOH (8:45:4); *F*, ethyl acetate–MeOH–AcOH (80:10:10); *G*, MeOH–acetone–triethanolamine (50:50:1.5); *H*, cyclohexane–acetone–Et_2NH (70:20:10); *I*, $CHCl_3$–Et_2NH (9:1); *J*, BuOH–5 *N* NH_4OH–MeOH (60:20:20). [b] Where no R_f value listed, the compound remained at point of application or it gave no reaction to reagents. [c] Two zones formed. [d] U.V. [e] I_2. [f] $KMnO_4$. [g] H_2SO_4 sprayed over $KMnO_4$.

For the partial resolution of nine preservatives Copius-Peereboom (141) used cellulose plates with a 20 cm. solvent travel. (Table XVI.) By using two developments on silica gel, Gossele (142) was able to separate these preservatives on a 20 × 20-cm. plate with temperature control below 22°. All of the compounds were observed under 366 mμ with a fluorescent indicator present, but other color reagents were used for additional differentiation.

Pinson (143) identified and determined four of the same compounds in codeine syrups and other syrups containing carboxymethylcellulose.

Antioxidants in vitamin A were detected after TLC by phosphomolybdic acid and ammonia vapor (144). Attaway (145) studied 60 flavor esters on silica gel using benzene or trifluorotrichloroethane–methylene chloride solvents and 5% vanillin in 96% H_2SO_4 reagent.

TABLE XVI—TLC OF PRESERVATIVES

Ref. →	(141)	(142)	(144)	(144)	(143)
Solvent[a] →	*A*	*B*	*C*	*D*	*E*
Adsorbent →	17	8	1	23	8
Compd.	$R_f \times 100$				
Benzoic acid	50	82			X
Sorbic acid	58	76			X
Salicylic acid	56	69			
Dehydroacetic acid	09	62			
Bromoacetic acid		43			
Propyl-*p*-HO-benzoate	90	30			X
Ethyl-*p*-HO-benzoate	86	25			
Methyl *p*-HO-benzoate	75	20			
p-HO-Benzoic acid	09	11			
o-Phenylphenol	95				
Hydroxyanisole			31		
Butyl hydroxytoluene			79		
Isoamyl gallate				61	
Nordihydroguaiaretic acid				45	

[a] *A*, BuOH–NH_3 35%–water (70:20:10); *B*, pet. ether–$CHCl_3$–formic acid (10:4:1); *C*, $CHCl_3$; *D*, $CHCl_3$ one dimension, then MeOH second dimension; *E*, *n*-pentane–AcOH (88:12).

TABLE XVII—TLC OF LOCAL ANESTHETICS

Ref. →	(42)	(42)	(42)	(42)	(42)	(146)	(146)	(146)	(146)	(146)	(146)	(146)	(146)	(146)	(146)	(147)
Solvent[a] →	A	B	C	B	D	E	F	G	H	I	J	K	L	M	N	O	P
Adsorbent →	2	2	2	4	4	13	13	13	13	13	13	13	13	13	13	13	1
Compd.	$R_f \times 100$																
Benzocaine	06	68	73	63	57	18	25	48			53	52	62	70	75	31	
Butacaine	08	63	71	59	51												
2-Chloroprocaine	03	53	52	46	26												
Cinchocaine						10	19	46	65	75	42	51	47	75	61	20	
Cocaine	58	57	64	26	10	17	34	65	75	82	57	67	60	80	70	30	X
Cyclomethycaine	66	45	42	46	29												
Dibucaine																	X
Diocaine						10	17	55	67	80	35	51	52	81	80	24	
Ethylaminobenzoate																	X
β-Eucaine						14	16	28	53	72	41	44	45	51	30	21	
Hexylcaine																	X
Hostocaine						10	17	26	42	68	27					05	X
Lidocaine	39	70	69	47	23	10	17	50	56	78	52	62	56	81	65	22	X
Larocaine						12	18	37	54	70	40	50	45	79	68	24	
Mesocaine						05	16	35	57	74	50	64	55	80	62	23	
Meprylcaine																	X
Onocaine																	X
Phenacaine	12	63	76	61	55	06	24	52	60	77	37	37	55	77	70	27	
Piperocaine	63	45	42	47	29												
Procaine	05	52	47	39	18	0	14	31	52	65	27	34	43	60	54	11	X
Proparacaine																	X
Psicaine						08	18	46	65	78	55	64	57	78	63	25	
Tetracaine	18	50	28	38	14	08	15	40	63	72	40	43	56	59	58	20	X
Tropacocaine																	X
Tutocaine						10	16	43	62	71	45	41	57	60	55	22	

[a] *A*, cyclohexane–benzene–Et_2NH (75:15:10); *B*, MeOH; *C*, acetone; *D*, 95% EtOH; *E*, benzene; *F*, benzene–EtOH (98:2); *G*, benzene–EtOH (95:5); *H*, benzene–EtOH (90:10); *I*, benzene–EtOH (80:20); *J*, $CHCl_3$; *K*, $CHCl_3$–EtOH (99:1); *L*, $CHCl_3$–BuOH (98:2); *M*, $CHCl_3$–acetone (1:1); *N*, ether; *O*, ether–pet. ether (1:1); *P*, benzene–acetone–NH_4OH (80:20:1).

LOCAL ANESTHETICS

A major contribution in the TLC of local anesthetics was made by Sarsunova (146). He recommended specific systems for pairs of compounds, including local anesthetics in a large group of drugs. Iodine and Dragendorff were effective reagents for these compounds (Table XVII). Fuwa (147), in a series of drug analyses by TLC, included 14 local anesthetics along with a study of hypnotics and anthelmintics. Other compounds were separated in BuOH–AcOH–H_2O (5:1:4) on silica gel (148).

ORAL HYPOGLYCEMIC DRUGS

In a series of papers on drug analyses Reisch (149) reported on the resolution of carbutamide, chlorpropamide, and tolbutamide with visualization by ninhydrin spray. Neidlein's (150) work included hypoglycemic compounds with other sulfonamides.

Strickland (151) studied the above compounds plus acetohexamide and phenformin HCl in six solvent systems. The R_f values cited in Table XVIII for his work (151) are the average of 50 measurements.

PSYCHOTROPIC DRUGS

In an early report on chromatography by paper, electrophoresis, and thin layer of phenothiazine drugs, Mellinger (152) found silica gel thin layer "by far, the best procedure for any phenothiazine drug." A comprehensive study of solvent systems was reported on 23 compounds (Table XIX). Fluorescence under 263 mμ gave a sensitive means of detection, but in the presence of acids, decomposition was evident. Light protected tanks were used for chromatography in acid systems. Various colors were obtained with 40% H_2SO_4. Addition of ferric salts or PDAB to the H_2SO_4 or a palladium chloride reagent gave no additional differentiation. Paulus (153) also observed a better color differentiation with H_2SO_4–EtOH 10% than with Dragendorff, $KMnO_4$, H_2O_2, and KNO_3 solutions. Margasinski (154) added iodoplatinate, H_2SO_4 16% + 1 ml. formalin to the list of color reagents. Adank (155) pointed out the utility of analyses of trace contaminants by TLC.

TABLE XVIII—TLC OF ORAL HYPOGLYCEMIC AGENTS

Ref. →	(149)	(150)	(151)	(151)	(151)	(151)	(151)
Solvent[a] →	A	B	C	D	E	F	G
Adsorbent →	1	6	1	1	1	1	1
Compd.	$R_f \times 100$						
Acetohexamide			52	67	49	50	42
Carbutamide	X	45					
Chlorpropamide	X		59	70	53	52	35
Phenformin HCl			03	52	24	32	03
Tolbutamide	X	54	75	76	65	66	33

[a] *A*, BuOH–$CHCl_3$–Et_2NH (7:7:1); *B*, BuOH–$CHCl_3$–acetone–Et_2NH (9:1:1:1); *C*, acetone–benzene–water (65:30:5); *D*, acetone–BuOH–water (20:50:30); *E*, BuOH saturated with water; *F*, BuOH–formamide–water (50:10:50) upper phase; *G*, dioxane–NH_3 0.88 sp. gr.–water (100:3:10).

TABLE XIX—TLC OF PSYCHOTROPIC DRUGS

Ref. →	(152)	(152)	(152)	(152)	(152)	(152)	(153)	(153)	(164)	(164)	(154)	(154)	(154)
Solvent[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
Adsorbent →	1	1	1	1	1	1	1	1	1	1	1	1	1
Compd.	$R_f \times 100$												
Acetophenazine	12	36	04		18	38							
Acetylpromazine													
Alimemazine													
Amitriptyline									X	X			
Aminopromazine													
Butyrylperazine	08	31	06	28	10	45							
Chlordiazepoxide								64					
Chlorpromazine	37	44	28	14	23	66	05	100	X	X	21	21	12
Chlorpromazine sulfoxide	05	10	01	09	05	27							
Chlorprothixine	34	78		20	36	88	69	100	X	X			
Dimethoxinate													
Dixyrazine													
Ethopropazine													
Fluphenazine	37	56	09	68	27	57							
Imipramine	45	55	24	13	16	66	47	100	X	X			
Isothypendyl	26	39	24	15	11	61							
Levomepromazine													
Mepazine	29	46	13	13	13	62							
Mepazine sulfoxide													
Meprobamate													
Methdilazine													
Methoxypromazine	15	26	12	12	09	45							
Methotrimeprazine													
Methylpromazine													
Nortriptyline													
Perphenazine	28	57	07	48	24	53					14	14	19
Pipamazine	38	71		41	37	79							
Prochlorpemazine													
Prochlorperazine	10	31	06	24	08	55							
Prochlorperazine sulfoxide													
Proketazine	19	45	06	53	25	44							
Promazine	16	31	12	11	11	50	37	92	X	X	21	21	12
Promazine sulfoxide													
Promethazine											12	23	18
Promethazine sulfoxide													
Prothipendyl	16	21	11	11	08	44							
Thiazinamine													
Thioridazine	24	39	14	24	15	64	45	100					
Thioridazine sulfoxide	03	13	01	09	03	33							
Thiopropazate													
Thiopropazine	64	79	49	65	53	81							
Thiethylperazine	14	50		23	13	61							
Triflupromazine	36	50	48	22	22	72					14	30	17
Triflupromazine sulfoxide													
Trimeprazine													
Trifluoperazine	18	33	09	34	12	51							
Trifluoperazine sulfoxide	03	11	02	17	03	31							
Trimeprazine													

[a] *A*, *tert*–BuOH–1 *N* NH_3 (90:10); *B*, *n*-PrOH–1 *N* NH_3 (88:12); *C*, ether saturated with water; *D*, 70% MeOH; *E*, 85% *n*-PrOH; *F*, *n*-BuOH saturated with 1 *N* NH_3; *G*, benzene–acetone–NH_3 25% (50:10:5); *H*, benzene–EtOH–NH_3 25% (50:10:5); *I*, BuOH–AcOH–H_2O (88:5:7); *J*, BuOH–AcOH–H_2O (65:15:20); *K*, acetone–ethyl acetate–EtOH 95% (5:4:1) saturated with NH_4 lactate pH 3; *L*, acetone–ethyl acetate–EtOH 95% (5:4:1) saturated with NH_4 lactate pH 7; *M*, acetone–

Continued ⟶

Imipramine HCl[1] was found to contain eight impurities with a composite total of less than 0.2%. Pharmacological evidence was obtained to show that the impurities would not cause unfavorable side effects at the low concentration present.

Cochin (95) in the development of methods for phenothiazine in body fluids preferred H_2SO_4 for color differentiation on 26 compounds including the sulfoxide metabolic products. Noirfalise (156) tried cellulose as an adsorbent and used I_2 vapor visualization in addition to others listed. Rusiecki (157) found the same problems with decomposition during exposure to light. With proper protection TLC was recommended for quality control of ampuls and suppositories for possible degradation products.

Heyndrickx (158) separated meprobamate from sulfathiazole for subsequent colorimetric assay. Hynie (159) used benzidine–KI reagent for meprobamate and metabolites in urine. Rusiecki (160) extracted chlorpromazine from decomposition products (157) and eluted TLC plates with MeOH for assay at 268 mμ. Noirfalise (161) continued

[1] Marketed as Tofranil by Geigy Pharmaceuticals, Ardsley, N. Y.

TABLE XIX—(*Continued.*)

Ref. →	(156)	(95)	(95)	(95)	(165)	(165)	(165)	(158)	(166)	(161)	(161)	(161)	(161)	(42)	(42)	(42)	(42)	(42)
Solvent[a] →	*N*	*O*	*P*	*Q*	*R*	*R*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*	*Z*	*AA*	*Z*	*AB*
Adsorbent →	17	1	1	1	1[b]	1[c]	1	1	1	1	1	1	1	2	2	2	4	4
Compd.	$R_f \times 100$																	
Acetophenazine														03	51	01	15	05
Acetylpromazine						65	54											
Alimemazine										39	64	64	39					
Amitriptyline		74	65	37										72	50	34	41	28
Aminopromazine	41									14	41	41	14					
Butyrylperazine																		
Chlordiazepoxide																		
Chlorpromazine	23	94	70	30		83	75			37	49	49	37	57	44	37	44	26
Chlorpromazine sulfoxide		26	47	19	86													
Chlorprothixine		80	73	46														
Dimethoxinate														24	31	13	33	13
Dixyrazine										61	39	39	61					
Ethopropazine														68	62	82	40	25
Fluphenazine	03	34	58	68		43	27			59	33	33	59	06	60	25	15	06
Imipramine		86	77	29										61	35	18	39	25
Isothypendyl														51	47	32	36	18
Levomepromazine						90	86			42	64	64	42					
Mepazine		88	57	29										55	49	37	43	21
Mepazine sulfoxide		16	59	24	74													
Meprobamate								75										
Methdilazine														44	29	14	39	18
Methoxypromazine														46	37	26	40	19
Methotrimeprazine														56	56	65	46	23
Methylpromazine														53	39	28	42	23
Nortriptyline										15								
Perphenazine														06	57	20	12	04
Pipamazine		12	72	56								43	34	0	60	32	48	27
Prochlorpemazine										44	23							
Prochlorperazine	13	70	27	32		43	27			59	33	33	59	46	41	14	07	02
Prochlorperazine sulfoxide		13	28	13	88													
Proketazine														04	57	17	12	04
Promazine		62	38	37								18	33	50	36	25	39	20
Promazine sulfoxide		17	46	15	70													
Promethazine	54	70	59	22		77	67			44	44	44	44	46	47	37	45	23
Promethazine sulfoxide		22	57	30	78													
Prothipendyl																		
Thiazinamine	64									03	03	03	03					
Thioridazine		97	65	20						32	43	43	32	52	45	31	41	27
Thioridazine sulfoxide																		
Thiopropazate		97	67	70														
Thiopropazine		69	33	40										44	66	67	30	11
Thiethylperazine														44	47	14	08	02
Triflupromazine		95	79	40			85	77						57	52	48	48	31
Triflupromazine sulfoxide		26	48	24	93													
Trimeprazine		96	64	30														
Trifluoperazine														45	49	19	10	02
Trifluoperazine sulfoxide																		
Trimeprazine														64	55	62	44	22

ethyl acetate–EtOH 95% (5:4:1) saturated with NH_4 lactate pH 9; *N*, 5% $(NH_4)_2SO_4$ saturated with iso-BuOH; *O*, benzene–dioxane–NH_3 (60:35:5); *P*, ethanol–AcOH–H_2O (50:30:20); *Q*, MeOH–BuOH (60:40); *R*, benzene–dioxane–NH_3 (10:80:10); *S*, $CHCl_3$–acetone (4:1); *T*, MeOH–$CHCl_3$ (1:2); *U*, acetone–MeOH (1:1); *V*, acetone–NH_3 (1:1); *W*, acetone–NH_3 (99:1); *X*, $CHCl_3$–MeOH (1:1); *Y*, cyclohexane–benzene–Et_2NH (75:15:10); *Z*, MeOH; *AA*, acetone; *AB*, EtOH 95%. [b] 30°. [c] 10°.

the series of papers on phenothiazine compounds and showed tables for color reaction with various compounds.

Reaction of psychotropic drugs on TLC with bromine and bromine + aniline were recorded by Clarke (162). Additional R_f values for phenothiazines on silica gel with a $CHCl_3$–EtOH (70:30) system were given by Moza (163).

STEROIDS

The section on steroids by Waldi in Stahl's book (1) followed by a series (13 pages) by Neher in Marini-Bettolo's book (22) on TLC of steroids and by Cavina and Vicari in Marini-Bettolo's book (22) on the qualitative and quantitative analyses of natural and synthetic corticosteroids by TLC (14 pages) covered the literature through 1962–1963. These authors all gave excellent details on techniques, reagents, and listings for specific separation. Heftmann's (167) article on TLC of steroids in chromatographic reviews (13 pages, 180 references) again surveyed earlier work and extended the review

through mid 1964. Stevens (168) covered a similar period (4 pages) with a more selective bibliography. Tschesche wrote a chapter (13 pages) in another book (169) on the same subject.

To bring the literature up to date from 1964 through 1965 is beyond the scope of this review. For example, there were at least 20 publications on the TLC of estrogens and androgens in urine.

Some publications (1964–1966) have been selected for the TLC of pure steroid compounds. They are listed in Table XX. A mass of R_f values and correlations with structure are available in these publications. Many of the means of visualization and solvent systems are listed in these references. Some articles on assay of steroids in pharmaceuticals during the same period are mentioned in the subsequent paragraphs.

Bican-Fister (180) applied a chloroform dilution of oil solutions of progesterone and testosterone to Silica Gel HF254. Development in cyclohexane–ether (8:2) and removal of U.V. absorbing spots permitted the quantitative assay of these compounds by the INH reaction with 98% recovery and relative standard deviation of about 3.6%. Cavina (181), with added sophistication, used continuous elution with petroleum ether–ethyl ether–AcOH (70:30:1 or 50:50:1) to separate testosterone propionate, progesterone, 19-nortestosterone propionate, and estradiol cyclopentylpropionate from oily solutions. The steroid spots were eluted with $CHCl_3$, evaporated, and dissolved in EtOH for measurement in U.V. from 225–250 mμ or by INH reaction. Recoveries of 95–99.4% were obtained. The U.V. method was found to be more precise than the INH reagent. Bennett (182) successfully separated some closely related steroids by an improved continuous development technique on 50 × 200 × 0.25-mm. Silica Gel G plates.

TABLE XX—TLC OF STEROIDS

Subject	Recent Lit. Ref.
Δ^4-3-Oxo-C_{21}-steroids (37 compd.) (means of visualization with 28 reagents)	(170)
Δ^4-3-Oxo steroids of androstane series (36 compd.)	(171)
Δ^5-3-Hydroxy-C_{19}-steroids (16 compd.)	(172)
Δ^4-3-Ketosteroids of pregnane series (37 compd.)	(173)
Saturated 21-deoxypregnane steroids (55 compd.)	(174)
Corticosteroids (11 compd.)	(175)
Corticosteroids (28 compd.)	(176)
Adrenal corticosteroids (26 compd.)	(177)
3-β-Hydroxy-Δ^5-steroids (13 compd. and 6 estrogens)	(178)
Δ^5-3-β-Hydroxysteroids (8 compd.)	(179)

Methyltestosterone and three related compounds were extracted from tablets and capsules with acetone and from liquids with $CHCl_3$ for subsequent TLC by Castren (183). After development in benzene–ethyl acetate (7:3), the methyltestosterone 2,4-dinitrophenylhydrazone was determined colorimetrically with accuracy of ± 5%.

Jacobsohn (184) photographed the H_2SO_4 color reaction zones of TLC plates of estrone, estradiol, and estriol, irradiated by U.V. The densities of spots were measured in a Photovolt densitometer and quantitative relationships of densities and concentrations were established.

Jensen (185) used TLC to study the stability of steroid ophthalmic solutions. $CHCl_3$ extracts of the solutions were evaporated and applied to Silica Gel G plates. Fluprednisolone (R_f 0.12) and the acetate (R_f 0.50) zones were removed and determined by blue tetrazolium to within ±2%. Comer (186) compared the results of the tetrazolium determination and a TLC scintillation method for measurement of the stability of ^{14}C-labeled fluorandrenolone cream. Jakovljevic (187) used TLC to verify a column chromatographic method for the same steroid. Tishler (188) separated the reaction products of the determination of methandrostenolone with MeOH–HCl solution and was able to postulate reaction mechanisms.

Schultz (189) applied TLC on silica gel, using (90:10) benzene–ether for development solvent, for the determination of 17α-ethynylestradiol-3-methyl ether (R_f 0.32) in the presence of 19-nortestosterone and 6-chloro-6-dehydro-17α-acetoxyprogesterone in tablets. The steroid zone was removed and measured at 278 mμ with accuracy of 95.5–98.5%.

Various colors formed initially, finally, and the colors in U.V. (366 mμ) with 50% H_2SO_4 spray and then heat at 78° were recorded (190) for 141 compounds.

SULFONAMIDES

Wollish (8) used the separation of five sulfonamides as examples in a review of advances in TLC in 1961. Klein (191) and Kho (192) used two-step systems for separation with diazotization for detection of N^1-substituted and bromocresol purple for the N^4-substituted sulfonamides. Bican-Fister (193) used alkaline coupling with betanaphthol after diazotization to detect 0.25 mcg. amounts after separation and the Bratton-Marshall reagent for quantitation after elution. In another study by Bican-Fister (194) the quantitative data showed a relative standard devia-

TABLE XXI—TLC OF SULFONAMIDES

Ref. →	(191)	(192)	(8)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)	(198)
Solvent[a] →	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Adsorbent →	1	1	1	13	13	13	13	13	13	13	13	13	13	13	13	13
Compd.	$R_f \times 100$															
Sulfacarbamide				16	33	66	71	41	34	32	29	19	13	37	45	63
Sulfacetamide	42	X		05	20	26	20	24	17	09	07	05	03	17	40	56
Sulfadiazine	47		40	09	18	08	03	35	50	38	33	31	23	25		
Sulfadimerazine																
Sulfadimethoxine																
Sulfadimidine				34	58	77	52	73	60	60	58	55	36	35	50	78
Sulfaethidol																
Sulfamerazine	57															
Sulfamethazine	64															
Sulfamethizole																
Sulfamethoxin				20	31	37	33	57	56	48	42	50	38	40		
Sulfamethoxydiazine																
Sulfamethoxypyrazine																
Sulfamethoxypyridazine				22		54	55	76	69	53	48	40	28	21	50	71
Sulfamethylpyrimidine																
Sulfanilamide	53	X		16	38	66	71	41	34	30	27	19	15	40	42	63
Sulfaguanidine					01	05	05	18	12	08	08	08	05	05	12	
Sulfaquinoxaline	66															
Sulfapyridine																
Sulfathiazole	50	X		08	22	03	05	34	25	12	10	06	05	06	38	55
Succinyl sulfacetamide		37														
Succinyl sulfathiazole		43		08	21	02	03	34	34	12	10	06	04	06	38	55
Succinyl sulfanilamide		49														
Phthalylsulfacetamide		51														
Phthalylsulfathiazole		61		08	08	04	07	34	34	12	10	34	04	05	38	55
Phthalylsulfanilamide		66														
Sulfisoxazole[b]			70													
Acetyl sulfisoxazole[c]			90													
Sulfadimethoxine[d]			80													
Sulfanilic acid			02													

Ref. →	(193)	(201)	(199)	(199)	(199)	(199)	(197)	(193)	(195)	(196)	(202)
Solvent[a] →	Q	R	S	T	U	V	W	X	Y	Z	AA
Adsorbent →	1	13	1	1	1	1	1	1	1	1	1
Compd.	$R_f \times 100$										
Sulfacarbamide											
Sulfacetamide	38	X					31		27		47
Sulfadiazine	50		48	42	87	35	39	53		61	65
Sulfadimerazine										72	
Sulfadimethoxine							67		47		72
Sulfadimidine			52	47	82	35					76
Sulfaethidol		X									
Sulfamerazine	59		45	45	87	45	44	60	35	67	
Sulfamethazine	64						52	72			
Sulfamethizole							25				41
Sulfamethoxin											
Sulfamethoxydiazine											
Sulfamethoxypyrazine							70				
Sulfamethoxypyridazine	68						61	38	40		
Sulfamethylpyrimidine							46				
Sulfanilamide	36		67	65		60	43	61	29	37	52
Sulfaguanidine	16	X	65	64	82	30	15	04	06		
Sulfaquinoxaline											
Sulfapyridine							37				69
Sulfathiazole	38	X	59	55	84	42	41	14	17	43	60
Succinyl sulfacetamide											
Succinyl sulfathiazole			61	58		41					
Succinyl sulfanilamide											
Phthalylsulfacetamide											
Phthalylsulfathiazole			63	61		45					
Phthalylsulfanilamide											
Sulfisoxazole[b]							51	51			
Acetyl sulfisoxazole[c]								81			
Sulfadimethoxine[d]											
Sulfanilic acid											

[a] *A*, $CHCl_3$–EtOH–heptane (1:1:1) plus 1.2% H_2O; *B*, MeOH–EtOH for 5 cm. (1:1) then *n*-propanol–.05 *N* HCl (4:1) for 5 cm.; *C*, $CHCl_3$–EtOH–heptane (1:1:1); *D*, benzene–EtOH (9:1); *E*, benzene–EtOH (8:2); *F*, ether–MeOH (9:1); *G*, ether–EtOH (9:1); *H*, $CHCl_3$–MeOH (80:15); *I*, $CHCl_3$–EtOH (80:15); *J*, $CHCl_3$–*n*-propanol (80:15); *K*, $CHCl_3$–isopropanol (80:15); *L*, $CHCl_3$–*n*-BuOH (80:15); *M*, $CHCl_3$–amyl aclohol (80:15); *N*, $CHCl_3$–acetone (1:1); *O*, $CHCl_3$–AcOH (95:5); *P*, $CHCl_3$–AcOH (90:10); *Q*, $CHCl_3$–MeOH (100:10); *R*, benzene–AcOH–MeOH (8.5:1:0.5); *S*, NH_3 30%–*sec*-BuOH–isopropanol–water (1.5:3.5:4.1); *T*, NH_3 30%–*sec*-BuOH–isopropanol–water (1.5:4:4:0.5); *U*, diethylamine (abs.)–isopropanol–water (1:5:4); *V*, diethylamine (abs.)–*sec*-BuOH–isopropanol–water (1.5:4:4:0.5); *W*, $CHCl_3$–BuOH–pet. ether (1:1:1); *X*, ether; *Y*, benzene–BuOH–pyridine (30:5:5); *Z*, $CHCl_3$–MeOH–$HCONMe_2$ (100:10:5); *AA*, $CHCl_3$–MeOH–water (160:40:2.5). [b] Marketed as Gantrisin by Roche Laboratories, Nutley, N. J. [c] Marketed as Acetyl Gantrisin by Roche Laboratories. [d] Marketed as Madribon by Roche Laboratories.

tion of about 4%. Tablets were extracted with 70% EtOH–NH_3 and the extract applied in three 3-μl. portions to Silica Gel G. Suppositories were extracted with petroleum ether–water–NH_3 and the aqueous layer applied to Silica Gel G. Suspensions were shaken with EtOH–NH_3 and centrifuged. The solution was applied to the TLC plates as in tablets. Pastor (195,196) separated and identified 11 sulfonamides.

Karpitschka (197) found better separation when EtOH in the Wollish system was replaced by butanol and a wedged-shaped adsorbent used.

Sarsunova (198) screened 13 systems for several components and made a table for the most useful systems for separation of specific pairs of sulfonamides. They used $CHCl_3$–EtOH (100:8) for separation of sulfathiazole, sulfadimidine, and sulfacetamide. For quantitative assay the tablets were extracted with acetone–ethanol and applied to Al_2O_3. After separation, the spots were located with I_2 vapor and removed with a vacuum filter, and the sulfonamides measured at 270 and 290 mμ. The relative standard deviations of 2.5%, 4.1%, and 3.2% were obtained for the three components with six replicates.

There have been so many solvent systems tried (see Table XXI) that it is difficult to find a new one. Even so, publications are still appearing with new combinations and color reagents. Other means of detection are 2% vanillin in acetic acid (199), coupling with dimethyl α-naphthylamine after nitrous acid (196) and 3% ninhydrin in BuOH (200). Other studies on TLC of sulfonamides include those of Poethke (201), Wehrli (202), Lin (203), and Fogg (204).

VITAMINS

A convenient starting point for a survey on TLC of vitamins is the chapter on vitamins in Stahl's book (1). Recent reviews by Katsui (205–207) on techniques, plates, adsorbents, solvent systems, and spray reagents, followed by reviews on fat-soluble and water-soluble vitamins, brought the literature up to date in 1964. This review will attempt to cover some of the work since that time.

Castren (208) was able to separate vitamins A, D, and E in multivitamin preparations after alkaline hydrolysis on silica gel. Oxidation products of vitamin A appeared on TLC of old preparations. Quantitative analyses of vitamins A and E by the linear relationship of area of spots, observed by U.V. or phosphomolybdic acid, to concentration were possible. Various components of vitamin A were observed on alumina (209) by fluorescence in U.V. Varma (210) studied the separation of vitamin A and related compounds in six systems and used one of these systems (*F* in Table XXII) for nonsaponifiable fractions of a liquid multivitamin sample to identify anhydrovitamin A, retrovitamin A alcohol, and vitamin A alcohol. John (211) observed the same compounds after TLC under U.V. and listed blue, green, or yellow colors formed with $SbCl_3$. Silica Gel G treated with PEG 200 was used (212) for the separation of vitamin A alcohol, D_2,D_3, and β-tocopherol.

David (213) used cellulose and other adsorbents and several solvent systems other than *O* in Table XXIII for TLC of thiamine and nitrates. A $Fe(CN)_3$ reagent was used for visualization.

Vorobéva (214) was able to separate vitamin B_{12}, factor B, and factor III on silica gel contain-

TABLE XXII—TLC OF A VITAMINS

Ref. →	(208)	(226)	(210)	(210)	(210)	(210)	(210)	(210)	(210)	(226)	(211)	(211)	(211)	(212)
Solvents[a] →	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*B*	*K*	*U*	*M*	*N*
Adsorbent →	1	1	13	13	13	13	13	13	13	13	1	1	1	15
Compd.	$R_f \times 100$													
Vit. A	X	X								X				
Anhydr. vit. A			63	90	97						93	82	82	
β-Carotene			06	80							100	100	100	
Retro-vit. A_1 acetate			0	36	90						70	47	49	
Vit. A_1 acetate				19	88									
Retro-vit. A_1 alcohol					12	36	42				20	08	08	32
Vit. A_1 alcohol					06	16	28	48						
Vit. A_1 epoxide					03	12		32						
Vit. A_2 alcohol					0	08	26	28	58					
Vit. A_1 acid						0		0	05		0	0	0	
Vit. A_1 palmitate											91	76	79	
Anhydro vit. A_2											87	69	61	

[a] *A*, benzene–ethyl acetate (7:3); *B*, $CHCl_3$; *C*, cyclohexane; *D*, cyclohexane–benzene (95:5); *E*, cyclohexane–MeOH (.25%); *F*, cyclohexane–MeOH (99:1); *G*, cyclohexane–MeOH (97:3); *H*, cyclohexane–EtOH (97:3); *I*, cyclohexane–EtOH (92:8); *K*, pet. ether–acetone (94:6); *M*, isooctane–acetone (97:3); *N*, pet. ether–benzene (1:1); *U*, hexane–acetone–MeOH (135:15:3).

ing gypsum and NaCN. Combustion of the organic material in a furnace allowed the detection of cobalt with β-nitroso-α-naphthol. The amount of cobalt could be determined from the eluted spots at 367 and 420 mμ.

Using basic alumina, Popova (215) separated additional analogs with a detection limit of 0.5 mcg. apparently based on the color of the compounds. The procedure was applicable to the control of the purity of concentrates of commercial preparations. The same authors (216) obtained improved separations on neutral alumina using NH_4OH to form cobalichromes.

Sasaki (217) found that cellulose MN300 gave good resolution of cyanocobalamin, hydroxycobalamin, and three coenzymes.

Chen (218) showed that fluorescent dyes sprayed on TLC plates of tuna oil gave less sensitivity to vitamin D_2, D_3, ergosterol, and 7-dehydrocholesterol than heating with H_2SO_4, but permitted a degree of qualitative differentiation. Pasalis (219) incorporated $AgNO_3$ and rhodamine into silica gel to separate some saturated and unsaturated esters of D_2 and D_3. Parekh (220) used TLC for the purification of ^{3}H-vitamin D_3 until all the U.V. fluorescent by-products were removed (Table XXIV). A quantitative assay of vitamin D in a pharmaceutical preparation was made possible by Heaysman (221) with wedges removed from the side of silica gel plates to form an elongated area for sample application and formation of concentric arcs following solvent development. Removal of the area containing vitamin D and subsequent rechromatography produced zones measured by the area of color found with H_2SO_4. The range found from eight tablet (800 units) assays was 700–920 units. The system of cyclohexane–ether (4:1) did not differentiate vitamins D_2 and D_3.

The TLC of tocopherols has been studied extensively and reported in recent publications. Skinner (222, 223) tabulated melting points, R_f, and spray reagents for many phenolic compounds related to tocopherols and their oxidation products and Sturm (224) published a quantitative determination of individual tocopherols. Peanut oils from various sources were assayed spectrophotometrically with bathophenanthroline–$FeCl_3$ after saponification and TLC. The average recovery of α-tocopherol was 93.2% ± standard deviation of 6.85%. Schmandke (225) obtained separation of α, γ, and δ-tocopherol on Al_2O_3–$ZnCO_3$ (3:1) plate with $CHCl_3$ as the solvent.

TABLE XXIII—TLC OF B VITAMINS

Ref. →	(208)	(226)	(213)	(214)	(215)	(216)	(217)
Solvents[a] →	*A*	*J*	*O*	*P*	*Q*	*R*	*S*
Adsorbent →	1	13	1	1	14	15	18
Compd.	$R_f \times 100$						
Vit. B_1		X	80				
Vit. B_1 PO_4			47				
Vit. B_1 $DiPO_4$			30				
Vit. B_1 Tri-PO_4			01				
Vit. B_6		X					
Nicotinamide		X					
Cyanocobalamin				X	62	46	X
Factor B				X	74		
Pseudo vit. B_{12}					25		
Factor B_{12}					46		
Factor V (nB)					12		
Factor A					37		
Hydroxycobalamin						30	X

[a] *A*, benzene–ethyl acetate (7:3); *J*, acetic acid–acetone–MeOH–benzene (5:5:20:70); *O*, *n*-propanol–PO_4 buffer pH 4.9–H_2O (60:20:20); *P*, water saturated with *sec*-BuOH; *Q*, BuOH–isopropanol–water (1:1:1); *R*, BuOH–isopropanol–water (1.5:1:1.25) NH_4OH to pH 8.5; *S*, *sec*-BuOH–0.1 *M* acetate buffer pH 3.5–MeOH (4:12:1).

QUANTITATIVE TLC

Many references were made to quantitative TLC methods under the various drug classes. Some other articles of special significance to pharmaceutical analysts are listed in Table XXV. The lack of precision and accuracy of the di-

TABLE XXIV—TLC OF D VITAMINS AND TOCOPHEROLS

Ref. →	(208)	(226)	(212)	(218)	(220)	(219)	(217)	(217)	(217)	(217)
Solvents[a] →	*A*	*B*	*N*	*T*	*U*	*V*	*B*	*X*	*B*	*B*
Adsorbent →	1	13	15	1	1	9	1	1	16	12
Compd.	$R_f \times 100$									
Vit. D	X	X								
Vit. D_2			47	32						
Vit. D_3			47	32	58					
7-Dehydrocholesterol				18	46					
Ergosterol				18						
D_2 stearate						79				
D_2 oleate						57				
D_2 linolenate						30				
α-Tocopherol	X	X	58				29	49	72	83
β-Tocopherol							20	33		
γ-Tocopherol							20	33	42	80
δ-Tocopherol							12	22	31	67

[a] *A*, benzene–ethyl acetate (7:3); *B*, $CHCl_3$; *N*, pet. ether–benzene (1:1); *T*, dichloromethane; *U* hexane–acetone–MeOH (135:15:3); *V*, hexane–benzene (1:2); *X*, pet. ether–ether (5:1).

TABLE XXV—QUANTITATIVE TLC

Subject	Ref.	Direct Measurement	Elution Measurement
Bile acids	(227)		U.V.
Cinchona alkaloids	(228)	Area by planimeter	
Cholesterol	(229)		Colorimetric
Coal tar	(230)	Spectrofluorometric	
Dyes	(231)	Spectral reflectance	
Furoic acid	(232)		U.V., I.R., GLPC
2-Nitro-4-acetamidophenetol	(233)		Polarographic
Noradrenaline	(234)	Area	Colorimetric
Opium alkaloids	(235)		U.V.
Phospholipids	(236)	Cellophane tape, then densitometer	
Review	(237)	Review	Review
Review	(238)	Wt. area relationship	
Selected pharmaceuticals	(239)	Area by Densicord model 542	
Senna glycoside	(240)		U.V.
Sorbic acid	(241)		Colorimetric
Steroids	(242)	Fluorescence recording photometer	
Technique	(243)	Reflectance spectroscopy	
Technique	(244)	Reflectance spectroscopy	
Technique	(245)	Fluorescent scanning photometer	
Technique	(246)	Scanning photometer	
Technique	(247)	Photometric color and fluorescence	
Technique	(248)		I.R.
Technique	(249)	Area	
Technique	(250)	Plate made transparent, then microphotometer	
Tropane alkaloids	(228)	Area by planimeter	

TABLE XXVI—RADIO-TRACER WITH TLC METHODS

Subject	Ref.	Direct Measurement	Elution Measurement
Benzoic acid-^{14}C	(251)	Methane flow counter	
Lipids-^{14}C,-^{3}H	(252)		Scintillation counter
Prochlorperazine	(253)		Scintillation counter
Sodium iodide-^{131}I	(254)	Radiochromatogram scanner	
Tritium-labeled compd.	(255)	Autoradiography	
Steroid cream-^{14}C	(186)		Scintillation counter

rect measurement instrumentation or the tedious nature of removal of adsorbent containing the desired compounds are the major disadvantages of quantitative TLC. This is probably the technology in TLC that will receive the most advances in the next few years. Table XXVI lists references on the application of radioactive tracers and TLC for analyses. This combination offers some exciting possibilities in the development of pharmaceutical assays, especially in the evaluation of stability.

REFERENCES

(1) Stahl, E., "Thin-Layer Chromatography—A Laboratory Handbook," Academic Press Inc., New York, N. Y., 1965.

(2) Bobbitt, J. M., "Thin-Layer Chromatography," 2nd printing, Reinhold Publishing Corp., New York, N. Y., 1964.

(3) Randerath, K., "Thin Layer Chromatography," translation by Libman, D. D., Academic Press Inc., New York, N. Y., 1963.

(4) Truter, E. V., "Thin Film Chromatography," Clever-Hume, London, England, 1963.

(5) Gänshirt, H., and Malzacher, A., *Arch. Pharm.*, **293**, 925(1960).

(6) Stahl, E., *Angew Chem.*, **73**, 646(1961).

(7) Stahl, E., *Z. Anal. Chem.*, **181**, 303(1961).

(8) Wollish, E. G., Schmall, M., and Hawrylyshyn, M., *Anal. Chem.*, **33**, 1138(1961).

(9) Demole, E., *J. Chromatog.*, **6**, 2(1961).

(10) Teijgeler, C. A., *Pharm. Weekblad*, **97**, 43(1962).

(11) *Ibid.*, **97**, 401(1962).

(12) Giacobazzi, C., and Gibertini, G., *Boll. Chim. Farm.*, **101**, 490(1962).

(13) Heftmann, E., *Anal. Chem., Pt. I*, **36**, 14R(1964).

(14) Russel, J. H., *Rev. Pure Appl. Chem.*, **13**, 15(1963).

(15) Zarnack, J., and Pfeifer, S., *Pharmazie*, **19**, 216 (1964).

(16) Mangold, H. K., Schmid, H. H. O., and Stahl, E., "Thin-Layer Chromatography," in Glick, D., "Methods of Biochemical Analyses," Interscience Publishers, New York, N. Y., 1964.

(17) Coombe, R. G., *Australian J. Pharm.*, **45**, S112 (1964).

(18) Heftmann, E., *Anal. Chem.*, **38**, 31R(1966).

(19) Teijgeler, C. A., *Pharm. Weekblad*, **99**, 101(1964).

(20) Baitsholt, A. D., "Bibliography of Pharmaceutical Applications of Thin-Layer Chromatography," Distillation Products Industries, Rochester, N. Y., 1966.

(21) Dallas, M. S. J., *J. Chromatog.*, **17**, 267(1965).

(22) Marini-Bettolo, G. B., "Thin-Layer Chromatography," Elsevier Publishing Co., New York, N. Y., 1964, p. 116.

(23) Shellard, E. J., *Lab. Pract.*, **13**, 290(1964).

(24) Feldmann, E. G., *J. Am. Pharm. Assoc.*, **NS6**, 582(1966).

(25) Waldi, D., Schnackerz, K., and Munter, F., *J. Chromatog.*, **6**, 61(1961).

(26) Stolman, A., "Progress in Chemical Toxicology," Academic Press Inc., N. Y., 1965, vol. 2, p. 321.

(27) Hohmann, T., and Rochelmeyer, H., *Arch. Pharm.*, **297**, 186(1964).

(28) McLaughlin, J. L., Goyan, J. E., and Paul, A. G., *J. Pharm. Sci.*, **53**, 306(1964).

(29) Ziner, M., and Baumgaertel, C., *Arch. Pharm.*, **297**, 158(1964).

(30) Sahli, M., and Oesch, M., *Pharm. Acta Helv.*, **40**, 25 (1965).

(31) French, W. N., and Wehrli, A., *J. Pharm. Sci.*, **54**, 1515(1965).

(32) Zarnack, J., and Pfeifer, S., *Pharmazie*, **19**, 222 (1964).

(33) Wasicky, R., *Anal. Chem.*, **34**, 1346(1962).

(34) Brochmann-Hanssen, E., and Furuya, T., *J. Pharm. Sci.*, **53**, 1549(1964).

(35) Ikram, M., Miana, G. A., and Islam, M., *J. Chromatog.*, **11**, 260(1963).

(36) Steele, J. A., *ibid.*, **19**, 300(1965).

(37) Vignoli, L., Guillot, J., Gouezo, F., and Catalin, J., *Ann. Pharm. Franc.*, **24**, 461(1966).

(38) Schwarz, V., and Sarsunova, M., *Pharmazie*, **19**, 267 (1964).

(39) Nakamura, G. R., *J. Assoc. Offic. Anal. Chemists*, **49**, 1086(1966).

(40) Pfeifer, S., *J. Chromatog.*, **24**, 364(1966).

(41) Kaess, A., and Mathis, C., *Ann. Pharm. Franc.*, **23**, 739(1965).

(42) Fike, W. W., *Anal. Chem.*, **38**, 1697(1966).

(43) Bayer, I., *J. Chromatog.*, **16**, 237(1964).

(44) Penna-Herreros, A., *ibid.*, **14**, 536(1964).

(45) Suszko-Purzycka, A., and Trzebny, W., *ibid.*, **17**, 114 (1965).

(46) *Ibid.*, **16**, 239(1964).

(47) Suszko-Purzycka, A., and Trzebny, W., *Chem. Anal.*, **9**, 1103(1964); through *Chem. Abstr.*, **63**, 431*c*(1965).

(48) Suszko-Purzycka, A., and Trzebny, W., *Poznan. Towarz. Przyjaciol Nauk, Wydzial Lekarsk. Prace Komisji Farm.*, **4**, 43(1966); through *Chem. Abstr.*, **65**, 8668*e*(1966).

(49) Weaver, W. J., *J. Pharm. Sci.*, **55**, 1111(1966).

(50) Guven, K. C., and Hincal, A., *Istanbul Univ. Eczacilik Fak. Mecmuasi*, **1**, 153(1965); through *Chem. Abstr.*, **65**, 8668*d*(1966).

(51) Kaess, A., and Mathis, C., *Ann. Pharm. Franc.*, **23**, 267(1965).

(52) Ikram, M., and Bakhsh, M. K., *Anal. Chem.*, **36**, 111 (1964).

(53) Chen, C., Chang, S., and Tai, J., *Yao Hsueh Hsueh Pao*, **13**, 131(1966); through *Chem. Abstr.*, **65**, 8673*c*(1966).

(54) Jakovljevic, I. M., Seay, L. D., and Shaffer, R. W., *J. Pharm. Sci.*, **53**, 553(1964).

(55) Farnsworth, N. R., and Hilinski, I. M., *J. Chromatog.*, **18**, 184(1965).

(56) Cone, N. J., Miller, R., and Neuss, N., *J. Pharm. Sci.*, **52**, 688(1963).

(57) Zeitler, H., *J. Chromatog.*, **18**, 180(1965).

(58) Conners, K. A., and Eriksen, S. P., *Am. J. Pharm. Educ.*, **28**, 161(1964).

(59) Fresen, J. A., *Pharm. Weekblad*, **99**, 829(1964).

(60) Savidge, R. H., and Wragg, J. S., *J. Pharm. Pharmacol.*, **17**, Suppl. 60S(1965).
(61) Thoma, F., *Arzneimittel-Forsch.*, **16**, 771(1966).
(62) Frodyma, M. M., Lieu, V. T., and Frei, R. W., *J. Chromatog.*, **18**, 520(1965).
(63) Gänshirt, H., *Arch. Pharm.*, **296**, 73(1963).
(64) *Ibid.*, **296**, 129(1963).
(65) Sarsunova, M., and Schwarz, V., *Pharmazie*, **18**, 34 (1963).
(66) Bailey, R. W., *Anal. Chem.*, **36**, 2021(1964).
(67) Shelly, N. E., *ibid.*, **38**, 935(1966).
(68) Mule, S. J., *ibid.*, **36**, 1907(1964).
(69) Tsuji, A., and Wada, A., *Eisei Shikensho Hokoko*, **82**, 73(1964); through *Chem. Abstr.*, **65**, 8668*g*(1966).
(70) Haefelfinger, P., Schmidli, B., and Ritter, H., *Arch. Pharm.*, **297**, 641(1964).
(71) Emmerson, J. L., and Anderson, R. C., *J. Chromatog.*, **17**, 495(1965).
(72) Kupferberg, H. J., Burkhalter, A., and Way, E. L., *ibid.*, **16**, 558(1964).
(73) Kutlu, H., *Istanbul Univ. Eczacilik Fak. Mecmuasi*, **1**, 128(1965); through *Chem. Abstr.*, **65**, 8670*g*(1966).
(74) Nicolaus, B. J. R., Coronelli, C., and Binaghi, A., *Farmaco (Pavia), Ed. Prat.*, **16**, 349(1961).
(75) Fischer, R., and Lautner, H., *Arch. Pharm.*, **294/66**, 1(1961).
(76) Nussbaumer, P. A., *Pharm. Acta Helv.*, **37**, 65(1962).
(77) *Ibid.*, **38**, 758(1963).
(78) Brodasky, T. F., *Anal. Chem.*, **35**, 343(1963).
(79) Akita, E., and Ikekawa, T., *J. Chromatog.*, **12**, 250 (1963).
(80) Nussbaumer, P. A., *Pharm. Acta Helv.*, **39**, 647 (1964).
(81) Sonanini, D., and Anker, L., *ibid.*, **39**, 518(1964).
(82) Kapadia, G. J., and Rao, G. S., *J. Pharm. Sci.*, **53**, 223(1964).
(83) Ito, Y., Namba, M., Nagahama, N., Yamaguchi, T., and Okuda, T., *J. Antibiotics, Ser. A*, **17**, 218(1964).
(84) Cassani, G., Albertini, A., and Ciferri, O., *J. Chromatog.*, **13**, 238(1964).
(85) Anderson, T. T., *ibid.*, **14**, 127(1964).
(86) Meyers, E., and Smith, D. A., *ibid.*, **14**, 129(1964).
(87) Nussbaumer, P. A., and Schorderet, M., *Pharm. Acta Helv.*, **40**, 205(1965).
(88) *Ibid.*, **40**, 477(1965).
(89) Nussbaumer, P. A., *ibid.*, **40**, 210(1965).
(90) Foppiano, R., and Brown, B. B., *J. Pharm. Sci.*, **54**, 206(1965).
(91) Rustici, L., and Ferappi, M., *Boll. Chim. Farm.*, **104**, 305(1965); through *Chem. Abstr.*, **63**, 11249*b*(1965).
(92) Gantes, P., Garinot, J. C., Barat, J., and Juhasz, J. P., *Ann. Pharm. Franc.*, **23**, 137(1965).
(93) Kline, R. M., and Golab, T., *J. Chromatog.*, **18**, 409 (1965).
(94) Simmons, D. L., Woo, H. S. L., Koorengevel, C. M., and Seers, P., *J. Pharm. Sci.*, **55**, 1313(1966).
(95) Cochin, J., and Daly, J. W., *J. Pharmacol. Exptl. Therap.*, **139**, 160(1963).
(96) Morrison, J. C., and Chatten, L. G., *J. Pharm. Sci.*, **53**, 1205(1964).
(97) Fike, W. W., and Sunshine, I., *Anal. Chem.*, **37**, 127 (1965).
(98) Cochin, J., and Daly, J. W., *J. Pharmacol. Exptl. Therap.*, **139**, 154(1963).
(99) Walker, W. H. C., and Wood, J. K., *J. Clin. Pathol.*, **18**, 482(1965).
(100) Bogan, J., Rentoul, E., and Smith, H., *Forensic Sci. J.*, **4**, 147(1964).
(101) Eberhardt, H., Freundt, K. J., and Langbein, J. W., *Arzneimittel-Forsch.*, **12**, 1087(1962).
(102) Shellard, E. J., and Osisiogu, I. U., *Lab. Pract.*, **13**, 516(1964).
(103) Petzold, J. A., Camp, W. J. R., and Kirch, E. R., *J. Pharm. Sci.*, **52**, 1106(1963).
(104) Kraus, L., and Vejrovska, E., *Cesk. Farm.*, **12**, 515 (1963); through *Anal. Abstr.*, **1965**, 2483.
(105) Sahli, M., and Oesch, M., *J. Chromatog.*, **14**, 526 (1964).
(106) Kiger, J. L., and Kiger, I. G., *Ann. Pharm. Franc.*, **22**, 477(1964).
(107) Ahmed, Z. F., El-Darawy, Z. I., Aboul-Enein, M. N., El-Naga, M. A. A., and El-Leithy, S. A., *J. Pharm. Sci.*, **55**, 433(1966).
(108) Stahl, E., and Kaltenbach, U., *J. Chromatog.*, **5**, 458 (1961).
(109) Steinegger, E., and van der Walt, J. H., *Pharm. Acta Helv.*, **36**, 599(1961).
(110) Reichelt, J., and Pitra, J., *Collection Czech. Chem. Commun.*, **27**, 1709(1962); through *Chem. Abstr.*, **62**, 11626*h* (1965).
(111) Duncan, G. R., *J. Chromatog.*, **8**, 37(1962).
(112) Faucennet, L., and Waldesbuehl, M., *Pharm. Acta Helv.*, **38**, 423(1963).
(113) Sonanini, D., *ibid.*, **39**, 673(1964).
(114) Momose, T., Matsukuma, T., and Okura, Y., *Yakugaku Zasshi*, **84**, 783(1964); through *Chem. Abstr.*, **61**, 11848*g*(1964).
(115) Lukas, G., *Sci. Pharm.*, **30**, 47(1962); through *Chem. Abstr.*, **57**, 12636*g*(1962).
(116) Corona, G. L., Raiteri, M., and Tieghi, G., *Farmaco (Pavia) Ed. Prat.*, **19**, 574(1964); through *Chem. Abstr.*, **62**, 5145*a*(1965).
(117) Noirfalise, A., *J. Chromatog.*, **20**, 61(1965).
(118) Beckett, A. H., and Choulis, N. H., *J. Pharm. Pharmacol.*, **15**, Suppl. 236(1963).
(119) Troup, A. E., and Mitchner, H., *J. Pharm. Sci.*, **53**, 375(1964).
(120) De Potter, W. P., Vochten, R. F., and De Schaepdryver, A. F., *Experientia*, **21**, 482(1965).
(121) Zemer, R. E., Analytical Laboratory, Eli Lilly and Co., Indianapolis, Ind., unpublished data.
(122) Haznagy, A., Szendrei, K., and Toth, L., *Pharmazie*, **20**, 541(1965).
(123) Boehme, H., and Kreutzig, L., *Arch. Pharm.*, **297**, 681(1964).
(124) Hoerhammer, L., Wagner, H., Bittner, G., and Graf, E., *Deut. Apotheker-Ztg.*, **105**, 827(1965).
(125) Haznagy, A., Szendrei, K., and Toth, L., *Pharmazie*, **20**, 651(1965).
(126) Hoerhammer, L., Wagner, H., and Kraemer-Heydweiller, D., *Deut. Apotheker-Ztg.*, **106**, 267(1966).
(127) Hoerhammer, L., Wanger, H., and Lay, B., *Pharmazie*, **15**, 645(1960).
(128) Kurono, G., Ogura, K., and Sasaki, K., *Yakugaku Zasshi*, **85**, 262(1965); through *Chem. Abstr.*, **62**, 15991*d* (1965).
(129) Moes, A., *J. Pharm. Belg.*, **19**, 173(1964).
(130) Atal, C. K., and Shah, K. C., *Indian J. Pharm.*, **28**, 162(1966).
(131) Margasinski, Z., Danielak, R., and Raflowska, H., *Acta Polon. Pharm.*, **23**, 35(1966); through *Chem. Abstr.*, **65**, 2066*a*(1966).
(132) Adam, R., and Lapiere, C. L., *J. Pharm. Belg.*, **19**, 79(1964); through *Chem. Abstr.*, **61**, 8134*f*(1964).
(133) Gasparic, J., and Gemzova-Taborska, I., *J. Chromatog.*, **12**, D9(1963).
(134) Gasparic, J., *Z. Anal. Chem.*, **218**, 113(1966).
(135) Synodinos, E., Kotakis, G., and Kokkoti-Kataki, E., *Chim. Chronika*, **28**, 77(1963); through *Chem. Abstr.*, **60**, 1089*e*(1964).
(136) Castagnola, V., *Boll. Chim. Farm.*, **102**, 784(1963).
(137) Breithurd, P., Puisieux, F., and Le Hir, A., *Ann. Pharm. Franc.*, **24**, 191(1966).
(138) Vakhtina, I. A., Okunev, P. A., and Tarakanov, O. G., *Zh. Analit. Khim.*, **21**, 630(1966); through *Chem. Abstr.*, **65**, 6279*f*(1966).
(139) Thoma, K., Rombach, R., and Ullmann, E., *Arch. Pharm.*, **298**, 19(1965).
(140) Hartsaw, P. E., Gonzales, D. R., Arnett, W. A., and Rutherford, B. S., Analytical Development Department, Eli Lilly and Co., Indianapolis, Ind., unpublished data.
(141) Copius-Peereboom, J. W., and Beekes, H. W., *J. Chromatog.*, **14**, 417(1964).
(142) Gossele, J. A. W., and Srebrnik-Friszman, S., *ibid.*, **23**, 305(1966).
(143) Pinson, R., Mirimanoff, A., and Kapetanidis, I., *Pharm. Acta Helv.*, **40**, 141(1965).
(144) Ishikawa, S., and Katsui, G., *J. Vitaminol.*, **12**, 112 (1966); through *Chem. Abstr.*, **65**, 7893*c*(1966).
(145) Attaway, J. A., Wolford, R. W., and Edwards, G. J., *Anal. Chem.*, **37**, 75(1965).
(146) Sarsunova, M., *Pharmazie*, **18**, 748(1963).
(147) Fuwa, T., Kido, T., and Tanaka, H., *Yakuzaigaku*, **24**, 123(1964); through *Chem. Abstr.*, **61**, 15934*f*(1964).
(148) Yatabe, M., and Oki, H., *Kagaku Keisatsu Kenkyusho Hokoku*, **17**, 167(1964); through *Chem. Abstr.*, **61**, 15933*f*(1964).
(149) Reisch, J., Bornfleth, H., and Tittel, G. L., *Pharm. Ztg. Ver. Apotheker-Ztg.*, **109**, 74(1964); through *Chem. Abstr.*, **63**, 16129*g*(1965).
(150) Neidlein, R., Kluegel, G., and Lebert, U., *Pharm. Ztg.*, **20**, 651(1965); through *Chem. Abstr.*, **63**, 6788*b*(1965).
(151) Strickland, R. D., *J. Chromatog.*, **24**, 455(1966).
(152) Mellinger, T. J., and Keeler, C. E., *J. Pharm. Sci.*, **51**, 1169(1962).
(153) Paulus, W., Hoch, W., and Keymer, R., *Arzneimittel-Forsch.*, **13**, 609(1963).
(154) Margasinski, Z., Danielak, R., Pomazanska, T., and Rafalowska, H., *Acta Polon. Pharm.*, **21**, 5(1964); through *Chem. Abstr.*, **62**, 8941*h*(1965).
(155) Adank, K., and Hammerschmidt, W., *Chimia (Aarau)*, **18**, 361(1964); through *Chem. Abstr.*, **62**, 6344*e* (1965).
(156) Noirfalise, A., and Grosjean, M. H., *J. Chromatog.*, **16**, 236(1964).
(157) Rusiecki, W., and Henneberg, M., *Acta Polon. Pharm.*, **21**, 23(1964); through *Chem. Abstr.*, **62**, 8940*h*(1965).
(158) Heyndrickx, A., Schauvliege, M., and Blomme, A., *J. Pharm. Belg.*, **20**, 117(1965); through *Chem. Abstr.*, **63**, 11256*a*(1965).
(159) Hynie, I., Konig, J., and Kacl, K., *J. Chromatog.*, **19**, 192(1965).
(160) Rusiecki, W., and Henneberg, M., *Acta Polon. Pharm.*, **23**, 117(1966); through *Chem. Abstr.*, **65**, 12061*e* (1966).
(161) Noirfalise, A., *J. Chromatog.*, **19**, 68(1965).
(162) Clarke, V., and Cole, E. R., *ibid.*, **24**, 259(1966).
(163) Moza, P. N., and Khajuria, G. S., *ibid.*, **24**, 261 (1966).
(164) Ferrari, M., and Toth, C. E., *ibid.*, **9**, 388(1962).
(165) Kozuka, H., and Isona, H., *Kagaku Keisatsu Kenkyusho Hokoku*, **17**, 353(1964); through *Chem. Abstr.*, **63**, 11257*g*(1965).
(166) Drabner, J., Bauer, H., and Schwerd, W., *Arch. Toxikol.*, **21**, 367(1966); through *Chem. Abstr.*, **65**, 14283*c* (1966).

(167) Heftmann, E., *Chromatog. Rev.*, **7**, 179(1965).
(168) Stevens, P. J., *Lab. Pract.*, **13**, 306(1964).
(169) Tschesche, R., Wolff, G., and Richert, K. H., in James, A. T., and Morris, L. J., eds., "New Biochemical Separations," Van Nostrand, London, England, 1964, pp. 247–260.
(170) Lisboa, B. P., *J. Chromatog.*, **16**, 136(1964).
(171) *Ibid.*, **19**, 81(1965).
(172) *Ibid.*, **19**, 333(1965).
(173) Lisboa, B. P., *Acta Endocrinol.*, **43**, 47(1963).
(174) Lisboa, B. P., *Steroids*, **6**, 605(1965).
(175) Yawata, M., and Gold, E. M., *ibid.*, **3**, 435(1964).
(176) Hall, A., *J. Pharm. Pharmacol.*, **16**, Suppl. 9T(1964).
(177) Quesenberry, R. O., and Ungar, F., *Anal. Biochem.*, **8**, 192(1964).
(178) Stárka, L., *J. Chromatog.*, **17**, 599(1965).
(179) Hagerman, D. D., and Spencer, J. M., *Steroids*, **4**, 547(1964).
(180) Bican-Fister, T., *J. Chromatog.*, **22**, 465(1966).
(181) Cavina, G., and Moretti, G., *ibid.*, **22**, 41(1966).
(182) Bennett, R. D., and Heftmann, E., *ibid.*, **21**, 488 (1966).
(183) Castren, E., *Farm. Aikakauslehti*, **74**, 189(1965); through *Chem. Abstr.*, **63**, 12974*h*(1965).
(184) Jacobsohn, G. M., *Anal. Chem.*, **36**, 275(1964).
(185) Jensen, E. H., and Lamb, D. J., *J. Pharm. Sci.*, **53**, 402(1964).
(186) Comer, J. P., and Hartsaw, P. E., *ibid.*, **54**, 524 (1965).
(187) Jakovljevic, I. M., Hartsaw, P. E., and Drummond, G. E., *ibid.*, **54**, 1771(1965).
(188) Tishler, F., and Brody, S. M., *ibid.*, **53**, 161(1964).
(189) Schultz, E. P., *ibid.*, **54**, 144(1965).
(190) Heftmann, E., Ko, S. T., and Bennett, R. D., *J. Chromatog.*, **21**, 490(1966).
(191) Klein, S., and Kho, B. T., *J. Pharm. Sci.*, **51**, 966 (1962).
(192) Kho, B. T., and Klein, S., *ibid.*, **52**, 404(1963).
(193) Bican-Fister, T., and Kajganovic, V., *J. Chromatog.*, **11**, 492(1963).
(194) *Ibid.*, **16**, 503(1964).
(195) Pastor, J., and Raimondi, R., *Trav. Soc. Pharm. Montpellier*, **23**, 220(1963); through *Chem. Abstr.*, **62**, 11634*b* (1965).
(196) Pastor, J., and Raimondi, R., *Bull. Soc. Pharm. Marseille*, **13**, 193, 197(1964); through *Chem. Abstr.*, **63**, 16133*d*(1965).
(197) Karpitschka, N., *Mikrochim. Ichnoanal. Acta*, **1963**, 157.
(198) Sarsunova, M., Schwarz, V., Feketeova, E., and Protiva, J., *Pharmazie*, **21**, 219(1966).
(199) Ritschel-Beurlin, G., *Arzneimittel-Forsch.*, **15**, 1247 (1965).
(200) Meyer-Dulheuer, K. H., and Ritter, E., *Pharm. Ztg.*, **110**, 260(1965); through *Chem. Abstr.*, **62**, 15994*h*(1965).
(201) Poethke, W., and Kinze, W., *Pharm. Zentralhalle*, **103**, 95(1964).
(202) Wehrli, A., *Can. Pharm. J. Sci. Sect.*, **97**, 208(1964).
(203) Lin, Y.-T., Wang, K.-T., and Yang, T.-I., *J. Chromatog.*, **20**, 610(1965).
(204) Fogg, A. G., and Wood, R., *ibid.*, **20**, 613(1965).
(205) Katsui, G., *Bitamin*, **29**, 147(1964); through *Chem. Abstr.*, **62**, 1516*a*(1965).
(206) Katsui, G., *Bitamin*, **29**, 211(1964).
(207) *Ibid.*, **29**, 300(1964).
(208) Castren, E., *Anales. Farm. Hosp.*, **8**, 31(1965); through *Chem. Abstr.*, **63**, 11254*f*(1965).
(209) Kuznetsova, L. M., and Kovalova, V. M., *Ukr. Biokhim. Zh.*, **36**, 302(1964); through *Chem. Abstr.*, **61**, 536*a* (1964).
(210) Varma, T. N. R., Panalaks, T., and Murray, T. K., *Anal. Chem.*, **36**, 1864(1964).
(211) John, K. V., Lakshmanan, M. R., Jungalwala, F. B., and Cama, H. R., *J. Chromatog.*, **18**, 53(1965).
(212) Richter, J., and Ropte, D., *Pharmazie*, **21**, 495 (1966).
(213) David, S., and Hirshfeld, H., *Bull. Soc. Chim. France*, **1963**, 1011.
(214) Vorobéva, L. I., *Mikrobiologiya*, **34**, 180(1965); through *Chem. Abstr.*, **62**, 12976(1965).
(215) Popova, Ya., Popov, Kh., and Ilieva, M., *J. Chromatog.*, **21**, 164(1966).
(216) *Ibid.*, **24**, 263(1966).
(217) Sasaki, T., *ibid.*, **24**, 452(1966).
(218) Chen, P. S., *Anal. Chem.*, **37**, 301(1965).
(219) Pasalis, J., and Bell, N. H., *J. Chromatog.*, **20**, 407 (1965).
(220) Parekh, C. K., and Wasserman, R. H., *ibid.*, **17**, 261 (1965).
(221) Heaysman, L. T., and Sawyer, E. R., *Analyst*, **89**, 529(1964).
(222) Skinner, W. A., and Parkhurst, R. M., *J. Chromatog.*, **13**, 69(1964).
(223) Skinner, W. A., Parkhurst, R. M., and Alaupovic, P., *ibid.*, **13**, 240(1964).
(224) Sturm, P. A., Parkhurst, R. M., and Skinner, W. A., *Anal. Chem.*, **38**, 1244(1966).
(225) Schmandke, H., *J. Chromatog.*, **14**, 123(1964).
(226) Ishikawa, S., and Katsui, G., *Bitamin*, **29**, 203(1964); through *Chem. Abstr.*, **61**, 15932*b*(1964).
(227) Gänshirt, H., Koss, F. W., and Morianz, K., *Arzneimittel-Forsch.*, **10**, 943(1960).
(228) Oswald, N., and Flueck, H., *Pharm. Acta Helv.*, **39**, 293(1964).
(229) Horvath, C., *J. Chromatog.*, **22**, 52(1966).
(230) Sawicki, E., Stanley, T. W., and Johnson, H., *Microchem. J.*, **8**, 257(1964).
(231) Frodyma, M. M., Frei, R. W., and Williams, D. J., *J. Chromatog.*, **13**, 61(1964).
(232) Millett, M. A., Moore, W. E., and Saeman, J. F., *Anal. Chem.*, **36**, 491(1964).
(233) Oelschlaeger, H., Volke, J., and Lim, G. T., *Arch. Pharm.*, **298**, 213(1965).
(234) Choulis, N. H., *Chim. Chronika* (*Athens, Greece*), **30**, 52(1965); through *Chem. Abstr.*, **63**, 13641*d*(1965).
(235) Mary, N. Y., and Brochmann-Hanssen, E., *Lloydia*, **26**, 223(1963).
(236) Morin, R. J., *Clin. Chim. Acta*, **13**, 395(1966); through *Chem. Abstr.*, **65**, 4240*b*(1966).
(237) Purdy, S. J., and Truter, E. V., *Lab. Pract.*, **13**, 500 (1964).
(238) Purdy, S. J., and Truter, E. V., *Chem. Ind.* (*London*), **1962**, 506.
(239) Morrison, J. C., and Orr, J. M., *J. Pharm. Sci.*, **55**, 936(1966).
(240) Longo, R., and Meinardi, G., *Boll. Chim. Farm.*, **104**, 503(1965); through *Chem. Abstr.*, **63**, 17799*h*(1965).
(241) Mueller, K. H., and Braxl, Ch., *Arch. Pharm.*, **299**, 560(1966).
(242) Ghiglione, C., Lebourhis-Sauer, J., and Fondarai, J., *Bull. Soc. Pharm. Marseille*, **14**, 183(1965); through *Chem. Abstr.*, **64**, 14968*c*(1966).
(243) Frodyma, M. M., and Frei, R. W., *J. Chromatog.*, **15**, 501(1964).
(244) Frodyma, M. M., Frei, R. W., and Williams, D. J., *ibid.*, **13**, 61(1964).
(245) Gordon, H. T., *ibid.*, **22**, 60(1966).
(246) Keyworth, D. A., and Swensen, R. F., *Talanta*, **13**, 829(1966).
(247) Klaus, R., *J. Chromatog.*, **16**, 311(1964).
(248) McCoy, R. N., and Fiebig, E. C., *Anal. Chem.*, **37**, 593(1965).
(249) Purdy, S. J., and Truter, E. V., *Analyst*, **87**, 802 (1962).
(250) Vioque, E., and Vioque, A., *Grasas Aceites* (*Seville, Spain*), **15**, 125(1964); through *Chem. Abstr.*, **62**, 7*c*(1965).
(251) Schildknecht, H., and Volkert, O., *Naturwissenschaften*, **50**, 442(1963); through *Chem. Abstr.*, **59**, 9365*e* (1963).
(252) Snyder, F., *Anal. Biochem.*, **9**, 183(1964).
(253) Seno, S., Kessler, W. V., and Christian, J. E., *J. Pharm. Sci.*, **53**, 1101(1964).
(254) Taylor, E. H., *ibid.*, **54**, 639(1965).
(255) Sheppard, H., and Tsien, W. H., *Anal. Chem.*, **35**, 1992(1963).

Use of Activation Analysis in Problems of Drug Control

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Two major problems in drug control are the manufacture of counterfeit drugs and the diversion of legitimate drugs into illegal underground channels of distribution. In this study the use of neutron activation analysis was investigated for identification of the source of a drug product by analysis of certain trace elements added to it during manufacture, or by analysis of natural trace elements or contaminants already present. The usefulness of added trace elements was found to depend on the levels of natural contaminants. A level too high of the latter would require the addition of larger amounts of additives than physiological considerations permit. Thus, each drug product should be considered individually, after first establishing its natural contaminant content. Samples of dextroamphetamine tablets and capsules from five manufacturers, along with common tablet and capsule ingredients, were analyzed for natural contaminants. In the group, 17 elements were detected in amounts of less than 1 part per billion to over 0.1 per cent. The contaminants present in the tablets and capsules from the five manufacturers indicated each product was readily distinguishable from the others.

THE INTRODUCTION of new classes of drugs, particularly barbiturates, tranquilizers, amphetamines, and more recently the hallucinogens, has created new and difficult problems of control for law enforcement agencies. The widespread abuse of these new products has been well documented in popular periodicals. The Food and Drug Administration (FDA) has estimated that half the barbiturates and amphetamines produced legally in the United States are diverted into illicit systems of distribution (1). This diversion is possible largely through pilferage and the smuggling of exported products back into the country (2). Legislation in the form of the Drug Abuse Control Amendments of 1965, enforced by the FDA, provides for safeguards in the distribution of depressant and stimulant drugs. A second aspect of the control problem is the small scale practice of drug counterfeiting, in which clandestinely manufactured imitations of brand name products are merchandised in normal channels (3).

Any aids in product source identification will obviously be of material assistance in the enforcement program. The present work explores various aspects of product source identification through the use of neutron activation analysis (NAA). One approach is to arbitrarily introduce minute quantities of trace elements, having no effect on drug quality, which will show up at any future time under NAA. Another approach is to investigate contaminants already present in the drugs in the hope of their proving distinctive with respect to origin.

According to Robinson (4), pharmaceutical manufacturers have long added small quantities of harmless substances to products for identification purposes. Such tracers, usually detected spectrographically, are considered by both manufacturer and the FDA to be trade secrets. The present study shows NAA to be a promising alternate tracer detection technique, with the additional prospect that natural contaminants of a product may also be used as a means of source identification. Others have reached similar conclusions. Pro (5) reports that he has suggested to the FDA a list of possible elements as markers.

NAA is a sensitive, powerful analytical tool, effective for about two-thirds of the elements. Activation is ordinarily carried out by placing the sample in a nuclear reactor, where the large quantities of thermal neutrons present create radioactive nuclides through an (n, γ) nuclear reaction. Analysis is achieved by measuring the radioactivity of the product nuclides after a radiochemical separation procedure, or by direct γ ray spectrometry, with a scintillation detector and multichannel analyzer. This work makes use of the latter. Basic principles and applications of NAA are amply described in the litera-

Received December 9, 1966, from the Bionucleonics Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907
Accepted for publication January 11, 1967.
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ture. Four recent reference books (6–9) list hundreds of applications in a variety of disciplines, but surprisingly few in the pharmaceutical sciences.

Using direct γ ray spectrometry without radiochemical separation, several investigators have presented evidence indicating the possibility that sources of phenobarbital and pethidine hydrochloride (10), moonshine whiskey (11), meprobamate and heroin (12), and raw opium (13) are readily distinguishable on the basis of the presence and relative concentration of trace elements. Schlesinger *et al.* (12) also report a private communication from Dr. V. P. Guinn saying that his group had used these techniques successfully with amphetamine tablets.

With these same techniques, Tuckerman *et al.* (14) have identified 13 trace elements in samples of 40 drugs and drug ingredients, in concentrations of 0.01 mcg./Gm. to 5%.

The arbitrary addition of tracers is applicable to a variety of drug products. The studies reported here, however, are concerned entirely with dextroamphetamine sulfate tablets and capsules, and diluents commonly employed in tablet and capsule manufacture.

EXPERIMENTAL

Systems of Additives—Minute quantities of trace elements could be added to drugs during manufacture either as a single element or in groups. The number of elements having the desired properties is quite limited and, if used singly, the list would be quickly exhausted. Consequently, group or multicomponent systems would be required if a large number of separate distinguishable tags was desired. It is believed that, if differentiation is to be based on quantitative measurement of the amounts of the additives present, such measurements could only have validity if expressed in relation to some internal standard included as an additive. Thus, it was decided to investigate a four-component system in which one of the components serves merely as a standard, against which the other three are varied and measured.

The amounts of additives to be used will depend on the quantities needed to override the radioactivity produced by natural contaminants in the drug product. It was essential, therefore, to determine the levels of natural contaminants. The second phase of this study was devoted to that end.

Selection of Additives—The basic equation for neutron activation is (15)

$$A_m = \frac{m\sigma f\phi N_0}{A}(1 - e^{-\lambda t_r}) \qquad \text{(Eq. 1)}$$

where A_m is the activity of the sample of mass m, σ the thermal neutron activation cross-section, f the fractional isotopic abundance, ϕ the thermal neutron flux, N_0 Avogadro's number, A the atomic weight, λ the radioactive decay constant, and t_r the time of irradiation. Criteria for the selection of an additive which follow from Eq. 1 are high natural isotopic abundance, high neutron activation cross section, and short half-life (but not shorter than about 30 min. to allow reasonable time for counting). Additional criteria used were a high γ ray yield per disintegration, a reasonably simple γ ray spectrum, freedom from energy overlap with other radionuclides of other additives, freedom from interfering activities of other isotopes of the nuclide selected as an additive, availability in the form of a fine, nonhygroscopic powder, availability as a chemical compound in which the other constituents do not appreciably activate under neutron bombardment, absence as a natural contaminant at least in comparable quantities, nontoxicity, and reasonable availability and cost.

Results of the survey of elements are shown in Table I. The sensitivity values were calculated using Eq. 1 plus application of the γ ray yield factor. The asterisk in the last column indicates whether the spectrum is complex, due either to the presence of a large number of γ ray peaks or to multiple activities from the various isotopes present in the element.

TABLE I—POSSIBLE IDENTIFYING MARKERS

Nuclide	Abundance, %[a]	Activation Cross-Section, Barns[a]	Half-Life[a]	γ Ray Yield/ Dis.[b]	Peak Energy, Mev.[c]	Sensitivity, mcg.[d]	Complex Spectrum[a, c]
^{55}Mn	100	13.3	2.57 hr.	1	0.84	5	
^{63}Cu	69.1	4.3	12.8 hr.	1.2	0.51	20	
^{75}As	100	5.4	26.4 hr.	0.40	0.56	134	
^{121}Sb	57.2	6.8	2.8 day	0.7	0.56	430	
^{127}I	100	5.6	25 min.	0.17	0.44	9	
^{138}Ba	71.7	0.5	84 min.	0.26	0.16	330	
^{139}La	99.9	8.2	40.2 hr.	1	1.60	108	
^{151}Eu	47.8	1400	9.2 hr.	0.025	0.97	12	*
^{152}Sm	26.6	140	47.1 hr.	1	0.10	30	
^{164}Dy	28.2	2100	2.32 hr.	[e]	0.098		
^{174}Yb	31.8	60	4.1 day	0.07	0.40	1900	*
^{175}Lu	97.4	35	3.7 hr.	[e]	0.088		*
^{179}Hf	13.8	65	5.5 hr.	0.80	0.44	19	*
^{187}Re	62.9	66	16.7 hr.	0.09	0.16	120	*
^{197}Au	100	96	2.7 day	0.98	0.41	20	

[a] From Koch (6). [b] From Strominger *et al.* (16). [c] From Heath (18). [d] Amount of element required for a 10-min. irradiation at a 10^{10} neutron flux to produce immediately 100 counts/sec at 100% counting efficiency. [e] Not known.

In selecting nuclides from Table I for a four-component system, several opposing factors must be considered. Manganese and copper have the desired properties, but are already known to be present to some extent in drugs (14). Arsenic might be thought unsuitable, even at submicrogram levels, because of its toxicity. Iodine has an undesirably short half-life for many experimental arrangements, and a photopeak overlapping that of gold, preventing their simultaneous use. The samarium peak overlaps that of dysprosium. Based on these considerations, the major portion of this work was done with the seven additives listed in Table II.

Sample Preparation—Samples for irradiation were prepared in the form of fine, dry powders sealed in plastic containers. Since quantities of an additive required for irradiation and counting were much less than 1 mg., a dilution technique was required to provide samples of known weight. Lactose powder was found to be sufficiently inert under activation to serve as the basic diluent, although, as Table VI shows, mannitol would have been preferred, had the fact been known at the time. Stock mixes of the seven additives in lactose were prepared in concentrations ranging from 0.01% for Dy_2O_3 to 2% for La_2O_3. Geometric dilutions were carried out with a spatula on a pill tile, with a mixing period of 10 min. after each successive blending of equal portions. After blending on the pill tile was complete, the mixture was transferred to a 22-ml. glass vial, where it was further agitated for 30 min.

The first stock mixes prepared exhibited a variation in specific activity under neutron activation, as shown in Table III by Au stock mixes A and B, and by Cu stock mix A. Although first thought to be due to inadequate mixing, this variability turned out to be related to the fineness of the additive powders. In the case of Au, mixes A and B were prepared from purchased powder, passed through a 100-mesh screen, with mix B being more thoroughly mixed than A. Mix C was passed through a 100-mesh screen. The CuO powder was used as purchased in mix A, and further subdivided with a mortar and pestle before preparing mix B. The La_2O_3 powder as purchased was noticeably finer than the other oxides, as Table III indicates.

Both Lucite and polyethylene vials were used for the irradiations. Because both showed slight Mn and Na contamination, samples were transferred to other vials before counting. To facilitate transfer, the sample was usually encased in a plastic film.[1] This was done by centering the weighed powder on a 3-in. disk of the film, gathering up the edges and twisting, and then holding the twisted portion about 2 mm. from a heated soldering pencil until fused.

Activation and Analysis—For the investigation of the scheme of additives, the Purdue University reactor (with an available flux of about 2×10^{10} thermal neutrons/cm.^{2}/sec.) was used. Studies on the natural contaminants were carried out, using a flux of about 5×10^{12}, in the CP-5 reactor at Argonne National Laboratory. Neutron flux values were determined by use of gold foil or gold powder flux monitors, along with calibration of the counting assembly using a calibrated ^{137}Cs source. The duration of all irradiations except the last, which was 105.6 hr., was 10 or 30 min. Counting was begun as soon as possible after removal from the reactor (about 1 hr. for the Purdue reactor and 3 hr. for the CP-5). Counting was delayed 1 week after the long irradiation, to permit decay of the ^{24}Na.

The counting system consisted of a NaI(Tl) crystal mounted in a commercial steel shield[2] having 6-in. walls and inside dimensions of 20 × 20 × 36 in. The detector output was fed into a 400-channel analyzer.[3] Readout was by a high speed printer,[4] writing at 5 channels/sec. Two different detectors were used. All measurements on the additives were made with a 2-in. well crystal. All spectra of natural contaminants were collected with a 4 × 4-in. crystal.

Spectra of single photopeaks of the additives were analyzed by summing the counts in the seven central channels and dividing by the count accumulation time, the exponential decay factor, and the additive weight. The result was the specific activity at the end of irradiation. In a mixture of radionuclides, this calculation was carried out only on the outermost peak. The peak was then removed by instrumental stripping, with previously prepared standards (8, 14), before analysis of the next lower energy peak. Thus, for each component of the mixture, the specific activity, corrected for decay, was determined. Studies of 14 mixtures resulted in the data contained in Table II.

Gold was selected as the internal standard against which the other three components were varied and

TABLE II—EXPERIMENTAL DETERMINATION OF MARKER SENSITIVITIES[a]

Additive	Radionuclide Marker	Specific Activity, Relative to Au[b]
MnO_2	^{56}Mn	1.38
CuO	^{64}Cu	0.437
La_2O_3	^{140}La	0.14[c]
Eu_2O_3	^{152m}Eu	4.01
Sm_2O_3	^{153}Sm	1.22
Dy_2O_3	^{165}Dy	69
Au	^{198}Au	1

[a] A 2-in. diameter × 2-in. thick well detector was used. [b] The specific activity of Au was 4.12×10^5 counts/min./mg. of Au, for a 30-min. irradiation at a flux of approximately 2.1×10^{10} neutrons/cm.^{2}/sec. Counting rate was based on a seven-channel peak, corrected for decay, at 5 kev/channel. [c] A 15-channel peak was used.

TABLE III—UNIFORMITY OF STOCK MIXES

Stock Mix (in Lactose)		Replicates Analyzed, No.	Specific Activity— Coeff. of Variation, %	Activity— Range, % of Mean
0.25% Au	A	6	9.7	43
	B	6	9.3	28
	C	7	2.2	5.3
1.0% CuO	A	8	7.3	21
	B	8	1.7	6.1
2.0% La_2O_3		8	1.4	4.8

[1] Handi-Wrap, Dow Chemical Co., Midland, Mich.

[2] Packard Instrument Co., Downers Grove, Ill.
[3] Radiation Instrument Development Laboratory, Melrose Park, Ill.; model 34-12B.
[4] Hewlett-Packard Co., Palo Alto, Calif.; model 562 A digital recorder.

measured. Reasons for the choice were its high sensitivity, single sharp photopeak, intermediate photopeak energy value, and longer half-life. For ^{165}Dy the X-ray peak was used since it predominated, but because of its low energy an analyzer resolution of 5 kev/channel was required.

Mix Combinations—In order to prove the efficacy of a four-component system, mix combinations of additives were prepared and further diluted with lactose, to simulate a batch of drug product. Samples were then taken for irradiation and measurement. It was considered desirable to determine the amount of change in any amount or level necessary for it to be positively identifiable as "different." Or, stated in another way, it was desirable to determine the number of distinguishable tags possible through varying the amounts of additives over an order of magnitude relative to Au. Results of measurements on the first three mix combinations are shown in Fig. 1. The solid lines represent the expected activities relative to Au, based on data similar to those in Table II. The data points show results obtained from actual measurement. The levels shown in Fig. 1 were selected to approximate an interval of $\sqrt{2}$ between levels. Each dot corresponds to a sample of the mix combination.

Difficulty in maintaining the intended level was encountered only with ^{56}Mn. This was found to be the result of a particle size too large in the MnO_2 powder, which was composed of hard granules as purchased and had to be subdivided before dilution.

Determination of Natural Contaminants—Eight samples of dextroamphetamine sulfate tablets and capsules from five manufacturers, along with 10 ingredients commonly used in amphetamine and other drug products, were irradiated in the Argonne CP-5 reactor. Each material was analyzed following two 30-min. and one 105.6-hr. irradiations. Samples approximately 100 mg. in size were sealed by heat in $^2/_5$ dr. polyethylene vials for the short irradiations, and quartz ampuls for the long one. Samples were weighed after counting.

Identification of contaminants was based on determination of the peak energy and half-life, with both being required. γ Ray spectrum catalogs of Crouthamel (17), and particularly Heath (18), were helpful here. Data reduction was carried out as for the additives, though subtraction of the adjacent continuum was usually necessary to determine the seven-channel-peak counting rate.

Standards were prepared and irradiated along with the unknowns, when their need became apparent from the previous irradiation. Amounts of contaminants present were determined by the comparator method described by Lyon (8). The radionuclides ^{46}Sc, ^{51}Cr, ^{59}Fe, ^{60}Co, ^{65}Zn, ^{122}Sb, ^{124}Sb, and ^{233}Pa have longer half-lives and were detected only after the long irradiation. Amounts of these elements present were calculated by an absolute method using Eq. 1, and published data on cross sections (6) and decay schemes (16). To relate photopeak counting rates to sample disintegration rates, it was necessary to determine the counting efficiency of the detector system. This was done in the following manner.

A calibrated ^{137}Cs source with a γ ray emission of 4.95×10^5 γ rays/min. was prepared. A count of this source immediately gave the counting efficiency for the energy 0.66 Mev. The values for other energies were calculated from absolute detection efficiencies and peak-to-total ratios published by Heath (18). Values of counting efficiency as a function of energy for three different detectors are plotted in Fig. 2. Data points on the 2-in. well crystal were established by direct experimental comparison to the other two crystals. The validity of the absolute method is supported by the cross checks shown in the data in Table IV.

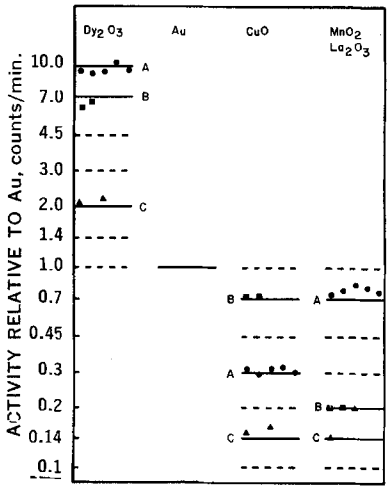

Fig. 1—Expected and measured levels of markers in three mix combinations, A, B, *and* C. *Key:* ●, A (*with* MnO_2); ■, B (*with* La_2O_3); ▲, C (*with* MnO_2).

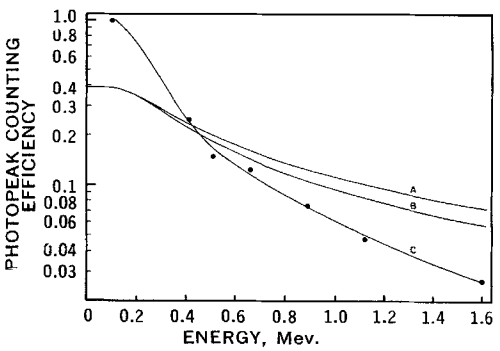

Fig. 2—Photopeak counting efficiencies for three different crystals. Source at minimum distance. Key: A, *4-in. crystal;* B, *3-in. crystal;* C, *2-in. well crystal.*

TABLE IV—MEASURED CONTAMINANT CONCENTRATIONS BY COMPARATOR AND ABSOLUTE METHODS

	Concn., mcg./Gm. of La in $CaSO_4 \cdot 2H_2O$	Au in Mannitol
Comparator method	0.17	
Comparator method involving two different irradiations	0.20	0.0014
Absolute method	0.16	0.0014

TABLE V—CONTAMINANTS IN COMMERCIAL DEXTROAMPHETAMINE TABLETS AND CAPSULES[a]

Element	Radionuclide Measured	Tablet Mfr. No. 1	Tablet Mfr. No. 2	Tablet Mfr. No. 3	Tablet Mfr. No. 4 Lot A[b]	Tablet Mfr. No. 4 Lot B	Tablet Mfr. No. 4 Lot C	Capsule Mfr. No. 4	Capsule Mfr. No. 5
Na	^{24}Na	400	200	300	200	200	200	60	60
Sc	^{46}Sc		0.008	0.02	0.02	0.02	0.01		
Ca	^{47}Ca		7%	4%	16%	16%	16%	13%	
Mn	^{56}Mn	0.9	10	0.7	2	1	1	0.06	0.1
Cu	^{64}Cu							1	2
Zn	^{65}Zn	30						2	2
Br	^{82}Br	2							0.5
Sr[c]	^{87m}Sr		600	300	800	800	800		
Sb[d]	^{122}Sb							0.03	0.07
La	^{140}La	0.01	0.02	0.02	0.04	0.05	0.03		
Au	^{198}Au							0.0005	

[a] Entries are in mcg./Gm. or per cent where designated. [b] Coded designation of lot numbers. Lot numbers were greatly different numerically, indicating lots manufactured at greatly differing times. [c] In every instance 64-day ^{85}Sr was also observed. [d] In every instance 60-day ^{124}Sb was also observed.

Application of Eq. 1 to contamination concentration determinations required measurement of the neutron flux for the long irradiation. This was done by six different determinations using La, Au, and Mn standards in two different irradiations, yielding as the result $(5.5 \pm 0.8) \times 10^{12}$ neutrons/cm.^{2}/sec.

Contaminants in Dextroamphetamine Tablets and Capsules—Contaminants observed in the dextroamphetamine sulfate tablets and capsules are shown in Table V. Polaroid photographs of samples of the spectra providing the data for Table V are shown in Fig. 3. Spectra of tablets from four different manufacturing sources, taken after a 30-min. irradiation and a 6-hr. waiting period, show the presence of only ^{24}Na, ^{56}Mn, and ^{87m}Sr, but in widely varying amounts. The longer lived nuclides are observable only with longer data collection times after the shorter ones have decayed. This effect is illustrated further with the capsules in Fig. 4, where *A* shows only the presence of ^{24}Na, ^{56}Mn, and ^{64}Cu. In *B*, the peak at 0.55 Mev. shows the presence of ^{82}Br in one manufacturer's product, but not in the other. In *C*, both sources show ^{65}Zn and ^{124}Sb, but ^{47}Ca is apparent in only the samples from manufacturer No. 4.

Contaminants in Drug Ingredients—Table VI shows contaminants present in a selection of nine commonly used tablet ingredients, and also in a commercial sample of pure dextroamphetamine sulfate crystals. Sodium was the one element found in all cases, but was present in a wide range of concentrations. Because the dextroamphetamine sulfate crystals were supplied by manufacturer No. 4, one might expect the same trace elements to appear in the tablets and capsules of manufacturer No. 4. That several do not is attributed to the fact that, because of sample sizes, the detection sensitivity for the pure dextroamphetamine sulfate sample is enhanced by a factor of about 50 over that of the tablets and capsules.

RESULTS AND CONCLUSIONS

Requirements for Additives—The experimental work has demonstrated that a four-component system of additives to provide product identification will work insofar as activation and counting techniques are concerned. In applying any such system to a particular drug product, the factor determining the amount of additives required will obviously be the principal activities produced by natural contaminants. For dextroamphetamine tablets, these activities would be due to Sr, Mn, and Na. Using a seven-channel-peak from a 4-in. detector at 10 kev/channel, the sensitivity ratios between ^{198}Au and ^{24}Na, ^{56}Mn, and ^{87m}Sr were 19, 0.16, and 29, respectively. If, in a four-component system, one assumes that Au used as an internal standard would need to produce a peak at least as large as that of any natural contaminants, when measured after a delay of the order of 3 hr., the Au concentrations required to match those of Na, Mn, and Sr for the tablets of Table V would be 13, 7, and 11 mcg./Gm., respectively. Such concentrations are unlikely to be granted official approval. A gain by a factor of 5–10 would be possible with an alternate approach using Sm_2O_3, Eu_2O_3, and CuO, together with Au, comprising the mixture, then waiting about 10 hr. until most of the ^{56}Mn and ^{87m}Sr had decayed, and removing the ^{24}Na by instrumental stripping. It is evident that

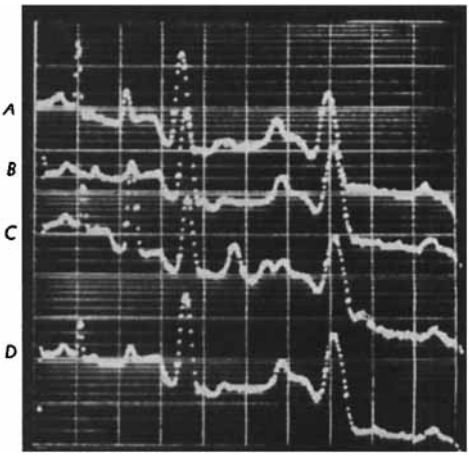

Fig. 3—Spectra of dextroamphetamine sulfate tablets from four manufacturers. Thirty-minute irradiation followed by 6-hr. decay; 0–4 Mev.; vertically displaced for clarity; scale of vertical axis is logarithmic. Key: A, manufacturer No. 4; B, manufacturer No. 1; C, manufacturer No. 2; D, manufacturer No. 3.

each drug product must be considered individually in designing a system of identifying additives, after first establishing the levels of natural contaminants.

Eu_2O_3 is suitable as an additive component, but only if irradiation time is in the range of 1 hr. or less, and assay made within about 10 hr. Though the spectrum is very complex, only the 9.3-hr. activity of ^{152m}Eu appears at first.

The number of separate identifiable tags possible under a four-component system also depends on how much the measurements are affected by natural contaminant activity. In a situation where one could use an arrangement such as shown in Fig. 1, 8^3 combinations are possible, if one includes the zero level. Not enough combinations were tried to be certain that all 512 combinations would be dis-

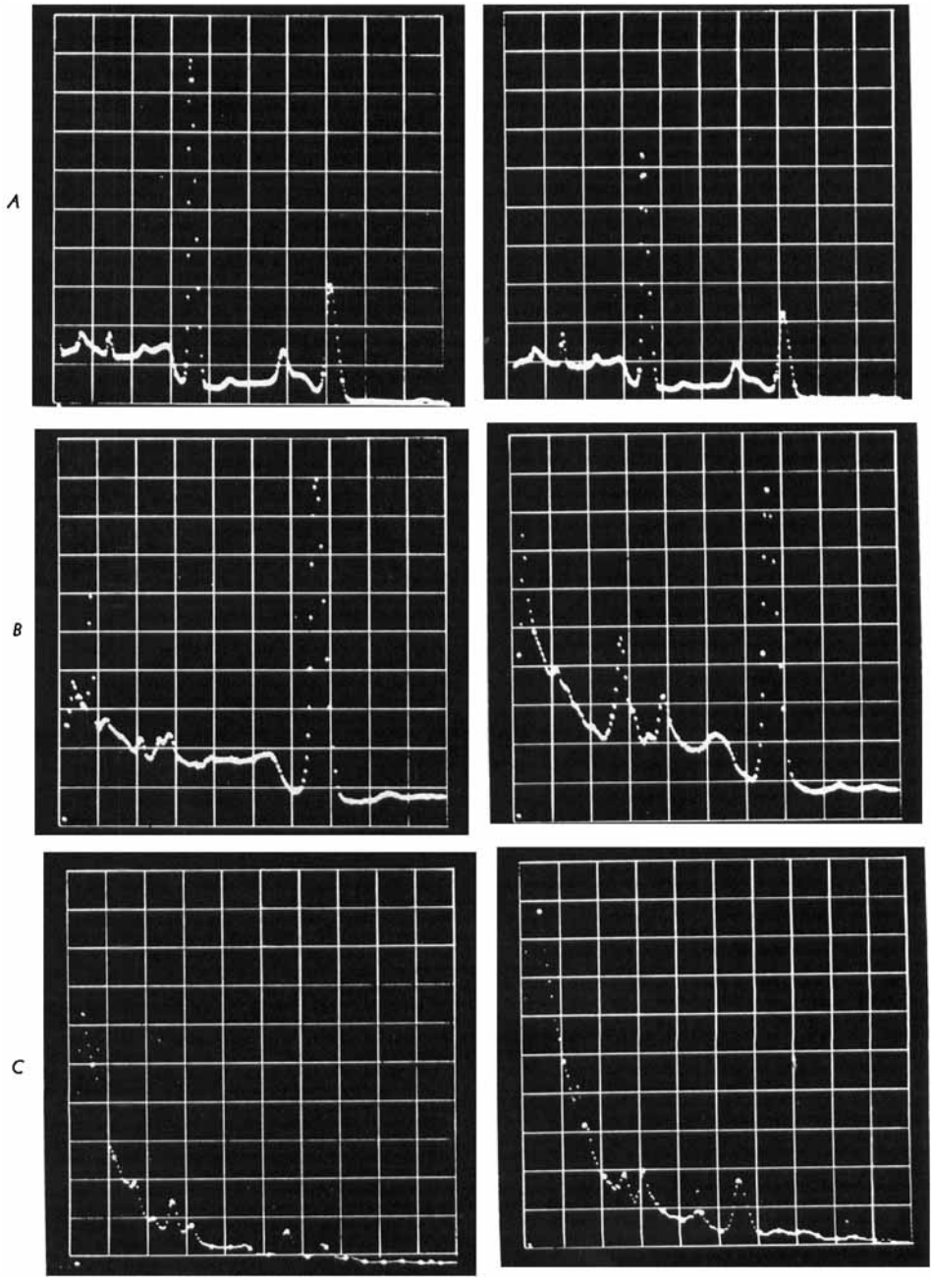

Fig. 4—Spectra of dextroamphetamine sulfate capsules from two manufacturers. Decay times: (manufacturer No. 4) A—7.3 hr., B—6.0 days, C—16 days; (manufacturer No. 5) A—7.7 hr., B—6.1 days, C—30 days. Scale of vertical axis is linear. Key: A, 0–4 Mev.; B, 0–2 Mev.; C, 0–2 Mev. Lefthand column, manufacturer No. 4; righthand column, manufacturer No. 5.

TABLE VI—CONTAMINANTS IN CERTAIN DRUG INGREDIENTS[a]

Element	Radionuclide Measured	Dextroamphetamine Sulfate	Mg Stearate	Wood Cellulose[b]	Microcrystalline Cellulose[c]	Mannitol	Calcium Sulfate Dihydrate	Dicalcium Phosphate Dihydrate	Lactose	Starch	Talc
Na	^{24}Na	10	700	600	6000	2	1	1000	40	200	400
Sc	^{46}Sc		0.07		0.001	0.001					0.7
Cr	^{51}Cr	0.2		2	0.07	0.4					
Mn	^{56}Mn	0.3		10		0.007	0.3	8		0.4	7
Fe	^{59}Fe	50	90	300		6					1000
Co	^{60}Co	0.1									
Cu	^{64}Cu	10		10							
Zn	^{65}Zn								0.6	20	
Br	^{82}Br									0.05	
Sr	^{87m}Sr						50				
Sb[d]	^{122}Sb	0.02	0.08	0.06	0.02	0.007			0.008		
La	^{140}La					0.006	0.2				0.4
Eu	^{152m}Eu						0.005				
Au	^{198}Au	0.0004			0.0004	0.001					
Hg	^{203}Hg		0.05							0.2	
Th	^{233}Pa										0.2

[a] Entries are in mcg./Gm. [b] Marketed as Solka-Floc by the Brown Co., Berlin, N. H. [c] Marketed as Avicel by the American Viscose Division, FMC Corp., Marcus Hook, Pa. [d] In every instance 60-day ^{124}Sb was also observed.

tinguishable; but it is evident that, with care, most could be used.

Single component additives have a distinct advantage. Quantitative differences would not be involved; only detection would be required for identification purposes, and far smaller amounts could be used. Table VI shows natural levels of Au of less than 1 part per billion were easily identified. Similar values would apply to other additives of comparable sensitivity.

Contaminants Detected—These results are generally consistent with those of Tuckerman *et al.* (14). In analyzing a somewhat different group of materials, the following additional trace elements were found: Cr, Fe, Zn, Sr, La, Eu, and Au. In the two compounds common to both studies, namely magnesium stearate and talc, these samples contained the same trace elements as those of Tuckerman, and in the same general levels; but other contaminants were also observed, as seen in Table VI.

Contaminants for Source Identification—Examination of Table V shows the six dextroamphetamine sulfate products from the five different manufacturers to be clearly and readily distinguishable from each other, on the basis of contaminants present and their relative concentrations. On the other hand, the three different lots of manufacturer No. 4, though apparently widely spaced in time, are so similar as to be indistinguishable. These results, then, provide further supporting evidence that natural contaminants may offer a means of determining the specific manufacturing source of amphetamine drug samples.

REFERENCES

(1) Davidson, B., *The Saturday Evening Post*, **23**(December 4, 1965).

(2) "Drug Abuse, a Manual for Law Enforcement Officers," Smith Kline & French Laboratories, Philadelphia, Pa., 1965.

(3) *Chem. Eng. News*, **38**, 27(October 24, 1960).

(4) Robinson, R. J., Bureau of Medicine, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C., personal communication.

(5) Pro, M. J., Research and Methods Evaluation, Internal Revenue Service, U. S. Treasury Department, Washington, D. C., private communication.

(6) Koch, R. C., "Activation Analysis Handbook," Academic Press Inc., New York, N. Y., 1960.

(7) Bowen, H. J., and Gibbons, D., "Radioactivation Analysis," Oxford University Press, New York, N. Y., 1963.

(8) Lyon, W. S., "Guide to Activation Analysis," D. Van Nostrand Co., New York, N. Y., 1964.

(9) Taylor, D., "Neutron Irradiation and Activation Analysis," D. Van Nostrand Co., New York, N. Y., 1964.

(10) Bate, L. C., and Pro, M. J., *Intern. J. Appl. Radiation Isotopes*, **15**, 111(1964).

(11) Pro, M. J., Schlesinger, H., and Cohan, M., *J. Assoc. Offic. Agr. Chemists*, **48**, 459(1965).

(12) Schlesinger, H. L., Pro, M. J., Hoffman, C. M., and Cohan, M. J., *ibid.*, **48**, 1139(1965).

(13) Leddicotte, G. W., Emery, J. F., and Bate, L. C., "The Assay, Characteristics, Composition and Origin of Opium I," Oak Ridge National Laboratory, Oak Ridge, Tenn., TM-1263, 1965.

(14) Tuckerman, M. M., Bate, L. C., and Leddicotte, G. W., *J. Pharm. Sci.*, **53**, 983(1964).

(15) Koch, R. C., "Activation Analysis Handbook," Academic Press Inc., New York, N. Y., 1960, p. 6.

(16) Strominger, D., Hollander, J. M., and Seaborg, G. T., *Rev. Mod. Phys.*, **30**, 585(1958).

(17) Crouthamel, C. E., "Applied Gamma Ray Spectrometry," Pergamon Press, New York, N. Y., 1960.

(18) Heath, R. L., "Scintillation Spectrometry Gamma Ray Spectrum Catalogue," vols. 1 and 2, 2nd ed., Atomic Energy Commission, IDO-16880, 1964.

Determination of Vitamin D in the Presence of Vitamin A by Cylindrical Thin-Layer Chromatography

By KENJIRO HIRAYAMA and KENZO INOUE

Vitamin A alcohol in vitamin D_2 preparations is converted to retinene by passage through a column of manganese dioxide, inactivated with acetone containing water, and eluted with the mixed solvent of acetone and petroleum ether (1:4). This eluate is used for cylindrical thin-layer chromatography (CTLC). The sample is applied to the thin layer under a stream of nitrogen gas, and the chromatogram is developed with chloroform. With this proposed procedure, no decomposition of vitamin D_2 took place and the recovery of vitamin D_2 from a vitamin A–D_2 preparation was about 97–102 per cent compared to untreated vitamin D_2.

THE FOLLOWING three points seem to be unresolved in the determination of vitamin D in pharmaceutical preparations using thin-layer chromatography (TLC).

(*a*) With commonly used adsorbents and developing solvents in TLC, vitamin A interferes with the isolation of vitamin D, due to the similarity of the R_f values of both vitamins. It is necessary, therefore, to convert vitamin A to substances that have R_f values different from that of vitamin D_2. In their earlier studies Barua and Rao (1) converted vitamin A to anhydrovitamin A with 0.033 *N* ethanolic hydrogen chloride. Later the same authors (2) reported its conversion to retinene on a column of precipitated manganese dioxide.

(*b*) Partial decomposition of vitamin D_2 occurs within a few minutes on a dry silica gel layer, but it is stable as long as the solvent is present. Therefore, if the ordinary thin-layer plate is used, it should be absolutely essential in the quantitative assay to place the plate in the developing chamber immediately after applying the sample solution—before the solvent (*e.g.*, petroleum ether) evaporates (3). However, this seems to be impossible in practice, since the solvent will evaporate during successive application on a single plate.

(*c*) To minimize errors and avoid concentration of sample extracts, it is very desirable that a large amount of sample solution be applied to the thin layer.

In the present paper, the conversion of vitamin A to retinene by a column of manganese dioxide according to Barua's method was studied in detail. Satisfactory results were obtained with the use of a new method of inactivating manganese dioxide so that the conversion of vitamin A to retinene was complete without alteration or adsorption of vitamin D_2.

Received October 11, 1966, from the Shonan Plant, Takeda Chemical Industries, Ltd., Fujisawa City, Japan.

Accepted for publication December 8, 1966.

The authors thank Mr. M. Iwanaga, for his advice and Mr. K. Hiruma for his cooperation in this work.

To apply a large amount (more than 1 ml.) of the sample solution and to prevent any decomposition of vitamin D_2 while applying the sample solution to the thin layer, a new apparatus was used for quantitative TLC. It was found that by using this apparatus and introducing nitrogen gas while applying the sample solution, no decomposition of vitamin D_2 took place.

After developing with chloroform, the vitamin D_2 zone is located between two visible yellow bands of vitamin A oxidation products. The use of an ultraviolet light is unnecessary.

The adsorbent between these two yellowish bands is scraped off while still damp with solvent (chloroform), and immediately eluted with ethyl ether. After evaporation of the ether, the residue is dissolved in ethylene dichloride for color development with antimony trichloride reagent (U.S.P. XVII).

EXPERIMENTAL

Apparatus—The cylindrical thin-layer chromatographic apparatus was previously described (4).

Reagents—Manganese dioxide, JIS 1st grade or extra pure; silica gel, Wakogel B-5 for TLC or Kieselgel G for TLC; calciferol, U.S.P. grade; antimony trichloride reagent, U.S.P. XVII vitamin D assay color reagent.

Other reagents (petroleum ether, acetone, chloroform, methanol, ethyl ether, ethylene dichloride, anhydrous sodium sulfate) were JIS special grade or guaranteed reagent.

Method—*Extraction*—To a convenient weight of the sample,[1] at least 0.1 Gm., equivalent to not less than 0.5 mg. (20,000 I.U.) of vitamin D_2, 20–30 ml. of water is added, and the fat-soluble materials (vitamin D_2 and A also included) are extracted with three 30-ml. portions of petroleum ether. If there is emulsifying agent in the sample, addition of acetone may be helpful for separation of the two phases.

The combined extracts are washed with water and dried over anhydrous sodium sulfate. The dry extract is evaporated under reduced pressure to

[1] This sample should contain enough vitamin A to provide the two yellowish pilot bands; add about 1000 I.U. of vitamin A alcohol, if necessary.

approximately 20 ml., transferred to a 25-ml. volumetric flask, and brought to volume with acetone.

Oxidation—*Preparation of the Column*—A column (1 cm. inside diameter) 3 cm. in height with manganese dioxide is prepared by mixing two different mesh sizes (equal parts by weight below 105 μ and 105–210 μ). The column is washed with 2 ml. of acetone containing 3% water, and then with two 1-ml. portions of acetone–petroleum ether (1:4).

Oxidation—Ten milliliters of the above dried extracted solution is transferred to the column and allowed to pass through dropwise. When all the solution has passed through, the column is washed with five 3-ml. portions of acetone–petroleum ether (1:4). During this procedure it is necessary to keep the solvent on the column. Collect the eluates in the same receiver and bring to 25 ml. with petroleum ether. This is the sample solution.

For samples of low vitamin D content (50–250 mcg.), the entire dried extract is evaporated almost to dryness and the residue transferred with acetone–petroleum ether (1:4) to the oxidation column. The eluate is evaporated under reduced pressure until free of solvent, the last few milliliters being removed in a current of nitrogen. The residue is redissolved accurately for cylindrical thin-layer chromatography.

Cylindrical Thin-Layer Chromatography (CTLC)—A volume (V_p ml.) of sample solution of at least 1.00 ml., equivalent to not less than 50 mcg., and preferably more than 2.00 ml., equivalent to a maximum of 100 mcg. of vitamin D_2, is pipeted into the thin-layer cylinder and allowed to exude to the outer thin layer. (Fig. 1.)

During the course of this procedure, nitrogen gas is introduced from the inlet (Fig. 2, a). In this experiment, "partial volume method" (see Fig. 2) is adopted for applying the sample solution.

Extraction from the Adsorbent—Immediately after development with chloroform, the colorless portion of the thin layer between the two yellowish ring bands (oxidation products of vitamin A alcohol), is scraped off with a flat spatula while rotating the thin-layer cylinder into a small flask containing a small amount of ethyl ether.[2]

The suspensions are vigorously shaken and rapidly filtered through a dry filter or centrifuged. This procedure is repeated using four or five 5-ml. portions of ethyl ether. Each filtrate or supernatant is collected in a 25-ml. volumetric flask and brought to volume with ether.

Determination of Vitamin D_2—An aliquot of the ether extract is pipeted into the test tube and the solvent perfectly evaporated to dryness *in vacuo* and with the aid of stream of nitrogen gas. The residue is redissolved in 3.0 ml. of ethylene dichloride, and the color is developed by mixing 3 ml. of antimony trichloride reagent. The absorbance is measured (A_{T500}) at 500 mμ 90 sec. after adding the reagent, and A_{T550} at 550 mμ after 120 sec.

RESULTS AND DISCUSSION

Stability of Vitamin D_2 Coloration with $SbCl_3$—In general, the absorbance at 500 and 550 mμ is measured 45 and 90 sec., respectively, after adding the reagent. But in this experiment the variation of absorbance with the time lapse from 45 to 120 sec. was very small (Fig. 3). Therefore, the A_{500} and A_{550} were measured after 90 and 120 sec., respectively, using the Zero method spectrophotometer.

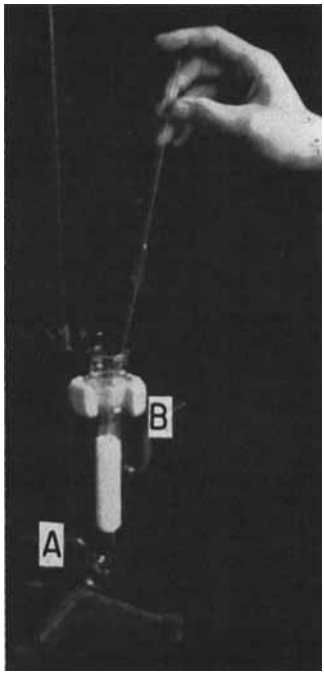

Fig. 1—Application of the sample solution to thin-layer cylinder.

Oxidation by Manganese Dioxide—The oxidation of vitamin A alcohol to retinene by manganese dioxide has been reported by Ball *et al.* (5) and Henbest *et al.* (6). These reports do not refer to the mesh size of the MnO_2. In the report of Barua *et al.*, precipitated MnO_2 was used, but the preparation and activation method was not described in detail.

In this experiment, investigation of mesh size and the inactivation method of MnO_2 are described.

Recovery Experiments with Pure Vitamin D_2 from the MnO_2 Column—A solution of vitamin D_2 in petroleum ether (equivalent to about 0.1 mg.) was pipeted into the prepared columns which contain various mesh sizes and heights of MnO_2 and was eluted with 20 ml. of each eluting solvent. In this procedure it is essential that the column never run dry; the moment the surface of the eluting solvent has dropped to the top of the column, fresh eluting solvent must be added. The eluate was collected in a 25-ml. volumetric flask and brought to volume with eluting solvent.

An aliquot of this solution equivalent to about 15 mcg. of vitamin D_2 was pipeted into the test tube and the vitamin D_2 determined as described above to obtain A_{T500} and A_{T550}. The A_{S500} and A_{S550} are determined for the D_2 solution (in petroleum ether) without passing through the MnO_2 column.

The recovery of vitamin D_2 from the MnO_2 was calculated from the following equation:

$$\text{recovery } (\%) = \frac{A_{T500} - A_{T550}}{A_{S500} - A_{S550}} \times 100$$

The results are shown in Table I.

[2] The most suitable solvents for extraction according to the literature are petroleum ether, ethyl ether, and chloroform. In this experiment ethyl ether was used.

The recovery experiments show (Table I) that vitamin D_2 was adsorbed on the column and cannot be eluted with *n*-pentane or petroleum ether as suggested in the literature (2).

Using the same procedure (1.5-cm. column of small mesh MnO_2), the inactivation of MnO_2 with water and eluting solvent was studied. The results are shown in Table II.

The recovery experiment shows (Table II) that vitamin D_2 is adsorbed firmly on the column of MnO_2 small mesh size, that the inactivation process with acetone containing water is necessary, and that the mixed solvent of acetone–petroleum ether (1:4) is superior to petroleum ether alone.

In the case of MnO_2 large mesh size, using the same procedure, the results are shown in Tables III and IV. The data in Table III are from untreated MnO_2, and the data in Table IV are those from inactivated MnO_2.

From the comparison of data in Tables III and IV, it is necessary to inactivate MnO_2. Inactivation method *A* is no different from *B* (*cf.* $\bar{A} = 99.13$; $\bar{B} = 99.27\%$). Washing method *A* is little different from *B* (*cf.* $\bar{A} = 99.47$; $\bar{B} = 98.93\%$). The results show that a little petroleum ether, used for washing the remaining acetone in the column, is superior to the excess; therefore, acetone–petroleum ether (1:4) should be used as the washing solvent.

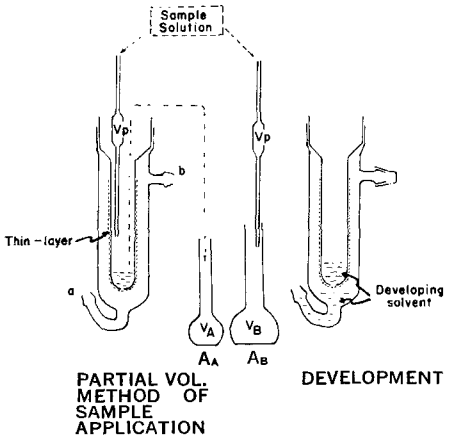

Fig. 2—Cylindrical thin-layer chromatography. The outside of inner cylinder (like a test tube), the bottom of which has a small hole (about 1 mm. in diameter) used for exuding the sample solution and developing solvent from the inside to outside, is covered with a thin layer of adsorbent. The sample solution is pipeted into the thin-layer cylinder and it exudes to the outer thin layer automatically. Thus, the sample makes the ring band around the bottom. The size of the ring band is regulated with the stream of introducing gas. In this case, if the gas flows very rapidly, the hole becomes plugged but if the gas flow is suitably regulated, the sample does not plug. The optional amount of sample solution (in this case the whole of solution exuding is total volume method) has exuded to the outer thin layer, then the remaining solution is removed, quantitatively diluted to a volume, and a color developed (or directly measured at suitable wavelength) for both solutions (original and diluted). The volume of the sample solution exuded to the outer thin layer is then calculated by difference.

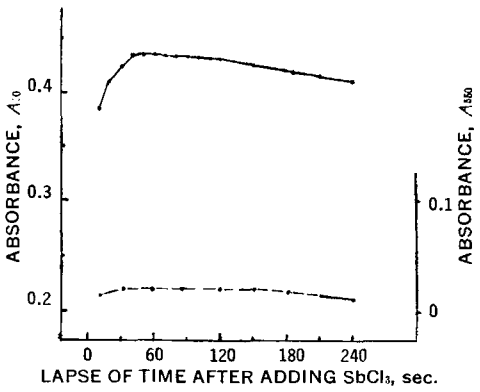

Fig. 3—Stabilities of vitamin D_2 color developed with $SbCl_3$. Key: top curve, A_{500}; bottom curve, A_{550}.

TABLE I—RECOVERY OF VITAMIN D_2 FROM MnO_2 COLUMN

MnO_2 Mfgr.	MnO_2 Mesh Size, μ	MnO_2 Ht., cm.	Eluting Solvent	Recovery, %
A	Below 105	1.5	Pet. ether	13.1
A	Below 105	1.5	*n*-Pentane	8.8
B	Below 105; 105–210 (1:1)	1.5	Pet. ether	14.8
B	Below 105; 105–210 (1:1)	1.5	*n*-Pentane	16.5
B	Below 105; 105–210 (1:1)	3.0	Pet. ether	0

TABLE II—RECOVERY OF VITAMIN D_2 FROM INACTIVATED MnO_2 (Below 105 μ) COLUMN (1.5 cm. HEIGHT) USING VARIOUS ELUTING SOLVENTS

Inactivation of MnO_2	Eluting Solvent	Recovery, %
1% water[a]	Pet. ether	0
5% water[a]	Pet. ether	30.3
No water	EtOH–pet. ether (1:9)	10.2
No water	EtOH–pet. ether (1:4)	10.7
No water	Acetone–pet. ether (1:9)	15.2
No water	Acetone–pet. ether (1:4)	44.1
No water	Acetone–pet. ether (3:7)	18.5
Acetone, 1 ml.	Pet. ether	17.5
Acetone, 1% water[b], 1 ml.	Acetone–pet. ether (1:4)	56.5
Acetone, 3% water, 1 ml.	Acetone–pet. ether (1:4)	59.5
Acetone, 5% water, 1 ml.	Acetone–pet. ether (1:4)	74.9
...	Ethyl ether	2.5

[a] Inactivation method with water: to 10 Gm. of MnO_2 in 30 ml. of petroleum ether, add 0.1 ml. or 0.5 ml. of water and vigorously shake for about 30 min. [b] Inactivation method with acetone containing 1–5% water: pour 1 ml. of acetone containing 1, 3, or 5% water into the MnO_2 columns soaked in petroleum ether.

In Tables I–IV, vitamin D_2 in petroleum ether is used. Results for vitamin D_2 in acetone–petroleum ether (1:4) using the column of below 105 μ: 105–210 μ (1:1) MnO_2, inactivation method *A*, and washing with two 1-ml. portions of acetone–petroleum ether (1:4), are shown in Table V. The only difference here is that the vitamin D was placed on the column in the acetone–petroleum ether (1:4) solution. The results are no different from those in Table IV.

Stability of Vitamin D_2 in Eluting Solvent—To vitamin D_2 in petroleum ether was added the eluting solvent which had passed through the column of MnO_2. This mixed solution was allowed to stand for various periods of time and the color developed by using the same procedure described above. The results are shown in Table VI.

After allowing to stand for 3 hr., an aliquot of each of the vitamin D_2 solutions *1–6* was evaporated to about 0.3 ml. and analyzed by TLC. Spots other than vitamin D_2 were not detected on the plate. The results show that the vitamin D_2 was not altered in these eluting solvents.

Oxidation of Vitamin A Alcohol by MnO_2—Vitamin A alcohol (about 5000–10,000 I.U.) in petroleum ether was poured into the column of MnO_2 with or without inactivation, and the eluate was evaporated to about 1 ml. under nitrogen gas. This concentrated solution was used for micro TLC[3] to determine the completeness of vitamin A alcohol oxidation. The results are shown in Table VII.

The results show that under the conditions for good recovery of vitamin D_2, almost all vitamin A alcohol is converted to retinene so that little vitamin A alcohol is detected on the thin layer.

The chromatogram of vitamin A and D_2 passing through the column of MnO_2 inactivated with 2 ml. of acetone containing 3% water is shown in Fig. 4. (Table VIII.)

Determination of Vitamin D_2 in the Presence of Vitamin A by CTLC—*CTLC of Vitamin D_2 Alone*—The vitamin D_2 in petroleum ether solution (about 50 mcg./ml.) (V_p ml.) was pipeted into the thin-layer cylinder and the vitamin D_2 was isolated according to the described procedure. The absorbance A_c was obtained (when dilution was V_c).

For an evaluation of the volume (V) of vitamin D_2 solution exuding through a hole to the outer thin layer, aliquots of V_A and V_B solutions (see Fig. 2) were evaluated with antimony trichloride and A_A, A_B were obtained.

Recovery (R) of vitamin D_2 from the thin layer against no treatment of vitamin D_2 was calculated by:

$$R(\%) = \frac{A_C \times V_C \times V_p}{A_B \times V_B \times V} \times 100 \quad \text{(Eq. 1)}$$

Equation 2 (volume determination) was combined with Eq. 1 to obtain Eq. 3

$$V(\text{ml.}) = \left(1 - \frac{A_A \times V_A}{A_B \times V_B}\right) \times V_p \quad \text{(Eq. 2)}$$

$$R(\%) = \frac{A_C \times V_C}{A_B \times V_B - A_A \times V_A} \times 100 \quad \text{(Eq. 3)}$$

The results are shown in Table IX.

The results show that if nitrogen gas was introduced using CTLC, decomposition of vitamin D_2 did not occur during the application of the sample solution to the thin layer.

Determination of Vitamin D_2 in Vitamin A and D_2 Emulsion by CTLC—To vitamin A emulsion containing gum arabic, vitamin D_2 in petroleum ether was added. Water and acetone were added and the vitamins A and D_2 extracted according to the method described.

For evaluation of the volume (V) of the sample extract solution exuding through a hole to the outer thin layer, absorbances of V_A and V_B solutions were measured spectrophotometrically at 328 mμ[4] and

TABLE III—RECOVERY OF VITAMIN D_2 FROM UNTREATED MnO_2[a]

Eluting Solvent	Recovery Av., % (n = 2)	Range, %
1, Ethyl ether	82.2	3.1
2, Acetone–pet. ether (1:4)	83.8	3.2
3, Acetone–*n*-pentane (1:4)	82.6	4.3
4, Acetone–ethyl ether (1:4)	80.9	4.5
5, MEK[b]–pet. ether (1:4)	67.7	4.0
6, MEK–ethyl ether (1:4)	74.0	7.9

[a] Equal part by weight below 105 and 105–210 μ. [b] MEK, methyl ethyl ketone.

TABLE IV—RECOVERY OF VITAMIN D_2 FROM INACTIVATED MnO_2 COLUMN USING ACETONE–PETROLEUM ETHER (1:4) AS ELUTING SOLVENT

	MnO_2 Mesh Size (Below 105 μ: 105–210 μ)	MnO_2 Ht., cm.	Conditioning of MnO_2 Inactivation Method[a]	Conditioning of MnO_2 Washing Method[b]	Recovery, %
1,	1:1	1.5	*A*	*A*	98.6
2,	1:1	1.5	*B*	*B*	98.7
3,	2:3	2.0	*A*	*B*	98.7
4,	2:3	2.0	*B*	*A*	100.9
5,	1:2	3.0	*A*	*A*	99.0
6,	1:2	3.0	*B*	*B*	98.1
7,	1:3	3.0	*A*	*B*	100.2
8,	1:3	1.3	*B*	*A*	99.4

[a] Method *A*: 2 ml. of acetone containing 3% water pass through the column. Method *B*: 1 ml. of acetone containing 5% water pass through the column. [b] Method *A*: two 1-ml. portions of pet. ether pass through the column. Method *B*: two 3-ml. portions of pet. ether after inactivation.

TABLE V—RECOVERY OF VITAMIN D_2 IN ACETONE–PETROLEUM ETHER (1:4) SOLUTION

	Eluting Solvent Acetone–Pet. Ether	Recovery, % Av.	Range, % n = 2
1,	1:9	99.2	1.2
2,	1:4	100.3	0.8
3,	3:7	98.9	1.0
4,[a]	1:4	98.0	2.1

[a] Without inactivation of MnO_2.

[3] Adsorbent, Silica Gel G (Wakogel B-5); developing solvent, chloroform; method, microchromatoplate according to Peifer (7); detecting method, U.V. light (365 mμ), $SbCl_3$ T.S., and concentrated H_2SO_4.

[4] The wavelength 328 mμ is the maximum of vitamin A palmitate. In this experiment, to avoid isomerization of vitamin D_2, the hydrolysis was not adopted for extraction of vitamin D_2 from the sample. Therefore, only vitamin A alcohol which existed in vitamin A palmitate was converted to retinene by MnO_2 and almost all vitamin A ester remained unchanged.

TABLE VI—STABILITY OF VITAMIN D_2 IN ELUTING SOLVENTS[a]

	Eluting Solvent Added	T_1 Initial	T_2 3 hr.	T_3 24 hr.	$\frac{T_1 - T_2}{T_1}$ (%) % Loss (3 hr.)	$\frac{T_1 - T_3}{T_1}$ (%) % Loss (24 hr.)
1	Acetone	0.4255	0.4255	0.419	0	1.5
2	Acetone, 1 ml. acetone–pet. ether (1:4)	0.4275	...	0.426	...	0.35
3	Acetone, 2 ml. acetone–pet. ether (1:4)	0.424	0.422	0.418	0.47	1.4
4	Acetone–pet. ether (1:9)	0.4275	...	0.422	...	1.3
5	Acetone–pet. ether (1:4)	0.426	0.425	0.427	0.23	−0.23
6	Acetone–pet. ether (3:7)	0.426	...	0.4245	...	0.35
S_T	Pet. ether	0.427	0.4265	0.4265	0.12	0.12
S_T	Acetone	0.4265	0.4250	0.421	0.35	1.3

[a] The data are represented as the absorbances ($A_{500} - A_{550}$). T_1, T_2, T_3: color developed immediately, 3 hr., and 24 hr., respectively, after mixing vitamin D_2 solution and eluted solvent.

TABLE VII—OXIDATION OF VITAMIN A ALCOHOL BY MnO_2

	MnO_2 Col. Ht., cm.	MnO_2 Col. Inactivation	Treated Vit. A Alc., I.U.	Eluting Solvent	Detection of Vit. A Alc.
1,	1.5	...	5×10^3	Pet. ether	−
2,	1.5	...	5×10^3	Acetone–pet. ether (1:4)	−
3,	1.5	...	10×10^3	Acetone–pet. ether (1:4)	−
4,	1.5	Acetone (1–3 ml.)	10×10^3	Acetone–pet. ether (1:4)	−
5,	1.5	Acetone, 3% water, 2 ml.	10×10^3	Acetone	±
6,	3.0	Acetone, 3% water, 2 ml.	10×10^3	Acetone	−
7,	1.5	Acetone, 3% water, 2 ml.	10×10^3	Acetone–pet. ether (1:4)	±
8,	3.0	Acetone, 3% water, 2 ml.	10×10^3	Acetone–pet. ether (1:4)	−

A_A and A_B were obtained, respectively. The volume (V) of the sample extracting solution exuded was calculated from Eq. 2.

The recoveries of vitamin D_2 from vitamin A and D_2 emulsion against untreated vitamin D_2 (standard) are shown in Table X.

These experiments show that the recoveries of vitamin D_2 from vitamin A–D_2 emulsion are excellent with the proposed procedure.

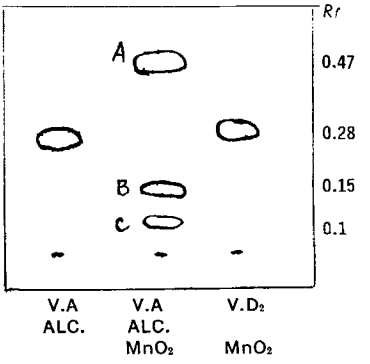

Fig. 4—Thin-layer chromatogram of oxidation products of vitamin A alcohol by MnO_2.

SUMMARY

It was found that vitamin D_2 was adsorbed on untreated MnO_2 and was not eluted with petroleum ether or pentane. Good recovery of the vitamin D_2 was obtained from water–acetone inactivated MnO_2 with an eluant of acetone in petroleum ether (1:4). Thus results were satisfactory.

Vitamin A alcohol having an R_f value similar to vitamin D_2 (R_f 0.28) was oxidized with inactivated MnO_2 on TLC into three yellowish bands which have R_f values of 0.47, 0.15, and 0.10.

Vitamin D_2 was located between the two yellowish bands which have R_f values of 0.47 and 0.15. It is unnecessary to use U.V. light to locate the bands.

By means of a cylindrical thin-layer chromatograph (4) with the introduction of nitrogen gas during the application of sample solution, the partial decomposition of vitamin D_2 was prevented.

With this technique it was found that the application of more than 1 ml. of sample solution, thus minimizing this source of error, was achieved without damaging the thin layer.

TABLE VIII—DETECTION OF OXIDATION PRODUCTS OF VITAMIN A ALCOHOL BY MnO_2 ON TLC PLATE

Spot	Daylight	U.V. Light	$SbCl_3$ T.S.	Conc. H_2SO_4
A	Yellow	Brown absorption	Blue	Red violet
B	Yellow	Brown absorption	Red	Red
C	Yellow	Brown absorption	Orange red	Blue
Vitamin A alc.	...	Yellow fluorescence	Blue	Blue

TABLE IX—RECOVERY OF VITAMIN D_2 FROM THE THIN LAYER

	Day of Expt. 1			2		
Repetition →	1	2	3	1	2	3
No. 1[a]		0.2495			0.280	
V_B - - - A_B No. 2[a]		0.320			0.350	
No. 3[a]		0.432			0.462	
V_A - - - A_A	0.356	0.210	0.3275	0.365	0.327	0.420
V (ml.)	0.8875	1.3438	0.9766	0.9571	1.0657	0.8000
V_C - - - A_C	0.280	0.425	0.310	0.332	0.378	0.281
Recovery, %	98.8	99.0	99.4	99.1	101.3	100.4
Av. %		99.07			100.27	
Range		0.6			2.2	

[a] Vitamin D_2 concentration of developed color solutions No. 1, 2, and 3 was 2, 2.5, and 3.3 mcg./ml., respectively. Absorbance data were represented as the value of $(A_{600} - A_{560})$.

TABLE X—RECOVERIES OF VITAMIN D_2 FROM A-D_2 EMULSION

	Day of Expt. 1			2		
Repetition→	1	2	3	1	2	3
Std. No. 1[a]		0.187			0.183	
A_S No. 2[a]		0.321			0.242	
No. 3[a]		0.461			0.326	
A_C	0.234	0.362	0.362	0.1745	0.236	0.333
V_A - - - A_A	0.332	0.2735	0.325	0.319	0.3125	0.292
V_B - - - A_B	0.318	0.312	0.3055	0.3115	0.3145	0.307
V (ml.)	0.9560	1.1234	0.9362	0.9759	1.0064	1.0489
Recovery, %	101.7	100.4	96.4	97.5	96.9	97.5
Av.		99.50			97.30	
Range		5.3			0.6	

[a] In the first day of experiment, vitamin D_2 concentration of developed color solution No. 1, 2, and 3 was 1.5, 2.5, and 3.5 mcg./ml., respectively, and in the second day, that of No. 1, 2, and 3 was 1.5, 2.0, and 2.5 mcg./ml., respectively.

REFERENCES

(1) Barua, R. K., and Rao, M. V. K., *Analyst*, **89**, 534 (1964).
(2) *Ibid.*, **90**, 571(1965).
(3) Bolliger, H. R., and König, A., *Z. Anal. Chem.*, **214**, 1(1965).
(4) Hirayama, K., *J. Chromatog.*, to be published.
(5) Ball, B., Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **42**, 516(1948).
(6) Henbest, H. B., Jones, E. R. H., and Owen, T. C., *J. Chem. Soc.*, **1957**, 4909.
(7) Peifer, J. J., *Mikrochim. Acta*, **1962/3**, 529.

Method for the Evaluation of Antihidrotic Substances in the Anesthetized Cat

By R. S. ALPHIN, DALE SAUNDERS, and JOHN W. WARD

An improved method using newly designed equipment is described for the evaluation of antihidrotic substances in the anesthetized cat. The substances to be tested were applied locally on the foot pads. The effect on the sweating induced by the administration of a pharmacological agent such as pilocarpine was then observed by recording changes in the moisture content of air passed at a constant flow over the foot pads. The method as described gave consistent and reproducible results with a substance known to produce antihidrotic effects.

FROM A SEARCH of the literature, there appear to be few methods available for investigating the pharmacological activity of substances on sweating in laboratory animals. Kuno (1) was among the first to use the moisture produced by the sweat glands as a measurement of sweating in man. The method had limitations since changes in sweat secretion could only be observed for a period of 5 min. Adams *et al.* (2) have

Received November 4, 1966, from the Department of Pharmacology, A. H. Robins Research Laboratories, Richmond, VA 23220
Accepted for publication January 9, 1967.
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REFERENCES

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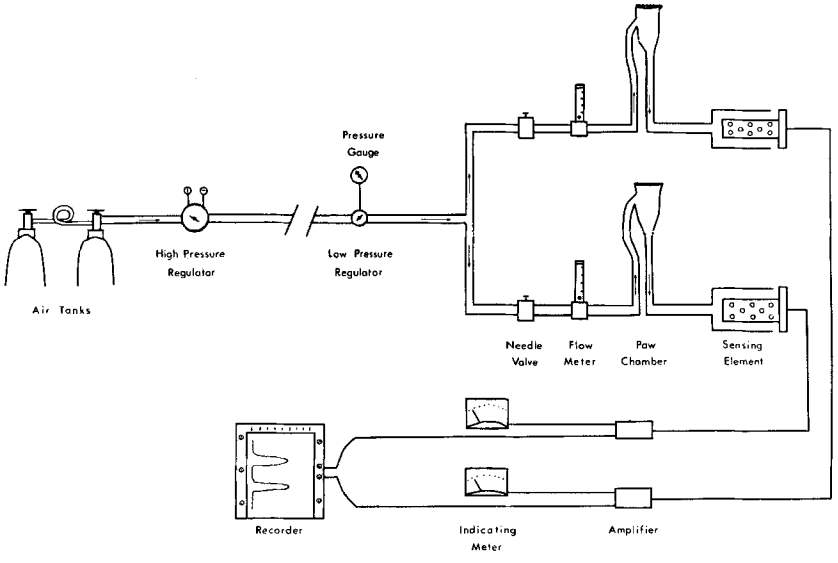

Fig. 1—Diagram illustrating the sweating rate measuring system. The sensing elements were partially enclosed to prevent accumulation of dust and other foreign material in the room air.

described the measurement of evaporative water loss by a thermal conductivity cell. Nakayama and Takagi (3) used, instead of the absorption of water as Kuno had done previously, a sensitive resistance hygrometer which enabled them to make a continuous recording of sweating. Bullard (4) later described a modification of the method used by Nakayama and Takagi for studies in man. The purpose of this report is to describe a method for the study of sweating, especially in cats. The method is a modification of the one described by Bullard (4) and which has been adapted for animal use. In principle, when air of any known low relative humidity is passed at a fixed and appropriate rate over the foot pads of cats, the change in the water content of the air is dependent upon the sweating rate.

MATERIALS AND METHODS

The sweating rate measuring system is shown diagrammatically in Fig. 1. The air (less than 5% relative humidity) supplied by storage tanks was reduced in pressure by high and low pressure regulators and fed into two separate flowmeters. By the use of needle valves at the flowmeters, the air flow could be adjusted and maintained at any desired rate. The air flow selected to be used in all of the experiments was 0.5 L./min. The air passed into a specially designed glass chamber which contained one of the cat's paws. The small chambers were not only designed to provide minimal air volume, but also large enough to permit the use of larger cats. The chamber dimensions were approximately 7 cm. in length and had an inside diameter of 4 cm. The air inlet and exit openings were 6 mm. inside diameter. The position of the paw was always such that the air inlet opening was opposite the foot pad surface. The cat's paw entered into the chamber through a small opening made in a rubber dam which was used to close the open end of the chamber. It was imperative that the position of the paw did not interfere with its blood circulation. This was accomplished by using a very flexible rubber dam with various size holes for entrance of the paw and to avoid inhibiting or occluding the local circulation. To insure that there was uniformity in distribution of air in the "paw chamber," the position of the paws inside the chambers was as nearly the same as possible. Figure 2 shows a close-up view of the cat's paw inside the chamber. After the air had passed over the foot pads at a known flow rate, it then passed into the humidity sensing elements (Hygrodynamics, Inc., Silver Spring, Md.). The principle of the sensing element is based on the ability of a hygroscopic film to change its

Fig. 2—The position of the cat's paws within the chambers. The small polyethylene cannula in the lower right was used for the intravenous injections via jugular vein.

Fig. 3—The entire system showing the cat; paws enclosed in the small glass containers.

electrical resistance instantly with microchanges in the relative humidity. The resistance change is then measured in terms of electric current flowing through the element and is indicated and recorded by suitable instruments. The hygroscopic film used in the sensing element is lithium chloride. The wide-range sensing element used is capable of detecting humidity changes in the range of 5 to 40%. The Hygrodynamics humidity indicator (catalog No. 15-3000) serves several purposes, that of an a.c. voltage source to the sensing elements, a meter showing the electrical current changes, and a d.c. signal source for recording purposes. The humidity changes may be visually recorded from the indicator meter. However, it is much simpler and less time consuming to use a suitable recording instrument. A Moseley (model 7100A) two pen strip chart recorder was used to record the results reported here. Each humidity sensing element contained a thermistor for temperature measurement. The elements were supplied with a graph of correction factors to be used between −40° and 120°F. The correction factors were added to or subtracted from the humidities read from the calibration curves depending upon the temperature at the level of the sensor. As the air flow rates are known, it may also be desirable to express the data as the amount of water vapor evaporated from a measured paw surface area per unit time. Lawton *et al.* (5) have used the following expression to determine the sweating rates:

$$\text{sweating rate (mg./min.)} = \text{air flow (L./min.)} \times \frac{\Delta RH}{100} \times \text{density of sat. steam (mg./L.)}$$

The entire system used for measuring the sweating response is shown in Fig. 3. The housing for the humidity sensing elements, flow and pressure regulators, amplifiers and meters was designed and made in the machine shop of these research laboratories. A heat lamp was installed, since it has been our experience that a more pronounced sweating response is obtained when heat is applied. The heat lamp consisted of a 125 w. infrared bulb contained in a reflector. It was placed about 18 in. directly above the animal's abdomen and remained in that position throughout the entire experiment. The heat lamp alone in this position never elicited a significant sweating response.

The pharmacological substances to be tested were dissolved in water at various concentrations. They were then applied (0.1–0.2 ml.) directly on the foot pads of the anesthetized (sodium phenobarbital, 125 mg./Kg. i.p.) cat by use of an applicator stick. The material was then allowed to dry before the paw was placed into the glass chamber. Another paw, enclosed in a separate chamber, served as a

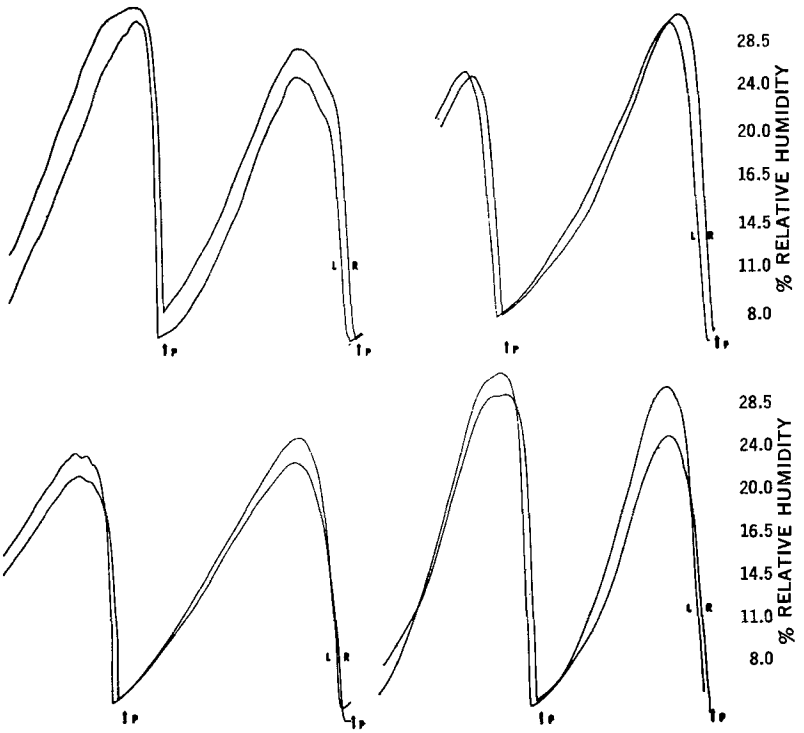

Fig. 4—Control sweating responses to the intravenous administration of pilocarpine (0.1 mg./Kg.) in four cats. The graphs read from right to left. The arrows indicate the time of pilocarpine injections. The time interval between the two injections was approximately 2 hr. Key: top right, cat No. 192-66; top left, cat No. 162-66; bottom right, cat No. 233-66; bottom left, cat No. 198-66.

control. Sweating was induced by the administration of pilocarpine hydrochloride (0.1 mg./Kg.) *via* a jugular vein previously cannulated with P.E. 50 tubing.

The duration of action produced by various substances can also be determined using this method simply by increasing the length of time between application and the injections of pilocarpine.

RESULTS

Figure 4 shows the sweating responses obtained in four cats following the intravenous administration of pilocarpine. In control experiments, the difference in sweating response between the two paws never varied more than 4 to 5%. This small difference made it possible to determine the effect of various substances, when applied topically, on the sweating response. The response to pilocarpine in the majority of tests has been consistent and reproducible over a period of about 5 to 6 hr. Whenever the time has been extended to 8 and 10 hr., the pilocarpine response may be diminished. This diminution in the response may be noted even though the body fluid lost through sweating and salivation is replaced during the entire experiment by the intravenous infusion of normal saline.

To illustrate that the system is capable of detecting differences in sweating rates, the effects of a commercially available aluminum salt preparation,[1] applied as previously described, are shown in Fig. 5. The aluminum salt preparation was applied 3–5 hr. prior to the injections of pilocarpine. It can be seen that the maximum relative humidity provided by the control paw was between 30 and 36%, whereas the treated paw showed values of between 12 and 15% following two injections of pilocarpine. Upon further extending the time between application and the injection of pilocarpine, the effect was greatly diminished or could no longer be demonstrated.

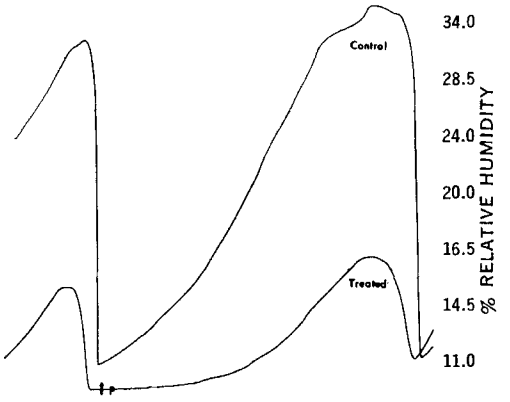

Fig. 5—The sweating response obtained after the local application to one foot pad of a commercial aluminum salt preparation 3–5 hr. prior to pilocarpine. The graph reads from right to left. The arrows indicate the time of the pilocarpine injections (0.1 mg./Kg. i.v.). Time interval between pilocarpine injections was approximately 2.5 hr. (cat No. 250-66.)

DISCUSSION

There have been few methods reported in the literature suitable for the rapid evaluation of pharmacological substances on sweating in laboratory animals. The adapted method of resistance hygrometry reported in this study makes it practical to investigate a relatively large number of such substances in anesthetized cats. The same general method has also been used in this laboratory to study the sweating response in dogs. The duration of the antihidrotic effects of a substance may be easily determined simply by varying the time between application and the injections of pilocarpine.

The choice of the anesthetic agent used is important in sweating rate studies involving cats. The longer-acting barbiturates are the most desirable since additional drug is usually avoided during the experiment. In most experiments involving the use of supplemental doses of a shorter-acting agent, there was an initial increase in the sweating response. The reason for the increased sweating rate following barbiturate administration is not clearly understood.

The sweating rate measuring system described is readily adaptable for the measurement of sweating produced under a variety of environmental conditions. Finger tip sweating in man has also been determined by this method.

[1] Marketed as Ban by Bristol-Myers Co., New York, N. Y.

REFERENCES

(1) Kuno, Y., "The Physiology of Human Respiration," Churchill, London, England, 1934.
(2) Adams, T., Funkhouser, G. E., and Kendall, W. W., *J. Appl. Physiol.*, **18**, 1291(1963).
(3) Nakayama, T., and Takagi, K., *Japan. J. Physiol.*, **9**, 359(1959).
(4) Bullard, R. W. J., *J. Appl. Physiol.*, **17**, 735(1962).
(5) Lawton, R. W., Prouty, L. R., and Hardy, J. D., *Rev. Sci. Instr.*, **25**, 370(1954).

Effect of Diester Type Plasticizers on the Differential Thermal Spectrum of a Polyvinyl Chloride System

By R. K. O'LEARY, J. FOY, W. L. GUESS, and J. AUTIAN

One of the most successful formulated plastics for medical and paramedical applications is the polyvinyl chloride (PVC) family. A variety of excellent properties is possible by incorporating other agents such as plasticizers into PVC. Plasticized systems are currently being used for flexible tubings, blood bags, various types of catheters, and protective materials. Because of the lack of published information on the effects of plasticizers on the thermal spectra of PVC and the paucity of data on this polymer using differential thermal analysis, a study was initiated to note what effects a group of plasticizers would have on the thermal behavior of formulated PVC systems. A group of diester plasticizers was used to note how each member of the series would influence the depolymerization process. Results of this study suggest a method of predicting whether a plasticizer will increase or decrease the tendency of the polymer to degrade.

THE RAPID increase in the use of polymeric materials to replace glass, rubber, and metals is very evident by observing the multitude of plastic products now in use in medicine and paramedical applications. Advancements in plastic technology have brought forth certain polymeric materials which require the introduction of an appreciable quantity of plasticizers to impart specific desired physicochemical and mechanical properties. Polyvinyl chloride, cellulose acetate, polyvinyl acetate, and nitrocellulose are a few examples of plastic materials which may contain one or more plasticizers.

In medical practice the plasticized polyvinyl chloride (PVC) has been and is currently being used widely for the construction of blood bags, tubings, devices for the administration of drug products, as collection devices for body fluids, and as storage containers for one or more products. This widespread acceptance of PVC has required much scrutiny in selecting plasticizers which will produce the desired processing and performance characteristics in the finished medical product. It is also understood that the plasticizers should be nontoxic when used for a medical application which might have contact with tissue or with a product to be administered to a patient (1, 2).

The addition of a plasticizer to polyvinyl chloride reduces the glass transition temperature (T_g) and thereby imparts various degrees of softness and flexibility. Since polyvinyl chloride materials must also contain a stabilizer to prevent or hinder degradation of the material during processing, it was felt that the various plasticizers might have different effects on influencing the stability of the plastic when thermal conditions are imposed. Few reports of a quantitative nature, however, have appeared in the literature in regard to the role of the plasticizer as a stabilizing agent in polyvinyl chloride. For this reason a study was undertaken to assess the relative heat stabilizing properties of a group of plasticizers on polyvinyl chloride when exposed to thermal treatments using differential thermal analysis (DTA).

EXPERIMENTAL

Materials and Apparatus—(*a*) Polyvinyl chloride.[1]

(*b*) Plasticizers: dimethyl phthalate, dibutyl phthalate, dioctyl phthalate, ditridecyl phthalate (W. R. Grace & Co., Hatco Chemical Division, Fords, N. J.), dioctyl adipate, didecyl adipate (Eastman Chemical Products, Inc., Kingsport, Tenn.), dihexyl azelate, dioctyl azelate (Emery Industries, Inc., Cincinnati, Ohio), dibutyl sebacate, dioctyl sebacate (Reichhold Chemical Co., Deecy Products Division, Cambridge, Mass.), dibenzyl sebacate, dibutyl suberate, dibutyl dodecanedioate (Wallace & Tiernan, Inc., Harchem Division, Belleville, N. J.), trioctyl trimellitate (Rubber Corporation of America, Hicksville, N. Y.), and trioctyl phosphate (Monsanto Co., St. Louis, Mo.).

(*c*) Stabilizer: a barium-cadmium carboxylate type stabilizer.[2]

(*d*) Polyvinyl chloride films: the films of the polymer are prepared by the plastisol fusion technique, *i.e.*, a colloidal dispersion of a high molecular weight vinyl chloride resin in the desired plasticizer is heated until all the plasticizer in the liquid phase is absorbed into a fused polymer. The loss of separate phases and interfacial boundaries produces a translucent plastic with a high tensile strength.

Received October 4, 1966, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78756

Accepted for publication December 15, 1966.

Presented to the Drug Standards, Analysis and Control Section, A. PH. A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported by contract PH 43-64-557 from the Division of Biologics Standards, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[1] Geon 121. High molecular weight. B. F. Goodrich Chemical Co., Cleveland, Ohio.

[2] Advastab BC-100 S-1, Advance Division, Carlisle Chemical Works, Inc., New Brunswick, N. J.

The formulation used in this study was: 100 Gm. PVC, 60 Gm. plasticizer, and 1.5 Gm. stabilizer.

The particular PCV[1] was chosen because its molecular weight is high enough to prohibit undue solvation by the plasticizer at normal temperatures, though assuring proper solvation at increased temperatures. Lower molecular weight resins were avoided because they are solvated at room temperatures such that the swelling resin particles raise the volume concentration of solid phase and the plastisol becomes an unworkable plastigel. The barium-cadmium carboxylate type was chosen as the stabilizer system for this study because it provides excellent heat and light stabilization in closed mold plastisol processing. Prior to molding the formulation, the plasticizer and stabilizer were placed in a Hobart model K45 mixer,[3] and the vinyl chloride resin added slowly until a uniform dispersion was produced (approximately 45 min. to 1 hr.). The formulations were stored for 24 hr. to achieve partial deaeration and then poured into preheated (165°) 40-mil thick molds and processed on a Dake press model 44–325[4] for 3.5 min. at 35,000 lb. pressure. The molds were then cooled to 120° and the films removed. All the formulations were conditioned 48 hr. at 22° and 50% relative humidity.

(*e*) Differential thermal analysis apparatus: the apparatus consists of a controlled atmosphere differential thermal analyzer with a quick cool furnace model F-1DF and two L&N Speedomax H strip chart recorders. This instrument is manufactured and described by Robert L. Stone Co., Austin, Tex. (3, 4).

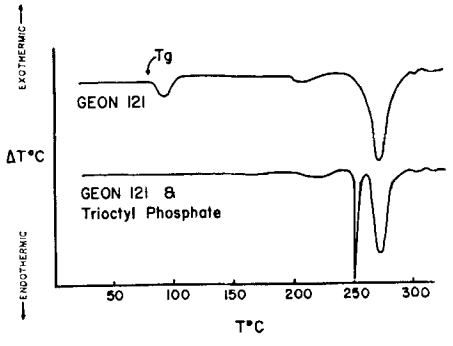

Fig. 1—DTA thermograms of plasticized and unplasticized polyvinyl chloride.

Effect of a Group of Plasticizers on the Dehydrohalogenation of PVC—One-milligram samples of each of the PVC formulas were run in the differential thermal analysis apparatus at a heating rate of 6.0°/min., using highly calcined, 80 mesh Al_2O_3 as the inert reference. Nitrogen was used as the dynamic gas in order that purely thermal effects might be distinguished from the oxidative transitions which will occur in an air atmosphere. In each instance four determinations were made for each plastic formula to insure the reliability of the results. Figure 1 illustrates the thermograms for an unplasticized PVC and for a plasticized PVC (trioctyl phosphate). As may be noted from the unplasticized PVC, a main glass transition temperature is apparent at 86.0° and a small depolymerization temperature occurs at 200.6°. No glass transition temperatures were noted for the plasticized resins in the temperature range studied (ambient to 300°). Figure 1 also reveals that in the plasticized PVC, the plasticizer (trioctyl phosphate) apparently "boils" off the plastic immediately prior to the dehydrohalogenation. None of the other plasticized resins followed this trend.

From each of the thermograms, the following information was derived: (*a*) the temperature of onset of dehydrohalogenation, (*b*) the peak temperature of dehydrohalogenation, and (*c*) the area under the curve corresponding to the dehydrohalogenation reaction. This area was then utilized to calculate the heat of dehydrohalogenation ($\Delta H°$) in a manner described in a previous report (5). Table I includes the onset and peak temperatures for all of the plastic formulas as well as the calculated heats of dehydrohalogenation.

In all experiments actual weights of the plastic samples were determined after dehydrohalogenation had occurred, and from this information it was possible to calculate the percentage weight loss included in Table I.

DISCUSSION

As has been indicated, the plasticized polyvinyl chlorides, at least in the temperature range studied, did not reveal any glass transition temperatures as the pure polymer did. In these cases, however, it may be assumed that the T_g values were present but occurred below ambient temperature or if they did occur above ambient temperature, they may not have been detected by the instrument.

A review of the results in Table I clearly reveals that the structure of the plasticizer can play a very important role in helping to stabilize the PVC resin. In the phthalate ester series, an increase in stability can be noted as the molecular weight of the plasticizer is increased. The most "protective" phthalate ester, and in fact the most protective plasticizer in the whole series, was the ditridecyl phthalate which had peak temperatures for dehydrohalogenation of 289.2° and a heat of dehydrohalogenation of 26.9 Kcal./mole, a value of 7 to 8 times that of the other phthalate esters and considerably higher than the $\Delta H°$ for any of the other plasticizers. Dibenzyl sebacate and trioctyl trimellitate had also high heats of dehydrohalogenations, 15.6 Kcal./mole and 20.6 Kcal./mole, respectively. Generally, these high heats of dehydrohalogenations indicate that greater stability is afforded the PVC resins when these are subjected to high processing temperatures. However, in the case of the dibenzyl sebacate there appeared to be an anomalous situation where the actual temperature for onset of dehydrohalogenation was depressed to a slight extent as compared to the other plasticized systems having high $\Delta H°$ values. Exact reasons for this depression are still not known.

It may be noted from the table that most of the plasticizers increase the temperature where dehydrohalogenation takes place, but for the most part once this temperature is reached, less energy ($\Delta H°$) is needed to bring about complete dehydrohalogenation.

The results of this study appear to demonstrate that higher molecular weight plasticizers have a

[3] Hobart Mfg. Co., Troy, Ohio.
[4] Dake Corp., Grand Haven, Mich.

TABLE I—EFFECT OF SELECTED ESTER TYPE PLASTICIZERS ON THE DEHYDROHALOGENATION TRANSITION OF POLYVINYL CHLORIDE

Plasticizer	Mol. Wt.	Onset[a] Temp., °C.	Peak[b] Temp., °C.	Heat of Dehydrohalogenation cal./Gm.	Kcal./mole	Polymer Wt. Loss, %
Pure polymer		243.4	278.8	30.9		
Dimethyl phthalate	194.18	258.2	266.9	16.2	3.15	76.4
Dibutyl phthalate	278.34	262.3	271.4	13.8	3.85	75.8
Dioctyl phthalate	390.53	271.4	277.9	10.9	4.27	75.9
Ditridecyl phthalate	530.0	273.8	289.2	50.7	26.9	65.5
Dioctyl adipate	370.56	271.2	275.9	10.6	3.95	76.0
Didecyl adipate	426.67	277.3	280.7	16.0	6.85	77.4
Dibutyl suberate	286.3	260.7	269.0	15.3	4.39	75.3
Dihexyl azelate	356.0	270.2	276.1	18.4	6.57	76.0
Dioctyl azelate	412.0	275.0	282.1	17.4	7.20	75.3
Dibutyl sebacete	314.0	263.5	271.7	12.6	3.95	76.0
Dioctyl sebacate	426.0	276.1	283.3	19.1	8.18	73.0
Dibenzyl sebacate	382.0	264.2	272.2	39.6	15.6	72.5
Dibutyl dodecanedioate	342.0	267.8	273.1	11.9	4.08	75.5
Trioctyl trimellitate	546.0	272.4	292.4	37.4	20.5	75.7
Trioctyl phosphate	362.40	265.4	269.6	16.7	6.07	75.5

[a] Onset temperature at which dehydrohalogenation takes place. [b] Peak temperature at which dehydrohalogenation takes place.

greater protective effect than the lower ones and that an increase in the hydrophobic moiety of the plasticizers plays an important role in the protective mechanism afforded to the PVC.

The use of differential thermal analysis for the evaluation of a group of plasticizers in regard to their protective effects under purely thermal conditions reveals that this technique can give considerable information in a relatively short period of time which should be helpful when a new plasticizer is to be considered for a polyvinyl chloride formula.

REFERENCES

(1) Calley, D., Autian, J., and Guess, W. L., *J. Pharm. Sci.*, **55**, 158(1966).
(2) Guess, W. L., O'Leary, R., Calley, D., and Autian, J., Society of Plastics Engineers, 22nd Annual Technical Conference, Session XXV, vol. XII, paper No. 4, 1966.
(3) Stone, R. L., *Anal. Chem.*, **32**, 1582(1960).
(4) Stone, R. L., *J. Am. Ceram. Soc.*, **35**, 76(1952).
(5) O'Leary, R. K., Foy, J., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **56**, 494(1967).

Application of 1-(*N*-β-Hydroxyethyl-4-piperidyl)-3-(4-piperidyl)-propane in the Mannich Reaction I

Substituted β-Aminoketones

By RAJENDRA S. VARMA and W. LEWIS NOBLES

The Mannich reaction has been successfully performed using 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(4-piperidyl)propane (4-DI-PIP-OL). A series of Mannich bases has been prepared employing 4-DI-PIP-OL and various ketones. Characteristic infrared absorption bands for OH, $\overset{+}{N}H$, $-\overset{O}{\overset{\|}{C}}-$ groups are recorded. The compounds are to be screened for possible pharmacological activity.

MANNICH BASES have been prepared (1–25) for pharmacological testing as antispasmodics, analgesics, local anesthetics, or chemotherapeutic agents. Such compounds are usually prepared by the condensation of formaldehyde (paraformaldehyde) with ammonia, a primary or secondary amine, and a compound containing at least one active hydrogen atom. The condensation reaction may be illustrated as in Scheme I.

Received October 14, 1966, from School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication December 15, 1966.
This investigation was supported in part by grant AI 04701 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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MANNICH BASES have been prepared (1–25) for pharmacological testing as antispasmodics, analgesics, local anesthetics, or chemotherapeutic agents. Such compounds are usually prepared by the condensation of formaldehyde (paraformaldehyde) with ammonia, a primary or secondary amine, and a compound containing at least one active hydrogen atom. The condensation reaction may be illustrated as in Scheme I.

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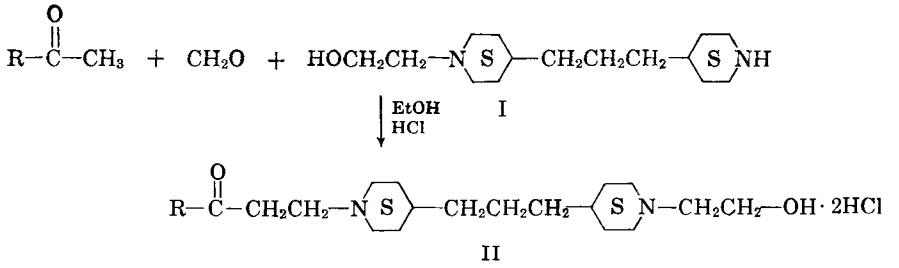
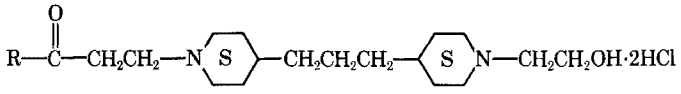


Scheme I

Mannich and Lammering (2) reported that β-piperidinopropiophenone hydrochloride possessed local anesthetic activity. Blicke and Blake (3) found that the corresponding Mannich base from 2-acetylpyrrole possessed marked activity of this type. Levvy and Nisbet (5) indicated that both piperidino and dimethylamino Mannich bases derived from 2-acetylthiophene demonstrated local anesthetic activity. Denton and his associates (6, 7) described the preparation and pharmacological evaluation of various β-dialkylaminopropiophenones and their derivatives. Many of these proved to be effective antispasmodics. Certain Mannich bases derived from α,β-unsaturated ketones have exhibited antibacterial activity (20). Many compounds prepared heretofore have possessed an alkylamino, dialkylamino, or azabicyclo amine as the amine moiety as well as a number of nitrogen heterocycles of various sizes. In this communication the Mannich reaction has been extended to include 4-DI-PIP-OL, a novel secondary amine. Recently it has been reported (26) that 1-(4-pyridyl)-3-(piperidyl)propane derivatives demonstrate anticonvulsant, antiparkinsonian, and antidepressant properties. In view of this and the multiplicity of pharmacological effects elicited by numerous ketonic Mannich bases, we have prepared a number of β-aminoketones of general structure II.

In a preliminary screening, compound *21* (Table I) has shown activity against both Gram-positive and Gram-negative bacteria. The detailed screening results will be published at a later date.

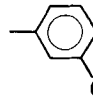
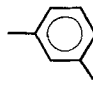
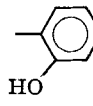
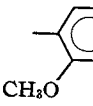
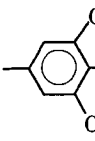
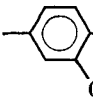
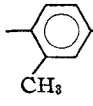
TABLE I—MANNICH BASE DIHYDROCHLORIDES



Compd.	R	M. p., °C.	Yield, %	Mol. Formula	Anal., %[d] Calcd.	Found
1	—C$_6$H$_5$ [a]	208–210	55	$C_{24}H_{40}Cl_2N_2O_2 \cdot {}^1/_2H_2O$	C, 61.51 H, 8.82 N, 5.98	61.57 8.87 6.14
2	—C$_6$H$_4$—CH$_3$ [a]	205–207	42	$C_{25}H_{42}Cl_2N_2O_2 \cdot {}^1/_2H_2O$	C, 62.23 H, 8.98 N, 5.81	62.32 8.66 6.12
3	—C$_6$H$_4$—NO$_2$ [a]	210–211	49	$C_{24}H_{39}Cl_2N_3O_4$	C, 57.14 H, 7.79 N, 8.33	57.26 8.25 7.98
4	—C$_6$H$_4$—F [a]	209–211	63	$C_{24}H_{39}Cl_2FN_2O_2$	C, 60.35 H, 8.23 N, 5.87	60.34 8.62 5.96
5	—C$_6$H$_4$—NO$_2$ [a]	190–192	50	$C_{24}H_{39}Cl_2N_3O_4$	C, 57.14 H, 7.79 N, 8.33	56.95 8.15 7.88
6	—C$_6$H$_4$—OCH$_3$ [a]	155–157	61	$C_{25}H_{42}Cl_2N_2O_3$	C, 60.77 H, 8.67 N, 5.67	60.70 8.82 6.02

(*Continued on next page.*)

TABLE I—(*Continued.*)

Compd.	R	M. p., °C.	Yield, %	Mol. Formula	Anal., %[d] Calcd.	Found
7	 [a]	194–196	63	$C_{24}H_{40}Cl_2N_2O_3$	C, 60.60 H, 8.67 N, 5.89	60.36 8.77 5.96
8	 [a]	182–183	53	$C_{25}H_{42}Cl_2N_2O_3 \cdot {}^1/_4H_2O$	C, 62.80 H, 8.96 N, 5.86	62.43 8.88 6.24
9	 [a]	201–203	58	$C_{24}H_{40}Cl_2N_2O_3$	C, 60.60 H, 8.67 N, 5.89	60.58 8.82 5.61
10	 [a]	125	30	$C_{25}H_{42}Cl_2N_2O_3 \cdot H_2O$	C, 59.15 H, 8.74	58.98 8.91
11	[c]	222–224	73	$C_{24}H_{39}Cl_3N_2O_2$	C, 58.35 H, 7.96 N, 5.67	58.25 7.76 5.86
12	[a]	227–229	48	$C_{24}H_{39}BrCl_2N_2O_2$	C, 53.52 H, 7.30 N, 5.20	53.39 7.31 5.03
13	[a]	193–194	52	$C_{30}H_{44}Cl_2N_2O_2 \cdot H_2O$	C, 65.07 H, 8.38 N, 5.06	65.05 8.53 5.20
14	[a]	189–192	49	$C_{26}H_{44}Cl_2N_2O_3$	C, 62.01 H, 8.81 N, 5.56	61.95 8.74 5.77
15	[a]	195–196	65	$C_{24}H_{44}Cl_2N_2O_3$	C, 60.60 H, 8.67 N, 5.89	60.46 8.54 5.93
16	 [a]	184–185	36	$C_{27}H_{46}Cl_2N_2O_5 \cdot {}^1/_2H_2O$	C, 58.07 H, 8.48 N, 5.02	58.00 8.92 5.34
17	[a]	182–185	37	$C_{25}H_{39}Cl_2F_3N_2O_2 \cdot {}^1/_2H_2O$	C, 55.95 H, 7.51 N, 5.22	55.55 7.77 5.89
18	 [a]	166–170	50	$C_{25}H_{42}Cl_2N_2O_3$	C, 61.33 H, 8.65 N, 5.72	61.21 8.79 5.91
19	[a]	218–220	50	$C_{25}H_{42}Cl_2N_2O_3$	C, 61.33 H, 8.65 N, 5.72	61.52 8.82 5.80
20	 [a]	178–180	30	$C_{25}H_{42}Cl_2N_2O_3$	C, 61.33 H, 8.65 N, 5.72	61.40 8.81 5.84
21	[b]	197–199	53	$C_{22}H_{38}Cl_2N_2O_2S$	C, 56.78 H, 8.23 N, 6.02	56.59 8.43 5.85

[a] Obtained by procedure *B*. [b] Obtained by procedure *A*. [c] Obtained by procedure *C*. [d] Microanalyses by Dr. A. Bernhardt, Mulheim (Ruhr), W. Germany.

EXPERIMENTAL

Melting points were taken in capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer using a Nujol mull.

1 - (N - β - Hydroxyethyl - 4 - piperidyl) - 3 - (4-piperidyl)propane Dihydrochloride—4-DI-PIP-OL, 25.4 Gm. (0.1 mole), was suspended in 25 ml. of ethanol. Concentrated hydrochloric acid (21 ml.) was added to it in small portions with cooling and shaking. After the additions were completed, some ethanol was distilled off under vacuum and 100 ml. of acetone was added to it. On refrigeration, there was obtained the desired dihydrochloride in nearly quantitative yield, which was recrystallized to analytical purity from ethanol–acetone, m.p. 205°.

Anal.—Calcd. for $C_{15}H_{32}Cl_2N_2O$: C, 55.04; H, 9.86; N, 8.56. Found: C, 54.76; H, 9.73; N, 8.58.

N,N′ - Methylenebis[1 - (N - β - hydroxyethyl-4 - piperidyl) - 3 - (4 - piperidyl)propane] Tetrahydrochloride—To a mixture of 10.16 Gm. of 4-DI-PIP-OL, 20 ml. of ethanol, and 8.4 ml. of concentrated hydrochloric acid was added 1.2 Gm. of paraformaldehyde and the resulting reaction mixture was heated under reflux for 4 hr. At the end of this period, the reaction mixture was cooled and poured into 100 ml. of acetone. The entire mass, on refrigeration overnight, gave a viscous liquid on decantation of the upper layer of solvents. This liquid was dissolved in 20 ml. of ethanol; 50 ml. of acetone was then added. This treatment gave a white solid which was subsequently recrystallized to analytical purity from ethanol–acetone, m.p. 181–185° (softens at 75°); yield, 9.5 Gm. (71%).

Anal.—Calcd. for $C_{31}H_{64}Cl_4N_4O_2$: C, 55.84; H, 9.68; N, 8.40. Found: C, 55.49, 55.61; H, 10.25, 10.15; N, 8.32.

Mol. wt. (Rast method)—Calcd. for $C_{31}H_{64}Cl_4N_4O_2$: 666.69. Found: 692.

Mannich Base Dihydrochlorides—Three general methods were employed to synthesize these compounds.

Procedure A—The aminoketones were prepared by the general procedure of refluxing 0.1 mole of the ketone with 0.1 mole of 4-DI-PIP-OL dihydrochloride and 4.5 Gm. of paraformaldehyde in 25 ml. of absolute ethanol containing 1 or 2 drops of concentrated hydrochloric acid for 15 min. Another 1.5 Gm. of paraformaldehyde was added and refluxing continued for 2 hr. At the end of this time the warm solution was poured into 50 ml. of acetone and the contents refrigerated overnight. The solid thus obtained was recrystallized from ethanol–acetone.

Procedure B—To a suspension of 0.1 mole of 4-DI-PIP-OL in 25 ml. of ethanol was added 21 ml. of concentrated hydrochloric acid in small portions with cooling and shaking. Four and five tenths grams of paraformaldehyde was then introduced into the reaction vessel followed by the appropriate ketone. The resulting reaction mixture was refluxed for 3–5 hr. During this time the entire mixture went into solution. In a few cases, an amorphous solid product separated out at the end of this period. The contents were then poured into 50 ml. of acetone and refrigerated overnight or until a solid product separated. The product was removed by filtration and recrystallized from ethanol–acetone.

Procedure C—This method is similar to that of *B* except that aqueous 37% formaldehyde solution is used in place of paraformaldehyde.

Attempted Mannich Condensation Using Methylenebis-1-(N-β-Hydroxyethyl-4-piperidyl)-3-(4-piperidyl)propane Tetrahydrochloride—Equimolar proportions of *p*-chloroacetophenone and methylenebis amine salt were refluxed in 25 ml. of ethanol for 4 hr. Working up the reaction mixture gave only unreacted methylenebis amine salt.

Infrared Spectra of the β-Aminoketone Dihydrochlorides—Spectra of these compounds have been run in Nujol mull. In Table II are recorded absorption bands for OH, $\overset{+}{N}H$, and $-\overset{O}{\overset{\|}{C}}-$ groups. Thompson and associates (27) have reported absorption spectra of a number of tertiary amine salts and our observations are in agreement with them. Mannich bases may be regarded as tertiary amines and they do give bands due to $\overset{+}{N}H$. The $\overset{+}{N}H$ peak is usually broad and falls in the range of 2700–2326 cm.$^{-1}$.

TABLE II—INFRARED ABSORPTION BANDS OF β-AMINOKETONE DIHYDROCHLORIDES

	cm.$^{-1}$		
Compd.[a]	OH	$\overset{+}{N}H$	$-\overset{O}{\overset{\|}{C}}-$
1	3226	2500	1695
2	3247	2564	1667
3	3226	2445	1684
4	3236	2469	1681
5	3226	2500	1689
6	3175	2632 (w)	1664
7	3333	2632 (w)	1667
8	3247	2494	1667
9	3226	2439	1639
10	3125	2632 (w)	1660
11	3226	2500	1689
12	3226	2506	1689
13	3175	2632 (w)	1672
14	3257	2469	1669
15	3380, 3140	2600	1667
16	3226	2564 (w)	1689
17	3333	2469	1669
18	3333	2667 (w)	1660
19	3279	2464	1684
20	3340	2600	1660
21	3236	2564	1669

[a] As in Table I.

DISCUSSION

4-DI-PIP-OL was converted to the dihydrochloride salt and condensed with various substituted ketones and formaldehyde. The source of formaldehyde was either paraformaldehyde or 37% aqueous formaldehyde solution. Attempts to prepare the Mannich bases using sym-trioxane were not successful. In most cases the condensation reaction was successful, and the products were easily isolated. In few cases, however, difficulty was encountered in

obtaining a pure sample. It was observed that some of the β-aminoketone hydrochlorides were very hygroscopic in the crude state, but this characteristic diminishes when the product is pure. All compounds were purified by crystallization from ethanol–acetone; some Mannich bases were not very soluble in ethanol; therefore, a few drops of water were added to facilitate dissolution. The yields were relatively low for most of the products obtained. Low yields may be attributed to the complexity of the products which arise in the Mannich reaction. Attempts to prepare Mannich bases using 3-acetylindole, 2-acetylpyridine, and 3-acetylpyridine were not successful.

It has been shown by Henry (28) that the reaction of two equivalents of an aliphatic amine with one equivalent of formaldehyde produces the methylenediamine. The work by Ingwalson (29) in attempts to prepare the Mannich base with *N*-phenylpiperazine, formaldehyde, and 2-nitropropane led to the conclusion that the only product of the reaction was bis-(*N*-phenylpiperazyl)-methane, as the properties of the product correspond with those reported by Prelog and Blazek (30). In our hands while attempting to prepare Mannich base from indanedione, formaldehyde, and 3-azabicyclononane hydrochloride, methylenebis (azabicyclononane) dihydrochloride was isolated and the expected Mannich base did not form (31). It is known (32–34) that methylenebis amines react with compounds possessing active hydrogens to produce normal Mannich bases. The authors have prepared the methylenebis amine salt of 4-DI-PIP-OL and attempted the condensation of it with 4-chloroacetophenone, but without success.

SUMMARY

Twenty-one β-aminoketone dihydrochlorides have been synthesized utilizing 4-DI-PIP-OL as a secondary amine and several substituted ketones as active hydrogen components under Mannich conditions for biological screening. Characteristic infrared absorption bands for OH, $\overset{+}{N}H$, and carbonyl groups are described.

REFERENCES

(1) Mannich, C., and Krosche, W., *Arch. Pharm.*, **250**, 647(1912); through *Chem. Abstr.*, **7**, 2746(1913).

(2) Mannich, C., and Lammering, D., *Ber.*, **55**, 3510 (1922).

(3) Blicke, F. F., and Blake, E. S., *J. Am. Chem. Soc.*, **52**, 235(1930).

(4) Nisbet, H. B., and Gray, C. G., *J. Chem. Soc.*, **1933**, 839.

(5) Levvy, G. A., and Nisbet, H. B., *ibid.*, **1938**, 1053.

(6) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, **71**, 2048(1949).

(7) Denton, J. J., Schedl, H. P., Neier, W. B., and Brookfield, M., *ibid.*, **72**, 3792(1950).

(8) Wilson, W., and Kyi, Z. Y., *J. Chem. Soc.*, **1950**, 1321.

(9) Weijlard, J., Orahovats, P. D., Sullivan, A. P., Jr., Purdue, G., Heath, F. K., and Pfistar, K., *J. Am. Chem. Soc.*, **78**, 2342(1956).

(10) Mercier, F., Mercier, J., and Sestier, M. R., *J. Physiol., Paris*, **45**, 186(1953); through *Chem. Abstr.*, **47**, 11527(1953).

(11) Issekutz, B., Sr., Porszasz, J., Issekutz, L., and Nador, K., *Acta Physiol. Acad. Sci. Hung.*, **6**, 95(1954); through *Chem. Abstr.*, **49**, 3394(1955).

(12) Da Re, P., Verlicchi, L., and Setnikar, I., *J. Org. Chem.*, **25**, 1097(1960).

(13) Bockstahler, E. R., and Wright, D. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 542(1957).

(14) Florestano, H. J., and Bahler, M. E., *ibid.*, **45**, 320(1956).

(15) Profit, E., *Chem. Tech. Berlin*, **4**, 241(1952); through *Chem. Abstr.*, **47**, 10531(1953).

(16) Profit, E., *Chem. Tech. Berlin*, **3**, 210(1951); through *Chem. Abstr.*, **46**, 688(1952).

(17) Varma, R. S., and Nobles, W. L., unpublished data.

(18) Schraufstatter, E., and Deutsch, S., *Chem. Ber.*, **81**, 489(1948).

(19) Profit, E., *Chem. Tech. Berlin*, **5**, 13(1953); through *Chem. Abstr.*, **48**, 7608(1954).

(20) Burckhalter, J. H., and Johnson, S. H., Jr., *J. Am. Chem. Soc.*, **73**, 4835(1951).

(21) Nobles, W. L., Britton, S. B., and Caldwell, H. C., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 641, 644(1954); Nobles, W. L., and Caldwell, H. C., *ibid.*, **44**, 273(1955); Nobles, W. L., and Britton, S. B., *ibid.*, **44**, 717(1955); Nobles, W. L., and Burckhalter, J. H., *ibid.*, **47**, 77(1958); Nobles, W. L., and Blanton, C. D., *J. Pharm. Sci.*, **51**, 878(1962); Nobles, W. L., and Luts, H. A., *ibid.*, **51**, 273(1962).

(22) Levvy, G. A., and Nisbet, H. B., *J. Pharmacol. Exptl. Therap.*, **65**, 129(1939); through *Chem. Abstr.*, **33**, 2985(1939).

(23) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Holcomb, W. F., and Rawlins, A. L., *J. Am. Chem. Soc.*, **68**, 1894(1946).

(24) Nobles, W. L., and Thompson, B. B., *J. Pharm. Sci.*, **53**, 1554(1964).

(25) Bruening, H. C., Darling, C. M., Magarian, R. A., and Nobles, W. L., *ibid.*, **54**, 1537(1965).

(26) *Neth. Appl.*, **6**, 508, 725(1966); through *Chem. Abstr.*, **64**, 15855(1966).

(27) Thompson, W. E., Warren, R. J., Eisdorfer, I. B., and Zarembo, J. E., *J. Pharm. Sci.*, **54**, 1819(1965).

(28) Henry, L., *Bull. Acad. Roy. Med. Belg.*, **26**, (No. 3) 200(1893).

(29) Ingwalson, R. W., M.S. Thesis, University of Florida, Gainesville, Fla., 1948.

(30) Prelog, V., and Blazek, Z., *Coll. Czech. Chem. Commun.*, **6**, 549(1934).

(31) Varma, R. S., and Nobles, W. L., *J. Pharm. Sci.*, **55**, 1451(1966).

(32) Butler, G. B., *J. Am. Chem. Soc.*, **78**, 482(1956).

(33) Lieberman, S. V., and Wagner, E. C., *J. Org. Chem.*, **14**, 1001(1949).

(34) Fernandez, J. E., Powell, C., and Fowler, J. S., *J. Chem. Eng. Data*, **8**, 600(1963).

Comparison of an Ultraviolet Spectrophotometric Assay and a Gas-Liquid Chromatographic Assay for 17α-Ethynylestradiol 3-Methyl Ether in Tablets

By ARVIN P. SHROFF and JOSEPH GRODSKY

Ultraviolet spectrophotometric and gas-liquid chromatographic methods for the analysis of tablets containing 17α-ethynylestradiol 3-methyl ether and 17α-ethynyl-19-nortestosterone have been developed. The results obtained by the two methods are compared. The gas-liquid chromatographic method is found to be superior to the ultraviolet method, especially on aged tablets. A complex from tablet excipients was found to interfere with the ultraviolet method and was identified by gas-liquid chromatography.

MORE THAN 5 million women in the U. S. and about 2 million women overseas are now using oral contraceptives. These oral contraceptives are composed of estrogen–progestin mixtures. Of the seven U. S. companies which offer 11 different dosage forms, eight forms contain the estrogen, 17α-ethynylestradiol 3-methyl ether (mestranol). This paper describes the development of ultraviolet spectrophotometric and gas-liquid chromatographic methods for the analysis of mestranol in tablets containing the estrogen and norethindrone and gives the results so obtained.

The steroidal estrogens, having the characteristic phenolic A-ring, have been quantitatively assayed by utilizing the well-known Kober reaction or various modifications thereof (1–4). Colorimetric determinations (5–11) based on the coupling of the phenolic hydroxyl group with diazotized amines and spectrofluorometric methods (12–14) have been reported for a number of estrogens. All these methods, though sensitive, lack to some degree the specificity required in the presence of other steroids and tablet excipients for a rapid and accurate analytical method.

Recently, a number of other methods have been employed including ion exchange (15) and paper chromatography (16). The inaccuracy of the latter method has been discussed by Schulz (17).

Klein and co-workers (18) have described the quantitative analysis of ethynylestradiol and methyltestosterone by ultraviolet spectrophotometry. Ethynylestradiol and mestranol have also been quantitatively determined by gas-liquid chromatography (17, 19, 20). The authors failed to duplicate the mestranol determination by GLC using the reported (17) parameters since the mestranol always decomposed in part to estrone methyl ether, thus making quantitation impossible.

This investigation describes an ultraviolet spectrophotometric assay and a gas-liquid chromatographic assay for mestranol. Both assays are performed in the presence of 17α-ethynyl-19-nortestosterone (norethindrone). In the GLC determination an XE-60 coated column and an internal standard was used. The steroids do not undergo any decomposition and can be separated without prior acylation or trimethylsilyl ether formation. Practical separation (by GLC) of steroids and a nonsteroidal substance having an U.V. interfering chromophore has also been achieved. The advantages and disadvantages of the two assay methods are also discussed.

EXPERIMENTAL

U.V. Assay Procedure

Apparatus—A Beckman model DU spectrophotometer with 1-cm. cells and a Cary recording spectrophotometer model 11.

Mestranol Standard Solution—Five milligrams of mestranol was accurately weighed into a 100-ml. volumetric flask and diluted to mark with spectral grade methylcyclohexane.

Method of Analysis for Standard Mestranol—The absorbance (at slit width 0.15 mm.) of the standard mestranol solution against spectral grade methylcyclohexane was measured at 316, 302, 287.7, and 278 mμ. The absorbance at 316 and 302 mμ was zero and the exact wavelengths of the peaks at 287.7 and 278 mμ were determined and their absorbance measured. The absorptivity (a) and the ratio of the two peaks (R) were calculated as follows:

$$a = \frac{A_{287.7} \times 100 \text{ ml.}}{\text{wt. in mg.}} \qquad R = \frac{A_{278}}{A_{287.7}}$$

Method of Analysis for Mestranol-Containing Tablets—A number of tablets containing about 200 mcg. of mestranol was put into a 29.5 × 80-mm. vial and treated with 4.0 ml. of water and 4.0 ml. of methylcyclohexane. The vial was shaken on a Burrell wrist-action shaker for 15–30 min. The mixture was centrifuged and a portion of the clear upper layer was transferred to a 1-cm. cell. The absorb-

Received August 24, 1966, from the Division of Organic Chemistry, Ortho Research Foundation, Raritan, NJ 08869
Accepted for publication January 18, 1967.
The authors thank Dr. I. Scheer for interest and encouragement and Mr. H. Barth, Quality Control Department, for technical assistance in obtaining ultraviolet analyses.

ance (at slit width 0.15 mm.) was read for the sample against spectral grade methylcyclohexane at 316, 302, 287.7, and 278 mμ. Two methods of calculation were used as follows: (*a*) algebraically, a straight line was extended through 316 and 302 mμ and the peak height at 287.7 mμ was measured from this base line. The micrograms of mestranol per tablet was calculated by the following formula (see Eq. 2 for the algebraic derivation)

$$[A^{\text{obs.}}_{287.7} - 2A_{302} + A^{\text{obs.}}_{316}] \times \frac{4.0 \text{ ml.} \times 1000}{a \times \text{No. of tablets}}$$

(*b*) Algebraically, a straight line was drawn from 302 mμ at a slope to make the ratio of A_{278} and $A_{287.7}$ parallel to the ratio R determined in the standard mestranol curve. The micrograms of mestranol per tablet was calculated by the following formula (see Eq. 11 for the algebraic derivation):

$$\frac{0.714}{1.714 - R}\,[2.4A^{\text{obs.}}_{287.7} - 1.4A^{\text{obs.}}_{278} - A^{\text{obs.}}_{302}] \times \frac{4.0 \text{ ml.} \times 1000}{a \times \text{No. of tablets}}$$

The average of the two values was used. If the difference between the two values was greater than 0.006%, then it was presumed that there was too much extraneous base line absorption and another assay was used.

GLC Assay Procedure

Instrument—F and M model 1609 with modified injection port. The modified injection port was made by F and M Scientific, Avondale, Pa., to eliminate the potential for inlet sample splitting and thus create less dead volume.

Column—A stainless steel column 21 × 1/4 in. i.d., packed with coated support, was employed.

Liquid Phase—A silicone gum nitrile (*e.g.*, F and M Scientific Corp. No. LP 125, designated as XE-60) was used.

Inert Support—Untreated siliconized diatomaceous earth[1] was suitable as support material. The column packing was prepared by coating the inert support with 4% XE-60 using distilled acetone as the solvent for the application of the liquid phase.

Column Conditioning—The stainless steel packed column was conditioned by passing nitrogen carrier gas for about 12–18 hr. at a rate of 72 ml./min. at a column temperature of 240°.

Instrumental Parameters—In the development of the analytical method a column temperature of 195°, inlet port temperature of 242°, and a detector temperature of about 300° were used. The flow rate was between 70–75 ml./min. for the carrier gas, nitrogen.

Mestranol Standard—Throughout this work commercial mestranol was used which has an *a*-value at 278 mμ of 6.5, in distilled ethyl alcohol.

Internal Standard—Throughout this work, estrone U.S.P. XVI was used.

Preparation of Column Packing—In a 500-ml. round-bottom flask, place 1 Gm. of XE-60 dissolved in 100 ml. of acetone. To this add 25.0 Gm. of untreated siliconized diatomaceous earth 60/80 mesh (F and M Scientific Corp. No. ST120) and evaporate the solvent under reduced pressure (preferably a rotary flash evaporator). Heat the powder on a water bath for about 2 hr. Collect the material which falls off with gentle tapping. The yield is about 24 Gm.

[1] Diatoport-S.

Standard Mestranol Solution—Weigh accurately about 25.0 mg. of reference standard mestranol in a 50-ml. volumetric flask and dilute to mark with ethyl alcohol.

Standard Estrone Solution—Place exactly 50.0 mg. of estrone U.S.P. XVI in a 50-ml. volumetric flask and dilute to mark with ethyl alcohol.

Preparation of a Typical Standard Curve—Pipet into three different vials exactly 1.0 ml. of the standard estrone solution. Evaporate to dryness over a steam bath and under a stream of nitrogen. To each vial add 0.80 ml. (about 400 mcg.), 1.0 ml. (about 500 mcg.), and 1.2 ml. (about 600 mcg.) of standard mestranol solution, respectively. Evaporate once again to dryness over a steam bath and under a stream of nitrogen. Dissolve each residue in 0.5 ml. of distilled acetone. Inject separately 6–7 μl. of each sample in the GC and adjust the attenuator to 320. Elute for at least 1 hr. Measure the areas of the two peaks separately after drawing a base line tangent to both minima of each peak. The area is obtained by multiplying the height of the peak by the width at one-half the peak height. The major peak close to the solvent front corresponds to mestranol and the one farthest away corresponds to estrone. A standard curve is obtained by plotting the ratio of the areas under mestranol and estrone *versus* the weight of mestranol and drawing the best straight line.

Analysis of Tablets—Place a number of tablets equivalent to about 1,000 mcg. of mestranol in a 29.5 × 80-mm. vial. To this add 10 ml. of distilled water and 10 ml. of methylcyclohexane (spectroquality reagent) by means of a pipet. Clamp the vial to a Burrel wrist-action type shaker and shake vigorously for 0.5 hr. and then centrifuge for 2 min. Pipet 5.0 ml. of the supernatant solution into a 21 × 50-mm. vial and evaporate to dryness over a steam bath and a stream of nitrogen. Add exactly 1.0 ml. of the standard estrone solution and once again evaporate as above. Treat this residue with 0.5 ml. of acetone and inject (6–7 μl.) into the GC. The attenuator is adjusted to 320. After at least 1 hr. the areas corresponding to the peaks of mestranol and estrone are calculated as indicated previously. (*Note:* There is an additional major peak due to norethindrone which appears between mestranol and estrone.) Using the ratio of these areas (mestranol/estrone) on the standard curve, find the amount of mestranol.

ALGEBRAIC DERIVATION FOR U.V. CALCULATIONS

Figure 1 represents an ultraviolet spectrum of norethindrone and Figs. 2 and 3 represent typical expanded scale spectra of mestranol. The algebraically extended lines in Figs. 2 and 3 are hypothetically magnified to explain the derivation for the two formulas used in mestranol calculation.

According to Fig. 2:

$$A^{\text{corr.}}_{287.7} = A^{\text{obs.}}_{287.7} - \text{PN} - A^{\text{obs.}}_{302} \qquad \text{(Eq. 1)}$$

Since triangle MPN = Δ302M316

$$\text{PN} = A^{\text{obs.}}_{302} - A^{\text{obs.}}_{316}$$

therefore,

$$A^{corr.}_{287.7} = A^{obs.}_{287.7} - 2A^{obs.}_{302} + A^{obs.}_{316} \quad \text{(Eq. 2)}$$

In Fig. 3:

$$A^{corr.}_{287.7} = A^{obs.}_{287.7} - X - A^{obs.}_{302} \quad \text{(Eq. 3)}$$

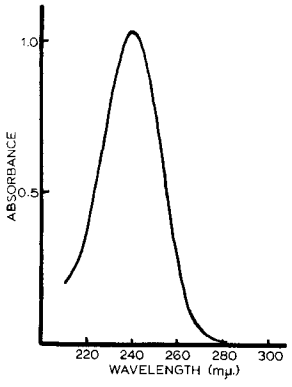


Fig. 1—U.V. absorption spectrum of norethindrone.

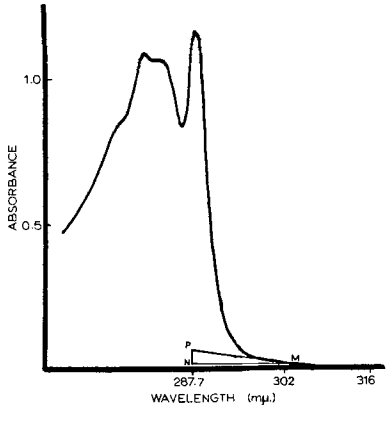


Fig. 2—U.V. spectrum of mestranol shown with the base line correction for Eq. 2.

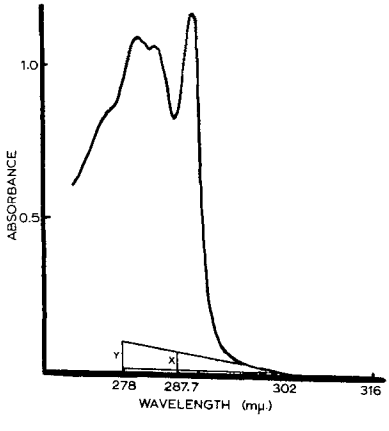


Fig. 3—U.V. spectrum of mestranol shown with base line correction for Eq. 11.

and

$$A^{corr.}_{278} = A^{obs.}_{278} - Y - A^{obs.}_{302} \quad \text{(Eq. 4)}$$

where

$$Y = \frac{302\ m\mu - 278\ m\mu}{302\ m\mu - 288\ m\mu} \times X \text{ (similar triangles)}$$

or

$$Y = 1.714\ x$$

Also Eq. 4 is equal to:

$$RA^{obs.}_{287.7} - RX - RA^{obs.}_{302} \quad \text{(Eq. 5)}$$

where R is the ratio of $A^{corr.}_{278}$ to $A^{corr.}_{287.7}$ and is obtained from a standard curve. By substituting the value of Y in Eq. 5 one obtains:

$$A^{obs.}_{278} - 1.714x - A^{obs.}_{302} = RA^{obs.}_{287.7} - RX - RA^{obs.}_{302} \quad \text{(Eq. 6)}$$

or

$$(1.714 - R)X = A^{obs.}_{278} - RA^{obs.}_{287.7} - A^{obs.}_{302} + RA^{obs}_{302}$$

and

$$X = \frac{A^{obs.}_{278} - RA^{obs.}_{287.7} - A^{obs.}_{302} + RA^{obs.}_{302}}{1.714 - R}$$

Substituting the value of X in Eq. 3 gives:

$$A^{corr.}_{287.7} = A^{obs.}_{287.7} - \left\{\frac{A^{obs.}_{278} + RA^{obs.}_{287.7} + A^{obs.}_{302} - RA^{obs.}_{302}}{1.714 - R}\right\} - A^{obs.}_{302} \quad \text{(Eq. 7)}$$

Multiplying Eq. 7 by $(1.714 - R)$ gives:

$$(1.714 - R)A^{corr.}_{287.7} = 1.714^{obs.}_{287.7} - RA^{obs.}_{287.7} - 1.714A^{obs.}_{302} + RA^{obs.}_{302} - A^{obs.}_{278} + RA^{obs.}_{287.7} + A^{obs.}_{302} + RA^{obs.}_{302} \quad \text{(Eq. 8)}$$

Solving Eq. 8 gives:

$$(1.714 - R)A^{corr.}_{287.7} = 1.714A^{obs.}_{287.7} - 0.714A^{obs.}_{302} - A^{obs.}_{278} \quad \text{(Eq. 9)}$$

Dividing Eq. 9 by (0.714) gives:

$$\left(\frac{1.714 - R}{0.714}\right)A^{corr.}_{287.7} = 2.4A^{obs.}_{287.7} - 1.4A^{obs.}_{278} - A^{obs.}_{302} \quad \text{(Eq. 10)}$$

or

$$A^{corr.}_{287.7} = \frac{0.714}{1.714 - R}\left[2.4A^{obs.}_{287.7} - 1.4A^{obs.}_{278} - A^{obs.}_{302}\right] \quad \text{(Eq. 11)}$$

RESULTS AND DISCUSSION

Tables I through IV summarize the data obtained in determining the accuracy, precision, and standard deviation for the ultraviolet spectrophotometric and gas-liquid chromatographic methods. The data indicate that both methods are in excellent agreement and can be used for routine assays. Therefore, several samples of formulation, prepared for clinical study, were subjected to the improved extraction procedure and analyzed by these two methods. The excipients of these formulated tablets consisted of lactose, cornstarch, magnesium stearate, and polyvinylpyrrolidone.

The results of the ultraviolet assay on tablets stored under various conditions are listed in Table V. The ultraviolet assay data on the original granulations included in the table agree with the theoretical amount of mestranol added. It is evident from the results that, on storage, there is an apparent decrease in mestranol content. This decrease could be either the result of an incomplete extraction or partial mestranol degradation or to extraneous material which gives a faulty algebraic base line. The latter enters into the calculation and could be responsible for low results.

To determine if mestranol was incompletely extracted or was partially degraded the gas-liquid chromatographic technique was used. Both pure and extracted mestranol showed no decomposition on the 4% XE-60 column. The tablets from the same lot used in the U.V. assay were subjected to the extraction procedure and analysis. The analysis by GLC was carried out using an internal standard technique. The data are summarized in Table VI. It is evident from the results that mestranol does not undergo any decomposition on storage, and that complete extraction was achieved.

The question of extraneous material being extracted from the tablets was also answered by the GLC technique. During the different GLC assays on aged tablets prominent unidentified peaks near the solvent front (Fig. 4) were noticed. These were negligible with fresh tablets. It had also been established that these peaks were not the normal degradation products of mestranol, norethindrone, and estrone. Working with the excipients of the tablets, these peaks were later identified as being generated from a complex formed between polyvinylpyrrolidone and magnesium stearate. This complex has an absorption in the ultraviolet which distorts the base line and invalidates both correction factors. The low results obtained in Table V are thus attributed to these base line distortions.

A comparison of the two methods suggests that the ultraviolet assay is not reliable for aged tablets because of the interfering chromophore. However, it is quite suitable for fresh tablets. The GLC assay is sensitive for both fresh and aged tablets. An additional advantage of the GLC technique is that it offers quantitation concurrently with fractionation, the latter being important in aged tablets. In conclusion, this study points out that mestranol does not undergo any degradation at various storage conditions.

TABLE I—ACCURACY OF THE U.V. METHOD OF ANALYSIS[a]

No.	Mestranol Added, mcg.	Mestranol Recovered,[b] mcg.	% Recovery
1	198.8	193.5	97.3
2	198.8	192.5	96.8
3	198.8	193.0	97.1
4	198.8	193.5	97.3
5	198.8	202.5	102.0

[a] $\bar{X}$ = 98.1%; σ = ±2.2%. [b] Average of two calculations.

SUMMARY

1. Ultraviolet spectrophotometric and gas-liquid chromatographic assay methods are described and compared.

2. The gas-liquid chromatographic assay method is shown to be sensitive for mestranol in both fresh and aged tablets.

3. A complex between polyvinylpyrrolidone and magnesium stearate is identified by GLC.

4. Mestranol does not undergo any decomposition when stored under various conditions.

TABLE III—ACCURACY OF THE GLC METHOD[a]

No.	Mestranol Added, mcg.	Mestranol Recovered, mcg.	% Recovery
1	493	481	97.6
2	493	495	100.4
3	493	485	98.4
4	500	495	99.0
5	500	501	100.2

[a] $\bar{X}$ = 99.1%; σ = ±1.2%.

TABLE IV—PRECISION OF THE GLC METHOD[a]

No.	1	2	3	4
Mestranol assayed, mcg.	103.7	106.4	105.6	102.0
No.	5	6	7	8
Mestranol assayed, mcg.	104.2	102.0	103.8	105.6

[a] $\bar{X}$ = 104.2; σ = ±1.64%.

TABLE V—MESTRANOL ASSAY OF VARIOUS TABLETS BY U.V.

Lot	Storage[a] Conditions	Original Granulation[b] U.V. Assay, mcg./Tablet	mcg./Tablet by U.V.	% Recovery
A	6 wk. at 100/80	51.1	42.5	83.2
B	6 wk. at 100/80	51.3	44.3	86.4
C	3 mo. at 100/80	51.3	42.8	83.4
D	6 mo. at 37°	80.0	71.7	89.6
E	7.5 mo. at RT	49.9	43.6	87.4
F	12 mo. at RT	56.0	51.2	91.4
G	12 mo. at RT	56.0	51.2	91.4
H	13 mo. at RT	51.5	45.6	88.5
I	18 mo. at RT	57.6	48.9	84.5

[a] 100/80 refers to 100°F. and 80% humidity. RT refers to room temperature. [b] Values within acceptable region of the theoretical value.

TABLE II—PRECISION OF THE U.V. METHOD OF ASSAY[a]

No.	1	2	3	4	5	6	7	8	9	10
Mestranol assayed, mcg.	98.6	100.1	101.4	101.7	101.4	99.6	99.2	98.9	101.0	99.7

[a] $\bar{X}$ = 100.16; σ = ±1.14.

TABLE VI—MESTRANOL ASSAY OF VARIOUS TABLETS BY GLC

Lot	Storage Conditions[a]	Original Granulation[b] U.V. Assay, mcg./ Tablet	mcg./ Tablet by GLC	% Recovery
A	6 wk. at 100/80	51.1	48.0	94
B	6 wk. at 100/80	51.3	51.8	100.9
C	3 mo. at 100/80	51.3	51.3	100.0
D	6 mo. at 37°	80.0	84.3	105.4
E	7.5 mo. at RT	49.9	51.6	103.4
F	12 mo. at RT	56.0	55.1	98.4
G	12 mo. at RT	56.0	55.0	98.2
H	13 mo. at RT	51.5	52.2	101.4
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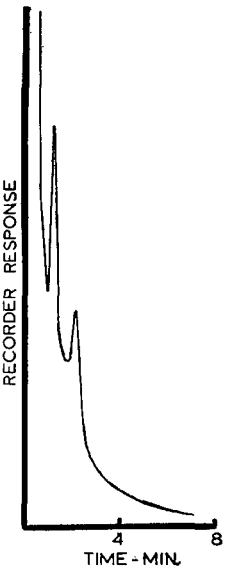

Fig. 4—GLC separation of a complex between polyvinylpyrrolidone and magnesium stearate.

REFERENCES

(1) Kober, S., *Biochem. Z.*, **239**, 209(1931).
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(4) McKerns, K. W., *Biochem. Biophys. Acta*, **69**, 417 (1963).
(5) Schmulowitz, J. J., and Wylie, H. B., *J. Lab. Clin. Med.*, **21**, 210(1935).
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(12) Slaunwhite, W. R., Engle, L. L., Olmsted, P. C., and Carter, P., *J. Biol. Chem.*, **191**, 627(1951).
(13) Boscott, R., *Nature*, **162**, 577(1948).
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(17) Schulz, E. P., *ibid.*, **54**, 144(1965).
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Diffusion of Drugs Across the Isolated Mesentery

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In order to obtain knowledge of the diffusion rates of pentobarbital, salicylic acid, and urea across the peritoneal membrane under ideal conditions, experiments with isolated rabbit mesentery were conducted. Diffusion coefficients of the drugs were applied to evaluate rates of dialysis at various conditions of pH. Pentobarbital and salicylic acid diffused across the isolated mesentery in both ionic and nonionic form, with no significant difference in rate due to pH. *In vivo* tests also make it appear that the ionized form of drugs may diffuse across the peritoneal membrane. These results suggest that the research for agents to promote dialysis should include substances other than alkalizing agents.

THE REMOVAL of drugs in poisonings by peritoneal dialysis has been the subject of many studies. Salicylates and barbiturates have been most commonly investigated due to the frequency with which they are involved in poisoning cases. The rates at which these drugs have been removed by peritoneal dialysis with the usual dialysis fluids have not been encouraging, and it is desirable that improved fluids and techniques be developed for this purpose. To proceed logically in this effort a more thorough knowledge is required of the mechanism by which drugs pass the membrane and the maximum rates which might be attained.

Berndt and Gosselin (1–3) indicated that the diffusion of ions and molecules through the peritoneal membrane may take place through pores.

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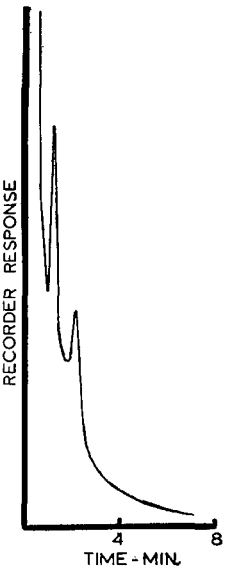

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Knochel *et al.* (4, 5) and Nahas and co-workers (6, 7) assumed that only the unionized forms of drugs pass the membrane and on this basis explained the effects of alkaline dialysis fluids on diffusion rates. Engel and Kerekes (8) claimed that the diffusion of a compound from the blood stream into the peritoneal cavity depends primarily on the chemical structure of the compound rather than its physical state.

The present investigation was undertaken to learn more of the nature of drug diffusion across the peritoneum *via* direct measurements on isolated membranes. The rabbit mesentery was selected for study, since the mesentery is the portion of the peritoneum thought to be most effective in dialysis. It was planned to determine the diffusion rates of pentobarbital and salicylate in solution at different pH values, thus to determine whether both ionized and unionized forms of the molecules diffuse. From the diffusion constants obtained, one might predict the maximum rates obtainable under ideal conditions. Urea, being unionized over a wide range of pH, was included in the study to evaluate direct effects of pH on the membranes.

MATERIALS AND METHODS

In order to obtain quantitative measurements on small volumes of dialysate, radioactive compounds were used: pentobarbital-2-^{14}C,[1] salicylic acid-1-^{14}C,[2] and urea-^{14}C.[3] Potassium chloride, A.R., was employed for calibration purposes. All other chemicals were of analytical reagent quality.

White grid Millipore membranes were selected for measurement of diffusion coefficients. It was felt that they would provide fast rates of diffusion and would fit the apparatus designed for animal membranes. The Millipore membranes used in this study had the following specifications: 37 mm. diameter, 0.45 ± 0.02 μ pore size, 150 ± 10 μ dry thickness, and porosity of 0.79 (9).

Isotonic solutions were used in all studies involving pentobarbital, salicylic acid, and urea. These solutions contained 0.005 moles/L. of $Na_2HPO_4 \cdot 7\ H_2O$, calculated quantities of 1 *N* HCl or NaOH to give the required pH, and sufficient NaCl to make the solution isotonic. The pH of all solutions were tested by measurement just prior to and following dialysis experiments.

The diffusion apparatus was constructed from two 125-ml. conical flasks. A 4-cm. tube with an expanded ground joint was sealed to the side of each flask. These joints could be clamped together, enclosing the membrane, by either a threaded or screw type clamp. The inside diameters of the joints were accurately measured. Stirring in both flasks was effected by glass stirrers driven by variable speed motors controlled by rheostats. The diffusion apparatus was mounted in a water bath at 37° ± 0.1°. A diagram of the diffusion apparatus is shown in Fig. 1.

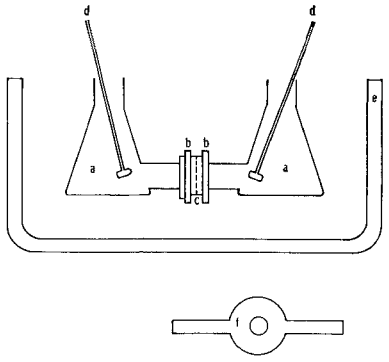

Fig. 1—Diffusion apparatus. Key: a, *conical flasks;* b, *aluminum clamp;* c, *membrane;* d, *glass stirrers;* e, *water bath;* f, *Teflon gaskets for mesentery membrane.*

EXPERIMENTAL PROCEDURE

Diffusion Through Millipore Membranes—A Millipore membrane, which had been previously soaked in water for at least 12 hr., was mounted between the two compartments and the joints were clamped. Then the apparatus was immersed in the water bath, leveled, and locked in position with a clamp. One hundred and fifty milliliters of potassium chloride solution in double distilled water, or of the drug in the isotonic solution of particular pH, was introduced into one compartment. A volume of double distilled water, in the case of KCl diffusion, or isotonic solution of the same pH, in the case of other substances, was introduced into the second compartment. Three different volumes, 150, 155, and 158 ml., were used in the latter compartment, depending on its capacity, in order to bring the liquid inside to the same level as that of the source solution. All solutions were equilibrated at 37° before use. The liquids in both compartments were stirred vigorously without creation of a vortex. Periods of 30 min. in the case of diffusion of potassium chloride, urea, and salicylic acid, and 90 min. in the case of pentobarbital, were allowed before the first sample was withdrawn from the sink compartment for analysis. Additional samples were removed every 10 min. for a period of 90 min. for potassium chloride diffusion, every 15 min. for a period of 2.5 hr. for urea, every 30 min. for a period of 4 hr. for salicylic acid, and every 30 min. for a period of 6 hr. for pentobarbital. Samples were withdrawn with a calibrated pipet which delivered 0.128 ml.

The Fajan's titration method (10) was used to assay potassium chloride samples. A manually operated microburet was utilized to deliver the 0.1 *N* silver nitrate titrant. Radioactivity measurement was employed with the samples withdrawn during diffusion of pentobarbital, salicylic acid, and urea. Each sample was added to a counting vial which contained 10 ml. of dioxane phosphor solution. The latter was prepared by dissolving 7.0 Gm. PPO, 2,5-diphenyloxazole, scintillation grade, 0.05 Gm. POPOP, (1,4-bis 2,5-diphenyloxazolyl)-ben-

[1] Supplied through the courtesy of Abbott Laboratories, North Chicago, Ill.
[2] Nuclear Chicago Corp., Des Plaines, Ill.
[3] Tracerlab, Waltham, Mass.

zene, scintillation grade, 50 Gm. of naphthalene, and 50 ml. of water in *p*-dioxane to make 1 L. The vials were counted in a Tri-Carb liquid scintillation counter using the average of three sets of counts. Suitable blanks and standards were counted with each set of tests. Results were calculated as the amount diffused over the given time interval.

Diffusion Through Isolated Mesenteric Membrane—Albino rabbits were sacrificed with intravenous pentobarbital. Immediately after death, the abdomen was opened and the broad, fan-shaped fold of the peritoneum connecting the convolutions of the jejunum and ileum with the posterior wall of the abdomen was separated. The separated tissue was immediately placed in normal saline solution and used for diffusion or stored in the refrigerator until used. When tissue was stored for longer than 24 hr., the saline was replaced daily. Membranes properly stored were not found to change in diffusion properties over periods up to 15 days. In several cases duplicate tests were performed on the same membrane to confirm this.

A piece of the mesenteric membrane freed from fat and blood vessels was mounted between a pair of Teflon gaskets, $^1/_{16}$ in. thickness, care being taken to avoid stretching the membrane. These gaskets had an outside diameter of $^7/_{16}$ in. and center opening which exposed an area of membrane of 1.266 cm.^{2} for diffusion.

The mesenteric membrane mounted between the Teflon gaskets was firmly clamped in the diffusion apparatus by means of a screw type clamp and the membrane was tested to insure that there was no leakage before use. The diffusion procedure was the same as previously described.

Since it was of some concern that the isolated membrane might in some way function differently from that in the live animal, a simple *in vivo* test was made to see whether both ionic and nonionic forms of a drug may diffuse. For this purpose it was decided to measure the transfer in the reverse direction, from peritoneum to blood, since pH of the dialysis fluid can be altered and buffered. Two tests were run on rabbits with pentobarbital in the dialysis fluid, using 100 mg. ^{14}C tagged pentobarbital in 150 ml. of intraperitoneal injection. In one rabbit an acid fluid consisting of isotonic phosphate buffer, pH 2.6, 0.15 *M*, was used; in the second an isotonic THAM solution, 0.15 *M*, was used. The concentration of nonmetabolized drug in the dialysate and pH of the dialysate were determined in samples removed in approximately 10-min. intervals over a period of about 100 min. Pentobarbital was measured by the method of Brodie *et al.* (11) but utilizing radioactive counting of the extracts rather than spectrophotometric measurement.

Results and Treatment of Data—The rate of diffusion between two well-stirred compartments separated by a membrane can be represented by Eq. 1. The detailed derivation of this equation is discussed by Lueck *et al.* (12).

$$\log_{10}\left[\left(1+\frac{V_1}{V_2}\right)C_1-\frac{V_1}{V_2}C_0\right]=-\left(\frac{V_1+V_2}{V_1V_2}\right)\frac{DA\epsilon}{2.303h}t+\log_{10}C_0 \quad \text{(Eq. 1)}$$

where

V_1 = volume of solution in source compartment in ml.,
V_2 = volume of solution in sink compartment in ml.,
C_1 = concentration of diffusing substance in source compartment at time, t,
C_0 = initial concentration of penetrant in source compartment,
D = diffusion coefficient of the penetrant in cm.^{2}/min.,
A = surface area of membrane in cm.^{2},
ϵ = porosity of membrane,
h = thickness of the membrane in cm.

In special cases where $V_1 = V_2$, Eq. 1 reduces to

$$\log_{10}(2C_1-C_0)=-\frac{2DA\epsilon}{2.303Vh}t+\log_{10}C_0 \quad \text{(Eq. 2)}$$

A plot of lefthand side of Eqs. 1 or 2 *versus* time should yield a straight line. From the slope of this line and known values of the cell constant the diffusion coefficient can be readily calculated.

The cell constant is obtained from diffusion measurements of a substance of a known diffusion coefficient with the same apparatus and membrane by dividing the slope of the line, from Eq. 1, by D. Thus,

$$\text{cell constant}=\frac{V_1+V_2}{V_1V_2}\cdot\frac{A\epsilon}{2.303h} \quad \text{(Eq. 3)}$$

or

$$\frac{2A\epsilon}{2.303hV} \quad \text{(Eq. 4)}$$

where $V_1 = V_2$.

The diffusion of 1 *M* potassium chloride through Millipore membranes was measured and using the known diffusion coefficient of 1.4356×10^{-3} cm.^{2}/min. (13) the cell constant was calculated. The mean value of nine experiments was 0.7264 cm.$^{-2}$ with a standard deviation of 0.0054 cm.$^{-2}$. With same apparatus and same type of membrane the diffusion of pentobarbital, salicylic acid, and urea was measured, and their diffusion coefficients were calculated as described above. These values, each the average of six measurements, were: for pentobarbital, 5.917×10^{-4} cm.^{2}/min.; salicylic acid, 6.901×10^{-4} cm.^{2}/min.; and urea, 1.018×10^{-3} cm.^{2}/min. Diffusion measurements at several pH values revealed no significant difference in diffusion coefficients with the Millipore membranes. A typical plot of diffusion data by Eq. 2 is presented in Fig. 2.

Measurements were then made of the diffusion of pentobarbital, salicylic acid, and urea through isolated mesentery at several pH values in isotonic solution. The data were plotted according to Eqs. 1 or 2, and results were calculated as permeability coefficients, $\frac{D\epsilon}{h}$, by dividing the slopes by the area of membrane exposed and multiplication by 2.303 and the appropriate volume factors. Coefficients obtained are presented in Tables I–III. Typical diffusion curves are shown in Figs. 3–5.

These data demonstrate that the three compounds diffused quite rapidly across the mesentery. For pentobarbital, the data indicated a slight

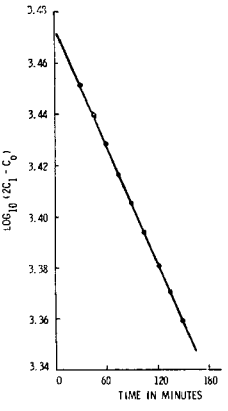

Fig. 2—Typical diffusion curve with Millipore membrane. Urea at pH 7.4.

decrease in permeability with increase in pH, but the differences are not significant when examined by statistical analysis. Using a value of 7.66 for the apparent dissociation constant of pentobarbital, from Krahl (14), it is estimated that the compound is completely dissociated at pH 11.7, 84.6% at pH 8.4, 35.5% at pH 7.4, and completely undissociated at pH 3.7. If the only species to diffuse were the undissociated molecule, one would expect great differences in diffusion rates with the different pH values used. Thus, one must conclude that both ionic and nonionic forms diffuse with approximately equal facility.

The diffusion rates of salicylic acid show that this compound diffused equally rapidly at pH 3.0 and 7.4. Salicylic acid is 50% dissociated at pH 3.0 and completely dissociated at pH 7.4. Thus, both species appear to diffuse equally well across the mesentery.

The permeability coefficients for urea at different pH values were essentially the same, as seen in Table III. This indicates that there was no appreciable effect of pH on the membranes, urea being undissociated at the pH range studies.

In several cases urea was run on membranes which had been previously used for pentobarbital. The ratios of diffusion rates of pentobarbital to urea were used to determine whether the membrane had been affected by the experimental conditions. These ratios were comparable to the ratio of the individual diffusion coefficients of the compounds, indicating no change in the performance of the membranes.

Although it is not known whether pores exist in the mesentery membrane, it is of interest to calculate the "apparent porosity" of these membranes. Such values give one a means of comparing penetrability of mesentery with other membranes and of predicting amounts which might diffuse under a variety of conditions. "Apparent porosities" can be calculated from average permeability coefficients, the measured diffusion coefficients for the three compounds, and the value of 38.5 μ for the thickness of the membrane (15). These calculations gave an average porosity of 29%, which is quite high as compared to most membranes. Calculating porosities from permeabilities with the different drugs, the values from pentobarbital data ranged from 22 to 36%, those from urea, 20 to 35%, and from salicylic acid, 29 to 39%. These differences in values for different drugs were not significantly greater than the variations between individual membranes with a single drug.

Results of the *in vivo* tests are presented in Table IV and curves of dialysate concentrations with time are shown in Fig. 6. From the data it is seen that the pentobarbital diffused rapidly at both high and low pH. This suggests that both ionic and nonionic forms diffuse, as was found with the isolated membrane.

TABLE I—PERMEABILITY COEFFICIENTS FOR PENTOBARBITAL THROUGH ISOLATED RABBIT MESENTERY AS A FUNCTION OF pH AT 37° C.

Series[a]	pH	Permeability Coefficient cm. min.$^{-1}$ × 10^3 Expt. *1*	Expt. *2*	Expt. *3*
B_{1-1}	3.7	55.8	53.9	...
B_{2-1}	3.7	37.4	52.8	...
	7.4	44.2	44.9	46.8
	11.7	40.7	43.0	44.2
B_{2-2}	3.7	50.1	45.7	...
	7.4	58.7	53.6	53.2
	8.4	43.0	48.5	...
B_{3-1}	3.7	34.0	...	...
	7.4	33.0	...	...
	8.4	32.0	...	...
B_{3-2}	3.7	42.5	...	...
	7.4	39.2	...	...
	8.4	37.5	...	...

[a] The first subscript represents rabbit number, and second subscript represents membrane number.

TABLE II—PERMEABILITY COEFFICIENTS FOR SALICYLIC ACID ACROSS ISOLATED RABBIT MESENTERY AS A FUNCTION OF pH AT 37° C.

Series[a]	pH	Permeability Coefficient cm. min.$^{-1}$ × 10^3
B_{4-1}	3.0	59.3
	7.4	66.0
B_{4-2}	3.0	60.6
	7.4	56.7
B_{5-1}	3.0	71.1
	7.4	68.0
B_{5-2}	3.0	53.6
	7.4	55.4

[a] First subscript represents rabbit number, and the second designates membrane number.

TABLE III—PERMEABILITY COEFFICIENTS FOR UREA THROUGH ISOLATED RABBIT MESENTERY AS A FUNCTION OF pH AT 37° C.

Series[a]	pH	Permeability Coefficient cm. min.$^{-1}$ × 10^3
B_{2-2}	7.4	79.9
B_{3-1}	7.4	52.3
B_{3-2}	7.4	67.8
B_{3-3}	3.7	69.5
	7.4	66.0
	8.4	68.6

[a] The first subscript represents rabbit number, and second subscript represents membrane number.

DISCUSSION AND CONCLUSIONS

This work clearly demonstrates that both ionic

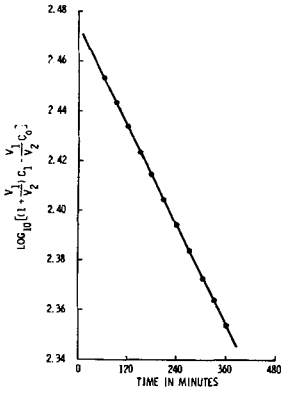

Fig. 3—Typical diffusion curve for pentobarbital with mesentery membrane, pH 7.4.

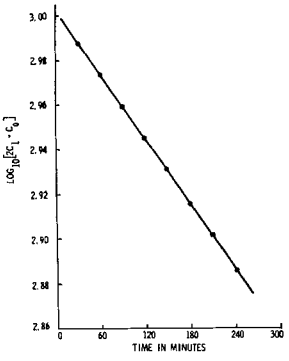

Fig. 4—Typical diffusion curve for salicylic acid with mesentery membrane, pH 7.4.

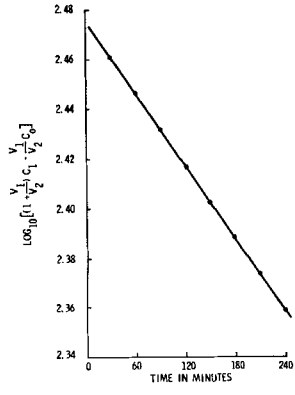

Fig. 5—Typical diffusion curve for urea with mesentery membrane, pH 7.4.

Table IV—Diffusion of Pentobarbital Across Peritoneum from Acid and Alkaline Peritoneal Fluid

Time, min.	Concn. Drug in Fluid, mcg./ml.	pH of Fluid	Concn. Unionized Drug, mcg./ml.
	THAM[a]		
0	666	8.7	59
15	347	8.6	35
25	261	8.5	34
35	168	8.2	37
45	120	8.0	38
55	89	8.0	28
65	73	7.8	31
75	64	7.8	28
85	55	7.8	24
95	40	7.7	19
	Acid Phosphate[b]		
0	666	2.6	666
8	533	2.6	533
15	443	2.6	443
25	341	2.6	341
35	302	2.6	302
60	270	2.6	270
70	224	2.6	224
80	222	2.6	222
90	209	2.6	209
100	188	2.6	188

[a] Animal No. 19, weight 4.18 Kg. [b] Animal No. 21, weight 3.56 Kg.

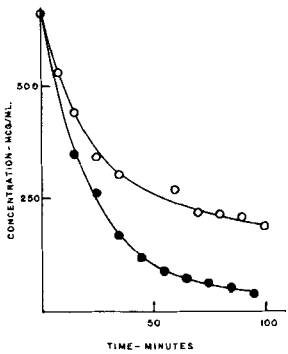

Fig. 6—Drop in concentration of pentobarbital in peritoneal fluid with time. Key: ○, acid fluid; ●, alkaline.

and nonionic forms of salicylate and pentobarbital diffuse across the isolated mesenteric membrane. Since the same buffer is on both sides of the membrane in the *in vitro* tests it is inconceivable that neutralization at the surface or within the membrane could occur. Whether this truly represents the transport in the live animal is difficult to test directly, since under all biological conditions the blood and extracellular fluid must be maintained at nearly normal pH, and the buffer substances of the blood may maintain neutrality within the membrane. The *in vivo* test strongly indicates, however, that the ionic form of pentobarbital does diffuse across the peritoneal membrane, since otherwise the concentration gradient of unionized molecules in the alkaline fluid would be low enough to have a pronounced effect on the dialysis rate. Rather than retard the diffusion, the THAM appeared to increase the rate. Other data (16) on effect of THAM on dialysis of urea show an increased rate of dialysis which cannot be attributed to pH effect.

These data suggest that the effects of THAM on rates of removal of drugs by peritoneal dialysis are not primarily a pH effect and that the search for new agents to improve the rates of dialysis should go beyond the testing of alkalizing substances.

REFERENCES

(1) Berndt, W. O., and Gosselin, R. E., *Am. J. Physiol.*, **200**, 454(1961).
(2) Berndt, W. O., and Gosselin, R. E., *Biochem. Pharmacol.*, **8**, 359(1961).
(3) Berndt, W. O., and Gosselin, R. E., *Am. J. Physiol.*, **202**, 761(1962).
(4) Knochel, J. P., Clayton, L. E., Smith, W. L., and Barry, K. G., *J. Lab. Clin. Med.*, **64**, 257(1964).
(5) Knochel, J. P., and Barry, K. G., *ibid.*, **65**, 361(1965).
(6) Nahas, G. G., Gjessing, J., Verosky, M., and Mark, L. C., *Trans. Am. Soc. Artif. Int. Organs*, **10**, 345(1964).
(7) Nahas, G. G., Gjessing, J., Giroux, J. J., Verosky, M., and Mark, L. C., *Clin. Pharmacol. Therap.*, **6**, 560(1965).

(8) Engel, D., and Kerekes, A., *Klin. Wochschr.*, **5**, 1709 (1962); through *Chem. Abstr.*, **21**, 133(1927).

(9) Millipore Technical Brochure, MF-64, Millipore Filter Corp., Bedford, Mass., 1964, pp. 2–7.

(10) Vogel, A. I., "A Textbook of Quantitative Inorganic Analysis," 3rd ed., John Wiley & Sons, Inc., New York, N. Y., 1961, pp. 79–81.

(11) Brodie, B. B., Burns, J. J., Mark, L. C., Lief, P. A., Berstein, E., and Papper, E. M., *J. Pharmacol. Exptl. Therap.*, **109**, 26(1963).

(12) Lueck, L. M., Wurster, D. E., Higuchi, T., Lemberger, A. P., and Busse, L. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 694(1957).

(13) Shenouda, L. S., "The Diffusion of Drugs and Chemicals Through Isolated Rabbit Mesentery," Doctoral Dissertation, The University of Michigan, Ann Arbor, Mich., 1965.

(14) Krahl, M. E., *J. Phys. Chem.*, **44**, 449(1940).

(15) Gosselin, R. E., and Berndt, W. O., *J. Theoret. Biol.*, **3**, 487(1962).

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Absorption and Activity of Some Derivatives of Griseofulvin

By L. J. FISCHER* and S. RIEGELMAN

Some derivatives of the 4′-keto group of griseofulvin were prepared which had increased water solubility and greater absorption potential. The derivatives, which exhibited little or no *in vitro* activity against *Microsporum gypseum*, were found to be converted to griseofulvin *in vivo*. After intravenous administration to rabbits, several of the derivatives were rapidly converted to griseofulvin. Griseofulvin plasma levels resulting from oral doses of the derivatives were found to be equal to, or in most instances significantly higher than, the levels produced by equal doses of griseofulvin, but of shorter duration. Evidence for the degradation of the derivatives in the gastrointestinal tract is presented.

PREVIOUS REPORTS have shown griseofulvin to be irregularly and incompletely absorbed from the gastrointestinal tract of man (1–3) and laboratory animals (4, 5). The incomplete absorption, which often produces low blood and tissue levels, appears to be a result of the slow rate of dissolution of the drug in the gastrointestinal fluids due to its extremely low solubility in water. Fischer and Riegelman (6) have shown that the solution-rate-limited absorption of griseofulvin in rabbits was by a pseudo zero-order process and that the drug was eliminated rather rapidly ($t^1/_2$ = 77 ± 14 min.) from the blood following intravenous injection.

Numerous attempts have been made to increase blood levels resulting from an oral dose of the drug. By increasing the specific surface area of orally administered griseofulvin threefold, many workers have reported experimental animal and human peak blood levels to be approximately twice those obtained with the same dose of regular particle size griseofulvin (7–14). However, Crounse (13) has observed that a double dose of microcrystalline griseofulvin did not significantly raise serum levels in some individuals and that these levels may still fall short of the proper inhibitory levels for the antibiotic. The administration of surfactants with an oral dose of griseofulvin generally has produced erratic results in animals (9, 12) and has not significantly increased blood levels in man (14). The oral administration of griseofulvin with lipids or after meals with high fat content has been shown to increase blood levels (9, 13, 15); however, the mechanism by which absorption is facilitated by lipids has not been clarified.

Although many attempts have been made to increase griseofulvin absorption by various methods, no previously published report has investigated the preparation and administration of derivatives of griseofulvin having increased water solubility and greater absorption potential. The derivatives were chosen so that active griseofulvin would be a possible product of their enzymatic metabolism in the body. The more water-soluble derivatives were expected to be absorbed more completely from the gastrointestinal tract, and if they were converted rapidly to griseofulvin in the body, increased blood levels of the antibiotic could be expected. In this manner the concept of drug latentiation was employed to increase plasma levels of griseofulvin following the oral administration of some derivatives of the 4′-carbonyl group of griseofulvin.

The derivatives of griseofulvin (I) selected to be prepared and tested were griseofulvin-4′-alcohol (II), griseofulvin-4′-oxime (III), griseofulvin-4′-carboxymethoxime (IV), and griseofulvin-4′-hemisuccinate (V). Griseofulvin-4′-

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Accepted for publication December 27, 1966.

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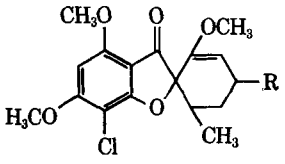
alcohol was selected for testing in this system because of a previous report suggesting *in vivo* activity in infected guinea pigs (16). Such secondary alcohols are converted to ketones or are conjugated in their normal routes of metabolism in the body (17). The hemisuccinate ester of griseofulvin-4′-alcohol, which will be called griseofulvin-4′-hemisuccinate for consistency, was prepared because its sodium salt could be administered in solution. The ester was expected to hydrolyze rapidly in the body to griseofulvin-4′-alcohol. Oximes also have been shown to be possibly converted to their corresponding ketones by enzymatic metabolism in the body (18). The carboxymethoxime derivative was chosen for testing because the molecule could be solubilized as the sodium salt and its metabolism could be expected to follow that of oximes.



R

I, =O

II, —OH

III, =N—OH

IV, =N—O—CH_2—COOH

V, —O—C(=O)—CH_2—CH_2—COOH

EXPERIMENTAL

Synthesis—The method of preparation of the griseofulvin-4′-alcohol (II) was that of Kyburz *et al.* (16). The method of synthesis of the griseofulvin-4′-oxime (III) was that of Oxford (19). For the details of the synthesis of compounds IV and V and the evaluation of the purity of each compound, the reader is referred to the thesis (20).

Determination of Water Solubility—An excess of pure crystalline drug was placed in a 40-ml. glass-stoppered centrifuge tube and a 25-ml. quantity of distilled water was added. The tube was stoppered, sealed with paraffin, and heated for a brief period at 45–55° prior to being placed in a water bath at 37 ± 1° for not less than 24 hr. Agitation was accomplished by mechanically rotating the tubes end over end at 30 r.p.m.

Samples were taken with a pipet plugged with glass wool and quickly diluted to the appropriate volume with distilled water. Griseofulvin and griseofulvin-4′-alcohol were determined spectrophotofluorometrically with excitation at 315 mμ and analyzing at 450 mμ. The griseofulvin-4′-alcohol samples were also assayed using a spectrophotometric method at 293 mμ. The oxime and carboxymethoxime derivatives were determined spectrophotometrically at 294 mμ.

Intravenous Administration and Sampling—The derivatives which were administered intravenously were given as a solution in 75% *N,N*-dimethylacetamide (DMA) containing 20 mg. of drug/ml. The administration and sampling techniques were the same as described by Fischer and Riegelman (6) for the i.v. administration of griseofulvin to rabbits.

Oral Administration and Sampling—New Zealand white rabbits were used for oral administration of the drugs. A control dose of microcrystalline griseofulvin[1] was prepared as a suspension in 0.05% polysorbate 80[2] and administered using the technique previously described by these authors (6). The low concentration of polysorbate 80 was used to facilitate wetting of the powders and was not expected to influence absorption of the drug. In testing for *in vivo* activity, a dose of griseofulvin was administered to each animal, usually as the first dose of the experiment, to obtain a control level of the drug in plasma. Griseofulvin-4′-alcohol and -oxime were also administered as suspensions made by triturating the crystalline drug with the appropriate amount of polysorbate 80 and gradually adding water while mixing to make a suspension containing 20 mg. of drug/ml. No attempt was made to control the particle size of the derivatives in suspension. Griseofulvin-4′-hemisuccinate and -carboxymethoxime were administered as their sodium salts dissolved in distilled water. The interval between doses to the same animal was at least 1 week after the griseofulvin blood level from the previous dose had returned to zero.

The oral doses were administered through a stomach tube to rabbits briefly anesthetized with halothane as previously described (6). Samples were taken by making a small cut in the marginal ear vein with a scalpel blade and allowing the blood to flow into a heparinized centrifuge tube. Plasma was separated from the blood samples by centrifuging at 3500 r.p.m. The rabbits were permitted normal food (Purina commercial rabbit chow) and water *ad libitum* during the experiment unless specified otherwise.

Microbiological Assay for Griseofulvin Activity—Activities of the griseofulvin derivatives on *Mycrosporum gypseum* were compared to the activity of griseofulvin on the same organism using the cylinder plate assay reported by Knoll, Bowman, and Kirshbaum (21). Activities were determined in phosphate buffer at pH 8.0 and in fresh rabbit plasma. The activity of griseofulvin in the presence of some of the derivatives was also tested. All activities reported were an average of three or more separate determinations.

After a dose of griseofulvin or a griseofulvin derivative, the plasma griseofulvin activity reported was a result of six to nine determinations on a particular plasma sample as described by Knoll (21) in the assay procedure.

Thin-Layer Fluorimetric Analysis—Griseofulvin and griseofulvin-4′-alcohol were determined quantitatively in plasma using the TLC fluorimetric assay reported by Fischer and Riegelman (22).

Griseofulvin could be separated from griseofulvin-4′-oxime in a plasma ether extract by using

[1] Griseofulvin (lot No. 0524, specific surface area 13.–1.7 M.²/Gm.) was obtained from McNeil Laboratories, Fort Washington, Pa.

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

a solvent system of 55% chloroform, 25% ether, and 20% acetone. Griseofulvin (R_f approximately 0.5), separated from the oxime derivative (R_f approximately 0.3) in the aforementioned solvent system, could be determined quantitatively using the TLC fluorimetric assay. It was found that standard griseofulvin readings were the same regardless of whether the ether–acetone (3:2) or the above-mentioned solvent system was used. Griseofulvin-4′-carboxymethoxime could also be separated from its parent compound by employing the 55% chloroform, 25% ether, and 20% acetone system. In this system the carboxymethoxime derivative would not move from the original spot while griseofulvin had an approximate R_f of 0.5.

Thin-layer chromatograms of plasma ether extracts were scanned with the adapted Photovolt TLC Densitometer to provide recording of the fluorescent spots over the entire length of the chromatogram. The scans were started on the original spot (labeled "S" on the TLC fluorimetric recordings) and were terminated when no more spots were observed or the fluorescent residue from plasma was detected near the end of the chromatogram. The heights of the observed peaks were proportional to the amount of fluorescent material on the spot when the previously described (22) application and development techniques were employed.

It should be noted that griseofulvin and griseofulvin-4′-alcohol concentrations determined by the TLC fluorimetric assay will be reported as mcg./ml. in plasma. Griseofulvin concentrations determined by the microbiological assay will be referred to as mcg./ml. of griseofulvin *plasma activity*. The activity, in this case, means the inhibition of growth of the test organism compared to griseofulvin, and not pharmacological activity as such.

RESULTS AND DISCUSSION

The griseofulvin-4′-derivatives which are shown in Table I exhibited varying degrees of increased water solubility. Reduction of the 4′-carbonyl group of griseofulvin to hydroxyl increased the solubility of the molecule approximately eighteen-fold. The solubility of the 4′-alcohol derivative was determined by fluorescence and ultraviolet methods and good agreement occurred between the different assays. A ninefold increase in solubility occurred for the 4′-oxime derivative, whereas the 4′-carboxymethoxime exhibited a somewhat smaller increase. The additional molecular weight of the 4′-carboxymethoxime molecule seems to account for a solubility lower than that of the oxime derivative. Because of its instability in aqueous solution, the solubility of griseofulvin-4′-hemisuccinate was not determined.

Griseofulvin-4′-oxime was the only derivative exhibiting *in vitro* antifungal activity, as shown in Table II. The activity of this derivative appeared to be somewhat characteristic upon observation of the zones of inhibition on the test plates. The zones consistently had a characteristic yellow color on their periphery which was not observed on the inhibition zones of griseofulvin. On closer examination, the oxime derivative appeared to inhibit production of aerial mycelium, and possibly sporulation, beyond the zone of total inhibition. Generally, the oxime appeared to be $^1/_7$th as active as griseofulvin on *M. gypseum* when tested in buffer and in serum. The combinations of griseofulvin and oxime tested produced slightly higher activity than griseofulvin alone; however, the oxime and griseofulvin activities were not proportionally additive. The griseofulvin alcohol and carboxymethoxime derivatives were found not to be active at the 50 mcg./ml. level. The hemisuccinate was not active at 10 mcg./ml. in buffer and was not tested further because its logical hydrolysis product, griseofulvin-4′-alcohol, was found not to be active.

The fact that little or no *in vitro* activity was exhibited by the derivatives was not surprising in view of the previous work on the structure–activity relationships of griseofulvin analogs. Crosse *et al.* (23) have studied the activity of more than 300 analogs of griseofulvin against dermatophytic and plant pathogenic fungi. Generally, it was found that substitutions on the griseofulvin molecule altered the spectrum and diminished the activity of the antibiotic. However, some substitutions at the 2′- and 3′-positions by groups which impart lipid solubility to the molecule increased and then decreased activity as a particular series was ascended. Many of the derivatives showed increased *in vivo* activity on plant and dermatophytic fungi (23–25). From the results of previous testing of griseofulvin analogs, it appears that increased *in vitro* activity

TABLE I—SOLUBILITY OF GRISEOFULVIN AND DERIVATIVES IN WATER AT 37°

Compd.	Method	Solubility, mcg./ml.
Griseofulvin (I)	Fluorescent	14.3
Griseofulvin-4′-alcohol (II)	Fluorescent	250.0
Griseofulvin-4′-alcohol	Ultraviolet	256.0
Griseofulvin-4′-oxime (III)	Ultraviolet	132.0
Griseofulvin-4′-carboxymethoxime (IV)	Ultraviolet	75.3

TABLE II—*In Vitro* ANTIFUNGAL ACTIVITY OF GRISEOFULVIN DERIVATIVES, TEST ORGANISM: *M. gypseum*

Amt./ml. Buffer	Griseofulvin Activity, mcg./ml.
Oxime, 10 mcg.	Inhibitory, but no definite zone
Oxime, 25 mcg.	3.6
Oxime, 50 mcg.	6.5
Oxime, 10 mcg., and griseofulvin, 5 mcg.	6.5
Oxime, 25 mcg., and griseofulvin, 5 mcg.	6.5
Alcohol, 50 mcg.	0
Carboxymethoxime, 50 mcg.	0
Hemisuccinate, 10 mcg.	0
Amt./ml. Rabbit Serum	
Oxime, 25 mcg.	3.4
Oxime, 50 mcg.	>5.0
Oxime, 25 mcg., and griseofulvin, 2.0 mcg.	2.7
Alcohol, 25 mcg., and griseofulvin, 2.0 mcg.	3.1

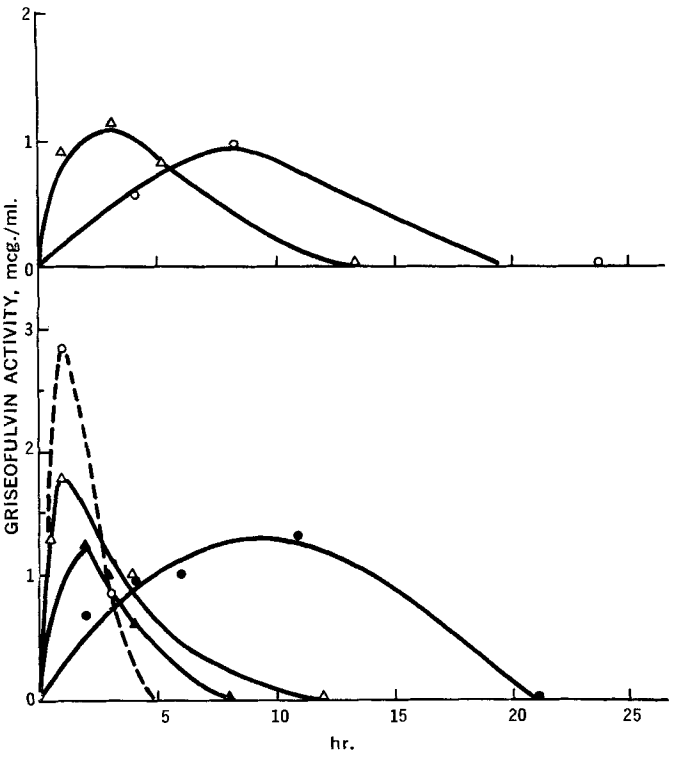


Fig. 1—Plasma griseofulvin activity-time curves resulting from oral administration of griseofulvin and derivatives. Top, rabbit 31. Key: ○, griseofulvin, 150 mg./Kg.; △, griseofulvin oxime, 150 mg./Kg. Bottom, rabbit 30. Key: ○, griseofulvin hemisuccinate, 150 mg./Kg.; △, griseofulvin alcohol, 200 mg./Kg.; ▲, griseofulvin alcohol, 150 mg./Kg.; ●, griseofulvin, 150 mg./Kg.

is accomplished by making particular substitutions on the molecule which increase lipid solubility; however, the more active compounds, due to their decreased water solubility, cannot be translocated efficiently *in vivo* to their site of action against the invading fungi. In such a situation the preparation of derivatives which have greater absorption potential and which will be converted to griseofulvin metabolically is a logical recourse to increase blood levels of the antibiotic.

The griseofulvin derivatives, which were prepared and tested as described, were administered to rabbits in order to evaluate their *in vivo* activity. The compounds, which showed little or no *in vitro* activity, were initially tested by assaying the plasma for antifungal activity following oral doses to test animals. As many derivatives as possible were administered to a particular animal to reduce the influence of individual animal variation on the results of the *in vivo* testing. An oral dose of griseofulvin (150 mg./Kg.) administered to rabbit 30 resulted in the typical plasma griseofulvin activity level (microbiological assay) shown in Fig. 1. A dose of griseofulvin-4′-alcohol (150 mg./Kg.) to the same animal reached a peak griseofulvin activity in 2 hr., which was approximately 8 hr. sooner than the corresponding dose of griseofulvin had reached its peak. The duration of activity, however, was much shorter when the alcohol derivative was administered. An increased dose of griseofulvin-4′-alcohol (200 mg./Kg.) produced a higher peak plasma activity of 1.8 mcg./ml. and again fell rapidly to zero at 12 hr. Griseofulvin-4′-hemisuccinate given in solution produced a rapid, higher peak plasma activity; however, the duration of activity was only 5 hr. Since these derivatives did not show griseofulvin activity *in vitro*, it appeared that conversion to griseofulvin had taken place in the body.

An oral dose of griseofulvin-4′-oxime (150 mg./Kg.) produced a more rapid but similar peak plasma griseofulvin activity compared to that produced by the same dose of griseofulvin, as represented by rabbit 31 in Fig. 1. Using the microbiological assay, it was not possible to determine definitely whether the plasma activity after a dose of the oxime derivative, which was slightly active *in vitro*, was due entirely to conversion or could be attributed in part to high blood levels of the derivative. The characteristic yellow color produced by the oxime derivative was observed on the periphery of zones of inhibition during the assay of plasma samples taken after an oral dose of oxime. Therefore, the activity appeared to be manifested in part by the oxime derivative. An oral dose of griseofulvin-4′-carboxymethoxime (200 mg./Kg.) did not exhibit plasma antifungal activity in any rabbit to which it was administered.

Following a dose of griseofulvin-4′-alcohol, the total fluorescence of the plasma was determined using the spectrophotofluorometric assay for griseofulvin (6). The fluorescence spectrum of the alcohol derivative is identical to the spectrum of griseofulvin, and the intensities were found to be additive in plasma. In three different animals given an oral dose of griseofulvin-4′-alcohol the peak plasma fluorescence time was between 4 and 8 hr., and at these times the plasma exhibited little or no antifungal activity as determined by the microbiological assay. Rabbit 30 shown in Fig. 1 (closed triangles) had peak fluorescence in the 8-hr. plasma sample which showed no griseofulvin plasma

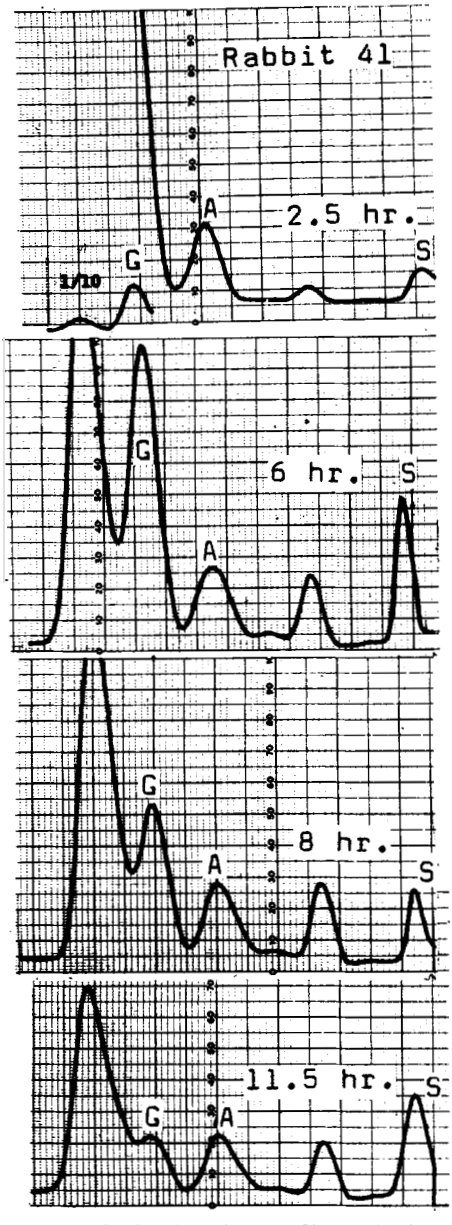


Fig. 2—TLC fluorimetric recordings of chromatograms containing fluorescent products extracted from 1 ml. of plasma following an oral dose of 200 mg./Kg. of griseofulvin-4′-alcohol.

activity. If conversion of the alcohol derivative to griseofulvin were taking place after absorption of the alcohol, and if only these two compounds were present in the blood, then peak plasma fluorescence should correspond to peak plasma griseofulvin activity.

In order to investigate the short duration of plasma griseofulvin activity, thin-layer chromatography was employed to separate the fluorescent constituents in the plasma following oral administration of griseofulvin-4′-alcohol. The TLC fluorimetric scans in Fig. 2 present the events which took place in the blood following an oral dose of 200 mg./Kg. of the alcohol derivative to rabbit 41. Each peak on the recording represents a fluorescent spot on the chromatogram. Fluorescent products in addition to griseofulvin (G) and griseofulvin-4′-alcohol (A) begin to make their appearance in the 2.5-hr. plasma sample. The three new fluorescent spots, one moving in front of griseofulvin and two trailing the alcohol derivative, are quite prevalent in the remaining plasma samples while griseofulvin plasma concentration falls. The appearance of other fluorescent substances in the plasma, besides the alcohol derivative and its parent compound, then, is accompanied shortly thereafter with falling griseofulvin levels. In rabbit 40, which showed high, somewhat prolonged griseofulvin levels, the appearance of the additional fluorescent substances was delayed, and they were initially observed beyond the 5-hr. sample. The discrepancy between peak plasma fluorescence and peak plasma griseofulvin activity, therefore, is due to the appearance of additional fluorescent products in the blood some time after an oral dose of griseofulvin-4′-alcohol.

Plasma samples from rabbit 39, given a 20 mg./Kg. intravenous dose of griseofulvin-4′-alcohol infused over a 15-min. period, were assayed for griseofulvin and its alcohol derivative using the TLC fluorimetric method (22). The first sample, taken 15 min. after completion of the infusion, exhibited the highest concentrations (Fig. 3). Thereafter, the plasma concentration of each substance decreased in a linear fashion when plotted against time on semilogarithmic paper. Griseofulvin, resulting from conversion of the alcohol derivative, had an apparent biological half-life of 70 min. in this animal. Griseofulvin-4′-alcohol in the plasma expectedly decreased more rapidly and had an apparent half-life of 28 min. A TLC fluorimetric scan of the extracted plasma samples showed that after an i.v. dose of the alcohol derivative only griseofulvin alcohol and griseofulvin were present in amounts detectable by the fluorescent technique. The fact that no additional fluorescent

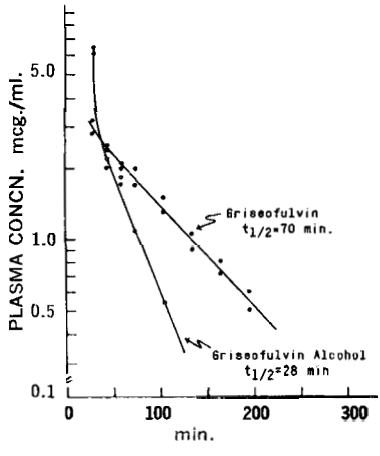


Fig. 3—Semilogarithmic plot of griseofulvin and griseofulvin-4′-alcohol plasma concentrations following an i.v. dose of griseofulvin-4′-alcohol, 20 mg./Kg., to rabbit 39.

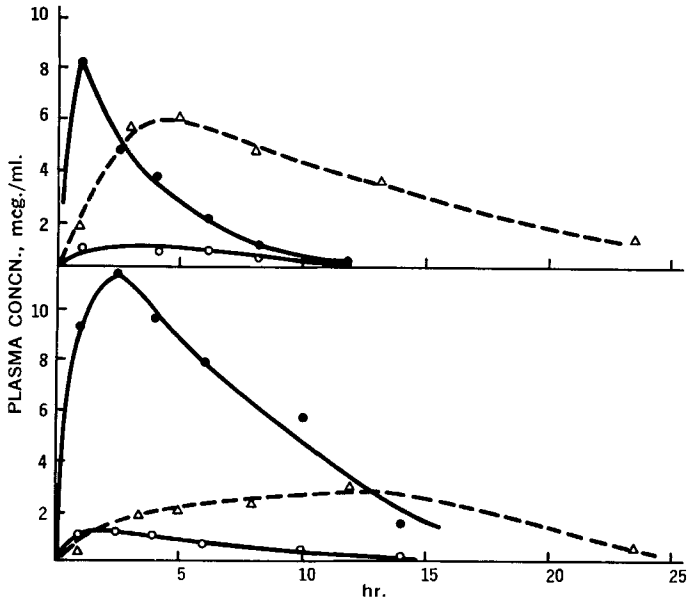


Fig. 4—Plasma concentration-time curves of griseofulvin and griseofulvin-4′-alcohol (TLC fluorimetric assay) following an oral dose of the alcohol derivative. A plasma level curve (broken line) from a control dose of griseofulvin is also shown. Oral dose—200 mg./Kg. griseofulvin alcohol. Key: ●, griseofulvin; ○, griseofulvin alcohol. Oral dose—200 mg./Kg. griseofulvin. Key: △, griseofulvin. Top, rabbit 41; bottom, rabbit 40.

products were found in the blood following i.v. administration of the alcohol derivative suggested that their appearance following an oral dose might be attributed to the degradation of the 4′-alcohol in the gastrointestinal tract.

An oral dose of griseofulvin-4′-alcohol (200 mg./Kg.) given to rabbit 40 (Fig. 4) produced high griseofulvin plasma levels and corresponding low griseofulvin-4′-alcohol levels as determined by the TLC fluorimetric assay. Griseofulvin reached an abnormally high plasma concentration of 11.5 mcg./ml. and had a duration of 20 hr. One week later the same animal was given an oral dose of griseofulvin (200 mg./Kg.) and exhibited a normal blood level picture as seen in the broken line curve on Fig. 4. This animal appeared to have an unusually high capacity for converting the alcohol derivative to griseofulvin. Another animal (No. 41) given a 200 mg./Kg. oral dose of the alcohol derivative exhibited the more typical blood level seen in Fig. 4. Plasma griseofulvin rose to a peak of 8.0 mcg./ml. in 1 hr. and decreased rapidly. The griseofulvin-4′-alcohol level again was consistently low. The control dose of griseofulvin produced rather high levels of griseofulvin (broken curve) showing this animal to be a good absorber of the drug.

It is particularly interesting to note that the level of griseofulvin-4′-alcohol, as represented by the data shown by rabbits 40 and 41 in Fig. 4, remains at a low, essentially constant level in the blood and at the same interval the griseofulvin level reaches a peak and rapidly falls. The alcohol derivative was shown to be approximately 18 times more soluble in water than griseofulvin and one can assume, therefore, that it should be more rapidly absorbed. The data obtained from the i.v. injection of the alcohol indicated that it has a very rapid elimination rate constant. The persistent griseofulvin-4′-alcohol blood level from an oral dose, then, must be due to a solution-rate-limited process where the low consistent blood level results from a steady state between the absorption and the rapid elimination processes. One then is left with the problem of how to explain why the griseofulvin blood levels (with a slower elimination rate) do not continue to persist at a high level as long as the griseofulvin-4′-alcohol remains in the blood and can be converted to griseofulvin.

Figure 5 shows plots of the heights of the fluorescence peaks found by a TLC fluorimetric scan, against time for one of the rabbits (No. 40) receiving an oral dose of griseofulvin-4′-alcohol. The curves give a semiquantitative measure of the appearance and elimination of all extracted and resolved fluorescent substances in the plasma follow-

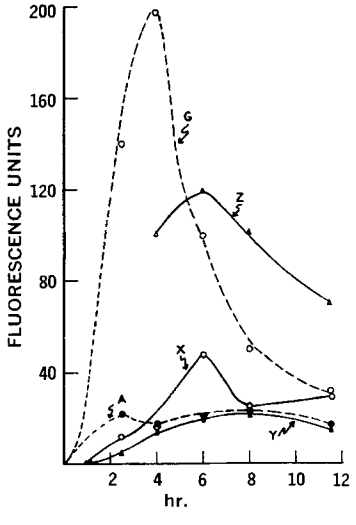


Fig. 5—Peak intensity of fluorescent spots from TLC fluorimetric scans of plasma extract chromatograms plotted against time. The rabbit (No. 41) received a 200 mg./Kg. oral dose of griseofulvin-4′-alcohol. Key: A, griseofulvin-4′-alcohol; G, griseofulvin; X, Y, and Z, unidentified compounds.

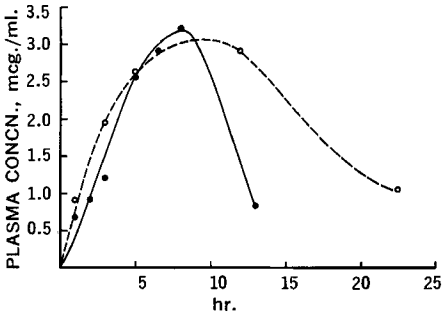


Fig. 6—Griseofulvin plasma concentration-time curves following single 200 mg./Kg. oral doses of griseofulvin (○) and griseofulvin-4′-oxime (●). (Rabbit 45.)

ing an oral dose of the alcohol derivative. From the curves, one observes that the unidentified spot, labeled X, which has the lowest R_f value, and another unidentified spot, labeled Y, trailing behind griseofulvin alcohol, appear after a short period of time and have consistently low values as does griseofulvin-4′-alcohol. The more intense spot (labeled Z in Fig. 5) moving slightly ahead of griseofulvin also has a later peak time than griseofulvin and has somewhat higher values than the other unidentified fluorescent products. It should be pointed out that none of these secondary fluorescent products are 6-demethyl griseofulvin, a metabolite of griseofulvin which does not fluoresce under these conditions and has a different R_f than any of the new compounds. Assuming that the fluorescent yield of each of the spots is not drastically different, it appears from the shapes of the curves that either substance X or Y could be converted to substance Z by a mechanism similar to the conversion of the alcohol derivative to griseofulvin. The proposed oxidative conversion of one of the assumed products (X or Y) to substance Z may compete with griseofulvin alcohol for enzymatic metabolism sites. This competition would explain the falling griseofulvin levels upon the appearance of the unidentified fluorescent substances. Even though the griseofulvin plasma level falls rapidly, it is obvious that some conversion of the alcohol is taking place as the apparent half-life of griseofulvin in the plasma after the peak level is approximately 2 to 4 hr., which is longer than the 77-min. half-life observed after an i.v. dose. It appears, then, that the short duration of griseofulvin plasma levels following an oral dose of the alcohol deriva-

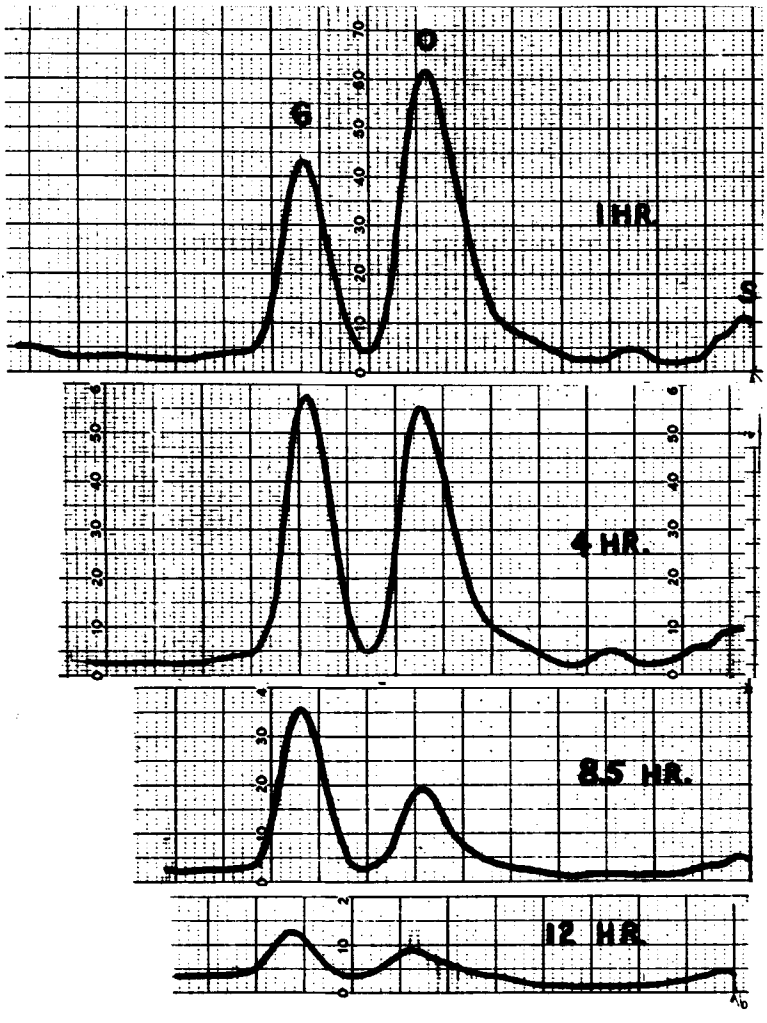


Fig. 7—TLC fluorimetric recordings of chromatograms showing fluorescent products extracted from plasma samples (1 ml.) following oral administration of 200 mg./Kg. of griseofulvin-4′-oxime. The fluorescent spot attributed to griseofulvin is labeled G, and that of the 4′-oxime as O. (Rabbit 46.)

tive was due to the degradation of the derivative in the gastrointestinal tract and the possible competition of the degradation products or products with griseofulvin-4′-alcohol for oxidative enzyme sites.

Due to the low fluorescence intensity of the oxime derivative, only griseofulvin plasma concentration was determined by the TLC fluorimetric method following administration of griseofulvin-4′-oxime to rabbits. The 4′-oxime derivative appeared to be converted to griseofulvin at a much slower rate than the 4′-alcohol. After intravenous administration of the 4′-oxime, low levels of griseofulvin from 0.38 to 0.6 mcg./ml. were observed in the plasma sample and during a 100-min. study reach a peak in 1 hr. Unlike the 4′-alcohol derivative, high griseofulvin plasma levels exhibiting apparent first-order elimination from the blood were not observed upon i.v. administration of the oxime. The oxime derivative was observed to be toxic when administered intravenously and the rabbit expired in 2 hr. Another animal given an identical i.v. dose of the oxime derivative exhibited a similar blood level and did not die during the experiment; however, heavy hemolysis was observed in the plasma samples of this animal.

An oral dose of griseofulvin-4′-oxime (200 mg./Kg.) to rabbit 45 produced a plasma griseofulvin curve (closed circles) similar in peak concentration, but of shorter duration than the control dose of griseofulvin (open circles) as seen in Fig. 6. As a result of oral doses of griseofulvin-4′-oxime the possible toxicity of this derivative was evidenced by the hemolysis of the early blood samples.

The slow conversion of the oxime derivatives to griseofulvin was seen in the TLC fluorimetric scans (Fig. 7) of plasma extracts after administration of a 200-mg./Kg. oral dose of griseofulvin-4′-oxime. In interpreting Fig. 7, it must be recalled that the oxime is approximately $^1/_{10}$th as fluorescent as griseofulvin. The earlier blood samples showed high levels of the oxime to be present along with lower levels of griseofulvin. From a preliminary extraction curve, there appeared to be approximately 15 to 20 mcg./ml. of the oxime derivative in the 4-hr. sample which contained 12 mcg./ml. of griseofulvin. Griseofulvin plasma concentration essentially decreased with the fall in oxime concentration and at 12 hr. only small amounts were present in the plasma. Oximes have been reported (18) to have other metabolites in addition to their corresponding ketones. It is probable, therefore, that griseofulvin is not the only metabolite of the oxime derivative. The spot trailing griseofulvin-4′-oxime on the 1- and 4-hr. TLC scans in Fig. 7 may be another metabolite or possibly a gastrointestinal degradation product of the oxime derivative. No attempts were made to characterize any metabolite other than griseofulvin.

Oral doses of griseofulvin-4′-carboxymethoxime have been shown to produce no *in vivo* griseofulvin activity, using the microbiological assay. In order to substantiate this finding, an intravenous dose (15 mg./Kg. in 75% DMA) of this derivative was given to rabbit 43. No evidence of hemolysis was observed in the blood samples taken from this animal. The first plasma sample, taken 15 min. after the 10-min. infusion of the drug, was found to contain only a trace amount of griseofulvin. A 30-min. sample exhibited no trace of the antibiotics. It was disappointing to observe that griseofulvin-4′-carboxymethoxime was not converted to griseofulvin after i.v. or oral administration. This derivative may be excreted rapidly by the kidneys, possibly by an active process, due to the similarity of the carboxymethoxime moiety to many substances actively secreted by the tubules. In addition, the $=N—O—CH_2—$ linkage in this compound may be stable to oxidative metabolism in the rabbit.

REFERENCES

(1) McNall, E. G., *Antibiot. Ann.*, **7**, 674(1959–1960).
(2) Crounse, R. G., *A.M.A. Arch. Dermatol.*, **87**, 176 (1962).
(3) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Antibiot. Chemotherapy*, **12**, 225(1962).
(4) Bedford, C., Busfield, D., Child, K. J., MacGregor, P. S., and Tomich, E. G., *A.M.A. Arch. Dermatol.*, **81**, 735 (1960).
(5) Davis, B., Child, K. J., and Tomich, E. G., *J. Pharm. Pharmacol.*, **13**, 166(1961).
(6) Fischer, L. J., and Riegelman, S., *J. Pharm. Sci.*, **54**, 1571(1965).
(7) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Nature*, **193**, 588(1962).
(8) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Antibiot. Chemotherapy*, **12**, 232(1962).
(9) Kramal, M., Dubuc, J., and Beall, D., *Can. J. Biochem. Physiol.*, **40**, 1449(1962).
(10) Kraml, M., Dubuc, J., and Gaudry, R., *Antibiot. Chemotherapy*, **12**, 239(1962).
(11) Kraml, M., Dubuc, J., and Dvornik, D., *A.M.A. Arch. Dermatol.*, **87**, 179(1963).
(12) Duncan, W. A. M., MacDonald, G., and Thornton, M. J., *J. Pharm. Pharmacol.*, **14**, 217(1962).
(13) Crounse, R. G., *A.M.A. Arch. Dermatol.*, **87**, 176 (1963).
(14) Marvel, J. R., Schlichting, D. A., Denton, C., Levy, E. J., and Cahn, M. M., *J. Invest. Dermatol.*, **42**, 197(1964).
(15) Crounse, R. G., *ibid.*, **37**, 529(1961).
(16) Kyburz, E., Geleick, H., Frey, J. R., and Brossi, A., *Helv. Chim. Acta*, **43**, 2083(1960).
(17) Williams, R. T., "Detoxication Mechanisms," John Wiley & Sons, Inc., New York, N. Y. 1959, p. 63.
(18) *Ibid.*, p. 166.
(19) Oxford, A. E., Raistrick, H., and Simonart, P., *Biochem. J.*, **33**, 240(1939).
(20) Fischer, L. J., Ph.D. Thesis, University of California, San Francisco, Calif., 1965.
(21) Knoll, E. W., Bowman, F. W., and Kirshbaum, A., *J. Pharm. Sci.*, **52**, 586(1963).
(22) Fischer, L. J., and Riegelman, S., *J. Chromatog.*, to be published.
(23) Crosse, R., McWilliam, R., and Rhodes, A., *J. Gen. Microbiol.*, **34**, 51(1964).
(24) Neves, H., *Trans. St. John's Hosp. Dermatol. Soc.*, **48**, 103(1962).
(25) Munro-Ashman, D., *ibid.* **48**, 108(1962).

Effect of Mescaline on Cardiopulmonary Dynamics

Method for Determination of Right Ventricular Pressure in the Guinea Pig

By VINCENT DE PAUL LYNCH, EMMETT CLEMENTE, and STEVEN CARSON*

The effect of mescaline on respiratory dynamics, right ventricular pressure, and the ECG in the guinea pig is presented. A procedure for the acquisition of normal right ventricular pressure is described. The mean value for the latter was 13.4 ± 4.6 mm. Hg. Mescaline in low dosages (1 to 20 mg./Kg.) produced a slight increase in heart rate; higher dosages (50 to 240 mg./Kg.) induced bradycardia and conduction disturbances. Respiratory rate, resistance of the airways, and minute volume increased significantly, while tidal volume and compliance decreased. The decrease in compliance was near maximum (−70 and −77 per cent) with high dosages (50 and 100 mg./Kg.). Right ventricular pressure increased, average 45 per cent, following moderate dosages of mescaline (25 mg./Kg.). Diphenhydramine and chlorpheniramine (range 0.4 to 0.7 mg./Kg.) in sufficient concentration to block the effect of histamine (4 mg./Kg.) were utilized to observe possible mescaline antagonistic activity. However, the antihistamines were ineffective against mescaline. It appears probable that mescaline does not owe its activity on these parameters to histaminic receptor stimulation.

ALTHOUGH the effects of mescaline on the central nervous system (CNS) are well documented, its mode of action is unclear (1–3). Unfortunately, the peripheral activities of mescaline have not been subject to as much critical evaluation. It must be emphasized that while analogies are often misleading and erroneous, when comparing peripheral to central effects, a valid working hypothesis may be obtained. Moreover, the current theory concerning the mechanism of action (central) of LSD had, in large part, developed from peripheral studies (4–7). In view of this concept, studies were conducted to observe the effect of mescaline on the cardiopulmonary system in the guinea pig. The investigation was divided into three phases: the effect of mescaline on (*a*) the electrocardiogram, (*b*) right ventricular pressure, and (*c*) respiratory dynamics.

Deniker (8), in studies designed to determine electrolyte alterations induced by mescaline in man, reported a significant decrease in serum potassium. Speck (9) determined heart rate and blood glucose levels in rats challenged with mescaline. She reported that both parameters decreased significantly following intermediate and high doses. Wolbach *et al.* (10) noted that mescaline had a variable effect on pulse rate in man.

The ECG was monitored in the present study since any of the above phenomena could be reflected in this parameter (11). Furthermore, at present there are no available data concerning the effect of mescaline on the guinea pig ECG.

Cochin *et al.* (12) conducted distribution studies in the dog and observed that mescaline entered ventricular tissue. While such findings do not signify that mescaline has an effect on cardiac tissue, it was necessary nevertheless to take cognizance of this possibility. This is true especially in light of reports that mescaline has a variable effect on pulse rate and blood pressure (8, 10, 13). Most investigators agree that systemic blood pressure rises following high doses of mescaline (9, 14–16). It has been established that changes in blood pressure are accompanied by alterations in cardiac activity and respiratory function. It was reasoned, therefore, that if right ventricular pressure was monitored, variations in all related systems would be manifested in this parameter. However, an investigation of this nature could not immediately be undertaken because there are no unequivocal data available regarding the normal variations and mean value of this parameter in the guinea pig. Consequently, this research was designed to obtain the latter information as an initial step. After this was accomplished, an evaluation of the effect of mescaline on the same system was commenced.

The availability of techniques for the cannulation of various vascular networks has facilitated the investigation of cardiopulmonary function (17, 18). Whereas most investigators have used the rat as the test animal (19, 20), the guinea pig was chosen in the present study because of its

Received October 3, 1966, from the Department of Pharmacognosy, Pharmacology and Allied Sciences, College of Pharmacy, St. John's University, Jamaica, NY 11432, and the * Food and Drug Research Laboratories, Inc., Maspeth, NY 11378

Accepted for publication December 30, 1966.

The authors acknowledge the American Medical Association, Education and Research Foundation, for support of a major portion of this work.

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

susceptibility to respiratory stress (21) in addition to the aforementioned reasons.

During the course of experiments involving the initial phase in this study, mescaline was observed to exert a potent effect on respiration. Doses comparable to those used in human experimentation, and previously reported to have little effect on this parameter in other species (10, 15, 22), were observed to cause a pronounced effect. Moreover, the induced changes closely resembled those produced by histamine (23). In view of these findings, this study was expanded ultimately to observe the action of mescaline on the cardiopulmonary system, its similiarity to histamine, and the possible antagonism of antihistamines upon induced alterations.

MATERIALS AND METHODS

Measurement of Right Ventricular Pressure—Adult male and female guinea pigs, weighing between 700 and 1000 Gm., were anesthetized intraperitoneally with sodium pentobarbital (35 mg./Kg.). The preparation of ventricular catheters and surgical procedures of Popovic (24) were modified in these studies according to the following procedures.

Ventricular catheters (polyethylene tubing, 0.011 in. i.d. × 0.024 in. o.d., No. P.E. 10, Clay-Adams) were prepared at least 24 hr. prior to intubation and allowed to soak in an antiseptic solution of benzalkonium chloride[1] (1:120). The tip of the catheter was shaped in the form of a right angle with the aid of a pencil soldering iron. The angled flexure was approximately 3 mm. from the tip. A small bulb (formed by rotating the catheter over a heat source) was placed a critical distance from the flanged tip of the catheter contingent upon the weight or size of the animal (Table I). The flange was produced with the soldering pencil and served the purpose of minimizing damage to the vessel linings (Fig. 1). The length of the catheter (20 cm.) was held constant so that changes in pressure could not be attributed to a variability in the tubing length. A 22-cm. stainless steel stylet (No. 32 gauge) was inserted into the catheter up to the flexure. The catheter was filled with heparinized saline (4 mg./ml.) and the external tip flame-sealed around the stylet.

An incision of approximately 2 cm. was made so that the midpoint of the incision coincided with the point at which the right transverse scapular vein joined the right external jugular vein. The jugular vein was dissected free and the cephalad portion permanently ligated while the peripheral end was secured with a loose ligature. The catheter was introduced distally into the vessel as close as possible to the junction of the right external jugular and right transverse scapular veins, and then advanced toward the heart. The loose ligature was secured after the bulb of the catheter had entered the vessel, the stylet removed, and the catheter flushed with a volume of saline slightly in excess of the capacity

[1] Marketed as Zephiran by Winthrop Laboratories, New York, N. Y.

Table I—Distance of Bulb from Catheter Tip

Body Wt., Gm.	Distance, cm.
700–750	3.9–4.1
750–800	4.1–4.3
800–850	4.3–4.5
850–900	4.5–4.7
900–950	4.8–5.0
950–1000	5.0–5.2

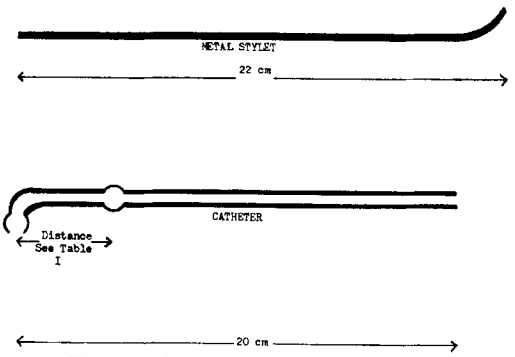


Fig. 1—Diagram of ventricular catheter.

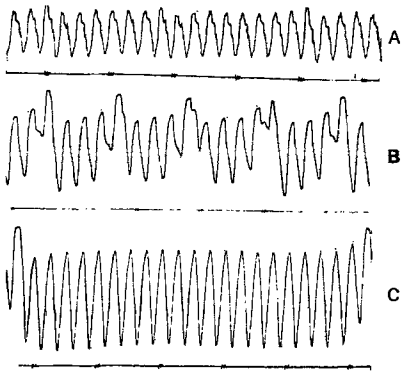


Fig. 2—Patterns of right ventricular pressure. Key: A, catheter in close proximity of pulmonary artery; B, catheter positioned in lower two-thirds of ventricular lumen; C, catheter positioned in upper third of ventricular lumen.

of the catheter (0.02 ml.). The validity of correct placement of the catheter was ascertained by raising the tip of the catheter approximately 5 cm. above the level of the heart and observing the ventricular pattern which was produced after the catheter was connected to a pressure transducer and monitored by a direct writing recorder (Fig. 2, C). It was then flushed with heparinized saline and sealed. This was accomplished by the application of a hemostat approximately 6 cm. from the tip of the catheter and the insertion of a 5-cm. piece of stainless steel wire (No. 30 gauge). This procedure eliminated the necessity of cutting the tip of the catheter before each pressure determination or blood sample withdrawal which would be required if heat sealing were used. The catheter was exteriorized by placing it into the lumen of a needle (No. 13 gauge) and pushing the needle underneath the skin around the

TABLE II—EFFECT OF ANTIHISTAMINES ON RIGHT VENTRICULAR PRESSURE CHANGES INDUCED BY MESCALINE AND HISTAMINE IN THE ANESTHETIZED GUINEA PIG

Compd.[a]	Dosage, mg./Kg.	Animals, No.	Treatment with Antihistaminics, mg./Kg., 15 min. Prior to Challenge	% Change in Right Ventricular Pressure at Max. Effect (Range)
Histamine	4.0	2	[c]	+70-75[b]
Diphenhydramine	0.7	1	[c]	No change
Histamine	4.0	1	Diphenhydramine 0.4	No change
Histamine	4.0	1	Diphenhydramine 0.7	+10.0
Chlorpheniramine	0.8	1	[c]	No change
Histamine	4.0	1	Chlorpheniramine 0.8	+20.0
Mescaline	25.0	6	[c]	+26-86[b]
Mescaline	25.0	1	Diphenhydramine 0.7	+50.0
Mescaline	25.0	1	Chlorpheniramine 0.4	+83.0

[a] Compounds were administered intraperitoneally as their salts. [b] Range value. [c] Animal not pretreated.

TABLE III—EFFECT OF DRUGS ON RESPIRATORY DYNAMICS IN THE GUINEA PIG

Animal, No. and Sex	Pretreatment with Diphenhydramine, mg./Kg.	Mescaline, mg./Kg.	Tidal Vol.	Respiratory Rate	min. Vol.	% Change from Control Compliance	Resistance
1M	[c]	5.0	±[d]	+	+	−33	+9
2M	−	25.0	±	±	±	−66	+140
3M	−	50.0	±	±	±	−70	+63
4M	−	100.0	−	+	+	−77	+200
5M	0.7	25.0	−	+	±	−64	+70
6M	0.7	25.0	±	NC	±	−60	+164
7M	0.7	−	NC[a]	NC	NC	+16[b]	+31[b]

[a] NC, no change. [b] Average value. [c] Compound not administered. [d] ±, fluctuation in response; increase followed by decrease.

neck until the desired area for immobilization was reached.

A Sanborn fluid pressure transducer, model 368B (range ±40 mm. Hg), was connected to a Sanborn recorder, model 350, for the sensing and recording of the ventricular pressure. Lead II was monitored with a Sanborn Twin-Viso recorder (model M-60-13000B, series 203). Connection between the catheter and transducer was accomplished according to the following procedure.

A needle adaptor was connected between the catheter and a short piece (5 cm.) of coiled polyethylene tubing (P.E. No. 10). A gasket was formed on the free end of the coiled tubing and sealed within a male-female B-D adaptor (size A for P.E. 10–50). The final connection was made by placing a male to male Luer adaptor between the female end of the B-D adaptor and the female adaptor of the transducer. The transducer was calibrated to record the pressures within the transducer limits at various attenuation settings.

After the surgical procedure had been completed, the animal was placed into a cylindrical Lucite chamber. The electrodes utilized for the conventional electrocardiographic (ECG) recordings were strategically placed in the chamber so that contact with the electrodes was achieved in the normal resting position. This apparatus facilitated simultaneous recording of right ventricular pressure and electrocardiographic leads I, II, or III. Provisions for obtaining intraventricular ECG recordings had been initiated, but were not completed during the study.

Drug Effects on Right Ventricular Pressures—Anesthetized guinea pigs, weighing between 750 and 1000 Gm., were utilized when studying the effect of antihistamines on the changes induced by histamine and mescaline on this parameter. A control period of 60 min. was maintained prior to injection of the test compound. Readings were obtained at a definite interval to correlate with those taken in the respiratory phases. Antihistamines (chlorpheniramine and diphenhydramine, dosage range 0.4 to 0.8 mg./Kg. i.p.) were administered to observe possible antagonistic action against histamine (4 mg./Kg.) and mescaline (25 mg./Kg.). The antihistamines were administered 15 min. prior to the histamine or mescaline challenge (Table II).

Electrocardiographic Recordings—Unanesthetized male and female guinea pigs, weighing between 700 and 1000 Gm., were used. To record the heart rate, the animal was confined in a plastic container in a prone position. The leads were secured to the forelegs so that lead I could be recorded. An arbitrary time period of 15 min. was allowed to elapse before the ECG recordings were begun. The animal was then removed from the container and injected intraperitoneally with mescaline or with the solvent (water). The latter served as the control. Various dosages of the drug were administered (ranging from 1 to 240 mg./Kg.), and the influence on the ECG was recorded at specific

TABLE IV—EFFECT OF MESCALINE ON THE RESPIRATORY RATE IN THE INTACT GUINEA PIG

	5–20 min.		5–60 min.	
	Mean Resp./ min.	S. D.	Mean Resp./ min.	S. D.
Control[a]	69.2		67.6	
mg./Kg.[b]				
5	90.4	± 7.94	87.2	± 7.55
25	98.0	±10.95	88.6	± 7.94
100	103.5	±12.92	96.5	±10.95

[a] Twelve animals in control group. [b] Four animals per dosage level.

TABLE V—EFFECT OF DIPHENHYDRAMINE ON RESPIRATORY RATE CHANGES INDUCED BY MESCALINE AND HISTAMINE IN THE UNANESTHETIZED GUINEA PIG

Compd.[a]	Dosage,[c] mg./Kg.	Treatment with Diphenhydramine, mg./Kg., 15 min. Prior to Challenge	% Change in Respiratory Rate at Max. Effect
Histamine	4.0	[b]	+20
Diphenhydramine	0.7	[b]	No change
Histamine	4.0	0.7	+6
Mescaline	25.0	[b]	+33
Mescaline	25.0	0.7	+36

[a] Compounds were administered intraperitoneally as their salts. [b] Compound not administered. [c] Two animals per dosage level.

intervals over a 240-min. period following injection.

Measurement of Respiratory Dynamics Utilizing the Amdur Technique—Tidal volume, respiratory rate, minute volume, compliance, resistance, and flow rate were recorded in the anesthetized guinea pig (weighing between 230–290 Gm.). The technique utilized was a modification of the Amdur method (25). Control readings were begun following a 30-min. recovery from catheterization. Control readings were taken every 3 min. for a period of 15 min. to insure parameter stability. The animal was then injected intraperitoneally with drugs of varying doses (Table III), and tracings were obtained at the specified intervals for a period of 1 hr.

Drug Effects on Respiration in the Intact Animal—In order to evaluate the effect of mescaline on the respiration of the intact animal, 12 animals in groups of four were given mescaline in doses of 5, 25, and 100 mg./Kg. intraperitoneally. Respiratory rate was counted at specific intervals over a 1-hr. period; the same procedure was followed for the control values (Table IV). Six animals were used to demonstrate the effect of diphenhydramine on the per cent change in the respiratory rate at peak responses to a histamine or mescaline challenge. The drugs and doses used are given in Table V.

RESULTS

Measurement of Right Ventricular Pressure—Normal right ventricular measurements were carried out in a series of 49 anesthetized guinea pigs. Electrocardiograms as well as right ventricular pressure was monitored in 16 animals at specific intervals for a period of 3 hr. Lead II was recorded simultaneously with the ventricular pressure at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. after cannulation. Due to instrument difficulty, ECG data have been compiled in nine of the 16 runs mentioned above.

Frequency distribution histograms were plotted for pressure values obtained immediately after cannulation (Fig. 3, A) and for the average value obtained during the 3-hr. exposure periods (Fig. 3, B). Histogram A exhibits a normal distribution pattern. The arithmetic mean was 13.4 mm. Hg, with a standard deviation of ±4.6 mm. Hg. Observation of the difference between the pressure immediately after cannulation and the average value after the 3-hr. exposure period was made and found in most instances to be small. In the majority of cases, the difference recorded was less than 1.0 mm. Hg (ranging from −1.1 to +2.5). Generally, when the difference was greater than +1.0 mm. Hg, the animal exhibited signs of partial recovery from the initial level of anesthesia. Marshall and Hanna (26) stated that under general anesthesia the mean arterial blood pressure in the guinea pig was lower than when the animal was conscious and active. More recently, Rudolph and Scarpelli (27) observed reduction in blood pressure after intravenous administration of pentobarbital in the unanesthetized dog. Popovic and Kent (28) have demonstrated the effect of anesthesia upon cardiac output presenting evidence that cardiac output is reduced under anesthesia. The ECG data and heart rate values obtained in these experiments correspond closely with those described by Lombard (29).

Drug Effects on Right Ventricular Pressure—Six anesthetized animals (35 mg./Kg. sodium pentobarbital) were injected intraperitoneally with mescaline and right ventricular pressure and lead II were monitored. In all cases a rise in pressure was noted in approximately 2 min. following injection. The rise in pressure correlates remarkably well with the onset of hyperpnea in the respiratory phase. The magnitude and duration of the pressure rise varied from animal to animal; however, the return to control values was initiated at about 30 min. after administration of the compound. During the entire test period there appeared compensatory changes, although this phenomenon was not observed during the control period (60 min.).

Animals exhibiting marked changes in pressure also demonstrated ECG alterations. In general, there was no significant change in pulse rate while marked changes occurred in conduction. One animal exhibited A-V block and inversion as well as diphasic "T" wave changes. Overall, the changes in ECG were not consistent. In some animals, the "T" wave was observed to reverse polarity and appear "tented," while in others it became more negative. However, the effect observed on the ECG was noted to occur between 5 and 10 min. after mescaline injection in all cases.

Three animals were injected with histamine. The ventricular pressure was observed to both rise and fall dramatically throughout the first 10 min. following administration. Following this phase, the pressure usually fell with an abrupt cessation of cardiac activity a few minutes thereafter. It is noteworthy to mention that this marked fluctuation in pressure was also observed with mescaline.

In order to assess the effect of antihistamines on

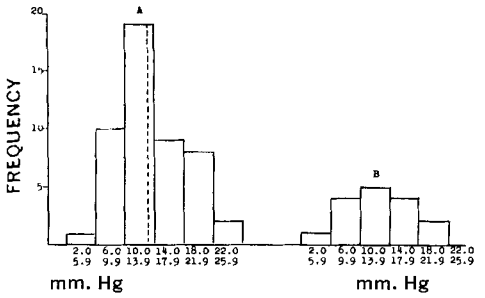


Fig. 3—Frequency histograms: ventricular pressures. Key: A, *values (49 animals) immediately after cannulation;* B, *average values for nine readings of right ventricular pressure for 3 hr. (16 animals);* - - - -, *mean pressure value (13.4 mm. Hg).*

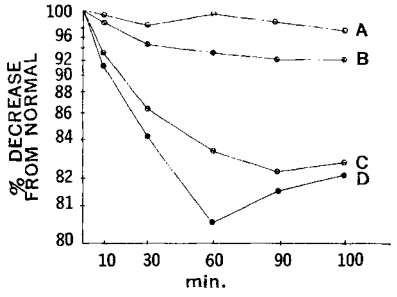


Fig. 4—Effect of mescaline on heart rate in the unanesthetized guinea pig. Key: A, *50 mg./Kg.;* B, *100 mg./Kg.;* C, *200 mg./Kg.;* D, *240 mg./Kg.*

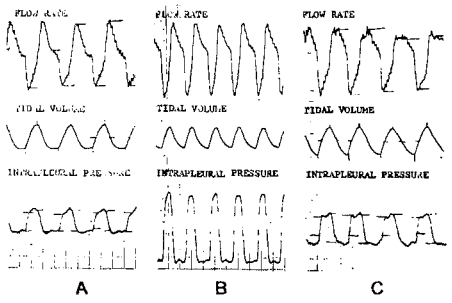


Fig. 5—Simultaneous effect of mescaline on various respiratory parameters in the unanesthetized guinea pig. Key: A, *control;* B, *test period, 5 min. following 100 mg./Kg i.p. of mescaline;* C, *recovery at 60 min.*

mescaline, the compounds were first tested on histamine challenged animals. As can be observed in Table III, the antihistamines blocked the previously pressor and lethal effects of histamine. The antihistamines did not effectually block the action of mescaline either on the rise in ventricular pressure or in respiratory rate, in dosages which were sufficient to curtail the effects of histamine. These findings are also borne out in the respiratory study on the intact animal.

Electrocardiographic Recordings—A series of 12 animals was used for this phase. Administration of mescaline in dosages of 1, 3, 8, 10, and 20 mg./Kg. did not exhibit a significant change in pulse rate or in wave characteristics when compared with control. However, when higher dosages were administered (50, 100, 200, and 240 mg./Kg.) there appeared to be a significant decrease in rate beginning at 10 min. and lasting for approximately 1 hr. (Fig. 4). In most of the latter cases, a "T" wave change was evident at 10 min. and was terminated at approximately 60 min. In general, low doses produced no significant change in the above parameters; high doses had a rather rapid but transient effect on pulse rate and "T" wave of the guinea pig ECG. A more critical evaluation of the electrical characteristics was limited due to instrument inefficiency.

Respiratory Data Utilizing the Amdur Technique—In all animals tested and with all dosages employed (5, 25, 50, and 100 mg./Kg.), there was exhibited an increase in respiratory rate and resistance; however, tidal volume and compliance decreased. Because of the marked increase in respiratory rate, the minute volume also increased. The changes in the above parameters occurred rapidly, usually within the first 3 min. The magnitude of the effect was observed to be dependent upon the dose. The maximum effect was demonstrated to range from 10 to 30 min. following administration of the compound. A return to control values usually began after 30 min. and was complete by 60 min. (Fig. 5). Furthermore, a considerable amount of compensation occurred throughout the test period, which was reflected in a "see-saw" activity of the respiratory rate and the dependent minute volume as well as in the other parameters monitored.

The most consistent change was observed in the compliance decrease. The per cent decrease in compliance at maximum effect for 5, 25, 50, and 100 mg./Kg. of mescaline was 33, 66, 70, and 77, respectively. It was noted on many occasions that the effect of this compound was in large part dependent upon the susceptibility of the animal, which in turn was dependent upon the physical state. Indeed, an animal with respiratory disturbances will, in many instances, not respond typically to the compound.

Pretreatment with diphenhydramine (0.7 mg./Kg.) did not alter the decrease in compliance following the administration of mescaline (25 mg./Kg.). Diphenhydramine alone was observed to cause either an increase or no change in compliance (Table II).

Drug Effects on Respiration in the Intact Animal—With all dosages employed, mescaline produced a significant increase in respiratory rate and activity of the animals. The effect was very rapid at all dose levels beginning at approximately 1 to 3 min. Termination of the effect was observed to occur between 20 and 30 min. after injection. The respiratory rate at the end of the test period (60 min.) was usually below control rate. This was also observed in the catheterized animals. The average per cent increase in respiratory rate for a total of 1 hr. at 5, 25, and 100 mg./Kg. was 23.2, 33.2, and 51.6, respectively. However, if only the first 20 min. were considered, the per cent increase was greater because the major effect of the compound was occurring during this period (Table IV). It is interesting to note that several animals in all dosage groups exhibited both coughing and very strong abdominal contractions. At times, the con-

tractions were so powerful as to propel the animal forward.

The effect elicited by mescaline on compliance of the lung and airway resistance is quite similar to that which has been observed for histamine (25). In order to observe the effect of antihistamine pretreatment on the respiratory changes produced by mescaline, without the influence of anesthesia and surgical trauma, several animals were given diphenhydramine (0.7 mg./Kg.) 15 min. prior to mescaline (25 mg./Kg.) or histamine (4.0 mg./Kg.). There were no significant changes induced by diphenhydramine alone; however, mescaline produced its characteristic symptoms with or without pretreatment of the antihistamine. Marked hyperpnea and coughing were noted within 5 min., the maximum occurring between 10 and 20 min. The effect of histamine was antagonized by the antihistamine (Table V).

DISCUSSION

Patterns of Right Ventricular Pressure—Observation of the pressure waves indicated that they could be grouped into three basic forms. The first demonstrated a typical peripheral pulse pattern (Fig. 2, A). The second showed a smoother wave with a slight flattening of the apical region (Fig. 2, B), and the third, which though similar to the second, was devoid of flattening of the apical region (Fig. 2, C). Dissection of the site of catheter insertion revealed that where the first wave formation was present, the catheter resided in the ventricle in proximity to the valve of the pulmonary artery. The other two pressure patterns differed only with respect to their location within the ventricular lumen, the former lying in the lower third and the latter in the upper third of the lumen. From an examination of the tracings of the former situation, the flattened apical region could be accounted for by assuming that at the peak of systole the catheter was forced against the ventricular wall (because of the narrowness of the lumen in that area) and resulted in a momentary interference with blood flow through the catheter. The apically flattened curves resulted from the catheter positioned in the narrower area of the ventricle, whereas the smooth waves described above resulted from the positioning of the catheter in the wider portion of the ventricle. The results indicated that slight movement of the catheter within the right ventricle may lead to a fourfold difference in pressure. There appeared to be an "optimal-position pressure relationship." When the catheter lay in the upper third of the ventricular lumen, the pressure was higher than that found when the catheters were positioned in the lower two-thirds of the ventricle.

In these studies, the ventricular pressure was determined to be less than 4.0 mm. Hg when the catheter tip lay under the papillary muscle. In this situation, the catheter could not, as a rule, be repositioned without damage to the overlying musculature. The ventricular pattern was characteristic when this occurred. The wave was small (low pressure) and considerably wider, whereas the apex of the wave was usually flattened. If the catheter was found to be pressing against the tissue anywhere within the ventricle, the wave formed was relatively wide and the pressure low (lower than 5.0 mm. Hg). In these studies, lead II was monitored simultaneously with pressure determination. These data are in accord with previously published reports.

Ventricular pressure variations reported in the literature seem to have been dependent upon positioning of the catheter as well as the methodological problems having little or nothing to do with the normal physiological state of the test animal.

The changes (decrease) in compliance observed with the higher dosages of mescaline (50 and 100 mg./Kg.) reflect near maximal decrease in this parameter (30–33). Concomitantly, the resistance in the airways increased. Moreover, because both effects occur at approximately the same time, it is difficult to assess the primary response. However, if mescaline exerts its respiratory effects in a fashion similar to other compounds, *e.g.*, serotonin or histamine (4), which induce a rapid and shallow respiratory pattern, one can attribute these effects to a primary change in compliance rather than resistance. If the respiration were deep and slow, this would tend to implicate the changes in resistance as the more susceptible function (31).

Decrease in compliance has been attributed to increased permeability and accumulation of blood in the pulmonary-capillary network of the lungs for histamine by Hughes, May, and Wittecombe (34, 35). However, spasm of the vascular network has also been demonstrated to cause the compliance to decrease. The effect of mescaline in low doses could be explained from the latter observations since the intrapleural pressure, although increased significantly, would at times approach control level. However, when higher doses were employed, the possible spasmogenic effect could not be compensated. Aviado (36) stated that methoxamine could influence pulmonary blood flow, but not directly influence the bronchiolar and pulmonary vascular smooth muscles. The consequences of a primary systemic pressor action, however, include reflex increase in vagal tone which can include bronchoconstriction. Moreover, the pressor effect of mescaline might serve to explain the changes observed, if an analogy can be made with methoxamine. Furthermore, Speck (9) demonstrated that bradycardia was produced following mescaline administration. This effect has been confirmed in this study. The possibility of reflex bronchoconstriction accounting, at least in part, for the action of mescaline on respiration has merit. Luduena, O'Malley, and Ogen (37) have demonstrated that lung edema can be produced by intravenous administration of epinephrine into rabbits. They attributed the edema to the intense and sustained high arterial blood pressure with increased left ventricular work load leading to elevated capillary pressure and then edema. It has been demonstrated that the edema produced in the dog by epinephrine is probably mediated by its peripheral action only.

Mescaline has been observed to increase systemic blood pressure in the cat (38), to cause severe peripheral vasoconstriction in the rat (9), and to produce constriction of various smooth muscle preparations *in vitro* (4, 6, 7); the latter effect has also been confirmed in this laboratory. The possibility exists that the lung edema observed in this study following intermediate and high dosages of mescaline (25 to 100 mg./Kg.) could be attributed to a similar mechanism as that postulated for epinephrine.

Because of the similarity with the observed respiratory change (in cannulated and intact animals) and the ventricular pressure between histamine and mescaline, antihistamines were employed in concentrations sufficient to block the effect of histamine. The results demonstrate that the antihistamines were not effective against mescaline; therefore, mescaline is probably not liberating histamine or stimulating histaminic receptor sites. It may be plausible that some of the effects on right ventricular pressure may be explained on a physical basis, due to the marked forcefulness induced by mescaline on respiratory movements. Moreover, this effect appears to be multifaceted: a combination of facilitated venous return, due to severe respiratory movements, increased peripheral vasoconstriction, and possibly a spasmogenic effect on the pulmonary vascular network.

Low doses of mescaline produced a slight increase in heart rate; however, higher doses produced bradycardia. The latter effect was also reported by Speck (9). Because the rise in blood pressure parallels the fall in heart rate, a baroreceptor mechanism appears to be, at least partly, responsible for the bradycardia. The effect of mescaline on the ECG was not remarkable at low dosages (1, 3, 8, 10, and 20 mg./Kg.). However, at higher doses in the intact and cannulated animal, conduction changes were evident. The most prominent effect was observed as an increase in positive polarity of the "T" wave. This effect is quite similar to those reported for various sympathomimetic amines, especially phenylephrine (39). The observed changes could have been mediated *via* a central and/or peripheral mechanism as has been demonstrated for phenylephrine. Because mescaline exerts a strong influence on the central nervous system, the effect on the cardiopulmonary system *via* central mechanisms cannot be dismissed. The "T" wave appeared to be "tented," a phenomenon observed when the serum potassium level is elevated (11). However, Deniker (8) reported that in humans, the serum potassium level fell within the first 10 min. and the effect was maximum between 30 and 60 min. following the intravenous administration of mescaline (10 mg./Kg.).

SUMMARY

The effect of mescaline on respiratory dynamics and the electrocardiogram in the unanesthetized guinea pig was determined. A procedure is described for the acquisition of normal right ventricular pressure in the anesthetized guinea pig and the action of mescaline on this parameter.

1. The mean value obtained for right ventricular pressure was 13.4 ± 4.6 mm. Hg.

2. Mescaline (25 mg./Kg.) and histamine (4.0 mm. Hg) caused a marked increase in right ventricular pressure. Pretreatment with antihistamines in sufficient concentration to curtail the effects of histamine did not antagonize the action of mescaline.

3. The action of mescaline on respiratory dynamics (compliance, resistance of the airways, tidal volume, minute volume, flow rate, and respiratory rate) in the unanesthetized animal resembled the effect of histamine. However, pretreatment with diphenhydramine did not alter these responses to mescaline. Mescaline, in various doses, including that used in human experimentation, produced a marked increase in the respiratory rate in the intact guinea pig. Diphenhydramine effectively antagonized the action of histamine but not of mescaline on this parameter in the intact animal.

4. Control ECG data obtained in these experiments are in accord with previously published reports. Mescaline did not significantly alter heart rate in low to intermediate dosages (1 to 25 mg./Kg.); however, bradycardia was apparent with higher dosages (50 to 240 mg./Kg.). Conduction disturbances were manifested at the high dose levels, the most consistent being a "T" wave alteration.

REFERENCES

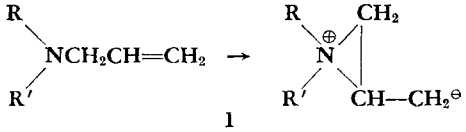
(1) Denber, H. C., Teller, D., Rajotte, P., and Kaufman, D., *Ann. N. Y. Acad. Sci.*, **96**, 14(1962).
(2) Giarman, N., and Freedman, D., *Pharmacol. Rev.*, **17**, 1(1965).
(3) Takeo, Y., and Himwich, H., *Science*, **150**, 1309 (1965).
(4) Gaddum, J. H., Hebb, C. O., Silver, A., and Swan, A., *Quart. J. Expll. Physiol.*, **38**, 255(1953).
(5) Gaddum, J. H., and Hameed, K. A., *Brit. J. Pharmacol.*, **9**, 240(1954).
(6) Åström, A., and Sameleus, U., *ibid.*, **12**, 410(1957).
(7) Costa, E., *Proc. Soc. Expll. Biol. Med.*, **91**, 39(1956).
(8) Deniker, P., *J. Nervous Mental Disease*, **125**, 427 (1957).
(9) Speck, L., *J. Pharmacol. Exptl. Therap.*, **119**, 78 (1957).
(10) Wolbach, A. B., Jr., Miner, E. J., and Isbell, H., *Psychopharmacologia*, **3**, 219(1962).
(11) Goldman, M., "Principles of Clinical Electrocardiography," 5th ed., Lange Medical Publications, Los Altos, Calif., 1965, pp. 377–380.
(12) Cochin, J., Woods, L. A., and Seevers, M. A., *J. Pharmacol. Exptl. Therap.*, **101**, 205(1951).
(13) Wolbach, A. B., Jr., Isbell, H., and Miner, E. J., *Psychopharmacologia*, **3**, 1(1962).
(14) Grace, G. S., *J. Pharmacol. Exptl. Therap.*, **50**, 359 (1934).
(15) Deniker, P., *J. Nervous Mental Disease*, **124**, 371 (1956).
(16) Parker, J. M., and Hildebrand, N., *Federation Proc.*, **21**, 419(1962).
(17) Halmagyi, D. J., Colebatch, H. J. H., and Starzecki, B., *J. Appl. Physiol.*, **19**, 105(1964).
(18) Popovic, V., *Am. J. Physiol.*, **207**, 1345(1964).
(19) Popovic, V., and Kent, K. M., *ibid.*, **207**, 767(1964).
(20) Weeks, J. R., and Davis, J. D., *J. Appl. Physiol.*, **19**, 540(1964).
(21) Carson, S., Goldhamer, R. E., Mackars, A., and Silson, J. E., *Arch. Environ. Health*, **11**, 635(1965).
(22) Friedhoff, A. F., and Goldstein, M., *Ann. N. Y. Acad. Sci.*, **96**, 5(1962).
(23) Amdur, M., and Mead, J., *Am. J. Physiol.*, **192**, 364 (1958).
(24) Popovic, V., and Popovic, P., *J. Appl. Physiol.*, **15**, 727(1960).
(25) Amdur, M., and Mead, J., *Proc. Third Natl. Air Pollution Symp.*, 1955.
(26) Marshall, L. H., and Hanna, C. H., *Proc. Soc. Expll. Biol. Med.*, **92**, 31(1956).
(27) Rudolph, A. M., and Scarpelli, E. M., *Am. J. Physiol.*, **206**, 1201(1964).
(28) Popovic, V., Kent, K., and Popovic, P., *Proc. Soc. Expll. Biol. Med.*, **113**, 599(1963).
(29) Lombard, E. A., *Am. J. Physiol.*, **171**, 182(1952).
(30) Carson, S., unpublished data.
(31) Mead, J., *J. Appl. Physiol.*, **15**, 325(1960).
(32) Murphy, S. D., and Uhlrich, C. E., *J. Am. Ind. Hygiene Assoc.*, **25**, 28(1964).
(33) Swann, H. E., Jr., Brunol, D., and Balchum, O., *Arch. Environ. Health*, **10**, 24(1965).
(34) Hughes, R., May, A. J., and Wittecombe, J. G., *J. Physiol.*, **142**, 306(1958).
(35) Cook, C. D., Mead, J., Schreiner, G., Frank, N., and Craig, J. M., *J. Appl. Physiol.*, **14**, 177(1959).
(36) Aviado, D. M., Jr., *J. Pharm. Sci.*, **51**, 191(1962).
(37) Luduena, F. P., O'Malley, E., and Ogen, I. H., *Arch. Intern. Pharmacodyn.*, **162**, 111(1959).
(38) Jacobsen, E., *Clin. Pharmacol. Therap.*, **4**, 480(1963).
(39) Keys, A., and Violante, A., *J. Clin. Invest.*, **21**, 1 (1942).

Synthesis of Possible Cancer Chemotherapeutic Agents Based on Enzyme Rationale VI

Allylamine Derivatives

By K. C. TSOU, SURESH B. DAMLE, R. W. CRICHLOW, R. G. RAVDIN, and HARRY W. BLUNT

The participation of electron pair on the nitrogen atom with the olefinic π electron in allylamines makes possible the formation of an ethylenimonium-carbanion pair (I), a reactive intermediate analogous to the ethylenimonium ions that are formed



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in nitrogen mustards. The opportunity of such participation decreases as the electron density of the nitrogen is suppressed by the formation of an amide. This interesting participation reaction suggested the evaluation of a number of allylamine amides so that they could be mediated by the difference in amidase activity between normal and neoplastic tissues. Physical data support this novel participation, and preliminary antitumor screening data of several compounds are reported. *N,N'*-Diallyl β-aziridino propionamide was found to be an effective agent against Dunning leukemia in both solid and ascites forms.

IN THE DESIGN and preparation of new drugs, one of the interesting approaches is to take advantage of the difference in enzyme activity between normal and neoplastic cells (1–3).

A scheme is proposed to develop cancer chemotherapeutic agents which are acted upon *in vivo* by enzymes to liberate compounds toxic to the tumor, if the enzyme concentration is higher in the tumor. (Scheme I, where A–B represents a drug that can be acted upon at the site indicated by the dotted line, and where the asterisk designates the toxic moiety.)

$$\underset{\text{less or nontoxic than } A}{A \text{ -}\!\!\!\!\!\!\text{:}\!\!\!\!\!\!\text{-} B} \xrightarrow[\text{enzyme}]{} \underset{\text{toxic agent}}{A^* + B}$$

Scheme I

If the enzyme level is lower in tumor, then a more toxic compound that can be detoxified by enzyme in the normal cell but not in tumor cell should be used. (Scheme II.)

$$\underset{\text{more toxic than } A \text{ or } B \text{ to tumor}}{A \text{ -}\!\!\!\!\!\!\text{:}\!\!\!\!\!\!\text{-} B} \xrightarrow[\text{enzyme}]{} \underset{\text{nontoxic}}{A + B}$$

Scheme II

Thus, in such synthetic work, one is confronted with both what enzyme difference should be used and with what toxic agents should be incorporated. The choice of cytotoxic moiety, hemisulfur mustard, $ClCH_2CH_2SCH_2CH_2OH$, was used in early work (2). This was based on the low hematopoietic effect of this compound as reported by Seligman, Rutenberg, Persky, and Friedman (4). Subsequent preclinical work indicated that hemisulfur mustard compounds often exhibit neurotoxicity (5) which could complicate the clinical management of such types of drugs. Thus, the effort has continued along the line of synthesis of compounds with reduced neurotoxicity and/or drugs that incorporate other one-arm alkylating agents, such as heminitrogen mustard[1] and aziridines (3). In addition, the authors also considered hexahydroazepine derivatives (6) as nonclassical alkylating agents. While much remains to be done in these compounds, the delay in hematopoietic effect in a pilot clinical trial (7) suggested that it would be worthwhile to consider also other nonclassical alkylating agents. The present paper reports a study of allylamine derivatives as potential alkylating agents, and the synthesis of allylamine amides as possible cancer chemotherapeutic agents based on the previously proposed rationale.

DISCUSSION

It is generally accepted that ethylenimonium ion is the active intermediate of nitrogen mustard

Received October 24, 1966, from the Harrison Department of Surgical Research, School of Medicine and Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104

Accepted for publication January 4, 1967.

Presented to the American Chemical Society, Atlantic City meeting, September 1965.

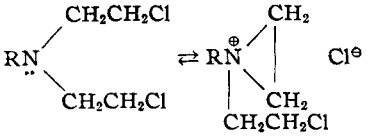
This investigation was supported by research grant CA-07339 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

The authors acknowledge the technical assistance of Mr. George Benson, Mr. H. Hoyt, and Mr. Jerry Ford, Dr. Gerald Peskin for advice in the dog experiments, and Dr. J. Venditti and Dr. H. Wood, Cancer Chemotherapy National Service Center, for the screening data.

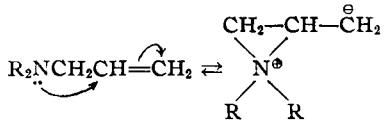
Previous paper: Tsou, K. C., and Su, H. C. F., *J. Med. Chem.*, 6, 693(1963).

[1] Tsou, K. C., unpublished data. Compounds cited in *Cancer Chemotherapy Rept.*, 1964.

(HN_2) (8) and that very likely biological alkylation is related to its formation (9). The formation of this transition state depends on the nucleophilic displacement of the nitrogen atom onto the carbon adjacent to the chlorine atom (Scheme III). Thus, it is not unreasonable to ask whether in allylamine derivatives a similar type of nucleophilic attack of electrons from the nitrogen atom could take place as shown in Scheme IV.



Scheme III



Scheme IV

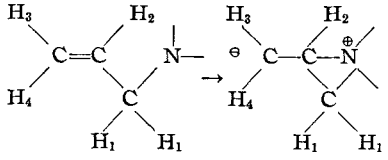
Such transition state could very well be favored if the carbanion could be stabilized by solvation as in the case of the chloride ion in HN_2. Thus, it is tempting to suggest that in the biological system allylamine might be stabilized through an internal ion pair and then act as potential alkylating agents. A careful investigation of the physical and chemical properties of some allylamine derivatives indeed suggested such possibilities could exist.

The pKa of allylamine (I), diallylamine (II), triallylamine (III), methyldiallylamine (IV), and dimethylallylamine (V) are listed in Table I (10). As the number of allyl group increases, the basicity decreases slightly in II, and significantly in III. This is in contrast to what is expected from the pKa values of ethylamine (10.63), diethylamine (10.93), and triethylamine (10.72), since increasing substitution generally results in slightly increased basicity. The substitution of methyl groups in IV and V also seems to result in a decrease in basicity, rather than the anticipated increase that one may expect from the hyperconjugation effect of the methyl group. It seems that these data could obviously be explained by an overlap of the electron pair on the nitrogen with the olefinic electrons. This is also suggested by their ultraviolet spectroscopy as shown in Table I, where additional suggestive evidence is apparent. Allylamine has a max. at 210 mμ which is clearly due to the participation of nitrogen electron with the π olefin, or a p–σ–π interaction in the triad =N—CH_2—CH= of diallylamine caused both bathochromic and hyperchromic effect as expected. The high intensity value (olefins are usually at 10–190 mμ) shows clearly that this is not due to the amine $n \rightarrow \sigma$ itself (usually ~1000) (11).

Examination of the infrared spectra of allylamine, diallylamine, and *N*-methyldiallylamine and other allylamines provides additional useful evidence of the formation of such "incipient ethylenimmonium ion." Thus, the strong 3075 cm.$^{-1}$ and 3005 cm.$^{-1}$ bands that are present in all aziridine derivatives are also in the substituted allylamines (12). In both diallylamine and methyldiallylamine, there was found the usual 2865 cm.$^{-1}$ methylene band and a very strong band at 2805 cm.$^{-1}$, but it is not so evident in monoallylamine. This 2805 cm.$^{-1}$ band can best be assigned as an unsymmetrical C—H stretching of the methylene group. The displacement of carbon 2–3 double bond to a single bond carbanion could contribute to an additional resonance vibration resulting in an increase in the intensity of this band.

A comparison of the nuclear magnetic resonance spectra of the allylamines reveals even more interesting data (Fig. 1).

As illustrated in Scheme V, if such intramolecular participation contributes to these compounds, the amine nitrogen should become less negative and the H_1 proton could be expected to shift to a higher field (or "curtain effect") (13). The chemical shifts



Scheme V

of H_1 in Table II indeed support this mechanism, especially when compared with propylamine. Thus, the theoretical H_1 value of dimethylallylamine (V) can be calculated from the Shoolery additive constant (14) to be 3.11, yet the found value is only 2.83. Correspondingly, an increase in carbanion character of the terminal allylic carbon is expected to cause chemical shifts of the terminal protons towards low field.[2] The slight increase in δ of H_3 in Table II obviously is in line with this expectation. There was no change in $J_{4,2}$ and $J_{3,2}$ in these compounds when compared with I in the liquid state, and there was also no significant change in J_{trans}/J_{cis} ratio. When one examines a molecular model, the approaching nitrogen to C_2 results actually in little change of the dihedral angle between $H_{4,2}$ and $H_{3,2}$; this is, very likely, why one may not expect any significant change in coupling constants.

More recently, Ferretti and Tesi (16) reported an observation of Prévost reaction in *N*-allylmorpholine in which they obtained a 2-morpholino-1,3-propandiol instead of the expected 1,2-diol. Independent of their work, a similar reaction of iodine chloride with *N*-allylpiperidine, followed by hydrolysis, has also been found by us to yield 2-piperididino-1,3-propanediol. These reactions thus provide indirect support for the formation of an ethylenimonium ion among allylamines.

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[2] The study of the chemical shift of proton attached to a carbanion has not hitherto been reported. It should especially be noted that the present case is not that of a delocalized allyl anion, CH_2=CH—$CH_2^{\ominus}$, where one might expect a shift to high field due to shielding. Where electron density can be localized on the terminal carbon, as in the present case, an anisotropic effect would predict a downfield shift. (See *Reference 13*, p. 176.) The nearest example the authors know of is the investigation of *N*-ethylallenimine by Roberts, who stated that the "vinyl proton does not change significantly with temperature" (15). While Roberts did not say whether it also shifted up or down slightly, it is important to note that the vinyl proton remained discrete, did not exchange with the ring proton, and therefore did not become involved in the following scheme:

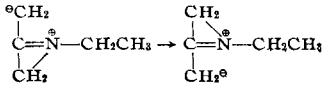


TABLE I—PHYSICAL PROPERTIES OF ALLYLAMINES

Compd.	B.p., ° C.	pKa[a]	U.V. Spectra[b] $\lambda_{max.}$ (mμ)	U.V. Spectra[b] $\epsilon_{max.}$	Retention Time,[c] sec.
Allylamine (I)	54–55	9.49	210.0	13,870	89
Diallylamine (II)	60–61.5	9.29	211.0	32,630	137
Triallylamine (III)	148–50.5	8.31	215.0	44,660	942
Methyldiallylamine (IV)	110.5–111.5	8.79	215.0	39,090	315
Dimethylallylamine (V)	60–61.5	8.72	213.0	26,550	120

[a] See *Reference 10.* [b] In 95% ethanol. [c] On amine-220 column (6 ft. × ¼ in., 5% on Chromosorb P, 60–80 mesh); column temperature, 78°C.; collector temperature, 56°C., injector, 153°C.; detector, 227°C.; helium flow, 60 ml./min.; 1 μl. injection.

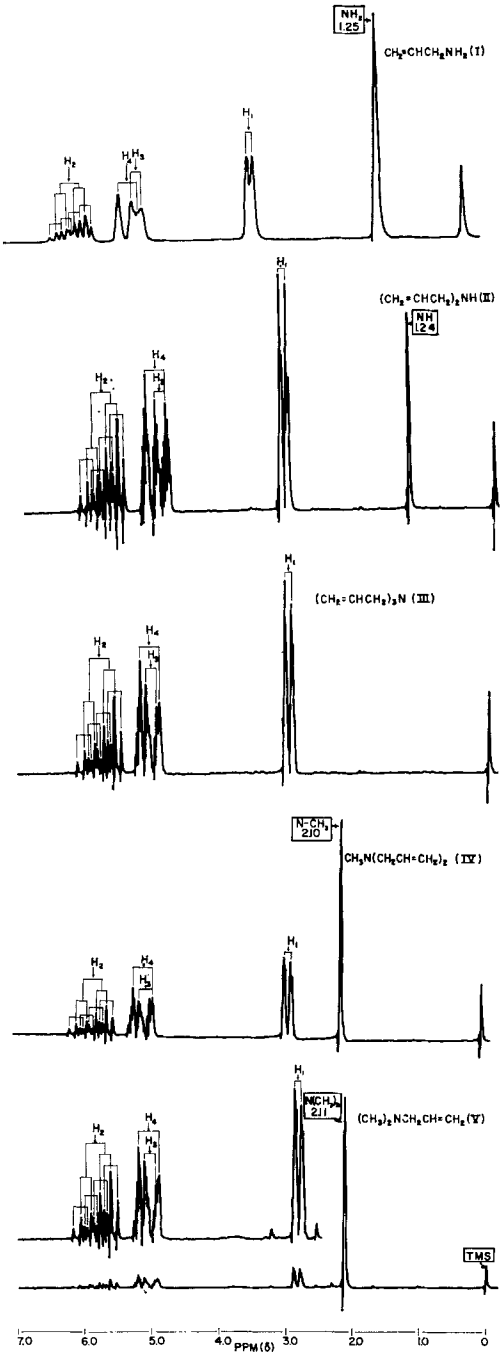


Fig. 1—Nuclear magnetic resonance spectra of allylamines.

While much of the assessment of the chemical data was in progress, exploratory screening of allylamine derivatives was simultaneously begun in different tumor systems through the generous cooperation of Cancer Chemotherapy National Service Center. Even though most of the data have appeared in their report (17), a discussion of these data in a summarized manner is certainly appropriate and not repetitious.

Allylamine, diallylamine, and triallylamine were compared in the sarcoma 180 system. The protocol of the screening has been described (17), and the results are summarized in Table III. It is of interest to note that diallylamine was found to have some tumor inhibitory activity in the sarcoma 180 system, even though the reproducibility in the confirmatory test was poor. No additional screening has been run in other systems.[8]

In another study, the authors were evaluating amidase hydrolysis of normal and neoplastic tissue on substrates of the *N*-acylaziridines (3), and occasionally high hydrolysis rate was noted in neoplastic tissue. First, if allylamine were to be incorporated in an amide, the electron density of the nitrogen atom would be suppressed and thereby reduce the opportunity for the allylamine to cyclize to an ethylenimonium ion. And yet, upon enzymatic hydrolysis, the allylamines could then undergo the incipient ethylenimonium ion formation as postulated.

In reviewing the literature on amidase activity, one noted that the hydrolysis of amides has been reported high in several tumors. Greenstein (18) reported fivefold increase in the hydrolysis activity of leucyl glycine in hepatoma over mouse liver. Goldbarg and Rutenberg (19) reported also the higher rate of hydrolysis of leucyl β-naphthylamide in cancer of the pancreas, using a method originally developed by Green, Tsou, Bressler, and Seligman (20). Burstone (21) and Hosoda, Takase, and Yoshida (22) also reported independently high activity in gastric cancer with the use of the same substrate. It thus seems of interest that a number of α- and β-amino diallyl amides might simulate closely the environmental requirement of the enzyme.

To survey the first requirement, *N*-allylacrylamide, *N,N'*-diallylacrylamide, and *N,N'*-diallylmethacrylamide were chosen for exploratory purposes. The use of acrylic acid derivatives was preferred because interesting results had been found when acrylamide (NSC No. 7785) was screened in its activity against adenocarcinoma 755. These allylamides were also compared in the CA-755 system. As can be seen readily (Table IV), *N,N*-diallylmethacrylamide did pass the primary screening

[8] One must add that the analogous methallylamines are not active in the CCNSC screening systems.

TABLE II—NUCLEAR MAGNETIC RESONANCE DATA OF ALLYLAMINES

Compd.	Chemical Shift[a] H_1	H_2	H_3	H_4	Coupling Constants[b] $J_{4,2}$	$J_{3,2}$	$J_{1,2}$	$J_{3,4}$
I	3.20	5.88	4.89	5.07	17.0	10.0	5.0	2.0
II	3.15	5.87	5.00	5.06	17.5	9.5	6.0	1.5
III	3.00	5.83	5.05	5.06	17.5	9.5	6.0	1.5
IV	2.90	5.80	5.00	5.02	17.5	9.5	6.0	1.5
V	2.83	5.87	5.03	5.06	17.5	9.5	6.0	2.0

[a] In p.p.m. from tetramethylsilane (TMS)$_1$ ± .02. [b] In c.p.s. ± 0.5.

TABLE III—COMPARISON OF ANTITUMOR ACTIVITIES OF ALLYLAMINE, DIALLYLAMINE, AND TRIALLYLAMINE IN SARCOMA 180

Dose, mg./Kg.	Survivors	Wt. Change, Gm. T/C[a]	Tumor Wt., mg. T/C[a]	% Inhibition
Allylamine, NSC No. 7600				
31.2	3/6	−3.0/−2.0	672/903	16
16.0	4/6	−1.0/−3.0	575/640	11
Diallylamine, NSC No. 20948				
415.0	2/6	+0.6/+3.2	430/1132	62
208.0	2/6	−2.6/+2.9	495/870	43
104.0	4/6	−1.9/+1.5	403/803	50
104.0	6/6	−2.4/−0.7	413/1339	69
104.0[b]	6/6	+2.3/+0.9	579/725	20
Triallylamine, NSC No. 32635				
500.0	4/6	−1.9/−2.1	415/711	42

[a] T/C, tumor/control. [b] In this group, one mouse has tumor weight 1090; one mouse did not respond (730).

TABLE IV—COMPARISON OF ACRYLAMIDE, *N*-ALLYLACRYLAMIDE, *N*-DIALLYLACRYLAMIDE, AND *N,N*-DIALLYLMETHACRYLAMIDE IN ADENOCARCINOMA 755

Dose, mg./Kg.	Survivors	Wt. Change, T/C[a]	Tumor Wt., mg., T/C[a]	% Inhibition
Acrylamide				
80	0/10	...	...	
40	8/10	1.7/3.2	146/627	77
40	9/10	0.1/0.9	238/645	64
40	10/10	2.3/3.1	533/757	30
20	10/10	2.1/2.4	2421/2795	14
N-Allylacrylamide				
55.8	10/10	−2.2/−0.3	1185/753	None
N,N-Diallyacrylamide				
110.0	9/10	−1.4/−0.5	784/948	19
N,N-Diallylmethacrylamide				
94	0/10	0.0/+4.4	0/1257	Toxic
47	7/10	−8.8/+2.2	39/711	94
47	9/10	−0.7/+2.2	1318/1817	27
47	10/10	+0.2/+2.3	1416/1830	23

[a] T/C, tumor/control.

system, even though it failed later in sequential tests. In the second group, one of the earliest compounds that we prepared is *N,N*-diallyl β-aziridinopropionamide (DAAP). It was prepared readily by the addition of ethylenimine to diallylacrylamide. While this series of compounds is still being extended, DAAP was found to be active in screening against the Dunning leukemia system (Table V), both in solid and ascites form. Curative doses

TABLE V—EVALUATION OF *N,N*-DIALLYL β-AZIRIDINOPROPIONAMIDE[a] IN DUNNING LEUKEMIA SYSTEM (SALINE, i.p.)

Dose, mg./Kg.	Survivors	Cures	Survival Time, Days	%
Solid (DA)				
40	6/6	6	30.0/10.0	300
20	6/6	6	30.0/10.0	300
10	6/6		21.0/10.0	210
5	6/6		16.0/10.0	160
Ascites (DL)				
40	6/6	5	30.0/13.5	222
20	6/6	6	30.0/13.5	222
10	6/6		25.0/13.5	185
5	6/6		17.5/13.5	129

[a] NSC No. 74431.

were effected in 20–40 mg./Kg. range. The remarkable low toxicity of DAAP was also found to be true in dog experiments where severe leucopenia were never noticed in 20–40 mg./Kg. dose in contrast to many known difunctional alkylating agents. Dimethyl β-aziridinopropionamide was also prepared in order to compare the alkylating effect of the β-aziridino group. Such a study will be reported elsewhere in a lymphoma system (23). The corresponding β-methylaziridino derivative was also made, but was found to be less effective.

It is obvious that much remains to be done to extend these observations and to verify our speculative rationale. Whether the low toxicity and effective antitumor activity of DAAP provides a worthwhile clue to our enzyme rationale remains to be shown. Such study is in progress.

EXPERIMENTAL

Allylamine, diallylamine, and triallylamine were obtained from the Shell Chemical Co. They were distilled free from inhibitor and sealed in ampuls under nitrogen for screening. For spectroscopic study, they were further purified by gas chromatography as shown in Table I.

N-Allylacrylamide and *N,N*-diallylacrylamide were obtained from the Monomer-Polymer Laboratory, Borden Chemical Co., and again purified just before sending to the screener.

N,N-Diallylmethacrylamide—To a solution of methacrylyl chloride (24) (20.92 Gm., 0.2 mole) in 50 ml. of dry benzene was added a mixture of diallylamine (19.5 Gm., 0.2 mole) and triethylamine (22.2 Gm., 0.2 mole) in 50 ml. of dry benzene. The addition was completed in 1 hr. The reaction mixture was stirred at room temperature overnight and filtered to remove triethylamine hydrochloride. The filtrate was evaporated under aspirator to

remove solvent and was distilled under high vacuum to yield 30.0 Gm. (89%) of the product, b.p. 90–92°/15 mm.

Anal.[4]—Calcd. for $C_{10}H_{15}NO$ (165.23): C, 72.69; H, 9.15; N, 8.48. Found: C, 72.57; H, 9.03; N, 8.19.

N,N-Diallyl β-Aziridinopropionamide—A 8.3-Gm. quantity of pure ethylenimine was added to 12 Gm. of *N,N*-diallylacrylamide (distilled, b.p. 108–110°/3.0 mm.) in 10 min. The solution was stirred for 2 hr. and allowed to stand overnight at room temperature. It was then distilled under reduced pressure to give 12.7 Gm. (80%) of the product, b.p. 110–114°/0.5 mm.; n_D^{20} = 1.4906; $d.^{25}$ = 0.9752. I.R. 3.17 μ (aziridine), 3.26, 3.32, 6.02, 6.78 μ.

Anal.—Calcd. for $C_{11}H_{18}N_2O$: C, 68.01; H, 9.34; N, 14.42. Found: C, 67.84; H, 9.41, N, 14.43.

N,N-Diallyl β-(2-Methyl)aziridinopropionamide—A 11.4 Gm. quantity of propyleneimine was added to 15.1 Gm. of *N,N*-diallylacrylamide in 10–15 min. with stirring and cooling. The solution was stirred continuously for 2 hr. and then allowed to stand overnight. The reaction mixture was then distilled under reduced pressure to yield 16.0 Gm. (77%) of *N,N*-diallyl β-(2-methyl)aziridinopropionamide, b.p. 101–102°/0.4 mm.; n_D^{20} = 1.4867; $d.^{26}$ = 0.9766.

Anal.—Calcd. for $C_{12}H_{20}N_2O$: C, 69.19; H, 9.68; N, 13.45. Found: C, 69.16; H, 9.90; N, 13.72.

N,N-Dimethyl β-Aziridinopropionamide—Ethylenimine (13.2 Gm., 0.3 mole) was added to a solution of dimethylacrylamide (14.9 Gm., 0.15 mole) in 30 min. with stirring. The mixture was allowed to stand overnight. The solvents were removed under aspirator and the product distilled under high vacuum to give 7.8 Gm. of the product, b.p. 71–72°/0.4 mm.; n_D^{20} = 1.4750; $d.^{24}$ = 0.9741.

Anal.—Calcd. for $C_7H_{14}N_2O$: C, 59.12; H, 9.92; N, 19.71. Found: C, 59.06; H, 9.89; N, 19.76.

Reaction of Allylpiperidine with Iodine Monochloride—To a benzene solution of ICl (1.6 Gm.) was added 1.3 Gm. of allylpiperidine slowly, and the reaction mixture was warmed for 10 min. and allowed to stand overnight. After removal of benzene, the residue turned reddish and failed to crystallize even though some yellow crystals appeared first. Purification of this oil was not successful. This mixture was then heated in an alkali suspension (4 *N* NaOH) and extracted with three 50-ml. portions of ether. The combined ether extract was dried with sodium sulfate and evaporated to dryness. An oily residue again resulted. This was distilled under reduced pressure to yield 0.6 Gm. of a lemon yellow oil, b.p. 149–152°/3 mm.; n_D^{27} = 1.4930, and analyzed to be 2-piperidino-1,3-propanediol (25).

Anal.—Calcd. for $C_8H_{17}NO_2$: C, 60.34; H, 10.77; N, 8.80. Found: C, 60.12; H, 10.52; N, 8.99.

[4] Microanalysis by Dr. S. M. Nagy, Belmont, Mass.

Similar reactions were carried out with methylallylamine, cyclohexylallylamine, and triallylamine, but the products were not separated as cleanly as in the case of allylpiperidine.

Gas chromatography was determined with a Wilken's Autoprep 700 under the conditions described in Table II.

Infrared spectra were determined on a Perkin-Elmer 421 diffraction-grating spectrophotometer, using 0.025 mm. KBr cell.

Nuclear magnetic resonance spectra were determined on a Varian HR-60 NMR spectrophotometer in the Department of Chemistry through the generous cooperation of Dr. C. C. Price.

Dog Toxicity Study of N,N-Diallyl β-Aziridinopropionamide—Three dogs each were given 20 and 40 mg./Kg. in a single injection of this compound in isotonic saline (50%) intravenously, and blood samples were withdrawn periodically and blood counts carried out in the Hematology Laboratory, University Hospital of Pennsylvania. All dogs survived. One dog was given 75 mg./Kg. This dog, however, died of distemper.

REFERENCES

(1) Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G., *Ann. Surg.*, **130**, 333 (1949).

(2) Tsou, K. C., Su, H. C. F., Segarbath, C., and Mirachi, U., *J. Org. Chem.*, **26**, 4987(1961).

(3) Tsou, K. C., Su, H. C. F., and Hoegerle, K., *J. Med. Chem.*, **6**, 435(1963).

(4) Seligman, A. M., Rutenberg, S. M., Persky, L., and Friedman, O. M., *Cancer*, **5**, 354(1952).

(5) Green, T. H., *Obstet. Gynecol.*, **13**, 383(1959).

(6) Tsou, K. C., and Hoegerle, K., *J. Med. Chem.*, **6**, 47(1963).

(7) Goodman, L. E., Bakal, D., Tsou, K. C., Kramer, S. P., Ulfohn, A., Gaby, S. D., Dorfman, H. E., Aybar, O., Candana, E., Aschi, M. G., and Seligman, A. M., *Cancer*, **18**, 3, 307(1965).

(8) Bartlett, P. D., Ross, S. D., and Swain, C. G., *J. Am. Chem. Soc.*, **71**, 1415(1949).

(9) Price, C. C., *Ann. N. Y. Acad. Sci.*, **68**, 663(1958).

(10) King, J. F., "Elucidation of Structure by Physical Methods," Bentley, D. W., ed., Interscience Publishers, New York, N. Y., 1963, pp. 337–338.

(11) Mason, S. F., *Quart. Rev.*, **15**, 287(1961).

(12) Steward, J. E., *J. Chem. Phys.*, **30**, 1259(1959).

(13) Pople, J. A., Schneider, W. G., and Bernstein, H. F., "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 166.

(14) Jackman, L. M., "Application of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, New York, N. Y., 1959, p. 59.

(15) Roberts, J. D., "Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York. N. Y., 1959, p. 76.

(16) Ferretti, A., and Tesi, G., *Tetrahedron Letters*, **1964**, 2975.

(17) Leiter, J., Bourke, A. R., Fitzgerald, D. B., Shepartz, S. A., and Wodinsky, I., *Cancer Res. (Suppl.)*, **22**, 221(1962).

(18) Greenstein, J. P., "Biochemistry of Cancer," Academic Press Inc., New York, N. Y., 1954, p. 379.

(19) Goldbarg, J. A., and Rutenburg, A. M., *Cancer*, **11**, 283(1958).

(20) Green, M. N., Tsou, K. C., Bressler, R., and Seligman, A. M., *Arch. Biochem. Biophys.*, **57**, 458(1955).

(21) Burstone, M. S., *J. Natl. Cancer Inst.*, **16**, 1149 (1956).

(22) Hosoda, S., Takase, S., Yoshida, K., and Tohoku, J., *Exptl. Med.*, **73**, 86(1960).

(23) Miller, E. M., and Tsou, K. C., *Proc. Am. Assoc. Cancer Res.*, 1966.

(24) Gotkis, D., and Cloke, J. B., *J. Am. Chem. Soc.*, **56**, 2710(1934).

(25) Cromwell, N. H., and Tsou, K. C., *J. Org. Chem.*, **15**, 1219(1950).

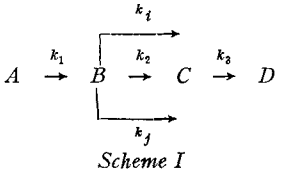
Method for Estimating Rate Constants for Absorption, Metabolism, and Elimination from Urinary Excretion Data

By JOHN G. WAGNER

A method is described for estimating the first-order rate constants for absorption, metabolite formation, and all-over loss of drug from the body if the time course of urinary excretion of the metabolite and an estimate of the rate constant for urinary excretion of the metabolite are available. The rate constants are estimated by drawing only two graphs and performing simple calculations which are described in detail. A simulation example is given where the rate constants were estimated with small errors when the rate constant for absorption was only twice the magnitude, and the rate constant for urinary excretion of metabolite only three times the magnitude of the rate constant for over-all loss of drug from the body. If the estimate of the rate constant for urinary excretion of metabolite is appreciably in error, the other rate constants are estimated with sufficient accuracy that they may be used as preliminary estimates for iterative computer programs which provide "least squares" fitting and which estimate all parameters simultaneously.

THE SAME type of equation used to construct absorption plots (1) may also be used to prepare plots of the cumulative amount or per cent of a metabolite formed as a function of time. Once such a plot is constructed, its resolution into components is the key to the complete elucidation of the kinetics of certain common pharmacokinetic models.

A common pharmacokinetic model is the catenary chain with parallel branches, such as shown in Scheme I where all the rate constants are first-order rate constants.



Scheme I

The most common situation would be the one where A represents a drug at the absorption site in the gastrointestinal tract in the case of oral administration or in the muscle in the case of intramuscular administration, B represents the drug, and C represents a metabolite (formed from the drug) in the blood and other fluids of distribution, and D represents the metabolite C in urine. The parallel paths, indicated by the arrows alongside the $B \rightarrow C$ step, represent other paths for disposal of the drug from the blood and other fluids of distribution. For example, k_i could be the first-order rate constant for urinary excretion of unchanged drug, and k_j could be the rate constant for formation of an additional metabolite. There could be other parallel paths in addition to the two shown, or there may be one or none. The number of paths does not influence the method to be described. Another possible situation equally amenable to the method is where A would represent the drug in blood and other fluids of distribution, B would represent a primary metabolite formed from A; this primary metabolite would be excreted in the urine (say the k_i path) but also would be converted to two other metabolites—one along the k_j path, the other being C; again, D represents the metabolite C in urine.

If the over-all rate constant for loss of B from the body is designated k, then, as Scheme I is written, $k = k_2 + k_i + k_j$. In other words, k is the sum of all the first-order rate constants involved in branches initiating at B. Let X_A, X_B, X_C, and X_D be the amounts in compartments A, B, C, and D, respectively, at time t and X_A^0 be the initial amount in A at time zero. The equations which give these amounts as a function of time, t, contain one exponential term for X_A, two exponential terms for X_B, and three exponential terms for X_C and X_D. The rate constants in the exponents of the exponential terms are k_1 for X_A, k and k_1 for X_B, and k, k_1, and k_3 for X_C and X_D. One can use the "feathering" or back-projection technique on a plot of the logarithm of the amount not excreted *versus* time. However, when three exponentials are involved, this method is subject to large errors; especially one often obtains very poor estimates of the larger two rate constants, which are usually k_1 and k_3.

The method to be discussed assumes there is available an approximation of the rate constant k_3; *i.e.*, the rate constant for urinary excretion of the metabolite C. This is sometimes available. For example, Levy (2) and Elliott (3) determined

Received November 16, 1966, from the Medical Research Division, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication December 20, 1966.

the first-order rate constant for urinary excretion of salicyluric acid in man, and Nelson *et al.* (4) determined the same rate constant for rats. Hence, the method to be proposed is applicable to the acetylsalicylic acid–salicylic acid–salicyluric acid system. It is always wise to estimate k_3 in a separate study since one should ensure that levels of C obtained, when A is administered, are not high enough to saturate the active secretory pathway in the kidney. One could determine k_3 for a metabolite of a drug administered to a human or animal, at the same time as the other rate constants were operative, by administering the drug in cold (nonradioactive) form and a dose of ^{14}C- or ^{3}H-labeled (radioactive) metabolite; the counting of radioactivity in the urine, and appropriate mathematical treatment, would yield the estimate of k_3; this estimate, employed with the data obtained by the normal chemical methods for C in the urine (compartment D), would provide estimates of k_1 and k.

The optimum method of estimating the constants X_A^0, k, k_1, k_2, and k_3 from a given set of X_D, t values is to estimate the constants simultaneously with an appropriate iterative digital computer program and a digital computer of sufficient capacity and speed. Such programs require preliminary estimates of the constants. The method to be proposed is an excellent one for obtaining such preliminary estimates, providing there is an adequate distribution and number of data points. A guide to obtaining an adequate distribution of data points will be the subject of a separate publication (5).

EXPERIMENTAL

Assume that $X_B = X_C = X_D = 0$ and $X_A = X_A^0$ at $t = 0$. The equations apply to all cases when no two or all of the rate constants k, k_1, and k_3 are exactly equal. Let X_D^∞ be the amount of metabolite (mass units) ultimately excreted in the urine. Urine should be collected for at least eight or nine half-lives of the drug to ensure that X_D, at this time, is close enough to X_D^∞. Then, for Scheme I, we have

$$\frac{dX_D}{dt} = k_3 X_C \qquad \text{(Eq. 1)}$$

$$X_C = X_A^0\left(\frac{k_2}{k}\right) \times \left[\frac{k_1 k e^{-kt}}{(k_1 - k)(k_3 - k)} - \frac{k_1 k e^{-k_1 t}}{(k_1 - k)(k_3 - k_1)} + \frac{k_1 k e^{-k_3 t}}{(k_3 - k_1)(k_3 - k)}\right] \qquad \text{(Eq. 2)}$$

$$X_D = X_A^0\left(\frac{k_2}{k}\right) \times \left[\frac{k_1 k_3(1 - e^{-kt})}{(k_1 - k)(k_3 - k)} - \frac{k k_3(1 - e^{k_1 t})}{(k_1 - k)(k_3 - k_1)} + \frac{k k_1(1 - e^{-k_3 t})}{(k_3 - k_1)(k_3 - k)}\right] \qquad \text{(Eq. 3)}$$

where t is time measured from time of administration, which is considered zero time. The cumulative amount of B converted to C in time T is given by

$$\frac{k_2}{k}\int_0^T \frac{dX_B}{dt}\cdot dt = X_C + X_D = \left[\left(\frac{1}{k_3}\cdot\frac{dX_D}{dt}\right) + X_D\right] \qquad \text{(Eq. 4)}$$

Substituting for X_C and X_D from Eqs. 2 and 3 into Eq. 4 and simplifying gives

$$\left[\left(\frac{1}{k_3}\cdot\frac{dX_D}{dt}\right) + X_D\right] = X_D^\infty \times \left[1 - \left\{\left(\frac{k_1}{k_1 - k}\right)e^{-kt} - \left(\frac{k}{k_1 - k}\right)e^{-k_1 t}\right\}\right]$$

when $k_1 > k$ (Eq. 5)

and $$\left[\left(\frac{1}{k_3}\cdot\frac{dX_D}{dt}\right) + X_D\right] = X_D^\infty \times \left[1 - \left\{\left(\frac{k}{k - k_1}\right)e^{-k_1 t} - \left(\frac{k_1}{k - k_1}\right)e^{-kt}\right\}\right]$$

when $k > k_1$ (Eq. 6)

where $X_D^\infty = X_A^0(k_2/k)$. It should be noted that Eqs. 5 and 6 are actually the same equation but, to avoid confusion, have been written so that the exponential terms are always positive with the given conditions.

Let

$$\%X_D = \frac{X_D}{X_D^\infty} \times 100 \qquad \text{(Eq. 7)}$$

and

$$Y = \left[\left(\frac{1}{k_3}\cdot\frac{\Delta\%X_D}{\Delta t}\right) + \widehat{\%X_D}\right] \qquad \text{(Eq. 8)}$$

$\frac{\Delta\%X_D}{\Delta t}$ represents the observed excretion rate measured over the interval $t_2 - t_1 = \Delta t$; this rate is an approximation of the derivative $\frac{dX_D}{dt}$. The $\%\hat{X}_D$, appearing in Eq. 8 is assumed to be the estimate of the cumulative per cent of metabolite excreted at the midpoint, T, of the excretion interval. In practice, it may be estimated from a smooth curve drawn through a plot of cumulative per cent excreted against time (*i.e.*, $\%X_D$ *versus* t).

Case When $k_1 > k$—From Eqs. 5 and 8, one can see that if $(100 - Y)$ is plotted on the logarithmic (base 10) scale of semilogarithmic graph paper against T, the line drawn through the terminal points will have a slope $-\frac{\hat{k}}{2.303}$. If this line is extrapolated back to $t = 0$, the intercept, I, will be (in natural numbers, not logarithms),

$$I \simeq 100\left(\frac{k_1}{k_1 - k}\right) \qquad \text{(Eq. 9)}$$

Rearrangement of Eq. 9 gives

$$\hat{k}_1 = \frac{\hat{k}}{1 - \frac{100}{I}} \qquad \text{(Eq. 10)}$$

If residuals, R, are taken, where

$$R = Ie^{-\hat{k}T} - (100 - Y) \qquad \text{(Eq. 11)}$$

and R is plotted on the logarithmic scale of the semilogarithmic graph paper against T, then the slope of the residual line will be $-\frac{\hat{k}_1}{2.303}$ and the value of R at $T = 0$ will be $I - 100$.

Case When $k > k_1$—Similarly, from Eqs. 6 and 8, the terminal line drawn through a semilogarithmic plot of $(100 - Y)$ against T will have a slope $-\frac{\hat{k}_1}{2.303}$ and an intercept, I', such that

$$I' \simeq 100\left(\frac{k}{k - k_1}\right) \qquad \text{(Eq. 12)}$$

Rearrangement of Eq. 12 gives

$$\hat{k} = \frac{\hat{k}_1}{1 - \frac{100}{I'}} \qquad \text{(Eq. 13)}$$

If residuals, R', are taken, where

$$R' = I'e^{-\hat{k}_1 T} - (100 - Y) \qquad \text{(Eq. 14)}$$

and, R is plotted on semilogarithmic paper against T, then the slope of the line will be $-\frac{\hat{k}}{2.303}$ and the value of R' at $T = 0$ will be $(I' - 100)$.

Hence, the equations provide a method of getting one estimate of the smaller of the two rate constants k and k_1, and two estimates of the larger of these two rate constants. It should be noted that one really does not know which is the smaller of the two, but the assumption is usually made that $k_1 > k$ unless there is evidence to the contrary.

The method of estimating k, k_1, and k_2 is then as follows.

(*a*) Measure urinary excretion rates long enough to obtain a good estimate of X_D^∞.

(*b*) Convert all X_D amounts to percentages according to Eq. 7.

(*c*) Calculate the percentage excretion rates, $\frac{\Delta \%X_D}{\Delta t}$, for each excretion interval.

(*d*) Estimate the $\%X_D$ values at the midpoints, T, of the excretion intervals from a $\%X_D$, t plot.

(*e*) Calculate the values of Y for each excretion interval according to Eq. 8.

(*f*) Plot $(100 - Y)$ on the logarithmic scale of semilogarithmic graph paper against T.

(*g*) Draw a straight line through the points at the tail end of this plot and extrapolate the line to $t = 0$.

(*h*) Multiply the absolute value of the slope of the line by 2.303 to obtain the estimate of the smaller of the two rate constants, k and k_1, with dimensions time^{-1}.

(*i*) Make the first estimate of the larger of the two rate constants, k and k_1, from the intercept of the terminal line using Eqs. 10 or 13.

(*j*) Estimate residuals for those points which are not on the terminal line using Eqs. 11 or 14.

(*k*) Plot the residuals on the logarithmic scale of the semilogarithmic graph paper against T.

(*l*) Either graphically determine the slope of the residual line to obtain the second estimate of the larger of the two rate constants, k and k_1, or, more elegantly, calculate the slope of the least squares line forced through the point at $t = 0$ by means of the formula

$$|\text{slope}| = \frac{\Sigma[\log_{10}(I - 100) - \log_{10}R] \cdot T}{\Sigma T^2} \qquad \text{(Eq. 15)}$$

(*m*) If all metabolites and unchanged drug have been measured in urine to "infinite time" (*i.e.*, for at least eight to nine half-lives, $0.693/k$, assuming k is smaller than k_1 and k_3), then k_2 may be estimated. Convert all the amounts of metabolites and unchanged drug excreted to "infinite time" to mass units equivalent to metabolite C; then the sum of these amounts is $\widehat{X_A}^0$. Or, if absorption is complete, then $X_A{}^0$ is equal to the dose administered, but this is not a good assumption in the usual case. Estimate k_2 from the relationship

$$\hat{k}_2 = \frac{X_D^\infty}{\widehat{X_A}^0} \cdot \hat{k} \qquad \text{(Eq. 16)}$$

The method is illustrated by simulations based on Scheme I.

Example 1—Assignments made were $X_A^\circ = 100$ units, $k_1 = 0.500$ hr.$^{-1}$, $k_2 = 0.150$ hr.$^{-1}$, $k = 0.250$ hr.$^{-1}$, and $k_3 = 0.75$ hr.$^{-1}$. Hence, $X_D^\infty = \frac{0.15}{0.25} \times 100 = 60$ units, and $k_1/k = 2$.

Example 2—Assignments were the same as in example *1* except it was assumed there was a 25% error in k_3 on the high side; *i.e.*, $k_3 = 1.0$ hr.$^{-1}$ was used instead of $k_3 = 0.75$ hr.$^{-1}$.

Example 3—Assignments made were $X_A^\circ = 100$ units, $k_1 = 1.250$ hr.$^{-1}$, $k_2 = 0.150$ hr.$^{-1}$, $k = 0.250$ hr.$^{-1}$, and $k_3 = 1.0$ hr.$^{-1}$. Hence $X_D^\infty = 60$ units and $k_1/k = 5$.

RESULTS

Example 1—Table I gives the detailed calculations made for example *1*. The observed data are represented by the t and X_D values listed in columns 1 and 3 of this table. The $\%\widehat{X_D}$ values at the midpoints, T, may be estimated from a smooth curve drawn through the data points such as shown in Fig. 1. The semilogarithmic plot of $(100 - Y)$ *versus* T is shown in Fig. 2. Due to the data points used, and since $k_1/k = 2$, there are only two points available to draw the appropriate terminal line. The tangent line should be drawn, and not a line through the last few points which form a slight curve, since $[d \log_{10}(100 - Y)/dT]_{T \to \infty} = -k/2.303$. There will usually be few points to estimate the terminal line when $2 \leq k_1/k < 3$ if $k_1 > k$ or when $2 \leq k/k_1 < 3$ if $k > k_1$.[1] When the ratio of rate constants is greater than these ranges, there will be more points for fitting the terminal line (*e.g.*, see example *3*).

The terminal line has a slope of -0.1095; hence $\hat{k} = 0.252$ hr.$^{-1}$. The real value is 0.250 hr.$^{-1}$. The intercept of the terminal line is 200; hence, $\hat{k}_1 = \frac{0.252}{1 - \frac{100}{200}} = 0.504$ hr.$^{-1}$. The real value is 0.500 hr.$^{-1}$. The slope of the residual line, forced through $\log_{10}(I - 100)$, is 0.2214; hence, $\hat{k}_1 = 0.510$ hr.$^{-1}$. Thus, the two estimates of k_1 agree quite closely.

Figure 3 is a plot of the cumulative amount of metabolite formed as a function of time. The points are the Y,T values used to make the estimates above. The line shown on the plot is the theoretical line obtained from

$$100 - \left[1 - \left(\frac{k_1}{k_1 - k}\right)e^{-kt} - \left(\frac{k}{k_1 - k}\right)e^{-k_1 t}\right]$$

[1] The method is practically useless when $1 < k_1/k < 2$, if $k_1 > k$, or when $1 < k/k_1 < 2$ if $k > k_1$ unless an extraordinarily good analytical method is available for the drug.

TABLE I—DETAILS OF ESTIMATION FOR EXAMPLE WHEN $k_1 = 0.50$ hr.$^{-1}$, $k = 0.25$ hr.$^{-1}$, AND $k_3 = 0.75$ hr.$^{-1}$

Time, hr. t	Mid-point Time, hr. T	X_D (units)	$= \frac{X_D}{X_D^\infty} \times 100$	$\frac{\Delta\%X_D}{\Delta t}$	Y[a]	$100 - Y$	$Ie^{-\hat{k}T}$[b]	R[c]	$\log_{10}R$	$\log_{10}(I - 100) - \log_{10}R$[d]
0		0	0		0	100.0	200.0	100.0	2.0000	0.
0.2	0.2	0.006	0.01	0.225	0.310	99.69	191.48	91.79	1.9628	0.0372
0.4		0.054	0.09							
0.6	0.6	0.162	0.27	0.775	1.303	98.697	171.56	72.86	1.8625	0.1375
0.8		0.240	0.40							
1.0	1.0	0.642	1.07	3.35	5.536	94.464	155.44	60.98	1.7852	0.2148
1.2		1.044	1.74							
1.6	1.6	2.148	3.58	5.4625	10.863	89.137	133.48	44.34	1.6428	0.3532
2.0		3.666	6.11							
2.2	2.2	4.560	7.60	7.950	18.20	81.80	114.88	33.08	1.5195	0.4805
2.4		5.574	9.29							
2.6	2.6	6.556	10.93	8.650	22.463	77.537	103.86	26.32	1.4203	0.5797
2.8		7.650	12.75							
3.2	3.2	10.03	16.716	10.1625	30.266	69.734	89.240	19.51	1.2902	0.7098
3.6		12.53	20.88							
3.8	3.8	13.85	23.08	10.875	37.58	62.42	76.78	14.36	1.1571	0.8429
4.0		15.14	25.23							
4.5	4.5	18.47	30.78	11.100	45.58	54.42	...	...	...	...
5.0		21.80	36.33							
5.5	5.5	25.03	41.71	10.570	58.80	41.20	...	...	...	...
6.0		28.14	46.90							
7.0	7.0	33.84	56.40	8.880	68.24	31.76	...	...	...	...
8.0		38.80	64.66							
10.	10.	46.40	77.33	5.285	84.376	15.624	...	...	...	...
12.		51.48	85.80							
14.	14.	54.73	91.21	2.200	94.143	5.857	...	...	...	...
16.		56.76	94.60							
18.	18.	58.03	96.71	0.850	97.843	2.157	...	...	...	...
20.		58.80	98.00							
22.	22.	59.28	98.80	0.315	99.22	0.780	...	...	...	...
24.		59.56	99.26							
∞	$X_D^\infty =$	60.00	100.00							

[a] $Y = \left[\frac{1}{k_3} \cdot \frac{\Delta\%X_D}{\Delta t} + \%\hat{X}_D\right]$. [b] $Ie^{-\hat{k}T}$ are the line values obtained from the terminal linear segment extrapolated to $t = 0$. (See Fig. 1.) In Fig. 1 the value of I is 200. [c] R is the residual, *i.e.*, $R = Ie^{-\hat{k}t} - (100 - Y)$. [d] The absolute value of the slope of the residual line (Fig. 1) is given by $|\text{slope}| = \frac{\hat{k}_1}{2.303} = \frac{\Sigma[\log_{10}(I-100) - \log_{10}R]T}{\Sigma T^2}$, where $\hat{k}_1 = (2.303)(|\text{slope}|)$ in hr.$^{-1}$.

which after substitution of the given constants yields

$$100 - [1 - \{2e^{-0.25t} - e^{-0.50t}\}]$$

One can see, indirectly, that with adequate and frequent sampling, the estimation of the derivatives, $\frac{d\%X_D}{dt}$, by the "excretion rates," $\frac{\Delta\%X_D}{\Delta t}$, is excellent except for the very initial part of the plot.

Example 2—This example was a test to see what the effect of a relatively large error in k_3 would be. From the terminal line a value of $\hat{k} = 0.254$ hr.$^{-1}$ was obtained. The intercept, I, was 234 from which $\hat{k}_1 = 0.449$ hr.$^{-1}$. Hence, use of a value of k_3, which was 25% higher than the true value, gave a value of $\hat{k}$ which was 1.6% higher than the true value, and a value of $\hat{k}_1$ which was 10.2% lower than the true value.

Example 3—In this example $k_1/k = 5$ and there are 11 points to determine the terminal line shown in Fig. 4. The least squares line for the $\log_{10}(100 - Y)$, T points in the range $2.2 \leq T \leq 22$ has a slope of -0.110 and an intercept of 127.6. Hence $\hat{k} = 0.253$ hr.$^{-1}$, obtained from the slope, and $\hat{k}_1 = 1.16$ hr.$^{-1}$, obtained from the intercept. The slope of the least squares line, forced through $\log_{10}(I-100)$, from residuals, corresponding to $T = 0.2$, 0.6, 1.0, and 1.6 hr., is -0.489; hence, $\hat{k}_1 = 1.13$ hr.$^{-1}$. The true values were $k = 0.250$ hr.$^{-1}$ and $k_1 = 1.25$ hr.$^{-1}$.

DISCUSSION

The method gives two estimates of the larger of the two rate constants k and k_1. Usually, but not always in pharmacokinetics, $k_1 > k$ and $k_3 > k$. In the absence of additional information, these are reasonable assumptions. Frequently in pharmacokinetic studies in which Scheme I applies, blood is sampled also and the blood and urine are analyzed for unchanged drug in addition to measurement of metabolite in urine as assumed in this report. If k is smaller than k_1 and k_3, the terminal linear segments of a plot of the logarithm of the plasma concentration of total (free and protein bound) unchanged drug against time and a plot of the logarithm of amount of unchanged drug not excreted *versus* time will also yield estimates of $k/2.303$.

If unchanged drug is measured in the urine, then application of the method of Wagner and Nelson (1) to these data should yield the same estimate of k_1, within error, as that obtained from the above method using data derived from measuring the metabolite in urine. A result such as this was found with the data of Levy (2).

When applying the above method to data derived from urine assays care must be taken in drawing the line through the terminal points of the semilogarithmic $(100 - Y)$, T plot. Terminal points

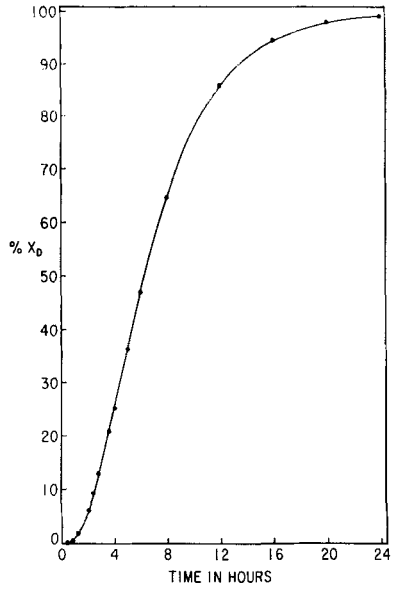


Fig. 1—The $\%X_D$ against time, t, plot for example 1. The points correspond to the cumulative per cent of the metabolite excreted to the end of the simulated urine collection periods at 0.4, 0.8, 1.2, 2, 2.4, 2.8, 3.6, 4, 5, 6, 8, 12, 16, 20, and 24 hr.

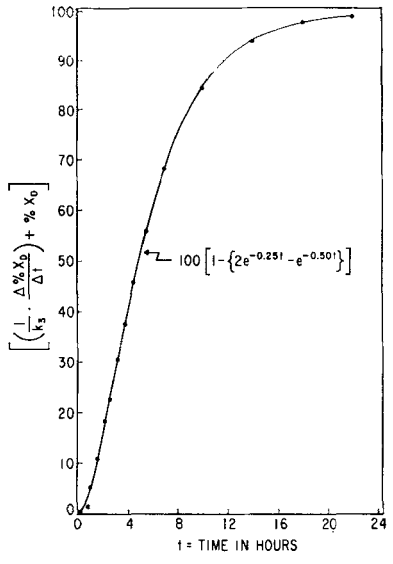


Fig. 3—A plot of the cumulative amount of metabolite formed against time for example 1. The points are the Y,T values listed in Table I from which the estimates of the rate constants were made. The line shown on the plot is the theoretical line for the model and assignments of the rate constants.

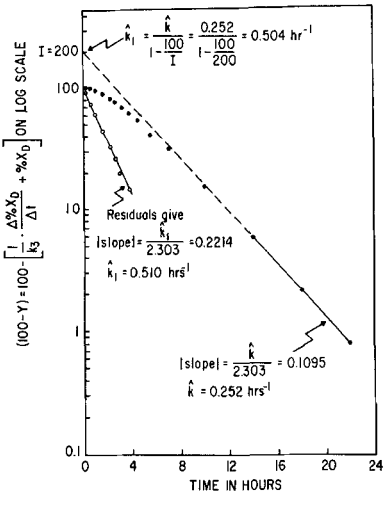


Fig. 2—Illustrating the semilogarithmic plots of (100−Y) and the residuals, R, against the midpoint, T, for example 1.

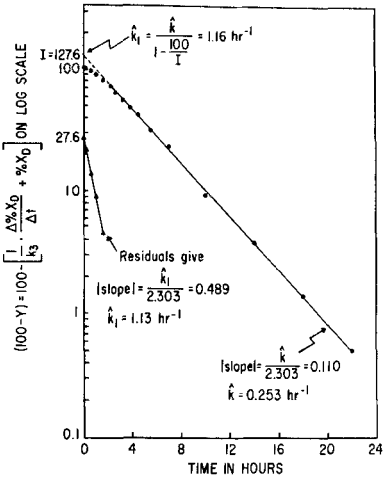


Fig. 4—Illustrating the semilogarithmic plots of (100−Y) and R against T for example 3.

on this plot will be derived from excretion rates which are relatively small in magnitude and which arise from urine collections obtained very late in the study and, hence, contain very little metabolite, if the ratio of the rate constants k_1 and k is between 1 and 3. The curvature of the points on the plot will indicate when this is the case. Assay error and the sensitivity of the assay will greatly affect the terminal points in these cases. The tendency will be for these inaccurate points to lie below the proper terminal line. The same problem exists in estimating the smallest of any two or more rate constants from the tail and of any type of semilogarithmic plot when the rate constants are within a factor of about 3 of each other. When applying the method to semilogarithmic $(100 - Y),T$ plots which have significant curvature, it is wise to make one, two, or more estimates of where the terminal line should be drawn and compare the results with other knowledge available about the particular system with which one is working.

It should be noted that the cumulative curves of

Figs. 1 and 3 arise from exponential equations only, *i.e.*, there are no zero-order rate constants involved. The curves are nearly linear for almost 50% of the ordinate scale. This phenomenon, and its implications in relation to recent publications concerning parallel zero-order and first-order kinetics (2, 4) will be the subject of a separate publication (6).

REFERENCES

(1) Wagner, J. G., and Nelson, E., *J. Pharm. Sci.*, **52**, 610(1963).
(2) Levy, G., *ibid.*, **54**, 959(1965).
(3) Elliott, H. C., *Proc. Soc. Exptl. Biol. Med.*, **121**, 861 (1966).
(4) Nelson, E., Hanano, M., and Levy, G., *J. Pharmacol. Exptl. Therap.*, **153**, 159(1966).
(5) Wagner, J. G., *J. Pharm. Sci.*, unpublished data.
(6) *Ibid.*, to be published.

Quantitative Evaluation of the Heat-Stabilizing Properties of Organotin Compounds in Rigid Polyvinyl Chloride Using Differential Thermal Analysis

By R. K. O'LEARY, J. FOY, W. L. GUESS, and J. AUTIAN

Rigid polyvinyl chloride bottles are in current use in Europe for a host of products including foods, drugs, and cosmetics. In this country, a number of pharmaceutical and cosmetic firms are exploring the possibilities of this plastic container for packaging of one or more products. Presently, a number of vinyl systems are effectively stabilized against thermal and radiation degradation by the use of organotin compounds. In order to ascertain the efficiency of organotins to stabilize rigid vinyls, a quantitative study was undertaken on a series of vinyl formulations containing various organotin compounds using differential thermal analysis. These techniques proved to be extremely helpful in evaluating the materials from both a qualitative and quantitative point of view. Furthermore, the same techniques appear to have great promise in predicting the most suitable plastic formula for achieving the most stable plastic item when thermal or radiation damage becomes a potential problem.

ONE OF THE most widely used polymers in the medical science field is polyvinyl chloride (PVC). Its primary application has been in the construction of blood bags, surgical tubings, containers for biological products, catheters, and a host of other items. As is well established, vinyl resins are inherently unstable materials and are prone to thermal and oxidative degradation (1). This instability is primarily due to the imperfections and anomalies on the polymer chain, such as unsaturated chain endings and chain branching and to certain incorporated oxygen functions such as hydroperoxy groups. During the heat processing and throughout the active life of the polymer, these structural abnormalities will be the initiating points of the degradation process. In 1936, Yngve discovered that certain organotin compounds proved effective in minimizing the deterioration of PVC (2). During the past 20 years hundreds of structurally modified organotin compounds have been used as PVC heat and light stabilizers. Much has been written on their mode of action, but even up to the present time no generally agreed theory has evolved.

Within the past several years, great commercial interest has been shown in the development of the clear, colorless, rigid polyvinyl chloride bottles which now enjoy a great deal of success in Europe as food and drug containers. Generally, it has been found that the organotin compounds are the most efficient stabilizers for the clear, colorless bottles, even though some question of toxicity may be raised if the organotin compound would be leached into a product (3). Extraction tests following generally accepted procedures, however, have demonstrated that the rigid vinyls have little propensity to release a constituent to an extracting medium, and thus the safety of the bottle may be quite acceptable for any intended drug or cosmetic use.

Since the stabilizers play a key role in producing an acceptable rigid polyvinyl chloride, and since the organotins appear to be in general the most suitable stabilizers, it was decided to undertake a study using differential thermal analysis to evaluate which of a group of organotin compounds might give the greatest protection against thermal degradation. As will be demonstrated, differential thermal analysis proved

Received October 4, 1966, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78756
Accepted for publication December 15, 1966.
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This investigation was supported by contract PH 43-64-557 from the Division of Biologics Standards, National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

Figs. 1 and 3 arise from exponential equations only, *i.e.*, there are no zero-order rate constants involved. The curves are nearly linear for almost 50% of the ordinate scale. This phenomenon, and its implications in relation to recent publications concerning parallel zero-order and first-order kinetics (2, 4) will be the subject of a separate publication (6).

REFERENCES

(1) Wagner, J. G., and Nelson, E., *J. Pharm. Sci.*, **52**, 610(1963).
(2) Levy, G., *ibid.*, **54**, 959(1965).
(3) Elliott, H. C., *Proc. Soc. Exptl. Biol. Med.*, **121**, 861 (1966).
(4) Nelson, E., Hanano, M., and Levy, G., *J. Pharmacol. Exptl. Therap.*, **153**, 159(1966).
(5) Wagner, J. G., *J. Pharm. Sci.*, unpublished data.
(6) *Ibid.*, to be published.

Quantitative Evaluation of the Heat-Stabilizing Properties of Organotin Compounds in Rigid Polyvinyl Chloride Using Differential Thermal Analysis

By R. K. O'LEARY, J. FOY, W. L. GUESS, and J. AUTIAN

Rigid polyvinyl chloride bottles are in current use in Europe for a host of products including foods, drugs, and cosmetics. In this country, a number of pharmaceutical and cosmetic firms are exploring the possibilities of this plastic container for packaging of one or more products. Presently, a number of vinyl systems are effectively stabilized against thermal and radiation degradation by the use of organotin compounds. In order to ascertain the efficiency of organotins to stabilize rigid vinyls, a quantitative study was undertaken on a series of vinyl formulations containing various organotin compounds using differential thermal analysis. These techniques proved to be extremely helpful in evaluating the materials from both a qualitative and quantitative point of view. Furthermore, the same techniques appear to have great promise in predicting the most suitable plastic formula for achieving the most stable plastic item when thermal or radiation damage becomes a potential problem.

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a very useful tool in deducing quantitatively the most suitable formula in a relatively short period of time.

EXPERIMENTAL

Materials and Apparatus—*Polyvinyl Chloride*—The PVC's used are listed with their corresponding weight average molecular weights.[1]

PVC Identification	Wt. Av. Mol. Wt.
PVC 65	62,000
PVC 105	83,000
PVC 161	91,000
PVC 120	107,000
PVC 123	114,000

Organotin Stabilizers—Di-*n*-butyltin diacetate, di-*n*-butyltin β-mercaptopropionate, di-*n*-octyltin β-mercaptopropionate, di-*n*-butyltin diisooctylthioglycolate, and bis-(di-*n*-butyltin monolauryl maleate) (Advance Division, Carlisle Chemical Works, Inc., New Brunswick, N. J.). Di-*n*-butyltin dilaurate, and di-*n*-butyltin maleate (M & T Chemicals, Inc., Rahway, N. J.). Di-*n*-butyltin diisooctyl maleate and di-*n*-butyltin dilauryl mercaptide (Cardinal Chemical Co., Columbia, S. C.).

Polyvinyl Chloride Films—In those cases where stabilizers are used, the polymer (600 Gm.) and the stabilizer (in the desired concentration) are placed into a 1-gal. laboratory Hobart mixer[2] and mixed for 30 min. The films of the polymers are prepared by milling them on a Farrell-Birmingham mill[3] having a chromium plated variable-speed roller. Prior to milling each film, the mill's rollers are carefully cleaned with practical grade stearic acid.[4] The temperature of the rollers is maintained at 175° and the duration of the milling is 5 min. During the final phase of the operation, the separation of the rollers is adjusted to furnish films having thicknesses in the range of 0.040–0.045 in. Cut samples $5^7/_8 \times 5^7/_8$ in. from the milled stock are then press polished on an electrically heated compression press[5] with platens cored for water cooling. The molds containing the samples are then heated for 3.5 min. at 35,000 lb. of pressure and rapidly cooled to 120° before removing the test sample from the mold. All samples are finally allowed to condition at room temperature and 50% relative humidity for 48 hr. prior to their use.

Differential Thermal Analysis Apparatus (DTA)—The apparatus consists of a combined controlled atmosphere differential thermal analyzer and effluent gas analyzer model KA-2-HD with a quick-cool furnace model F-1DF, and two L&N Speedomax H strip chart recorders. This instrument is manufactured and described by Robert L. Stone Co., Austin, Tex. (4, 5).

The sample holder assembly, model SH-M4CAZ, consists of two separate, circular Platinel differential thermocouples and a Chromel-Alumel furnace couple which is utilized for programming as well as recording the temperature of the furnace. The sample holder and recorder system permit the recording of two separate thermograms simultaneously. The recorder is linear in time only and has a chart speed of 1.0 in./min. The sample and reference compartments are cylindrical aluminum pans with a diameter of 3.5 mm., a depth of 1.5 mm., and a weight of 1.4 mg. The temperature scale of the instrument is calibrated using the known crystallographic inversion temperature of ammonium thiocyanate (88°) and the melt transition temperature of tin (231.8°). The heat transfer coefficient for the differential thermocouple is determined by utilizing the heat of fusion of tin (14 cal./Gm.). The calibration is performed utilizing a linear programming rate of 12.6°/min. and nitrogen as the dynamic gas to prevent any oxidative effects. The material under study is placed in the sample container and no reference material is used. The K value determined is 3.09×10^{-7} cal./mm.^{2}. Various range settings of the d.c. amplifiers provide identical K values. All areas are measured in triplicate with a plane planimeter and the results are averaged.

Effect of Molecular Weight on Transition Temperatures—In order to establish certain standards or guidelines in regard to temperatures at which transitions occur in polyvinyl chlorides of the different weight average molecular weights, samples of each of the polymers were run in the differential thermal analysis instrument at a heating rate of 6.0°/min. Highly calcined, 80-mesh alumina (Al_2O_3) was used as the reference. In all determinations exactly 1-mg. samples were employed. Nitrogen was the dynamic gas on the DTA runs, and helium was used for the effluent gas analysis.

Figure 1 illustrates a typical thermogram with the several transition temperatures. It will be noted that two transition temperatures are evident. The lower transition temperature may be considered as the glass transition temperature and may further be referred to as the main glass transition temperature (T_g). These T_g values for the various molecular weights are shown in Table I and reveal that this transition temperature increases from 80.9° for the lowest molecular weight (62,000) to a high of 87.3° for the highest molecular weight (114,000). A second transition temperature, which presently will be referred to as a depolymerization transition temperature (T_t), is seen at 200.6° for all of the polymers, regardless of weight. From the various thermograms, it was possible also to evaluate the peak temperature at which dehydrohalogenation took place as well as calculate the standard heat of transition of $\Delta H°$ for the dehydrohalogenation process. The method of calculation for $\Delta H°$ is included under *Appendix*. These values and constants are also included in Table I, and it appears that as the weight average molecular weight increases, the polymer has greater thermal stability. This is, however, an assumption since there are some polymers which show better thermal stability to active burning and produce thermograms that indicate a lower decomposition temperature. The reason for this is that some combustibles or noncombustibles are released at a lower temperature; a protective char is formed on the surface of the polymer which prevents further active burning.

Figure 2 illustrates the thermograms of the dehydrohalogenation transitions of the polymers studied. It should be noted that the lower molec-

[1] PVC is trademarked as Vygen by the General Tire & Rubber Co., Akron, Ohio, who also supplied the weight average molecular weight data.
[2] Model K 45, Hobart Mfg. Co., Troy, Ohio.
[3] Farrell-Birmingham Co., Ansonia, Conn.
[4] Eastman Chemical Co., Rochester, N. Y.
[5] Dake Corp., model 44-325, Grand Haven, Mich.

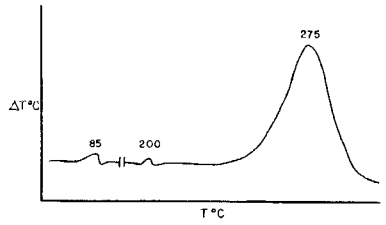

Fig. 1—Differential thermal analysis thermogram of polyvinyl chloride in a nitrogen atmosphere.

TABLE I—EFFECT OF MOLECULAR WEIGHTS ON TRANSITION TEMPERATURE AND DEHYDROHALOGENATION PROCESS OF POLYVINYL CHLORIDES

Wt. Av. Mol. Wt.	Glass Transition Temp., °C. T_g	T_t	Dehydrohalogenation Peak Temp.	ΔH (Kcal./mole)	Effluent Gas Peak, mm.²/mg. of Sample
62,000	80.9	200.6	275.9	20.3	212
83,000	82.8	200.6	282.1	25.8	267
91,000	83.9	200.6	283.2	29.2	300
107,000	85.8	200.6	284.6	30.4	330
114,000	87.3	200.6	285.2	33.2	369

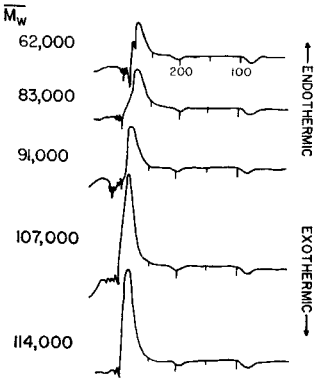

Fig. 2—The effects of molecular weight on the polyvinyl chloride thermogram.

ular weight polymers have their HCl evolution transitions interrupted by multiple exothermic reactions typical of bond scission. The higher molecular weight polymers do not reveal this type of polymer chain degradation, but merely exhibit large dehydrochlorination endotherms.

As each of the samples were run, there was a simultaneous recording of the effluent gas evolution, and as may have been anticipated, the greater the molecular weight, the greater will be the volume of HCl released for a unit weight of polymer. The quantity of HCl released with each polymer is included in Table I but is expressed as the area under the curve rather than in units of volume.

Effect of a Group of Dialkyltin Stabilizers on Glass Transition Temperatures—A series of experiments was conducted with a group of organotin compounds at three different concentrations (1.5, 3.0, and 5.0 p.h.r.) using one molecular weight polymer (114,000). The highest molecular weight polymer was used in this case because the previous experiment demonstrated that the most thermally stable polymer was the polymer having the weight average molecular weight of 114,000. The glass transition temperatures for these formulations have been included in Table II. In every case except one (dibutyltin dilauryl mercaptide at 1.5) the glass transition temperature was reduced below the 87.3° T_g for the pure polymer. Furthermore, the decrease continued as the concentration of the stabilizer was increased. These T_g values demonstrate that for this group of stabilizers, a plasticizing effect is noted, and that the extent of plasticization is dependent upon the compound and its concentration.

TABLE II—EFFECT OF DIALKYLTIN STABILIZERS ON GLASS TRANSITION TEMPERATURE OF A POLYVINYL CHLORIDE (114,000)[a]

Stabilizer	Concn. Level In Polymer	Transition Temp., °C.
Dibutyltin diacetate	1.5	83.3
	3.0	81.5
	5.0	74.1
Dibutyltin diisooctyl thioglycolate	1.5	80.3
	3.0	75.8
	5.0	71.3
Dioctyltin β-mercaptopropionate	1.5	83.7
	3.0	84.8
	5.0	83.9
Dibutyltin β-mercaptopropionate	1.5	82.6
	3.0	84.3
	5.0	85.5
Dibutyltin diisooctyl maleate	1.5	81.5
	3.0	78.7
	5.0	73.6
Bis(dibutyltin mono-) lauryl maleate	1.5	81.9
	3.0	78.1
	5.0	76.9
Dibutyltin dilauryl mercaptide	1.5	88.7
	3.0	77.0
	5.0	72.9
Dibutyltin dilaurate	1.5	79.1
	3.0	76.4
	5.0	76.9
Dibutyltin maleate	1.5	83.9
	3.0	82.6
	5.0	81.9

[a] T_g of pure polymer is 87.3°C.

Effect of a Selected Group of Dialkyltin Stabilizers on Dehydrohalogenation—Since the main purpose of heat stabilizers is to prevent or retard degradation of polymers when exposed to heat, differential thermal analysis was conducted on polyvinyl chloride films containing two concentrations of a specific organotin compound. In these films only the lowest molecular weight (62,000) polymer was used since, as has been revealed before, this material has the least thermal stability of the group of polymers studied. Four organotin compounds were used and these were based upon those which have or are known to be the most used compounds in vinyl formulations requiring a high degree of heat stability. From the various thermograms, the onset temperature of dehydrohalogenation and the temperature of peak degradation were found. Heats of dehydrohalogenation were also calculated and, from the effluent gas recording, the peak area of gas evolved was evaluated. All these values or constants are included in Table III.

TABLE III—EFFECT OF SELECTED DIALKYLTIN STABILIZERS ON THE DEHYDROHALOGENATION OF POLYVINYL CHLORIDE

Stabilizer	Concn.	Onset of Transition, °C.	Peak Transition, °C.	Heat of Transition, cal./Gm.	Peak Area of Effluent Gas Analysis, mm.²/mg. of Sample
Pure polymer	0	221.0	275.9	20.31	212
Dibutyltin diisooctyl thioglycolate	1.5	266.3	284.3	13.17	
	3.0	271.2	285.2	11.58	370
Dioctyltin β-mercaptopropionate	1.5	263.3	284.8	15.80	
	3.0	258.7	290.4	12.89	300
Dibutyltin β-mercaptopropionate	1.5	261.4	284.3	18.03	
	3.0	276.5	287.6	15.17	360
Dibutyltin maleate	1.5	276.8			
	3.0	277.2		[a]	400

[a] Thermogram produced multiple endo- and exotherms from 276.8° to 326.3°C.

DISCUSSION

Differential thermal analysis permits the evaluation of a final plastic material such as polyvinyl chloride and allows in a relatively short period of time an assessment of how a number of parameters will influence the final product. In the case of this report differential thermal analysis was conducted under an inert environment (nitrogen) to eliminate oxidative effects.

As Table I shows, the main glass transition temperature increases with an increase in molecular weight. This results from a decrease in the polymers' fractional free volume. Generally, the glass transition temperature is considered as that point in the temperature scale where a material will be glass-like (below T_g) or rubber-like (above T_g). Perhaps a better way to think about the glass transition temperature is that as the T_g decreases a more pliable material will result.

Increase in the molecular weights of the pure polymers (Table I) had very profound effects upon retarding the dehydrohalogenation of the polymer. The dehydrohalogenation transition can be considered as the temperature where rapid polymer degradation starts to take place and thus may be related to the stability of the polymer. As may be seen from Table I, the heat requirement for dehydrohalogenation is increased by 13 cal./Gm. when the molecular weight of the PVC is increased from 62,000 to 114,000. In applications where heat treatments (for example to produce a certain device) reach temperatures above normal processing conditions, a higher molecular weight polymer will be the most suitable, providing all other factors are constant.

As has been reported before (see Table II), various organotin compounds incorporated in a specific polyvinyl chloride will reduce the glass transition temperature. The ability of these compounds to influence the T_g may on occasions be beneficial as for example when there is a need for a more ductile plastic, but without the desire to add any plasticizer. In this case, the addition of dibutyltin diisooctyl thioglycolate reduced the T_g from 87.3°, for the pure polymer, to 80.3° at a 1.5 concentration or to 71.3° for a concentration of 5.0. Compare the values just quoted to those values for dioctyltin β-mercaptopropionate. At a concentration of 1.5 the dioctyltin β-mercaptopropionate reduces the T_g to 83.7°, but further increase of the stabilizer to 3.0 or 5.0 has little additional effect on the T_g. These decreases in T_g may be attributed to a plasticizing effect on the polymer such that an increase in fractional free volume results (6,7).

Differential thermal analysis also was very valuable in conveying at which temperatures rapid dehydrohalogenation of the polymers will occur with some of the most widely used organotin compounds. As can be seen in comparing the compounds in Table III, in each instance the tin compounds were able to increase the temperature of dehydrochlorination from 221° for the pure polymer to values of above 260° (approximately). An interesting result, not readily explainable, is the reduction of the heat of dehydrohalogenation from 20.3 cal./Gm. to values of nearly half. In other words, these values (ΔH) indicate that once the plastic has reached a temperature where rapid dehydrohalogenation is to commence, less energy will be needed to bring about the degradation. The authors have reported this same phenomenon when different plasticizers were added to PVC (8).

The analysis of the data gathered from the use of the differential thermal analysis instrument for the limited number of polymers reported in this paper demonstrates that the technique can become a powerful tool for laboratories wishing to evaluate the stability of plastic materials, particularly the vinyl types. The results of the experiments reported here confirm the ability of organotin compounds to protect rigid polyvinyl chloride at thermal histories which would be destructive to the pure polymers. This fact is not a new revelation but the use of the differential thermal analysis instrument permits quantitizing results; thus, a more logical and scientific assessment of vinyl formulations can take place, replacing more laborious techniques currently used.

APPENDIX

The heat of dehydrohalogenation may be calculated by the expression (9–11):

$$\text{peak area} = \int_{t_1}^{t_2} \Delta T dt = \frac{HM}{g\tau_s} \quad \text{(Eq. 1)}$$

t_1 = onset temperature of transition,
t_2 = sample temperature equals reference temperature,
ΔT = differential temperature,

M = mass of the reactive sample,
g = constant dependent on the furnace and sample holder geometry,
τ_s = thermal conductivity of the sample.

If the instrument is calibrated with a suitable standard, such as tin whose heat of fusion is 14 cal./Gm., then g and τ_s can be reduced to one constant, K, the heat transfer coefficient of the system.

K is determined for each thermocouple by means of the following equations which describe the response of the system:

$$\frac{\text{area}}{\text{Gm.}} = \frac{\text{area of fusion transition of tin} \times \text{range setting } (\mu\text{v.})}{\text{wt. of tin, Gm.}} \quad \text{(Eq. 2)}$$

$$\frac{\Delta H_f \text{ of tin (cal./Gm.)}}{\text{area/Gm.}} = \frac{\text{cal.}}{\text{area}} = \frac{\text{cal.}}{\text{mm.}^2} = K \quad \text{(Eq. 3)}$$

The heats of transitions can thus be determined by the following equation:

$$\Delta H_f = \frac{K \times \text{peak area in mm.}^2 \times \text{range setting } (\mu\text{v.})}{\text{sample wt., Gm.}} \quad \text{(Eq. 4)}$$

REFERENCES

(1) Chevassus, F., and DeBroutelles, R., "The Stabilization of Polyvinyl Chloride," St. Martina Press, Inc., 1963.
(2) Yngve, V., U. S. pat. 2,219,463 (October 29, 1941).
(3) Guess, W. L., O'Leary, R. K., Calley, D., and Autian, J., "Parenteral Toxicity of a Series of Commercially Available Dioctyl and Dibutyl Tin Stabilizers Used in PVC Formulations," presented to the 22nd Annual Technical Conference of the Society of the Plastics Engineers, Montreal, Quebec, Canada, March 1966.
(4) Stone, R. L., *Anal. Chem.*, **32**, 1582(1960).
(5) Stone, R. L., *J. Am. Ceram. Soc.*, **35**, 76(1952).
(6) Nielsen, L. E., "Mechanical Properties of Polymers," Reinhold Publishing Corp., New York, N. Y., 1962, pp. 11–46.
(7) Shen, M. C., and Tobolsky, A. V., "Glass Transition Temperatures of Polymer," presented to the American Chemical Society Annual Symposium, Philadelphia, Pa., April 1964.
(8) O'Leary, R., Foy, J., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **56**, 453(1967).
(9) Spiel, S., *U. S. Dept. Interior, Bur. Mines., RI 3764*, 1944.
(10) *Ibid., Tech. Paper 664*, 1945.
(11) Vold, M. J., *Anal. Chem.*, **21**, 683(1949).

Dissolution of Sodium Salicylate from Tablet Matrices Prepared by Wet Granulation and Direct Compression

By EDWARD MARLOWE* and RALPH F. SHANGRAW

A quantitative evaluation of dissolution of active ingredients from tablets is discussed. An investigation of filler-binder, lubricant, disintegrating agent, and hardness on the dissolution rate of sodium salicylate from tablet bases was studied by means of a 4 × 3 × 2 × 2 factorial experiment and analysis of variance. The utilization of a filler, spray-dried lactose, which required no preliminary granulation, gave significantly faster release rates than granulations prepared with acacia mucilage, starch paste, or ethylcellulose. The presence of a disintegrating agent notably affected the dissolution rate of the active ingredient, especially where wet granulation was employed. The release of sodium salicylate from several bases was a result of the interaction of many variables and was not dependent solely upon the effect of an individual component.

THE EFFECT of formulation and processing factors on the dissolution rate of active ingredients from compressed tablets has been the subject of a number of reports. Wensley (1), using a modification of the Gershberg-Stoll apparatus, investigated the effects of granulating agents and pressure on the dissolution rate of sodium bicarbonate tablets. Kadar and Walker (2) studied the *in vitro* release of sulfathiazole from compressed formulations using a modification of the U.S.P. XV tablet disintegration apparatus. The effects of particle density and neutral ionic and nonionic compounds on the dissolution rate of slightly soluble acidic drugs were determined by Parrott *et al.* (3). In a later study by the same workers (4) the influences of bases and buffers on rates of dissolution of acidic solids were investigated. Levy (5–7) studied the effect of formulation factors on the dissolution rate of the active ingredient. Included in the factors studied were the agitation intensity, granule size, starch concentration, compression pressure, and lubricants.

With the advent of new machines and materials, a great many tablets formerly prepared by wet granulation can now be manufactured by direct compression. Various direct compression fillers have been evaluated: spray-dried lactose by Gunsel and Lachman (8) and by Duvall *et al.* (9), microcrystalline cellulose by Reier and Shangraw (10), amylose by Kwan and Milosovich

Received January 18, 1965, from the School of Pharmacy, University of Maryland, Baltimore, MD 21201
Accepted for publication January 9, 1967.
Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.
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M = mass of the reactive sample,
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REFERENCES

(1) Chevassus, F., and DeBroutelles, R., "The Stabilization of Polyvinyl Chloride," St. Martina Press, Inc., 1963.
(2) Yngve, V., U. S. pat. 2,219,463 (October 29, 1941).
(3) Guess, W. L., O'Leary, R. K., Calley, D., and Autian, J., "Parenteral Toxicity of a Series of Commercially Available Dioctyl and Dibutyl Tin Stabilizers Used in PVC Formulations," presented to the 22nd Annual Technical Conference of the Society of the Plastics Engineers, Montreal, Quebec, Canada, March 1966.
(4) Stone, R. L., *Anal. Chem.*, **32**, 1582(1960).
(5) Stone, R. L., *J. Am. Ceram. Soc.*, **35**, 76(1952).
(6) Nielsen, L. E., "Mechanical Properties of Polymers," Reinhold Publishing Corp., New York, N. Y., 1962, pp. 11–46.
(7) Shen, M. C., and Tobolsky, A. V., "Glass Transition Temperatures of Polymer," presented to the American Chemical Society Annual Symposium, Philadelphia, Pa., April 1964.
(8) O'Leary, R., Foy, J., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **56**, 453(1967).
(9) Spiel, S., *U. S. Dept. Interior, Bur. Mines., RI 3764*, 1944.
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Dissolution of Sodium Salicylate from Tablet Matrices Prepared by Wet Granulation and Direct Compression

By EDWARD MARLOWE* and RALPH F. SHANGRAW

A quantitative evaluation of dissolution of active ingredients from tablets is discussed. An investigation of filler-binder, lubricant, disintegrating agent, and hardness on the dissolution rate of sodium salicylate from tablet bases was studied by means of a 4 × 3 × 2 × 2 factorial experiment and analysis of variance. The utilization of a filler, spray-dried lactose, which required no preliminary granulation, gave significantly faster release rates than granulations prepared with acacia mucilage, starch paste, or ethylcellulose. The presence of a disintegrating agent notably affected the dissolution rate of the active ingredient, especially where wet granulation was employed. The release of sodium salicylate from several bases was a result of the interaction of many variables and was not dependent solely upon the effect of an individual component.

THE EFFECT of formulation and processing factors on the dissolution rate of active ingredients from compressed tablets has been the subject of a number of reports. Wensley (1), using a modification of the Gershberg-Stoll apparatus, investigated the effects of granulating agents and pressure on the dissolution rate of sodium bicarbonate tablets. Kadar and Walker (2) studied the *in vitro* release of sulfathiazole from compressed formulations using a modification of the U.S.P. XV tablet disintegration apparatus. The effects of particle density and neutral ionic and nonionic compounds on the dissolution rate of slightly soluble acidic drugs were determined by Parrott *et al.* (3). In a later study by the same workers (4) the influences of bases and buffers on rates of dissolution of acidic solids were investigated. Levy (5–7) studied the effect of formulation factors on the dissolution rate of the active ingredient. Included in the factors studied were the agitation intensity, granule size, starch concentration, compression pressure, and lubricants.

With the advent of new machines and materials, a great many tablets formerly prepared by wet granulation can now be manufactured by direct compression. Various direct compression fillers have been evaluated: spray-dried lactose by Gunsel and Lachman (8) and by Duvall *et al.* (9), microcrystalline cellulose by Reier and Shangraw (10), amylose by Kwan and Milosovich

Received January 18, 1965, from the School of Pharmacy, University of Maryland, Baltimore, MD 21201
Accepted for publication January 9, 1967.
Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.
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(11), and anhydrous lactose by Batuyios (12).

However, wet granulation continues to be widely employed, and proponents of this process argue that dissolution of active ingredients from granulated matrices is equal or may be superior to that from direct compression matrices. The object of this investigation was to study the individual effects and possible interactions of filler, binder, disintegrating agent, lubricant, and pressure on the release of an active ingredient from tablets prepared by wet granulation and compare such tablets with those made by direct compression. By means of statistical analysis, the magnitude of the individual factors as well as interactions were determined.

EXPERIMENTAL

Apparatus and Method—Many methods have been utilized for studying dissolution rates from tablets. The apparatus used in this study was similar to the one utilized by Patel and Foss in their studies on binding (13). A typical cell is shown in Fig. 1. Various membranes were studied in an attempt to find one which would give rapid equilibrium and at the same time provide adequate filtration. Many membranes were found to be unsatisfactory because they exhibited extremely long equilibrium times, possessed poor wet strengths, or gave rise to cloudy solutions. However, four thicknesses of a thin paper membrane[1] proved to overcome these difficulties. As no apparatus or procedure can exactly duplicate *in vivo* conditions, all dissolution studies are relative, and the most important considerations are ones of reproducibility, practicality, and reasonableness. The apparatus and method appeared to qualify in these respects.

The plastic cell was assembled, 15 ml. of distilled water placed on each side of the membrane, and the entire assembly placed in a water bath maintained at 37°C. When the experimental temperature was reached, two tablets from each batch were inserted into one side of the cell. The cell was rotated in the water bath at a rate of 15 r.p.m. At specified intervals, 1-ml. samples were removed from the cavity on the other side of the membrane, appropriately diluted, and assayed spectrophotometrically.

The dissolution procedure is not as simple as would be desirable. The manual removal of samples and their assay are time consuming, and interruption of the rotation of the cell is necessary while samples are withdrawn. Even though the tablet remains immersed in the liquid and the removal procedure standardized, there is a chance for a slight distortion (reflected in a delay) in the dissolution profile. Obviously, an automatic sampler and analyzer would be advantageous.

Formulation and Preparation of Tablets—Sodium salicylate was chosen as an active ingredient due to its solubility characteristics and ease of assay. Test solutions were diluted with distilled water and absorbance read on a Beckman DU spectrophotometer at 296 mμ. It was found that approximately 6 min. were required to obtain equilibrium when a solution of sodium salicylate was placed into one side of the dissolution cell. Although a 6-min. equilibrium time is longer than might be ideally desirable, a high percentage of the drug does pass the membrane rapidly, and the delay is not unreasonable when comparable *in vivo* absorption conditions are considered.

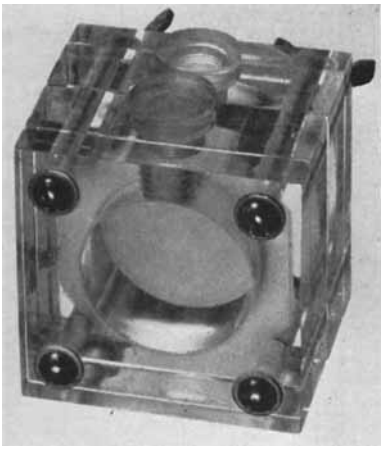

Fig. 1—Plastic dissolution cell.

Tablets were prepared containing 50 mg. of sodium salicylate made up to a total weight of 400 mg. with binder, lubricant, disintegrating agent, and filler. Two fillers (lactose and spray-dried lactose), three binding solutions (5% ethylcellulose in alcohol, 10% acacia mucilage, and 10% starch paste), and three lubricants (1% magnesium stearate, 1% hydrogenated vegetable oil,[2] and 3% of a soluble lubricant composed of a 1:1:2 ratio of DL-leucine, calcium benzoate, and polyethylene glycol 4000) were studied. Absence of a disintegrating agent was compared to 10% cornstarch. Since no granulation was necessary with spray-dried lactose, only 5% starch was added, and the difference in weight was made up with additional spray-dried lactose. The sodium salicylate, starch, and lubricant were blended with the spray-dried lactose, and tablets compressed directly.

The tablets prepared by wet granulation were manufactured by first blending the lactose, sodium salicylate, and one-half the starch (when present), and then moistening the powder blend with the appropriate binding solution. An attempt was made to maintain an equal amount of solid binder in the final granulation. The wet mass was forced through a No. 8 screen and allowed to dry overnight in an oven at 120°F. The dry granules were passed through a No. 12 screen and the lubricant and remaining starch blended into the mixture. Tablets were compressed to two different hardnesses (Strong-Cobb 3.5 and 6.5) on a Stokes model A-3 single punch tablet press.

Tablets were prepared utilizing all possible combinations of filler-binders (lactose–ethylcellulose, lactose–starch paste, lactose–acacia, and plain spray-dried lactose), lubricants, disintegrators, and hardnesses, giving a 4 × 3 × 2 × 2 factorial experiment with 48 batches of tablets. In order to

[1] Marketed as Tricolator by Tricolator Manufacturing Co., Inc., Bellmore, N. Y.

[2] Marketed as Sterotex by Capitol City Products, Columbus Ohio.

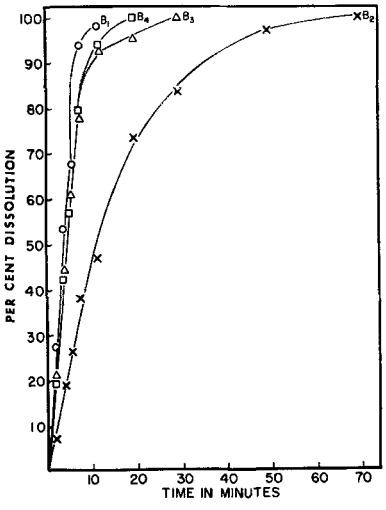

Fig. 2—Comparison of filler-binders in a tablet formula containing starch, magnesium stearate, and 3.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

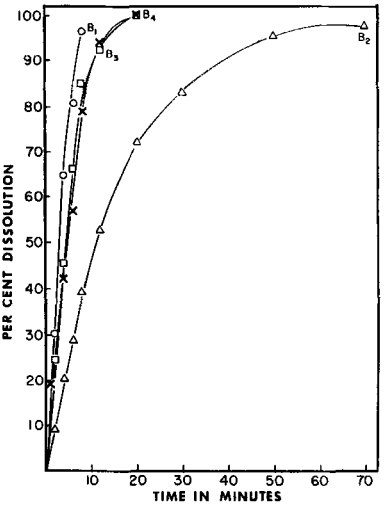

Fig. 4—Comparison of filler-binders in a tablet formula containing starch, hydrogenated vegetable oil, and 3.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

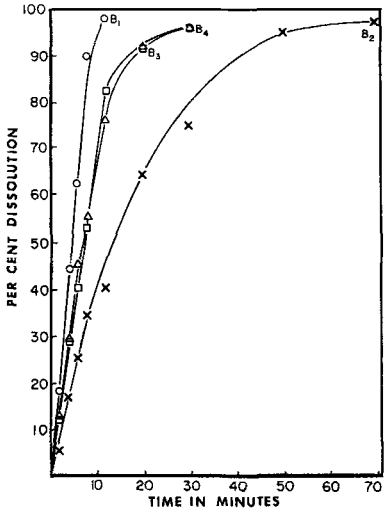

Fig. 3—Comparison of filler-binders in a tablet formula containing starch, magnesium stearate, and 6.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

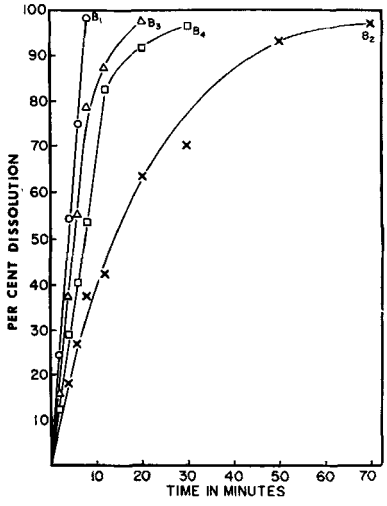

Fig. 5—Comparison of filler-binders in a formula containing starch, hydrogenated vegetable oil, and 6.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

eliminate variables other than those under consideration, attempts were made to standardize the amount of active ingredient in each tablet as well as tablet weight and thickness.

Results and Treatment of Data—The per cents of dissolution of sodium salicylate from the various tablet formulations are shown in Figs. 2–8. Tablets prepared by direct compression with spray-dried lactose uniformly exhibited more rapid dissolution patterns. In all cases except where no disintegrating agent was employed (Fig. 8), dissolution was complete within 12 min. This includes the uniform delay necessary for equilibrium which is inherent in the experimental method employed. All tablets prepared by wet granulation displayed slower release rates, particularly during the latter stages. The ethylcellulose binding solutions gave the slowest release rates in all cases where disintegrating agents were employed, but no real differences could be noted when a disintegrating agent was not used. In general, an increase in tablet hardness retarded dissolution, while the presence of starch dramatically increased it.

In order to give the data mathematical significance

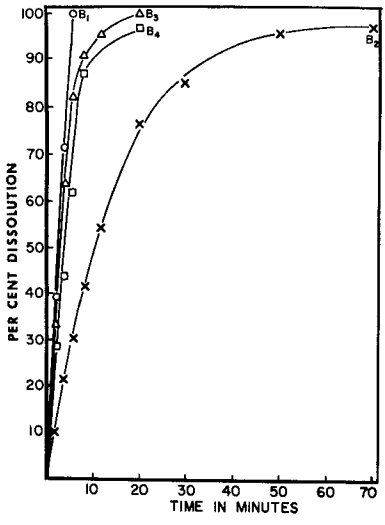

Fig. 6—Comparison of filler-binders in a tablet formula containing starch, soluble lubricant, and 3.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

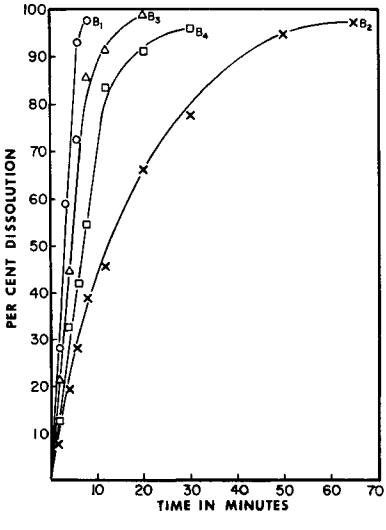

Fig. 7—Comparison of filler-binders in a tablet formula containing starch, soluble lubricant, and 6.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

and to point up interactions between formulation components, an analysis of variance was carried out. The method used for analysis was a $4 \times 3 \times 2 \times 2$ factorial experiment representing four filler-binders, three lubricants, two disintegrating agents, and two compression hardnesses. In order to make use of all the data collected at all nine time intervals, it would have been necessary to calculate a $9 \times 4 \times 3 \times 2 \times 2$ factorial experiment. Because this would have been cumbersome to handle and necessitate the use of a computer, a single time was selected.

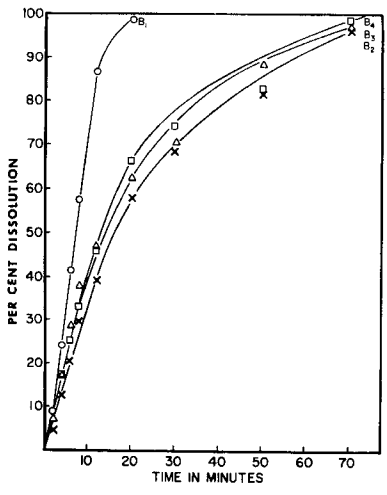

Fig. 8—Comparison of filler-binders in a tablet formula containing no starch, magnesium stearate, and 6.5 S.-C. hardness. Key: B_1, *spray-dried lactose;* B_2, *ethylcellulose and lactose;* B_3, *acacia mucilage and lactose;* B_4, *starch paste and lactose.*

The 8-min. per cent dissolutions chosen as data from that time interval were influenced less by the membrane equilibrium time and had not reached 100% for most formulations. The analysis of variance is shown in Table I.

DISCUSSION

From Table I it can be seen that filler-binders and presence of a disintegrating agent are significant at a 5% level. This was to be expected from visual examination of the data. No two factor interactions were significant at the 5% level which can be attributed to the large mean squares associated with the three factor interactions, two of which (binder–hardness–disintegrating agent and binder–disintegrating agent–lubricant) were significant. These are exactly the same three factor interactions found significant by Kwan *et al.* (14) in their study using disintegration times as end points. By utilization of a t test, it was shown that spray-dried lactose and acacia–lactose give significantly different mean availability times. The calculated t value was 3.93 as compared to a significant value at 95% of 2.02. Since acacia–lactose gave the second fastest availability rate, it can be concluded that spray-dried lactose is significantly superior to the other filler-binders tested in this study.

Analysis of variance was then repeated utilizing only the data obtained from the three filler-binders which required prior granulation, *i.e.*, acacia–lactose, starch–lactose, and ethylcellulose–lactose. The binder effect previously obtained was no longer significant, indicating no substantial difference between acacia, starch, and ethylcellulose. This can be attributed to the lack of differences when no disintegrating agent is employed, as it appears obvious that when a disintegrating agent is used (Figs. 2–7) ethylcellulose gives a much slower release rate. The same three factor interaction, binder–hardness–disintegrating agent, again was shown to be significant. More important, the two factor interaction, binder–disintegrating agent,

TABLE I—ANALYSIS OF VARIANCE OF PER CENT DISSOLUTION OF SODIUM SALICYLATE AFTER 8 min.

Source	d.f.	Mean Sq.	*F*vs I[e]	*F*va R[f]	$F_{.05}$	$F_{.10}$
B[a]	3	611,879	4.65[g]		2.88	2.25
H[b]	1	49,729	<1.00			
D[c]	1	628,148	4.77[g]		4.12	2.86
L[d]	2	43,373	<1.00			
B × N	3	12,124	<1.00			
B × D	3	62,733	2.62[h]		3.20	2.44
B × L	6	8,200	<1.00			
H × D	1	22,576	<1.00			
H × L	2	1,504	<1.00			
D × L	2	617	<1.00			
B × H × D	3	11,774		13,41[g]		
B × H × L	6	624		<1.00	4.76	3.29
B × D × L	6	11,061		12.60[g]	4.28	3.05
H × D × L	2	445		<1.00		
Residual	6	878				
Total	47					

[a] Three filler-binder combinations. [b] Four hardness. [c] Five disintegrating agents. [d] Six lubricants. [e] *F* determined by the combined mean square of the interactions. [f] *F* determined by the mean square of the residual. [g] Significant at 5% level. [h] Significant at 10% level.

became significant. This can be attributed to the large effect the disintegrating agent had on the acacia and starch granulations as opposed to the small effect on the ethylcellulose.

Tablet hardness did not have a significant effect in either study. However, hardness did appear to affect the rate of dissolution from those formulations containing starch. Analysis of variance of 8-min. dissolution data from all tablets containing starch was calculated. Hardness still did not show up as a significant factor, although the binder–hardness interaction was extremely significant. One of the reasons for lack of significance was the softness of tablets obtained from both pressure settings. It is probable that tablets of both hardnesses possessed sufficient pore spaces to promote capillary water penetration and subsequent tablet rupture. The high interaction term can be attributed in large measure to the little effect hardness had in spray-dried lactose tablets, even in the presence of starch.

In order to further substantiate that the presence of a disintegrating agent was significant, a *t* test was carried out on the mean per cent dissolution of sodium salicylate from 6-min. samples containing starch and no starch. The difference between the means was found to be significant, the calculated *t* value being 2.64 as compared to a table value of 2.01 for significance at the 95% level.

One further study was conducted to show the importance of binders on dissolution for all values of time. In this case, the per cent availabilities of all the filler-binders were compared at different time intervals, keeping pressure constant (high), lubricant the same (magnesium stearate), and including starch in the formulations. Analysis of variance indicated that binders were even more significant when compared over all the time intervals. Of course, time showed up highly significant as would be expected.

In order to make a more meaningful study, the disintegration times of all batches prepared were determined by the U.S.P. XVI method. An analysis of variance of the mean disintegration times was computed. The same single effects, filler-binder and disintegrating agents, appeared significant at the 95% level as well as the two factor interaction of filler-binder and disintegrating agent. However, no three factor interactions were significant due to the fact that the residual or error term against which three factor interactions were tested was large. This can be attributed to the large inherent error in disintegration time determinations which arise from indistinct end points and operator variability. The statistical analysis of the U.S.P. disintegration results failed to demonstrate the interplay between component variables present in normal formulations and reflected in *in vitro* release data.

In an attempt to summarize the preceding experiments, the mean dissolution per cents for 8-min. samples and the mean disintegration times are presented in Tables II and III. Both tables denote analogous results indicating that an 8-min. dissolution test gives the same indications of component effects as much longer disintegration times. The spray-dried lactose showed superior release rates followed by acacia–lactose, starch–lactose, and ethylcellulose–lactose.

In order to present these dissolution data in a manner which more closely approximates disintegration times, an attempt was made to determine the mean 100% dissolution times. However, this end point did not always give good comparable data as can be seen from the dissolution curves in Figs. 2–8. Thus, the times necessary for samples to reach 80% dissolution were chosen for comparison and the means of these values are presented in Table IV. These figures substantiate the same order of dissolution previously described. However, the difference between acacia and starch as binders is negligible. It is quite apparent from Tables III and IV that the time for 80% availability appears to differ significantly from the mean disintegration time. Even taking into consideration a delay in equilibrium time, the dramatic increase in the amount of time necessary to obtain only 80% dissolution points up the inadequacy of the U.S.P. XVI disintegration method for determining active ingredient release patterns.

One of the objects of this investigation was to study the effect of a water-soluble lubricant com-

TABLE II—AVERAGE PER CENT DISSOLUTION OF 8-min. SODIUM SALICYLATE SAMPLES

Filler-Binder		Pressure		Disintegrators		Lubricants	
Spray-dried lactose	88.8	Low	62.0	No starch	47.4	Mg stearate	53.2
5% Ethylcellulose and lactose	34.6	High	55.6	Starch	70.2	Hydrogenated vegetable oil	59.7
10% Acacia mucilage and lactose	59.3					Soluble lubricant	63.5
10% Starch paste and lactose	52.4						

TABLE III—MEAN DISINTEGRATION TIMES (sec.) OF SODIUM SALICYLATE TABLETS

Filler-Binder		Pressure		Disintegrators		Lubricants	
Spray-dried lactose	221	Low	404	No starch	662	Mg stearate	689
5% Ethylcellulose and lactose	922	High	641	Starch	382	Hydrogenated vegetable oil	446
10% Acacia mucilage and lactose	435					Soluble lubricant	370
10% Starch paste and lactose	510						

TABLE IV—AVERAGE TIME (sec.) OF 80% DISSOLUTION OF SODIUM SALICYLATE

Filler-Binder		Pressure		Disintegrators		Lubricants	
Spray-dried lactose	390	Low	1122	No starch	1644	Mg stearate	1290
5% Ethylcellulose and lactose	1992	High	1320	Starch	798	Hydrogenated vegetable oil	1260
10% Acacia mucilage and lactose	1266					Soluble lubricant	1104
10% Starch paste and lactose	1230						

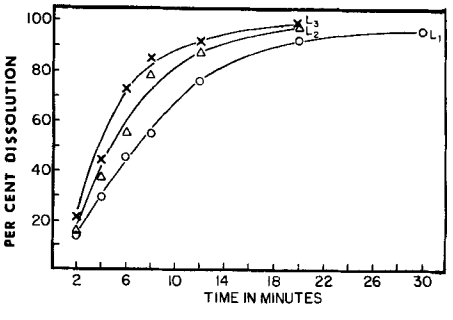

Fig. 9—Comparison of lubricants in a tablet formula containing acacia mucilage, starch, and 6.5 S.-C. hardness. Key: L_1, *magnesium stearate;* L_2, *hydrogenated vegetable oil;* L_3, *soluble lubricant.*

posed of a DL-leucine, polyethylene glycol 4,000, and calcium benzoate on the availability of active ingredients. It was hoped that the soluble lubricant would increase dissolution over the more conventionally used stearates. However, lubricant variation did not appear significant in any of the studies. As shown in Fig. 9, which represents the data in a typical formulation containing starch, acacia–lactose, and compressed at high pressure, the soluble lubricant exhibited the fastest dissolution rate, followed by hydrogenated vegetable oil and then magnesium stearate. This pattern, although showing only slight differences, was consistent throughout all the work.

The results of the investigation indicate that for this particular active ingredient and the various factors studied, direct compression uniformly gives rise to faster dissolution rates than wet granulation. In addition, direct compression is clearly less time consuming and requires less equipment than wet granulation. On the other hand, both acacia and gelatin gave tablets which exhibited acceptable dissolution profiles when a disintegrating agent was present, indicating that wet granulation is still an effective means of preparing powders for compression. It appears, however, that economics alone will lead to a steady decrease in the utilization of wet granulation procedures, and the process will eventually be confined to those drugs which cannot be adapted to direct compression techniques.

SUMMARY AND CONCLUSIONS

1. An apparatus and procedure for the determination of the *in vitro* dissolution of active ingredients from tablets is described.

2. The effect of filler-binders, lubricants, disintegrating agents, and hardness on the dissolution of sodium salicylate from tablet bases was studied by means of a $4 \times 3 \times 2 \times 2$ factorial experiment.

3. Tablets prepared by direct compression with spray-dried lactose exhibited faster dissolution rates for sodium salicylate than tablets prepared by wet granulation.

4. Dissolution of an active ingredient from a tablet matrix is generally not dependent solely on the effect of a single component or production

specification, but is the result of the interaction of many variables.

REFERENCES

(1) Wensley, W. R., Elowe, L. N., and Walker, G. C., *Can. Pharm. J.*, **92**, 141(1959).
(2) Kadar, D., and Walker, G. C., *Bull. Ontario College of Pharmacy*, **10**, 44(1961).
(3) Parrott, E. L., Wurster, D. E., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 269(1955).
(4) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.*, **47**, 376(1958).
(5) Levy, G., *J. Pharm. Sci.*, **52**, 1039(1963).
(6) Levy, G., Antkowiak, J. M., Procknal, J. A., and White, D. C., *ibid.*, **52**, 1047(1963).
(7) Levy, G., and Gumtow, R. H., *ibid.*, **52**, 1139(1963).
(8) Gunsel, W. C., and Lachman, L., *ibid.*, **52**, 178(1963).
(9) Duvall, R. N., Koshy, K. T., and Dashiell, R. E., *ibid.*, **54**, 1196(1965).
(10) Reier, G., and Shangraw, R., *ibid.*, **55**, 510(1966).
(11) Kwan, C., and Milosovich, G., *ibid.*, **55**, 340(1966).
(12) Batuyios, N. H., *ibid.*, **55**, 727(1966).
(13) Patel, N. K., and Foss, N. E., *ibid.*, **53**, 94(1964).
(14) Kwan, K. C., Swart, F. O., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 236(1957).

Preliminary Investigation of the *Z*-Value Measure of Relative Solvent Polarity in Micellar Solubilization

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The *Z*-value method for determining solvent polarity has been applied to surfactant solutions containing camphor and 2-heptanone. The surfactants used were partially purified polysorbate 80, potassium laurate, and dodecyltrimethylammonium chloride. As the surfactant concentration increased the *Z*-value decreased, indicating that the solubilized ketones encountered an environment of decreasing polarity. In the polysorbate 80 system 2-heptanone appeared to be solubilized deeper in the micelle interior than camphor. Both ketones in 0.2 *M* potassium laurate solutions were solubilized in more highly polar environments than in 0.2 *M* dodecyltrimethylammonium chloride solutions.

SURFACTANTS, as agents for making aqueous solutions of poorly water-soluble substances, continue to be of interest in pharmaceutical as well as many other systems. The solute is considered to be solubilized by the less polar micellar pseudophase, and this type of solubilization has been shown to affect the chemical and biological properties of the solubilizate. Compounds are thought to be solubilized in various regions of the micelle: on the surface, in the palisade layer, or in the lipophilic micellar core (1, 2). It is logical to assume, therefore, that solubilized molecules would encounter regions of varying polarity.

Riegelman *et al.* (3) have shown that the ultraviolet absorption spectra of solubilized compounds alter upon solubilization and have drawn certain conclusions regarding the micellar region of solubilization from the spectra of solubilized systems. The ultraviolet spectral investigations of Sasaki *et al.* (4) indicated that the micellar interior in solubilized systems does contain some water.

Although polarity is reflected by a number of measurements on bulk liquids, such as dielectric constant and refractive index, the correlation of such measurements with the liquid's solvent properties has been shown to have many limitations. No single measurement appears to be valid in more than a general way for predicting solubility and the effect of a given solvent on the properties of its solutions. Several investigators have attempted to improve the situation by developing empirical methods to assess the relative polarity of a series of solvents (5–9). One of these, the *Z*-value method developed by Kosower (9), appeared to be promising enough for use in examining micellar solubilized systems and was the subject of this study.

Based upon the assumption that the first layers of solvent around a molecule or ion will be appreciably different in dielectric constant from that measured for the bulk solution, Kosower originated the *Z*-value method for measuring the relative polarity of the layer of solvent molecules immediately surrounding the solute molecule (cybotactic region). The *Z*-value was defined as the energy of electronic transition corresponding to the charge-transfer absorption band of 1-ethyl-4-carbomethoxy pyridinium iodide. The shift of this band to longer wavelengths, occurring with solvents of decreasing polarity, was found to correlate well with other phenomena, such as rates of hydrolysis and keto–enol equilibria, which were known to be sensitive to solvent polarity. Subsequently, many substances were found to exhibit solvent-sensitive absorption spectra and

Received September 13, 1966, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication January 18, 1967.
Abstracted in part from a thesis presented by Steven G. Bjaastad (deceased) to the Graduate School, University of Washington, in partial fulfillment of Doctor of Philosophy degree requirements.

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REFERENCES

(1) Wensley, W. R., Elowe, L. N., and Walker, G. C., *Can. Pharm. J.*, **92**, 141(1959).
(2) Kadar, D., and Walker, G. C., *Bull. Ontario College of Pharmacy*, **10**, 44(1961).
(3) Parrott, E. L., Wurster, D. E., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 269(1955).
(4) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.*, **47**, 376(1958).
(5) Levy, G., *J. Pharm. Sci.*, **52**, 1039(1963).
(6) Levy, G., Antkowiak, J. M., Procknal, J. A., and White, D. C., *ibid.*, **52**, 1047(1963).
(7) Levy, G., and Gumtow, R. H., *ibid.*, **52**, 1139(1963).
(8) Gunsel, W. C., and Lachman, L., *ibid.*, **52**, 178(1963).
(9) Duvall, R. N., Koshy, K. T., and Dashiell, R. E., *ibid.*, **54**, 1196(1965).
(10) Reier, G., and Shangraw, R., *ibid.*, **55**, 510(1966).
(11) Kwan, C., and Milosovich, G., *ibid.*, **55**, 340(1966).
(12) Batuyios, N. H., *ibid.*, **55**, 727(1966).
(13) Patel, N. K., and Foss, N. E., *ibid.*, **53**, 94(1964).
(14) Kwan, K. C., Swart, F. O., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 236(1957).

Preliminary Investigation of the *Z*-Value Measure of Relative Solvent Polarity in Micellar Solubilization

By STEVEN G. BJAASTAD and NATHAN A. HALL

The *Z*-value method for determining solvent polarity has been applied to surfactant solutions containing camphor and 2-heptanone. The surfactants used were partially purified polysorbate 80, potassium laurate, and dodecyltrimethylammonium chloride. As the surfactant concentration increased the *Z*-value decreased, indicating that the solubilized ketones encountered an environment of decreasing polarity. In the polysorbate 80 system 2-heptanone appeared to be solubilized deeper in the micelle interior than camphor. Both ketones in 0.2 *M* potassium laurate solutions were solubilized in more highly polar environments than in 0.2 *M* dodecyltrimethylammonium chloride solutions.

SURFACTANTS, as agents for making aqueous solutions of poorly water-soluble substances, continue to be of interest in pharmaceutical as well as many other systems. The solute is considered to be solubilized by the less polar micellar pseudophase, and this type of solubilization has been shown to affect the chemical and biological properties of the solubilizate. Compounds are thought to be solubilized in various regions of the micelle: on the surface, in the palisade layer, or in the lipophilic micellar core (1, 2). It is logical to assume, therefore, that solubilized molecules would encounter regions of varying polarity.

Riegelman *et al.* (3) have shown that the ultraviolet absorption spectra of solubilized compounds alter upon solubilization and have drawn certain conclusions regarding the micellar region of solubilization from the spectra of solubilized systems. The ultraviolet spectral investigations of Sasaki *et al.* (4) indicated that the micellar interior in solubilized systems does contain some water.

Although polarity is reflected by a number of measurements on bulk liquids, such as dielectric constant and refractive index, the correlation of such measurements with the liquid's solvent properties has been shown to have many limitations. No single measurement appears to be valid in more than a general way for predicting solubility and the effect of a given solvent on the properties of its solutions. Several investigators have attempted to improve the situation by developing empirical methods to assess the relative polarity of a series of solvents (5–9). One of these, the *Z*-value method developed by Kosower (9), appeared to be promising enough for use in examining micellar solubilized systems and was the subject of this study.

Based upon the assumption that the first layers of solvent around a molecule or ion will be appreciably different in dielectric constant from that measured for the bulk solution, Kosower originated the *Z*-value method for measuring the relative polarity of the layer of solvent molecules immediately surrounding the solute molecule (cybotactic region). The *Z*-value was defined as the energy of electronic transition corresponding to the charge-transfer absorption band of 1-ethyl-4-carbomethoxy pyridinium iodide. The shift of this band to longer wavelengths, occurring with solvents of decreasing polarity, was found to correlate well with other phenomena, such as rates of hydrolysis and keto–enol equilibria, which were known to be sensitive to solvent polarity. Subsequently, many substances were found to exhibit solvent-sensitive absorption spectra and

Received September 13, 1966, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication January 18, 1967.
Abstracted in part from a thesis presented by Steven G. Bjaastad (deceased) to the Graduate School, University of Washington, in partial fulfillment of Doctor of Philosophy degree requirements.

were utilizable for Z-value determinations. In 1961 Kosower showed that the low intensity n to π^* electronic transitions of cyclic ketones were sensitive to changes in solvent polarity and one, cyclohexanone, could be used as a secondary standard for determining solvent Z-values (10).

In applying the Z-value method to solubilized systems it was assumed that the electronic transitions, as reflected by the ultraviolet absorption peak shifts, are due to the same forces of interaction between the solute and its cybotactic region in solubilized systems as in simple binary alcohol–water systems. In absence of information to the contrary, it was further assumed that the electronic transitions in the solute depend only upon the Z-value of the solvent, thus making the solubilizate behavior regular and predictable.

The study reported here involved the application of the Z-value method to the examination of the environment of several ketones in aqueous surfactant solutions. The surfactants chosen were an anionic agent, potassium laurate; a cationic agent, dodecyltrimethylammonium chloride; and a nonionic agent, polysorbate 80. Two micellarly solubilized ketones, camphor and 2-heptanone, were used and two ketones with appreciable water-solubility, cyclohexanone and norcamphor, were also used for comparison. Subsequent to this investigation, the behavior of camphor in polysorbate 20 solutions was studied (11).

EXPERIMENTAL

Materials—The surfactants, potassium laurate, dodecyltrimethylammonium chloride, and partially purified polysorbate 80, were prepared by a previously described method (12). Norcamphor (Aldrich Chemical Co.) and D-camphor (Eastman Organic Chemicals) were purified by sublimation immediately before use. The cyclohexanone and 2-heptanone (Matheson, Coleman and Bell) were purified by preparation of the bisulfites, regeneration with alkali, and fractional distillation. Solvents of reagent grade were not further purified. The n-hexanone was fractionally distilled after washing with acid permanganate. Acetonitrile was dried over P_2O_5 and fractionally distilled. Ethanol was redistilled at atmospheric pressure.

Surfactant solutions of the ketones which were saturated were prepared by adding an excess of solute and shaking for 24 to 48 hr. at room temperature. For spectral readings the solutions were filtered into the absorption cells through a Swinney filter adapter (0.45-μ disk) attached to a hypodermic syringe. Solutions not saturated were made of such concentration that the absorbance maxima could be readily determined.

Procedure—Spectral measurements were made in matched cylindrical absorption cells (10 and 1 mm. light path) with a model 14 Cary recording spectrophotometer. The reference cell for each measurement contained only the solvent or surfactant solution. The absorption maxima were determined by placing the solvent in each of the matched absorption cells and a solvent–solvent base line was recorded between 310 mμ and 250 mμ. The cell in the sample compartment was then removed, emptied, and filled with the ketone-containing solution. After the chart paper and wavelength scale were returned to their starting positions and the sample cell replaced, the spectrum between 310 mμ and 250 mμ was recorded at a scanning speed of 0.25 mμ/sec. The location of the spectral peak was noted, and a 10-mμ spectral recording was made over the region including the peak at a scanning speed of 0.05 mμ/sec. The slow speed 10-mμ recording was repeated twice and values of the three determinations were averaged. Repeated observations showed that this method gave maxima of $\pm$0.4 mμ precision.

This procedure gave the following absorption maxima for the ketones in simple aqueous solution: camphor, 284.1 mμ; norcamphor, 281.6 mμ; 2-heptanone, 272.2 mμ; and cyclohexanone, 277.1 mμ.

The energy of the n–π^* transition (E_t) was calculated from the wavelength of the absorption maxima ($\lambda_{max.}$) through the use of the relationship (13):

$$E_t = 2.859 \times 10^4/\lambda_{max.\ (in\ m\mu)}\ \text{Kcal./mole}$$

Solvent polarity (Z) values for the solvents used in this study were obtained from the published works of Kosower (9, 10, 14) or were determined from the measured absorption maximum of dissolved cyclohexanone. The Z-value of a given solvent could be calculated from the equation expressing its linear relationship with the transition energy of cyclohexanone:

$$E_t = 0.14650\ Z + 89.234\ (10)$$

The solvents used and their Z-values are listed in Table I.

RESULTS AND DISCUSSION

Ketones in Simple Solvent Systems—For each of the ketones, camphor, norcamphor, and 2-hepta-

TABLE I—Z-VALUES OF SELECTED SOLVENTS

Solvent	Z Value 25°
Water, distilled	94.6[a]
Methanol	83.6[a]
Ethanol	79.6[a]
Isopropanol	76.6[a]
Acetonitrile	71.3[a]
Diethylether, water saturated	66.1[b]
Chloroform	63.2[a]
n-Hexane	60.4[b]
56% Methanol	90.9[a]
75% Methanol	87.8[a]
80% Methanol	87.1[a]
90% Methanol	85.5[a]
92.5% Methanol	84.9[a]
95% Methanol	84.5[a]
70% Ethanol	84.4[a]
80% Ethanol	84.8[a]
90% Ethanol	82.5[a]
95% Ethanol	81.2[a]

[a] Values obtained from *References 9, 10, 14.* [b] Values experimentally determined.

TABLE II—REGRESSION EQUATIONS Z *Versus* E_t

D-Camphor	
Aqueous	$E_t = 0.1284Z + 88.25$ ($r^a = 0.95$)
Nonaqueous	$E_t = 0.03805Z + 95.61$ ($r = 0.97$)
Norcamphor	
Aqueous	$E_t = 0.1662Z + 85.67$ ($r = 0.98$)
Nonaqueous	$E_t = 0.1006Z + 90.96$ ($r = 0.98$)
2-Heptanone	$E_t = 0.1071Z + 94.98$ ($r = 0.98$)
Cyclohexanone[b]	$E_t = 0.1465Z + 89.23$

[a] r = correlation coefficient. [b] From *Reference 10.*

none, the absorption maxima in the solvents listed in Table I were carefully measured, and a plot of E_t *versus* Z was made. With 2-heptanone, only a single linear correlation was evident; however, with camphor and norcamphor two linear correlations were found. One line described the correlation in water-containing solvent systems and another the anhydrous systems. Table II indicates the results of the least squares regression analysis of the data for the ketones used in this study.

Ketones in Aqueous Surfactant Systems—Initially the absorption maxima for various ketones were measured in partially purified polysorbate 80 solutions to determine whether the method would show lower polarity regions for the solubilized ketones than for those which were water-soluble. Because of their appreciable water-solubilities, norcamphor and cyclohexanone were examined in less than saturated solutions. The plot of Z *versus* polysorbate 80 concentration for saturated solutions of camphor and 2-heptanone is shown in Fig. 1.

The Z-values for norcamphor and cyclohexanone in polysorbate 80 solutions up to 2% showed an environment of essentially the same polarity as water, indicating that significant quantities were not included in the micellar phase. Camphor and 2-heptanone, both possessing low water-solubility, would be expected to be included in the micelles, and the Z-value of the surfactant system indicates an increasingly less polar environment as the surfactant concentration increased. At low surfactant concentrations both ketones behaved as if in an aqueous environment, however.

The lower polarity of the environment of the ketones may come about through the increased concentration of micelles at higher surfactant concentrations, increased micellar organization, or deeper penetration of the micelles by the ketone molecules. Interpretations of X-ray data (2, 15) suggest that micellar organization does increase with concentration. The relative regions of solubilization in the micelle can be deduced by comparing the changing Z-values of the two ketones. With increasing concentrations of polysorbate 80, 2-heptanone apparently encounters a less polar environment than camphor, hence it is probably deeper within the micelle. One would expect 2-heptanone, a rather flexible molecule, to be able to penetrate the micellar interior farther than camphor, a bulky molecule of rigid spherical shape. Possible multilayer adsorption or tail-to-tail adsorption following initial adsorption, as described by DeLuca and Kostenbauder (16), also might account for the less polar environment of 2-heptanone, however.

The decrease in Z-value did not appear to depend upon the concentration of solubilizate, even though the saturated solutions with the lower Z-values do contain higher solubilizate concentrations. When spectra of solutions containing a constant concentration of camphor (1 mg./ml.) were measured, the Z-values were the same as in the saturated solutions

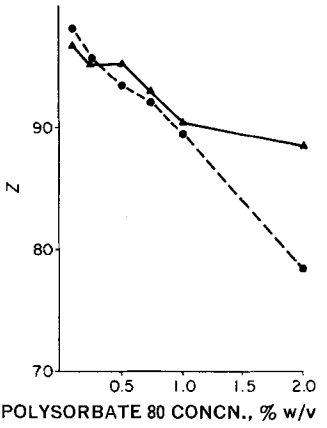

Fig. 1—Solvent polarity (Z) versus *polysorbate 80 concentration for saturated solutions of* D-*camphor* (▲) *and 2-heptanone* (●).

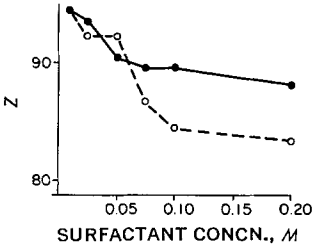

Fig. 2—Solvent polarity (Z) versus *molar surfactant concentration for* D-*camphor in potassium laurate solutions* (●) *and in dodecyltrimethylammonium chloride solution* (○).

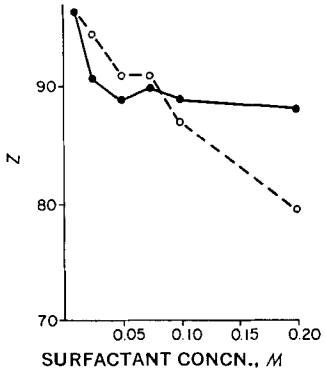

Fig. 3—Solvent polarity (Z) versus *molar surfactant concentration for 2-heptanone in potassium laurate solutions* (●) *and dodecyltrimethylammonium chloride solutions* (○).

within experimental error. Bjaastad and Brown (11) also found constant Z-values for varying camphor concentrations in polysorbate 20 solutions.

The behavior of the Z-value with concentration was also examined with the anionic surfactant, potassium laurate, and the cationic agent, dodecyltrimethylammonium chloride. The data from saturated camphor solutions were plotted for Fig. 2, and the data for saturated 2-heptanone solutions were plotted for Fig. 3.

Examination of Figs. 2 and 3 shows that the environmental polarity of the solubilized ketones begins to fall off above the CMC of both ionic surfactants (about 0.02 M). Dodecyltrimethylammonium chloride solutions exhibit the less polar cybotactic regions at high surfactant concentrations. In the potassium laurate solutions, however, the Z-value appears to attain a plateau. Klevens (2) has reported that potassium laurate achieves "full colloidal form" at about 0.15 M.

The Z-value method of measuring the polarity of the cybotactic region for micellar systems containing solubilizates appears to show promise. The polarity revealed by the method seems to conform to current micellar theories. Experimental refinements through the use of cells with shorter light paths may make possible the study of higher surfactant concentrations and solubilizates with higher molar absorptivities. Since the Z-value is an empirical measure, studies involving its use probably will not have great theoretical significance; however, further investigation of the method is warranted to gain more knowledge of solubilization in complex micellar systems.

REFERENCES

(1) McBain, M. E. L., and Hutchunson, E., "Solubilization and Related Phenomena," Academic Press Inc., New York, N. Y., 1955.
(2) Klevens, H. B., *Chem. Rev.*, **47**, 1(1950).
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(13) Bauman, R. P., "Absorption Spectroscopy," John Wiley & Sons Inc., New York, N. Y., 1962, pp. 7–8.
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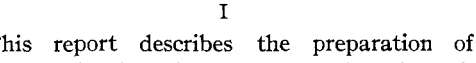
Preparation of Tritium-Labeled Compounds II

Chlorphenesin Carbamate by Exposure to Tritium Gas and Determination of Intramolecular Distribution of Tritium

By RICHARD C. THOMAS, DONALD R. BUHLER, and GEORGE J. IKEDA

Incorporation of stably bound tritium in purified chlorphenesin carbamate was 141 μc. per curie-day exposure. The intramolecular distribution of radioactivity was determined by a combination of biological and chemical degradation methods. Aromatic substitution predominated, accounting for 95 per cent of the tritium; two-thirds of this was located *ortho* to the chlorine substituent. The remaining 5 per cent of the radioactivity resided at the number one position of the alkoxy side chain.

CHLORPHENESIN CARBAMATE[1] [3-(*p*-chlorophenoxy)-1,2-propanediol 1-carbamate; I] is a new, centrally acting skeletal muscle relaxant recently discussed by Matthews *et al.* (1).



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This report describes the preparation of labeled chlorphenesin carbamate by the tritium gas exposure method of Wilzbach (2) and determination of the intramolecular distribution of tritium in the resulting product.

This compound was prepared in a radioactive form to facilitate a study of its metabolism, part of which has recently been reported (3, 4). Exchange labeling by exposure to tritium gas was employed since previous experience (5) in this laboratory, as well as in others, with compounds of comparable structure indicated that incorporation of tritium would be adequate and purification would be feasible.

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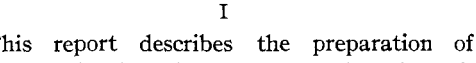

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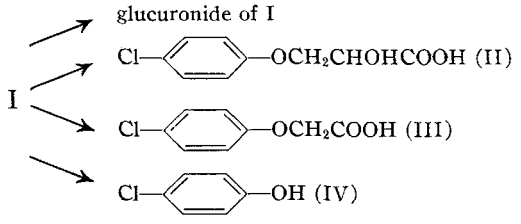
this compound. The location of tritium on the side chain was determined by measuring the specific activities of the metabolites isolated from urine. Thus, the differences in molar specific activities of I and II, II and III, and III and IV represent tritium incorporation on the number one carbon atom (and amide nitrogen), the number two carbon atom, and the number three carbon atom, respectively. Bromination of one of the metabolites, *p*-chlorophenol (IV), was employed to locate tritium on the aromatic ring. The specific activity of the bromination product, 2,6-dibromo-4-chlorophenol (V), represents tritium incorporation *ortho* to the chlorine substituent, whereas the difference in specific activities of IV and V represents tritium incorporation *ortho* to the alkoxy side chain.



Major Metabolites of Chlorphenesin Carbamate (I) in the Rat (3)

Scheme I

EXPERIMENTAL

Radioactivity Measurements—All counting was performed with a Tri-Carb[2] model 314X or 314EX2A liquid scintillation spectrometer at −8° under conditions suitable for measuring tritium. Appropriate aliquots of samples were dissolved in 15 ml. of diotol scintillator (6) [toluene–dioxane–methanol (350:350:210 by volume) containing 73 Gm. of naphthalene, 4.6 Gm. of 2,5-diphenyloxazole, and 0.08 Gm. of 1,4-bis-2-(5-phenyloxazolyl)-benzene per L.]. The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of tritium-labeled toluene and results then expressed as microcuries (μc.) or disintegrations per minute (d.p.m.). Paper chromatograms were scanned for radioactivity with a Forro[3] 2-π or 4-π radiochromatogram scanner.

Paper Chromatography—Chromatograms were developed by the descending method using Whatman No. 2 paper in the following systems: (*a*) 1-butanol–water (4:1 by volume); (*b*) 1-butanol–piperidine–water (81:2:17 by volume); (*c*) Bush B-3 (7), benzene–Skellysolve C–methanol–water (333:667:800:200 by volume); (*d*) Mattox BFW (8), paper impregnated with formamide–methanol (1:1 by volume), mobile phase butyl acetate–formamide–water (100:5:5 by volume); and (*e*) Zaffaroni type paper (9), impregnated with formamide–methanol (1:1 by volume), mobile phase benzene–chloroform (1:1 by volume) saturated with formamide.

[2] Packard Instrument Co., Downers Grove, Ill.
[3] Forro Scientific Co., Evanston, Ill.

Exposure to Tritium and Purification of Chlorphenesin Carbamate—A 2-Gm. sample of finely pulverized chlorphenesin carbamate was exposed to 4 c. of carrier-free tritium gas at room temperature in the dark for 32 days under approximately 0.2 Atm. tritium pressure. Following removal of the tritium gas, the crude sample was dissolved in ethanol and the resulting solution was evaporated to dryness *in vacuo*. Labile tritium was completely removed by repeating this procedure twice. The specific activity of the product at this stage was 76 μc./mg. This material was recrystallized twice from ethanol with the aid of charcoal to yield 1.55 Gm. of product having a specific activity of 27.3 μc./mg. Paper chromatography in the butanol–water, the butanol–piperidine–water, and the Bush B-3 systems, followed by scanning for radioactivity, showed 70% of the radioactivity apparently associated with chlorphenesin carbamate and the remaining 30% associated with an impurity less polar than the desired product. The sample was dissolved in methylene chloride and the solution was applied to a 2.8 × 122 cm. adsorbent magnesium silicate[4] column. The column was developed by the gradient technique using 2 L. of 20% acetone in Skellysolve B and 2 L. of 75% acetone in Skellysolve B while collecting 100-ml. fractions. The product emerged at approximately 45% acetone in Skellysolve B and comprised three major weight fractions. Each of these fractions revealed a single radioactive zone corresponding to chlorphenesin carbamate when subjected to paper chromatography as previously described. However, the three fractions had specific activities of approximately 9.5, 10.2, and 22.9 μc./mg., respectively, indicating the presence of a high specific activity impurity not resolved from the desired product by the three paper chromatography systems employed. This was confirmed in that paper chromatography in the Mattox BFW and the Zaffaroni systems revealed two radioactive zones, one of which corresponded to chlorphenesin carbamate, in the cases of the two fractions having the higher specific activities. The fraction having a specific activity of 9.5 μc./mg. revealed a single radioactive zone corresponding to the desired product. This fraction was diluted with an equal weight (0.590 Gm.) of nonradioactive chlorphenesin carbamate and recrystallized from ethanol–water to yield 1.00 Gm. of product having a specific activity of 4.5 μc./mg. and a melting point of 89–89.5° (capillary, uncorrected; authentic chlorphenesin carbamate had the same melting point). The ultraviolet and infrared spectra of the product corresponded to those of authentic chlorphenesin carbamate. The material showed a single radioactive zone corresponding to the mobility of the desired product in each of the five previously mentioned paper chromatography systems.

Anal.—Calcd. for $C_{10}H_{12}ClNO_4$: C, 48.89; H, 4.93; Cl, 14.43; N, 5.70. Found: C, 48.53; H, 5.17; Cl, 14.57; N, 5.72.

Biological Degradation of Tritium-Labeled Chlorphenesin Carbamate—The isolation and characterization of the major urinary metabolites of tritium-labeled chlorphenesin carbamate in rats have been described (3). The specific activities of the

[4] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.

isolated and purified chlorphenesin carbamate (I), *p*-chlorophenoxylactic acid (II), and *p*-chlorophenoxyacetic acid (III), recovered from the urine of rats which had received an oral dose (200 mg./Kg.) of the labeled chlorphenesin carbamate, were determined, and are listed in Table I.

The specific activity of I, recovered from the urine following hydrolysis of the glucuronide conjugate, was 5.8×10^7 d.p.m./m*M*. This was somewhat less than the value of 5.9×10^7 d.p.m./m*M* for the administered drug. The difference probably reflects a slight loss of tritium label *in vivo* (3).

The specific activity of the volatile metabolite, *p*-chlorophenol (IV), was estimated to be 5×10^7 d.p.m./m*M* on the basis of spectrophotometric determination of concentration in a chloroform solution. For an accurate determination of its specific activity, a nonvolatile derivative was prepared that could be further purified and would not lose exchangeable tritium during its formation. Thus, a portion of radioactive IV was condensed (as described in detail for its chemical degradation below) with chloroacetic acid under basic conditions to yield a small amount of crude III. The derivative was purified by recrystallization from methylene chloride to yield 1 mg. of III. This material was further purified by thin-layer chromatography on silica gel GF[5] using the system chloroform–methanol–formic acid–water (1000:100:4:96 by volume). The radioactive zone comprising III was eluted with 0.1 *N* HCl and the acid solution was extracted four times with chloroform. The concentration of III in the extract was determined by measuring its absorption at 227 mμ using the absorptivity for standard III at this wavelength (a = 62.76). The radioactivity was determined on duplicate aliquots of the extract and the specific activity of III then calculated. The value of 5.5×10^7 d.p.m./m*M* for the specific activity of IV determined in this manner is reported in Table I.

Chemical Degradation of p-Chlorophenol—A portion of the chloroform solution of radioactive IV, containing 7.1 mg. and 3.0×10^6 d.p.m. isolated from the urine of rats in the study described above (3), was mixed with 180 mg. of nonradioactive IV[6] carrier, and the chloroform solution was then extracted twice with 2-ml. portions of 1 *N* NaOH solution, and finally with 10 ml. of water. The combined basic extracts and washing were acidified with dilute H_2SO_4 and the solution was subjected to steam distillation. The distillate, containing the purified, diluted IV, was acidified with dilute H_2SO_4 and extracted five times with chloroform. The organic phase was washed with water to yield a chloroform solution of radioactive, diluted IV containing 2.74×10^6 d.p.m.

As a check on the reliability of the dilution and distillation procedures, the concentration of dilute IV in the chloroform extract was calculated from a measurement of its absorption at 283 mμ using the absorptivity of authentic IV at this wavelength (a = 14.16). The solution was found to contain 160 mg. of diluted IV, thus giving a calculated specific activity of 2.2×10^6 d.p.m./m*M*. For an accurate determination of its specific activity, however, one-half of the chloroform solution containing 80 mg. (0.62 m*M*) and 1.36×10^6 d.p.m. of tritium-labeled diluted IV was added to 0.3 ml. of 5 *N* NaOH (1.5 m*M*) and the chloroform was removed *in vacuo*. Chloroacetic acid (79 mg., 0.75 m*M*) and 0.8 ml. of water were then added to the mixture which was heated under reflux for 6 hr. The cooled reaction mixture was then diluted with 10 ml. of water, acidified with dilute H_2SO_4, and extracted four times with ethyl ether. The ether solution was in turn extracted with 10 ml. of 0.2 *M* $NaHCO_3$ followed by water. The combined basic extract and washing were acidified with dilute H_2SO_4 and then extracted four times with ethyl ether. The combined ether extracts were washed with water and then evaporated to dryness *in vacuo* and the crude diluted III was recrystallized twice from methylene chloride to yield 54 mg. of crystals, m. p. 156–157° [capillary, uncorrected; lit. (3) value 157–158°]. The infrared spectrum of the purified material was identical with that of authentic III. The specific activity of diluted III (and therefore of diluted IV) was 2.13×10^6 d.p.m./m*M*, a value in excellent agreement with that (2.08×10^6 d.p.m./m*M*) calculated based on dilution of IV with the carrier.

Anal.—Calcd. for $C_8H_7ClO_3$: C, 51.49; H, 3.78; Cl, 19.00. Found: C, 51.54; H, 4.02; Cl, 19.50.

The second half of the chloroform solution of tritium-labeled diluted IV, containing 1.36×10^6 d.p.m., was extracted with 2 ml. of 1 *N* NaOH and then washed twice with 2-ml. portions of water. The combined basic extract and washings were adjusted to pH 7 with 1 *N* HCl and approximately 2 ml. of a bromine solution (800 mg. KBr dissolved in 5 ml. of water and containing 0.16 ml. of bromine) was added dropwise with stirring until a yellow color persisted (10). The white precipitate was removed by filtration, washed with water, and then recrystallized twice from methanol–water with the aid of charcoal to yield 74 mg. of crystals having a melting point of 89–90° [capillary, uncorrected; lit. (10) value 90°]. The infrared spectrum of the product was identical with that of authentic 2,6-dibromo-4-chlorophenol (V). Previous NMR studies carried out on nonradioactive V, similarly pre-

TABLE I—SPECIFIC ACTIVITIES OF PURIFIED URINARY METABOLITES AND THEIR DERIVATIVES FROM RATS FOLLOWING ORAL ADMINISTRATION OF TRITIUM-LABELED CHLORPHENESIN CARBAMATE[a]

Compd.	Specific Activity d.p.m. × 10^7/m*M*	% of Total Radioactivity
Chlorphenesin carbamate (I)[b]	5.8	100
p-Chlorophenoxylactic acid (II)	5.5	95
p-Chlorophenoxyacetic acid (III)	5.5	95
p-Chlorophenol (IV)[c]	5.5	95
2,6-Dibromo-4-chlorophenol (V)[d]	3.6	62

[a] Male Wistar rats received an oral dose of tritium-labeled chlorphenesin carbamate (200 mg./Kg.) with a specific activity of 5.9×10^7 d.p.m./m*M*. [b] Excreted as the glucuronide conjugate. [c] Specific activity determined as *p*-chlorophenoxyacetic acid prepared by condensation of the metabolite IV with chloroacetic acid. [d] The specific activity of V has been corrected for the dilution of IV with nonradioactive carrier during the preparation of V.

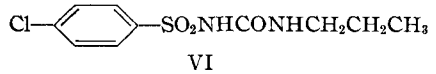
[5] Brinkmann Instrument Co., Great Neck, N. Y.
[6] Distillation Products, Inc., Rochester, N. Y.

pared, confirmed the structural assignment.[7] The specific activity of the purified, diluted V was 1.41 × 10^6 d.p.m./m*M*. For purposes of comparison, the specific activity of diluted V has been corrected for dilution of IV with nonradioactive carrier and the value of 3.6 × 10^7 d.p.m./m*M* is reported in Table I.

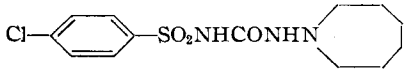
Anal.—Calcd. for $C_6H_3Br_2ClO$: C, 25.17; H, 1.05. Found: C, 25.52; H, 1.12.

RESULTS AND DISCUSSION

Incorporation of tritium into chlorphenesin carbamate was satisfactory, giving a specific activity of 9.0 μc./mg. (corrected for dilution with nonradioactive carrier). The incorporation of stably bound tritium was thus 141 μc./curie-day exposure, based on the weight of material exposed, the amount of tritium used, the length of the exposure, and the specific activity of the purified product. This falls within the range obtained (67–165 μc./curie-day exposure) for a series of seven sulfonylurea hypoglycemic agents (exposed as sodium salts), having structures somewhat similar to chlorphenesin carbamate (5). Two of the most closely related sulfonylureas, chlorpropamide (VI) and glypinamide (VII),



VI



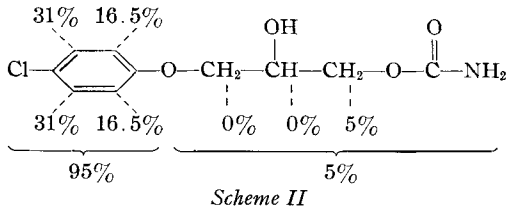
VII

incorporated 161 and 137 μc. of tritium per curie-day exposure, respectively.

The radiochemical purity of compounds labeled by exposure to tritium gas must be carefully determined, as first pointed out by Wilzbach (2). During the exposure, impurities having extremely high specific activities often are formed in trace amounts by weight. Although these high specific activity impurities have little effect on the chemical purity of the product, they can drastically reduce its radiochemical purity. In the present work, three paper chromatography systems showed the presence of one impurity in the tritiated chlorphenesin carbamate prior to column chromatography and no impurities in the major weight fractions eluted from the column. However, the specific activities of the column fractions varied indicating the presence of a second high specific activity impurity, resolved from chlorphenesin carbamate by the column, but not by the three paper chromatography systems. The presence of this impurity was then confirmed with two additional paper chromatography systems.

The data in Table I show the distribution of stably bound tritium in chlorphenesin carbamate exposed to tritium gas to be as shown in Scheme II. The side chain thus contains only 5% of the total molecular radioactivity. This must represent incorporation of tritium on the number one carbon or the amide nitrogen since this amount of tritium was lost upon oxidation of chlorphenesin carbamate to *p*-chlorophenoxylactic acid. Nuclear magnetic

[7] Determined in $CDCl_3$ with a Varian model A-60 instrument using tetramethylsilane internal standard by Dr. G. Slomp and his associates.



Scheme II

resonance studies (11), however, have demonstrated that the amide hydrogens of chlorphenesin carbamate are readily exchangeable with solvent, so tritium in this position would have been lost during purification of the labeled material. Consequently, the side chain tritium label must reside at the number one carbon atom.

The preferential incorporation of tritium into aromatic positions is in agreement with the results of Wilzbach (2) and Ache *et al.* (12) for toluene, Cacace *et al.* (13) for anisole, Meshi and Sato (14) for benzoylvaline, Krizek *et al.* (15) for diethyl benzylacetamidomalonate, and Golder *et al.* (16) for 2,6-di-*tert*-butyl-4-methylphenol. In the latter case, however, there was not a great difference between incorporation of tritium in aromatic positions and in the 4-methyl group. This may be due to shielding of the aromatic positions by the *tert*-butyl groups.

The aromatic ring, accounting for 95% of the molecular radioactivity of chlorphenesin carbamate, contained nearly twice as much tritium *ortho* to the chlorine substituent as *ortho* to the alkoxy side chain.

The distribution of tritium in several other disubstituted benzene derivatives, tritiated by the gas-exposure method, has been reported. Anthranilic acid (17) preferentially incorporates tritium *ortho* to the amino group, whereas salicylic acid (14) preferentially incorporates tritium *ortho* and *meta* to its hydroxyl group; 39% *ortho* and 48% in one or both of the two *meta* positions.

Previous studies with monosubstituted benzene derivatives, tritiated by exposure to tritium gas, have shown that tritium is preferentially incorporated in *ortho* and *para* positions regardless of whether the substituent is nucleophilic or electrophilic. Thus, nitrobenzene (13), chlorobenzene (13), toluene (2, 12), and benzoic acid (14) favor *ortho* incorporation, and anisole (13) favors *para* incorporation. Furthermore, Cacace *et al.* (13) have shown that total incorporation of tritium in the aromatic ring also is nearly independent of the chemical nature of the substituent present; nitrobenzene, chlorobenzene, and anisole, exposed to tritium under identical conditions, attain similar specific activities.

These results suggest that a homolytic, rather than a heterolytic, mechanism is involved in the incorporation of tritium into aromatic positions. Garnett *et al.* (17) proposed such a mechanism to explain the distribution of tritium in anthranilic acid. Ache *et al.* (12) suggested a relationship between incorporation of tritium in aromatic positions and homolytic aromatic substitution. As shown in Table II, a good correlation indeed exists, except for the *meta*/*para* substitution ratio in the case of nitrobenzene.

Pratt and Wolfgang (18) and Wexler (19) have systematically studied the exchange of tritium gas

TABLE II—COMPARISON OF TRITIATION[a] AND HOMOLYTIC AROMATIC SUBSTITUTION[b]

Reaction	% *ortho* Substitution				
	$PhCH_3$	PhCl	$PhOCH_3$	$PhNO_2$	PhCOOH
Tritiation	54, 56	50	40	58	58
Phenylation	71	62	67	62.5	...
Methylation	56.5	64	74	65.5	...
	meta/para Substitution Ratio				
Tritiation	1.92, 1.34	2.10	1.22	3.44	1.04
Phenylation	1.32	1.71	1.20	0.36	...
Methylation	1.56	2.27	1.36	0.21	...

[a] From *References 2, 12, 13, 14.* [b] Fieser, L. F., and Fieser, M., "Advanced Organic Chemistry," Reinhold Publishing Corp., New York, N. Y., 1961, p. 638.

with methane in the gaseous phase. They concluded that methane incorporates tritium by several mechanisms, all involving heterolytic reactions of positively charged transient species. It would be difficult to apply these mechanisms to more complex systems, particularly to a condensed phase system such as chlorphenesin carbamate.[8] However, it seems unlikely that an electrophilic reaction mechanism could explain the distribution of tritium on the aromatic ring of chlorphenesin carbamate or the other aromatic compounds discussed above.

REFERENCES

(1) Matthews, R. J., DaVanzo, J. P., Collins, R. J., and VanderBrook, M. J., *Arch. Intern. Pharmacodyn.*, **143**, 574(1963).
(2) Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013(1957).
(3) Buhler, D. R., *J. Pharmacol. Exptl. Therap.*, **145**, 232(1964).
(4) Buhler, D. R., *Biochem. Pharmacol.*, **14**, 371(1965).
(5) Thomas, R. C., and Ikeda, G. J., *J. Pharm. Sci.*, **55**, 112(1966).
(6) Herberg, R. J., *Anal. Chem.*, **32**, 42(1960).
(7) Bush, I. E., *Biochem. J.*, **50**, 370(1952); Reineke, L. M., *Anal. Chem.*, **28**, 1853(1956).
(8) Mattox, V. R., and Lewbart, M. L., *Arch. Biochem. Biophys.*, **76**, 362(1958).
(9) Zaffaroni, A., *Recent Progr. Hormone Res.*, **8**, 51 (1953).
(10) Kohn, M., and Rosenfeld, A., *Monatsh. Chem.*, **46**, 101(1925).
(11) Slomp, G., Baker, R. H., Jr., and MacKellar, F. A., *Anal. Chem.*, **36**, 375(1964).
(12) Ache, H. J., Herr, W., and Thiemann, A., in "Tritium in the Physical and Biological Sciences," vol. 2, International Atomic Energy Agency, Vienna, Austria, 1962.
(13) Cacace, F., Guarino, A., Montefinale, G., and Possagno, E., *Intern. J. Appl. Radiation Isotopes*, **8**, 82(1960).
(14) Meshi, T., and Sato, Y., *Bull. Chem. Soc. Japan*, **37**, 683(1964).
(15) Krizek, H., Verbiscar, A. J., and Brown, W. J., *J. Org. Chem.*, **29**, 3443(1964).
(16) Golder, W. S., Ryan, A. J., and Wright, S. E., *J. Pharm. Pharmacol.*, **14**, 268(1962).
(17) Garnett, J. L., Law, S. W., and Till, A. R., *Australian J. Chem.*, **18**, 297(1965).
(18) Pratt, T. H., and Wolfgang, R., *J. Am. Chem. Soc.*, **83**, 10(1961).
(19) Wexler, S., *ibid.*, **85**, 272(1963).

[8] It is interesting to note that the distribution of tritium in chlorobenzene is essentially the same whether it is tritiated in the gaseous phase or in the presence of excess liquid chlorobenzene (13).

Drug Standards

Outline of Details for Official Microbiological Assays of Antibiotics

By AMIEL KIRSHBAUM and BERNARD ARRET

Tables are presented listing pertinent data required for 57 different microbiological assays, including those for all antibiotics approved for human use in the United States.

In 1959, the authors published an "outline" for assaying antibiotics (1). Development of new methods, modifications and improvements of old ones, and development of methods for antibiotics which have since been discovered necessitate a revision and updating of that publication. As in the earlier article, pertinent information has been tabulated as a ready guide for the analyst performing microbiological assays of antibiotics. Complete details for the assays are not given since these tabulations are prepared for those already familiar with the basic methods. Procedural information can be found in the Code of Federal Regulations (2).

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PREPARATION OF MICROBIAL SUSPENSIONS

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TABLE I—TEST ORGANISMS FOR VARIOUS ASSAYS

		ATCC No.
(*A*)	*Staphylococcus aureus*	6538P
(*B*)	*Sarcina subflava*	7468
(*C*)	*Sarcina lutea*	9341
(*D*)	*Staphylococcus epidermidis*	12228
(*E*)	*Saccharomyces cerevisiae*	9763
(*F*)	*Bordetella bronchiseptica*	4617
(*G*)	*Bacillus cereus* var. *mycoides*	11778
(*H*)	*Bacillus subtilis*	6633
(*I*)	*Klebsiella pneumoniae*	10031
(*J*)	*Escherichia coli*	10536
(*K*)	*Streptococcus faecalis*	10541
(*L*)	*Micrococcus flavus*	14452
(*M*)	*Microsporum gypseum*	14683
(*N*)	*Sarcina lutea*	9341a

described (3). A modification of this procedure is used for preparing and standardizing most of the microbial suspensions, as follows.

Maintain the test organism on slants of medium No. 1 and transfer to a fresh slant once a week. Using 3 ml. of U.S.P. saline T.S., wash the organism from the agar slant (which has been incubated for 24 hr. at 32–35°) onto a large agar surface of medium No. 1 such as that provided by a Roux bottle containing 300 ml. of agar. Incubate for 24 hr. at 32–35°. Wash the resulting growth from the nutrient surface, using 50 ml. of sterile U.S.P. saline T.S.

Determine the dilution of the suspension with saline that will give a 25% light transmission reading at a wavelength of 580 mμ in a spectrophotometer

TABLE II—MEDIA

Medium	Ingredients	Gm.
(*1*)[a]	Peptone	6
	Pancreatic digest of casein	4
	Yeast extract	3
	Beef extract	1.5
	Dextrose	1
	Agar	15
	Distilled water to make	1000 ml.
	pH 6.55 ± 0.05 after sterilization	
(*2*)[a]	Same as medium *1*, except omit pancreatic digest of casein and dextrose	
(*3*)[a]	Peptone	5.0
	Yeast extract	1.5
	Beef extract	1.5
	Sodium chloride	3.5
	Dextrose	1.0
	Monobasic potassium phosphate	1.32
	Dibasic potassium phosphate	3.68
	Distilled water to make	1000 ml.
	pH 7.0 ± 0.05 after sterilization	
(*4*)[a]	Same as medium *1*, except omit pancreatic digest of casein	
(*5*)[a]	Same as medium *2*, except the pH is adjusted so that it is 7.9 ± 0.1 after sterilization	
(*8*)[a]	Same as medium *2*, except the pH is adjusted so that it is 5.65 ± 0.05 after sterilization	
(*9*)[a]	Pancreatic digest of casein	17.0
	Papaic digest of soy bean	3.0
	Sodium chloride	5.0
	Dibasic potassium phosphate	2.5
	Dextrose	2.5
	Agar	20
	Distilled water to make	1000 ml.
	pH 7.25 ± 0.05 after sterilization	
(*10*)[a]	Same as medium *9*, except use 12 Gm. instead of 20 Gm. of agar. Boil to dissolve the ingredients; then add 10 ml. of polysorbate 80.	
(*11*)[a]	Same as medium *1*, except the pH is adjusted so that it is 7.9 ± 0.1 after sterilization.	
(*13*)[a]	Peptone	10
	Dextrose	20
	Distilled water to make	1000 ml.
	pH 5.65 ± 0.05 after sterilization	
(*18*)[b]	Same as medium *11*, except boil to dissolve the ingredients; then add 20 ml. of polysorbate 80.	
(*19*)[b]	Peptone	9.4
	Yeast extract	4.7
	Beef extract	2.4
	Sodium chloride	10.0
	Dextrose	10.0
	Agar	23.5
	Distilled water to make	1000 ml.
	pH 6.1 ± 0.1 after sterilization	
(*20*)[b]	Dextrose	40.0
	Peptone	10.0
	Chloramphenicol	0.05 (activity)
	Agar	15.0
	Distilled water to make	1000 ml.
	pH 5.65 ± 0.05 after sterilization	
(*21*)[b]	Dissolve 10 mg. of cycloheximide per ml. of distilled water and sterilize by filtration through a membrane filter having a porosity of 0.22 mμ. Aseptically, add 20 ml. of the sterile solution to each liter of medium *20* which has been autoclaved and cooled to 50°.	

[a] Numbers correspond to those used in "Assay Methods of Antibiotics" Grove, D. C., and Randall, W. A., Medical Encyclopedia Inc., New York, N. Y., 1955, p. 220. [b] Numbers assigned by authors.

TABLE III—BUFFERS

Buffer		
I	**1% Phosphate Buffer, pH 6.0 ± 0.05**	
	Dibasic potassium phosphate	2 Gm.
	Monobasic potassium phosphate	8 Gm.
	Distilled water to make	1000 ml.
III	**0.1 M Phosphate Buffer, pH 7.9 ± 0.1**	
	Dibasic potassium phosphate	16.73 Gm.
	Monobasic potassium phosphate	0.523 Gm.
	Distilled water to make	1000 ml.
IV	**0.1 M Monopotassium Phosphate Buffer, pH 4.5 ± 0.05**	
	Monobasic potassium phosphate	13.6 Gm.
	Distilled water to make	1000 ml.
VI	**10% Phosphate Buffer, pH 6.0 ± 0.05**	
	Same as I, except use 10 times as much of each salt.	
X	**0.2 M Phosphate Buffer, pH 10.5 ± 0.1**	
	Dibasic potassium phosphate	35 Gm.
	10 *N* sodium hydroxide	2 ml.
	Distilled water to make	1000 ml.

such as a Bausch & Lomb Spectronic 20, using a round cell with an inside diameter of 1.2 cm.; a Beckman model B spectrophotometer, using a 1.0-cm. square cell; or any other suitable instrument. Use U.S.P. saline as the blank for all instruments. Determine the amount of suspension to be used by test plates or test broth (2, 3).

The test organisms used for the various assays are listed in Table I. The media and buffers are listed in Tables II and III, respectively.

DETAILS OF ASSAY PROCEDURES

Pertinent information for cylinder plate assays is given in Table IV and for turbidimetric assays in Table V.

The third column of the tables, titled "Inoculum," lists the dilution of the stock suspension necessary to obtain 25% light transmission and, in parentheses, the volume of suspension to be added to each 100 ml. of seed medium for plate assays or broth for turbidimetric assays. Those volumes followed by *Footnote b* are added from a dilution of the stock suspension; those without the footnote are added directly from the stock suspension. For example, for penicillin G, the suspension requires a 1 + 19 dilution to give 25% light transmission, and 1.0 ml. of the stock suspension is used per 100 ml. of seed agar. It is stressed that these values are guides only, and each laboratory should perform its own standardization.

For those assays to which the uniform procedure

TABLE IV—PLATE DIFFUSION ASSAYS

Antibiotic	Test Organism	Inoculum	Medium Base	Medium Seed	Initial Soln. of Std.	Buffer for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Ampicillin	*C*	1 + 39 (0.5)	*11*	*11*	100 mcg./ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35
Amphomycin	*L*	1 + 34 (0.5)	*2*	*1*	100 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	37
Amphotericin B	*E*	1 + 29 (0.2)	None	19[a]	1000 mcg./ml. in dimethylsulfoxide	X	0.51, 0.7, *1.0*, 1.4, 1.96	30
Bacitracin	*B*	1 + 29 (0.3)	*2*	*1*	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Bacitracin	*L*	1 + 29 (0.3)	*2*	*1*	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Carbomycin	*C*	1 + 39 (0.2)	*11*	*11*	10,000 mcg./ml. in methanol. Then dilute to 100 mcg./ml. in distilled water	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Cephaloridine	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Cephalothin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Chloramphenicol	*C*	1 + 39 (2.0)	*2*	*1*	10,000 mcg./ml. in ethanol	I	32, 40, *50*, 62.5, 78.1	32–35
Chlortetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.01 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Cloxacillin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Sodium colistimethate	*F*	1 + 19 (0.1)	*9*	*10*	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37
Colistin	*F*	1 + 19 (0.1)	*9*	*10*	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37
Cycloserine	*A*	1 + 19 (0.04)	*2*	*1*	1000 mcg./ml. in distilled water	I	32, 40, *50*, 62.5, 78.1	30

(*Continued on next page.*)

TABLE IV—(*Continued.*)

Antibiotic	Test Organism	Inoculum	Medium Base	Medium Seed	Initial Soln. of Std.	Buffer for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Dactinomycin	*H*	Sec. 148u.1	*5*	*5*	10,000 mcg./ml. in methanol	III	0.5, 0.71, *1.0*, 1.41, 2.0	37
Dicloxacillin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Dihydrostreptomycin	*H*	Sec. 141c.101	*5*	*5*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Erythromycin	*C*	1 + 39 (1.5)	*11*	*11*	10,000 mcg./ml. in methanol	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Gentamicin	*D*	1 + 14 (1.5)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Griseofulvin	*M*	Sec. 148g.1	*20*	*21*	1000 mcg./ml. in dimethylformamide[d]	III	3.2, 4.0, *5.0*, 6.25, 7.81	30 (48 hr.)
Kanamycin	*A*	1 + 19 (0.4)[b]	*11*	*11*	1000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Lincomycin	*C*	1 + 39 (1.5)	*11*	*11*	1000 mcg./ml. in distilled water	III	1.28, 1.6, *2.0*, 2.5, 3.12	32–35
Methicillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	6.4, 8.0, *10.0*, 12.5, 15.6	32–35
Nafcillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	1.28, 1.6, *2.0*, 2.5, 3.12	32–35
Neomycin	*A*	1 + 19 (0.4)[b]	*11*	*11*	1000 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35
Neomycin	*D*	1 + 14 (1.0)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Novobiocin	*D*	1 + 14 (4.0)	*11*	*11*	10,000 mcg./ml. in ethanol	III	0.32, 0.4, *0.5*, 0.625, 0.781	32–35
Nystatin	*E*	1 + 29 (1.0)	None	*19*[a]	1000 mcg./ml. in dimethylformamide[d]	VI	12.8, 16, *20*, 25, 31.2	30
Oleandomycin	*D*	1 + 24 (1.0)	*11*	*11*	10,000 mcg./ml. in ethanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Oxacillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Oxytetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Paromomycin	*D*	1 + 24 (2.0)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Penicillin G	*A*	1 + 19 (1.0)	*2*	*1*	1000 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Phenethicillin	*C*	1 + 39 (0.5)	*11*	*11*	1000 units/ml. in III	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35
Phenoxymethylpenicillin	*A*	1 + 19 (1.0)	*2*	*1*	1000 units/ml. in methanol	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Polymyxin	*F*	1 + 19 (0.1)	*9*	*10*	10,000 units/ml. in distilled water	VI	6.40, 8.0, *10.0*, 12.5, 15.6	37
Streptomycin	*H*	Sec. 141c.101	*5*	*5*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Tetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Triacetyloleandomycin	*D*	1 + 24 (1.0)	*18*	*18*	1000 mcg./ml. in 80% isopropanol	X	9.6, 12, *15*, 18.8, 23.4	37
Tylosin	*N*	1 + 39 (1.0)	*11*	*11*	10,000 mcg./ml. in methanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Vancomycin	*G*	Sec. 148s.1	*8*[c]	8	1000 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	30
Viomycin	*H*	Sec. 148t.1	*5*[c]	*5*	1000 mcg./ml. in III	III	32, 40, *50*, 62.5, 78.1	37

[a] No base layer is used. Add 8 ml. of inoculated seed layer only. [b] Use indicated dilution of stock suspension. [c] Use 10 ml. instead of 21 ml. [d] Solution is further diluted in solvent to 20 times the final concentration of each concentration of the standard curve so that each final dilution is 1 + 19 with indicated buffer.

does not apply, reference is made to the appropriate section of the Antibiotic Regulations (2).

In Table IV, under the column headed "Medium," there are subheadings "Base" and "Seed." Unless otherwise specified, 21 ml. of base agar and 4 ml. of seed agar should be used for each plate. For turbidimetric assays in Table V, 9 ml. of broth should be used.

The column marked "Initial Soln. of Std." indicates the method of getting the standard into solution. Unless a concentration of 1000 mcg. or units or less per milliliter is indicated, the solution

TABLE V—TURBIDIMETRIC ASSAYS

Antibiotic	Test Organism	Inoculum	Medium	Initial Soln. of Std.	Diluent for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Candicidin	*E*	1 + 29 (0.2)	*13*	1000 mcg./ml. in dimethylsulfoxide	Distilled water	0.03, 0.042, *0.06*, 0.084, 0.12	25
Chloramphenicol	*J*	1 + 19 (0.7)	*3*	10,000 mcg./ml. in ethanol	I	2.0, 2.24, *2.5*, 2.79, 3.12	37
Chlortetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.01 *N* HCl	IV	0.036, 0.047, *0.060*, 0.077, 0.099	37
Demethylchlortetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.06, 0.077, *0.10*, 0.129, 0.167	37
Dihydrostreptomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in distilled water	Distilled water	24, 26.7, *30*, 33.6, 37.5	37
Doxycycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.08, *0.10*, 0.125, 0.156	37
Gramicidin	*K*	Overnight broth culture (1.0)	*3*	1000 mcg./ml. in 95% ethanol	95% ethanol	0.028, 0.034, *0.040*, 0.048, 0.058	37
Methacycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.01 *N* methanol soln. prepared with concentrated hydrochloric acid	IV	0.036, 0.047, *0.060*, 0.077, 0.099	37
Oxytetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Rolitetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in methanol	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Streptomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in distilled water	Distilled water	24, 26.7, *30*, 33.6, 37.5	37
Tetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Triacetyloleandomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in 80% isopropanol	I	15, 19.5, *25*, 32, 41.2	37
Tyrothricin	*K*	Overnight broth culture (1.0)	*3*	1000 mcg./ml. in 95% ethanol	95% ethanol	0.14, 0.17, *0.20*, 0.24, 0.29	37
Viomycin	*I*	1 + 24 (0.1)	*3*	5000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37

should immediately be further diluted with the indicated buffer to obtain a stock solution of convenient concentration.

The entries under "Final Concn. for Std. Curve" are self-explanatory, except that the italicized concentration in each instance is the reference concentration. Authentic standards are supplied by the U.S.P. or N.F. for those antibiotics listed in these compendia.

SUMMARY

Five tables have been presented listing pertinent data required for 57 different microbiological assays, including those for all antibiotics approved for human use in the U. S. For those analysts already familiar with basic procedures, the tables give all of the information necessary for the performance of the assays.

REFERENCES

(1) Kirshbaum, A., and Arret, B., *Antibiot. Chemotherapy*, 9, 613(1959).

(2) Code of Federal Regulations, Title 21, Part 130 to end, January 1, 1966, U. S. Government Printing Office, Washington, D. C.

(3) Kirshbaum, A., Kramer, J., and Garth, M. A., *Antibiot. Chemotherapy*, 12, 545(1962).

Improved Assay for Dibasic Calcium Phosphate

By LARRY M. KLEINMAN* and HERBERT SCHRIFTMAN

Phosphate ion interference in chelometric titration of calcium can be eliminated by extracting the anion as phosphomolybdic acid. A titrimetric procedure for calcium ion in dibasic calcium phosphate is described. Thermogravimetric analysis shows that dibasic calcium phosphate dihydrate is thermally stable to at least 80°, obviating conversion to the pyrophosphate before assay. The proposed procedure has advantages of speed and accuracy over the U.S.P. assay.

DIBASIC CALCIUM phosphate is an important raw material used in medicine as a calcium replenisher. It has extensive use in pharmacy as an excipient, where it is especially valuable in tablets made by direct compression. Although the official compendia have adopted chelometric titrations for assay of most calcium salts (1), the classical oxalate precipitation and permanganate titration were retained in U.S.P. XVII (2) for dibasic calcium phosphate because phosphate ion interferes with the direct titration of calcium. A procedure which circumvents this interference is reported here.

Assay of dibasic calcium phosphate by the U.S.P. method was found cumbersome and slow in these laboratories, and the values obtained were biased at the low end of the specification. Ion-exchange separation of calcium and phosphate (3) proved unsatisfactory due to incomplete elution of calcium from the resin. Phosphate can be assayed gravimetrically as magnesium pyrophosphate (4), but that procedure has no advantage over the present U.S.P. assay.

Solvent extraction of phosphate as phosphomolybdic acid (5, 6) removes the interference to chelometric titration of calcium, and the yellow color of the extract affords a means for quantitative spectrophotometric determination of phosphate ion as well as obviating an identification test for phosphate. Although quantitation of both the cation and anion are possible, titration of the calcium suffices for quality control of the salt.

In the U.S.P. assay, the salt is dried at 800–850°, and results are reported on the dried basis. In employing this procedure one can omit the drying step because thermogravimetric analysis indicates (*a*) dihydrate is stable to at least 80° and (*b*) samples showed about 1% or less moisture. However, the addition of a simple "loss on drying" or a thermogravimetric determination can provide the data necessary for obtaining an accuracy of better than 1%.

Received June 22, 1966, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication November 17, 1966.

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The authors express their sincere appreciation to Dr. Thomas H. Simon for the thermogravimetric analyses of the salt and to Dr. Lester Chafetz for his encouragement and assistance in the preparation of this manuscript.

EXPERIMENTAL

Materials and Supplies—Dibasic calcium phosphate dihydrate U.S.P., hydroxy naphthol blue indicator (U.S.P. XVII, p. 1068), concentrated, 10%, and 1 *N* hydrochloric acid, 40% sodium molybdate, "mixed solvent" (1:1 *n*-butanol–chloroform), 10% sodium hydroxide, exsiccated sodium phosphate N.F., 0.05 *M* disodium ethylenediaminetetraacetate (EDTA), a Beckman DU spectrophotometer, a duPont Thermogravimetric analyzer, model 950, and standard laboratory glassware were used in this work.

Determination of Calcium—Accurately weigh about 1000 mg. of dibasic calcium phosphate dihydrate,[1] and transfer it to a 100-ml. volumetric flask. Add 30 ml. of 10% hydrochloric acid to dissolve the salt, and dilute the solution to the mark with water. Transfer a 5.0-ml. aliquot of the solution to a 250-ml. separator containing 20 ml. of water, 3 ml. of 40% sodium molybdate, and 2 ml. of concentrated hydrochloric acid. Extract the yellow phosphomolybdic acid with three 20-ml. vols. of mixed solvent. Wash the combined extracts with 10 ml. of 1 *N* hydrochloric acid, and add the washings to the aqueous layer.

Adjust the pH of the aqueous layer to 12.5 by adding about 20 ml. of 10% sodium hydroxide. Add 300 mg. of hydroxy naphthol blue indicator, and titrate the violet solution to a persistent blue end point with standard 0.05 *M* EDTA.

$$\% \ CaHPO_4 \cdot 2H_2O = V \times M \times 172.09 \times 100/5 \times 100/\text{mg. sample}$$

where V = volume of titrant used, and M = molarity of titrant.

Thermogravimetric Analysis—Samples were heated at rates and spans described in Figs. 1 and 2.

RESULTS

Comparison with U.S.P. Assay—Two lots of dibasic calcium phosphate dihydrate were assayed by the proposed and U.S.P. procedures by two different analysts on different days. The results are presented in Table I.

No interference from chlorides, alkali metal cations, or fluoride was encountered in the chelometric assay of dibasic calcium phosphate in a formulation.

[1] Compensate for weight of water which can be expeditiously removed by drying for 3 hr. at 60° *in vacuo*.

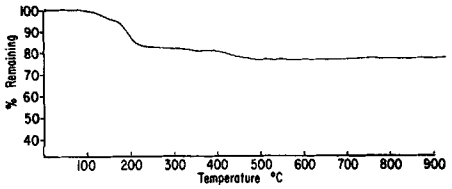


Fig. 1—Thermogram of dibasic calcium phosphate dihydrate U.S.P. over a 900° range. A loss in weight of sample is not noted until a temperature close to 100° is reached. The sample then continues to lose weight up to 500°, at which point no further loss is evident. The per cent remaining was measured as 78, which corresponds to the percentage of anhydrous calcium phosphate present in the dihydrate salt. The equipment was operated at a rate of 25° per minute.

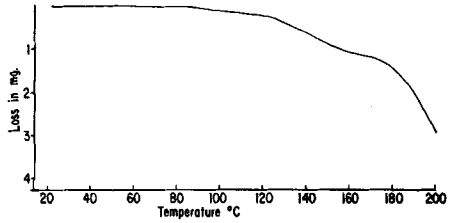


Fig. 2—Similar to Fig. 1, except that the temperature scale was expanded over the 0–200° span at a rate of 20° per minute. This thermogram indicates no loss in weight of sample below 82°.

SUMMARY AND CONCLUSIONS

1. A chelometric titration procedure for dibasic calcium phosphate has been described which compares favorably with the U.S.P. assay in simplicity, speed, and accuracy.

2. Extraction of phosphate as phosphomolybdic acid by an *n*-butanol–chloroform (1:1) mixture eliminates the interference of the anion in the titration of the calcium ion and obviates identification test B in the U.S.P. monograph for the salt.

3. Thermogravimetric analysis of samples indicated that the dihydrate is stable to at least 80° and their moisture content was 1% or less, thus justifying the elimination of the drying step which is required in the U.S.P. method. Furthermore, ignition of sample converts the salt to the pyrophosphate which would obviate the use of the proposed procedure.

4. A thermogravimetric determination of free moisture content provides the correction factor for obtaining accuracy of better than 1%. Alternatively, a loss on drying technique (see Table II), although not as accurate, gives a reasonable indication of free moisture present.

TABLE I—COMPARISON OF PROPOSED AND U.S.P. CALCIUM ASSAYS

Lot	Analyst	U.S.P.	% $CaHPO_4 \cdot 2H_2O$ —Proposed Method— As Is	Anhydrous[a]
A	1	98.3	99.5	100.5
		98.2	99.8	100.8
			99.8	100.8
			100.2	101.2
			98.6	99.6
			99.5	100.5
			99.8	100.8
			99.3	100.3
	2	96.0	99.0	100.0
			99.0	100.0
			99.8	100.8
B	1	98.6	99.8	100.3
			100.0	100.5
			100.0	100.5
			100.3	100.8
			100.9	101.4
			98.9	99.4
	2	99.0	99.5	100.0
		97.8	100.0	100.5
			100.2	100.7
C	3		100.2	...
D	3		99.1	...
E	3		99.8	...
			As is	Anhydrous
Av.	Lot *A* =		99.5 ± 0.56 (S.D.)	100.5 ± 0.56
	Lot *B* =		100.0 ± 0.63 (S.D.)	100.5 ± 0.63

[a] Moisture determined by thermogravimetry. (See Table II.)

TABLE II—COMPARISON OF MOISTURE DETERMINATION BY LOSS ON DRYING (LOD) (60° FOR 3 hr. UNDER VACUUM) AND THERMOGRAVIMETRIC ANALYSIS (TGA)

Lot	LOD, %	TGA, %
A	0.93	1.09
B	0.62	0.51

REFERENCES

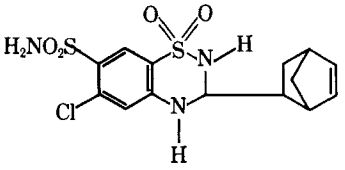
(1) Butler, A. Q., Maurina, F. A., and Morecombe, F. A., *J. Pharm. Sci.*, **54**, 134(1965).
(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 98.
(3) Kindt, B. H., Balis, E. W., and Liebhafsky, H. A., *Anal. Chem.*, **24**, 1501(1952).
(4) Lundell, G. E. F., and Hoffman, J. I., *J. Assoc. Offic. Agr. Chem.*, **8**, 184(1924).
(5) Brunisholz, G., Genton, M., and Plattner, E., *Helv. Chim. Acta*, **36**, 782(1953).
(6) Collier, R. E., *Chemist-Analyst*, **43**, 41(1954).

Qualitative and Quantitative Tests for Cyclothiazide

By EDWARD F. SALIM* and W. W. HILTY†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

6 - CHLORO - 3,4 - DIHYDRO - 3 - (5 -NORBORNEN-2 - YL) - 7 - SULFAMOYL - 1,2,4 - BENZOTHIADIAZINE-1,1-DIOXIDE; $C_{14}H_{16}ClN_3O_4S_2$; mol. wt. 389.88. The structural formula of cyclothiazide may be represented as:



Physical Properties—Cyclothiazide occurs as a white to nearly white, practically odorless powder, and melts with decomposition at about 220° (U.S.P., class I). It is practically insoluble in water and in chloroform, sparingly soluble in alcohol, and freely soluble in acetone and in methanol.

Identity Tests—A 1 in 100,000 solution of cyclothiazide in methanol exhibits ultraviolet absorbance maxima at about 271 mμ [absorptivity (*a*) about 57] and 315 mμ, and absorbance minima at about 242 and 295 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of cyclothiazide in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Transfer 20 mg. of cyclothiazide to a test tube and dissolve in 5 ml. of methanol. Add 1 ml. of hydrochloric acid and warm on a steam bath for 5 min.: the liquid acquires a blue color and a yellow fluorescence.

Purity Tests—Determine the water content of cyclothiazide by the titrimetric (Karl Fischer) method: not more than 1% is found.

Char about 1 Gm. of cyclothiazide, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%.

Assay—Transfer about 400 mg. of cyclothiazide, accurately weighed, to a 125-ml. conical flask, and dissolve in 40 ml. of ethylenediamine. Add 5 drops of a solution of *o*-nitroaniline in benzene (1 in 100), and titrate with 0.1 *N* sodium methoxide, using a magnetic stirrer and taking precautions against absorption of atmospheric carbon dioxide as by the use of an atmosphere of nitrogen. Perform a blank determination and make any necessary correction. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 19.49 mg. of $C_{14}H_{16}ClN_3O_4S_2$. The amount of cyclothiazide found is not less than 97% and not more than 101.5%, calculated on the anhydrous basis.

Received September 26, 1966, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION, Washington, DC 20037

Accepted for publication December 9, 1966.

† Eli Lilly and Co., Indianapolis, IN 46206. Eli Lilly and Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The authors express their appreciation to J. M. Woodside and E. G. Spinks, Eli Lilly and Co., and Carolyn Damon and Hannah Klein, the Drug Standards Laboratory, for valuable contributions to the monograph.

DOSAGE FORMS OF CYCLOTHIAZIDE

Cyclothiazide Tablets

Identity Test—The solution prepared from the tablet sample in the *Assay* exhibits absorbance maxima and minima at the same wavelengths as the cyclothiazide standard solution.

Assay—Weigh and finely powder not less than 20 cyclothiazide tablets. Weigh accurately a portion of the powder, equivalent to about 2 mg. of cyclothiazide, and transfer to a separator. Add 5 ml. of water and extract with four 25-ml. portions of a mixture of chloroform–methanol (4:1), filtering each portion through chloroform-washed cotton into a suitable beaker. Evaporate the combined extracts on a steam bath in a current of air to dryness. Transfer the residue quantitatively with the aid of about 100 ml. of methanol to a 200-ml. volumetric flask, dilute to volume with methanol, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of cyclothiazide, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 271 mμ, with a suitable spectrophotometer, using methanol as the blank. Calculate the quantity, in milligrams, of $C_{14}H_{16}ClN_3O_4S_2$ in the portion of tablets taken by the formula $0.2C \times (A_u/A_s)$, in which C is the exact concentration of the standard solution, in mcg./ml., A_u is the absorbance of the sample solution, and A_s is the absorbance of the cyclothiazide standard solution. The amount of cyclothiazide found is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Cyclothiazide[1] is an oral diuretic agent useful in

[1] Marketed as Anhydron by Eli Lilly and Co., Indianapolis, Ind.

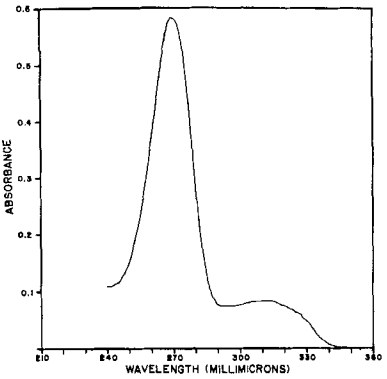


Fig. 1—Ultraviolet absorption spectrum of cyclothiazide in methanol (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

conditions in which increased urinary excretion of sodium chloride and water is desirable. It is also effective for reduction of hypertension, alone or in combination with other antihypertensive agents.

Identity Tests—The identity tests included for the drug substance are satisfactory for distinguishing cyclothiazide from official compounds of similar structure. The ultraviolet absorption spectrum of cyclothiazide is significantly different from the spectrum of chlorothiazide in methanol (single absorbance maximum at 280 mμ) for identification purposes. The color and fluorescence observed when cyclothiazide is warmed in hydrochloric acid solution are not characteristic of chlorothiazide or hydrochlorothiazide and constitute a differentiating test.

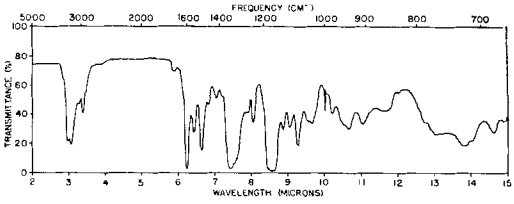


Fig. 2—Infrared spectrum of cyclothiazide in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Quantitative Tests—The nonaqueous titration of cyclothiazide with sodium methoxide using *o*-nitroaniline as the indicator gave an average value of 99.4 ± 0.3%.[2] The assay recoveries are calculated on an equivalent based on two titratable groups in the molecule for the conditions specified. Cyclothiazide dissolved in dimethylformamide and titrated with 0.1 *N* sodium methoxide to a thymol blue end point exhibits only one acidic group and a corresponding equivalent of 38.99 mg./ml. of titrant. Analysis of commercial tablets by the spectrophotometric method gave an average value of 99.2 ± 1.2%[2] of the labeled amount of cyclothiazide. The suitability of the procedure was verified by an average recovery of 100.3 ± 0.6%[2] of a standard cyclothiazide solution carried through the extractive steps as included in the tablet assay.

[2] Maximum deviation from the mean value.

Colorimetric Determination of Iodochlorhydroxyquin and Diiodohydroxyquin

By JOHN J. WINDHEUSER and DIAN Y. CHU

A method has been developed for the extraction and determination of 8-hydroxyquin derivatives utilizing cupric ion as a chelating agent. The method is highly sensitive and simple to carry out. The effect of time, pH, and cupric ion concentration has been investigated.

ALTHOUGH introduced into use as medicinal agents more than a half century ago, the halogenated derivatives of 8-hydroxyquinoline are still of significance today. The analysis of these drugs has been carried out by a number of methods. Fresenius (1) reported a colorimetric method by formation of a colored complex with ferric ion in a glacial acetic acid. The system suffered from a marked sensitivity to variation in the moisture content of the system. Haskins and Luttermaser (2) modified the system by dissolving the drugs or dried extracts from urine in 2-methoxymethanol[1] forming ferric complexes which were reported to be less sensitive to moisture variations. Both systems had the problem of interference by other phenolic compounds which might be present in the systems. Official compendia have utilized extraction techniques followed by halogen determination (3), precipitation as a metal chelate (4), and ultraviolet absorption (5). The halogen determination and metal chelate precipitation methods lack the sensitivity of the spectrophotometric methods. On the other hand, the direct spectrophotometric method suffers from interference by other absorbing species.

In this communication the development of an analytical procedure based on the formation of a

Received November 7, 1966, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication December 20, 1966.

[1] Marketed as Methyl Cellusolve by Union Carbide Corp., New York, N.Y.

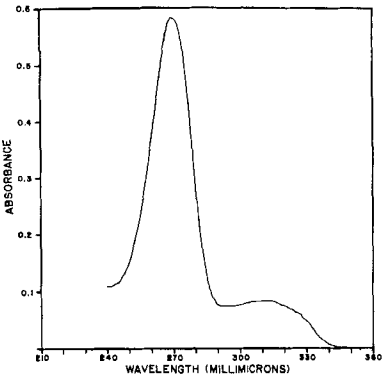


Fig. 1—Ultraviolet absorption spectrum of cyclothiazide in methanol (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

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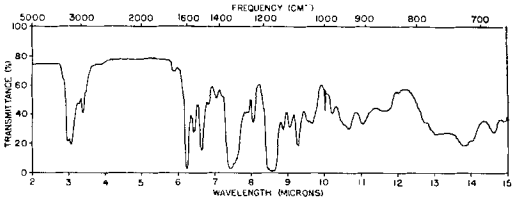

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A method has been developed for the extraction and determination of 8-hydroxyquin derivatives utilizing cupric ion as a chelating agent. The method is highly sensitive and simple to carry out. The effect of time, pH, and cupric ion concentration has been investigated.

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[1] Marketed as Methyl Cellusolve by Union Carbide Corp., New York, N.Y.

copper chelate which is readily extractable into chloroform yielding a stable and highly reproducible yellow color, proportional to the 8-hydroxyquinoline derivative present, is reported. This highly sensitive procedure is simple and rapid and readily adaptable to unit dose analysis

EXPERIMENTAL

Reagents—Iodochlorhydroxyquin U.S.P. and diiodohydroxyquin U.S.P. Cupric sulfate solution, 0.1% $CuSO_4 \cdot 5H_2O$ reagent grade, was dissolved in distilled water. All other reagents used were of analytical reagent grade. Spectrophotometric measurements were carried out using 5-cm. cells in a Cary model 14 or 15 spectrophotometer.

Standard Solutions—Twenty milligrams of iodochlorhydroxyquin or 20 mg. of diiodohydroxyquin are dissolved in sufficient 3 *N* hydrochloric acid to make 100.0 ml.

Analytical Procedure—Prepare a solution of the drug to be assayed in such a manner in 3 *N* hydrochloric acid so that 5.0 ml. contain approximately 1 mg. of the active substance. In the case of tablets, the drug can be extracted from a powdered aliquot of a single tablet or a composite sample of the desired number with 3 *N* hydrochloric acid. In the case of a cream, the required amount of cream equivalent to 20 mg. of the drug is weighed and treated in the manner described below. A sample containing the equivalent of 20 mg. of the drug is placed into a glass-stoppered conical flask and 75 ml. of 3 *N* HCl is added and the mixture shaken for 30 min. on a mechanical shaker. The solution is filtered into a 100-ml. volumetric flask and the flask rinsed with sufficient 3 *N* HCl and passed through the filter to make 100.0 ml. of test solution.

Pipet 5 ml. of the test solution (in the case of diiodohydroxyquin a 1-ml. sample is used) into a 125-ml. separator and add enough 1 *N* sodium hydroxide to make 50 ml.; add 0.5 ml. of the 0.1% copper sulfate solution, mix well, and extract with 25-ml., 15-ml., and 10-ml. fractions of water-saturated chloroform. Adjust the volume of the chloroform extract to 50 ml. and determine the absorbance of the solution at 430 mμ in a 5-cm. cell against a water-saturated chloroform blank. The concentration of the sample can be calculated as follows:

$$\left(\frac{A_{\text{sample}}}{A_{\text{std.}}}\right) \times (\text{concn. of the std.}) = \text{concn. of the test soln.}$$

From this the concentration in the dosage form can be readily calculated.

Figure 1 shows the absorption curve of the iodochlorhydroxyquin–copper complex and the diiodohydroxyquin complex showing essentially the same characteristics with maxima at 430 mμ.

DISCUSSION

Figure 2 depicts the adherence to Beer's law of the absorbance of the complex as a function of drug concentration. At values of greater than 0.6 mg./50 ml. of diiodohydroxyquin, there is a negative deviation due to the insolubility of the copper complex. The method was applied to solutions of the pure drugs, oral tablets, vaginal tablets, and creams and showed a standard deviation of ± 1.1%.

Investigation of Variables—*Effect of Time on the Color Stability*—Samples of iodochlorhydroxyquin were assayed and the absorbances determined immediately and at varying time intervals up to 96 hr. The results as shown in Fig. 3 indicated no change in absorbance over the time of the observations.

Effect of pH of the Test Solution—A 5.0-ml. sample containing 1.035 mg. of iodochlorhydroxy-

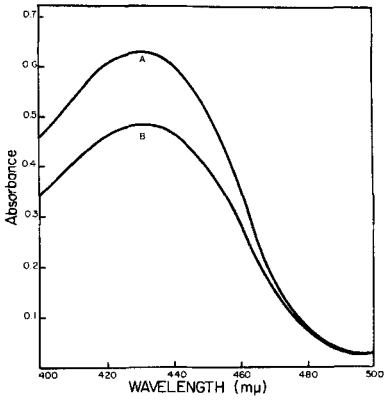


Fig. 1—Absorption spectra of the copper (II) chelate of iodochlorhydroxyquin (A) *and diiodohydroxyquin* (B) *in chloroform. Different concentrations were used to allow ready comparison of the curves.*

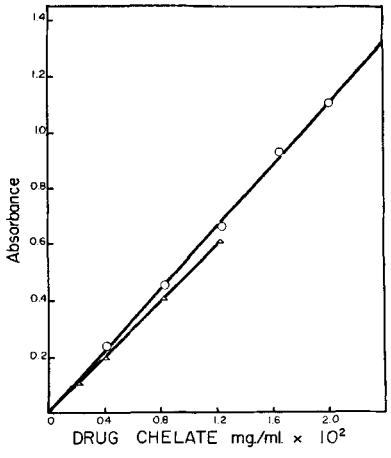


Fig. 2—Beer's law plot for iodochlorhydroxyquin and diiodohydroxyquin copper (II) chelates in chloroform at 430 mμ using 5-cm. cells. Key: ○, *iodochlorhydroxyquin;* △, *diiodohydroxyquin.*

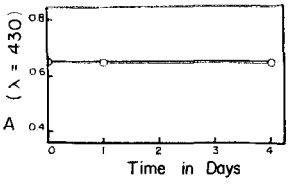


Fig. 3—The time-stability of the absorbance of the copper (II) chelate of iodochlorhydroxyquin in chloroform.

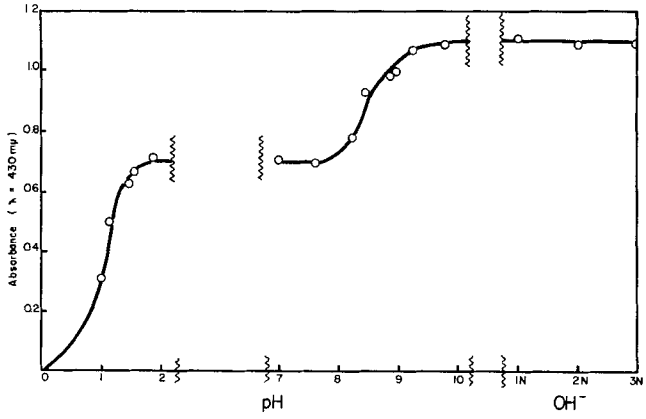


Fig. 4—pH profile of the extraction of iodochlorhydroxyquin–copper (II) chelate by chloroform.

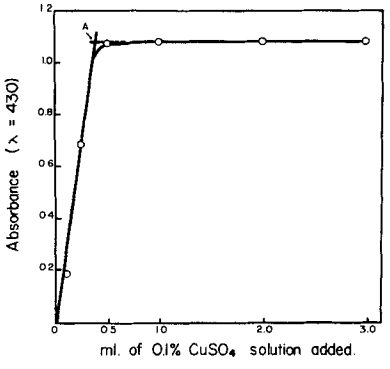


Fig. 5—The effect of cupric ion concentration on the formation and extraction of the iodochlorhydroxyquin–copper (II) chelate at constant drug and hydroxyl ion concentration.

quin in 3 *N* HCl was diluted to 50 ml., the pH being adjusted by the addition of 1 *N* NaOH, and then extracted as under the general procedure. The absorbance was determined at 430 mμ. Figure 4 shows the results, indicating that the system is highly pH dependent with increasing absorbance at higher pH. Attempts were made to find invariant absorbance at higher hydroxyl ion concentration. As shown in Fig. 4 trials using 1, 2, and 3 *N* sodium hydroxide to dilute the test sample prior to extraction produced invariant results. Based upon this, 1 *N* sodium hydroxide was used for all dilutions prior to extraction. From the pH-extraction profile, it would appear that only the ionized form of the drug formed the copper chelate. In the intermediate pH range, the profile is not presented, due to the marked variation of results which might possibly be attributed to the insolubility of the drug at these pH values. It might be suggested that in this region the neutral form of the drug itself can also be extracted into the chloroform layer. At low pH the absorbance of the chloroform layer goes to zero due to nonextractability of the protonated drug. It is obvious from the profile that the most accurate and reproducible results are obtained at high hydroxyl ion concentration.

Effect of Copper Concentration—To determine the dependence of the extractable chelate formation on the molar ratio of cupric ion to iodochlorhydroxyquin, an experiment was conducted at constant drug concentration and varying the copper concentration.

As shown in Fig. 5, the absorbance of the extracted chelate reaches a maximum and further addition of copper sulfate does not result in increased absorbance as might be anticipated. Since all work was carried out with concentrations of iodochlorhydroxyquin less than 1 mg./50 ml. of aqueous sample, 0.5 ml. of 0.1% copper sulfate solution was utilized in this study. It is obvious that if higher concentrations were utilized, the copper sulfate concentration would have to be increased.

The stoichiometry of the complex can be estimated from Fig. 5 by extending the horizontal and vertical lines to point A, the point at which equivalance is reached. It was found that at this point 1.6×10^{-6} moles of Cu^{++} ion had been added to the system containing 3.3×10^{-6} moles of iodochlorhydroxyquin. On the assumption that the complexation reaction is essentially complete, the ratio of Cu^{++} to drug would be 1:2.06 which is in good agreement with the 1:2 complex suggested by others (6) for the interaction of cupric ion with 8-hydroxyquinoline.

SUMMARY

Based upon the formation of an extractable chelate between 8-hydroxyquinoline derivatives and cupric ion, an assay has been developed for several medicinally important drugs. The method is simple, rapid, and highly sensitive. Interference from other drugs or dosage form adjuvants has been minimized. The method is suitable for unit dosage form analysis.

REFERENCES

(1) Fresenius, P., *Suddeut. Apoth. Ztg.*, **79**, 68(1939).
(2) Haskins, W. T., and Luttermaser, G. W., *Anal. Chem.*, **23**, 456(1951).
(3) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 316.
(4) *Ibid.*, 16th rev., 1960, p. 357.
(5) *Ibid.*, 17th rev., 1965, p. 317.
(6) Ishibashi, M., Suito, E., and Sekido, E., *Nippon Kagaku Zasshi*, **78**, 1788(1957).

Automated Analysis of Erythromycin

By NORBERT R. KUZEL and HENRY F. COFFEY

An automated method has been developed for the analysis of erythromycin by adaptation of the long-established manual U.V. procedure. The method uses a basic automatic analyzer (Technicon AutoAnalyzer) arrangement and a Hitachi Perkin-Elmer spectrophotometer. The method features the concept of the simultaneous determination of a sample and its associated blank in a dual-channel arrangement. A unique cell positioner controlled by a modified liquid sampler enables the use of only one spectrophotometer and one recorder to monitor both channels. In this manner critical phasing problems have been eliminated. The system is capable of making at least 300 measurements per man per day. This method has been developed for use in fermentation broths, process samples, purified materials, and dosage forms.

ERYTHROMYCIN[1] is a broad spectrum antibiotic that has been marketed for many years. Kuzel *et al.* (1) published a spectrophotometric method for the manual determination of this antibiotic. This method is still used routinely in these laboratories. The method is useful for the determination of erythromycin in dosage forms, process samples, and fermentation broths. This manual method is based on a mild alkaline hydrolysis of erythromycin producing a chromophore which has an absorbance peak at 236 mμ. To determine materials other than erythromycin which have an absorbance at 236 mμ, the erythromycin is blanked out by a mild acid treatment. The absorbance difference between the two determinations has been shown to be due to erythromycin alone even in the case of degraded samples or in fermentation broths.

Since sample loads had become increasingly large, it became necessary to consider an automated method. It was decided to attempt automation of the spectrophotometric method because of the long history of reliability of this method. Certain requirements were imposed on the automated method. A 1-hr. service is often required on certain samples, so it became imperative that samples and blanks be determined simultaneously. Second, it was desired to duplicate as much as possible the manual method and this involved contending with high blanks in fermentation samples. The first requirement necessitated the use of either two separate analytical trains or a dual-channel system. The second requirement would tend to eliminate differential spectrophotometry since phasing problems become very serious with two highly absorbing solutions and a relatively low net absorbance. The purpose of this paper is to describe a dual-channel system for the analysis of erythromycin, but more importantly to describe a generalized approach to precise, dual-channel spectrophotometry using only one spectrophotometer and one recorder.

Received December 27, 1966, from the Analytical Research Department, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication February 14, 1967.
Presented in part to the Technicon Symposium, "Automation in Analytical Chemistry," New York, N.Y., October 17, 1966.

[1] Marketed as Ilotycin by Eli Lilly and Co., Indianapolis, Ind.

EXPERIMENTAL

Apparatus—The analytical system consisted of the following modules: (*a*) automatic sampler[2] (modified), proportioning pump, and heating baths (modified) from Technicon Instruments, Inc., Chauncey, N.Y.; (*b*) a Hitachi Perkin-Elmer spectrophotometer model 139; (*c*) micro flow cells 9120-NO5, from A. H. Thomas; and (*d*) a Honeywell Electronic 15 recorder (linearized).

Procedure—Figure 1 shows a flow diagram of the automated method including all pump tubing sizes. A sample is drawn from a liquid sampler and is split into two equal portions.

One half of the sample is drawn into the sample channel shown in the lower portion of the figure, and the other half to the blanking channel shown in the upper portion of the figure. The sample channel will be described first. Sodium hydroxide (0.25 *N*) is diluted with water, and the diluted alkali is added to the sample stream, segmented with air, and mixed. The stream is heated in one coil of a 65° heating bath, cooled, and debubbled just before it enters flow cell A of the spectrophotometer.

The other half of the sample is diluted with water, and 0.5 *N* sulfuric acid is added through an H3 fitting. The stream is segmented with air, mixed, and the erythromycin inactivated in the 43° heating bath fitted with 19 ft. of 0.076 i.d. Teflon tubing. As the stream leaves the inactivation bath, 0.35 *N* sodium hydroxide is added to adjust the stream to the same alkalinity as the sample stream. The blank stream enters the second coil of the 65° heating bath, is subsequently cooled and debubbled, and enters flow cell B of the spectrophotometer.

The streams are drawn through the flow cells by pumping a controlled amount from the flow cells while the excess solution and air flow to waste just

[2] AutoAnalyzer Sampler II.

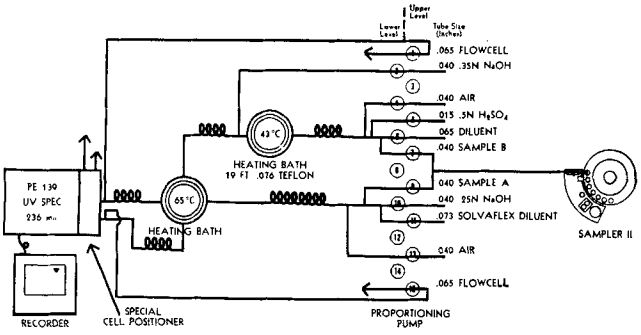


Fig. 1—Flow diagram of the automated method for erythromycin.

ahead of the flow cells. The two channels are alternately monitored in the spectrophotometer at 236 mμ by use of a unique cell positioner. The cell positioner is controlled by a modified liquid sampler.

Standards are run about every 10 or 20 samples, since the stability of the system has been excellent. Reference standard erythromycin base is dissolved in about 10 ml. of methanol and diluted to a concentration of about 250 mcg./ml. with pH 7.0 phosphate buffer. Only one concentration of standard is used since the response of the system is linear. Samples are diluted to about the same concentration as the standard, with the exception of fermentation broths, which are diluted with water so that the maximum absorbance of the sample does not exceed the absorbance range of the analytical system. In these laboratories, this is equivalent to about 20–100 mcg. of erythromycin per ml. of diluted broth. Duplicate cups of sample solutions are generally placed in the liquid sampler, and the results of the duplicates are averaged for calculation purposes. The sampler is operated at 20 samples per hour with a 1:2 sample rinse ratio, so that about 10 samples and blanks in duplicate are processed each hour. Polyoxyethylene (23) lauryl ether[3] is added to deionized water, and this solution is used as the diluent in the automated method.

RESULTS AND DISCUSSION

The automated method follows Beer's law over the entire absorbance range of the system, and the steady state response is very stable. In fact, the steady state has been used to follow long-term hydrolysis curves of several esters of erythromycin in a dynamic system. Figure 2 is a photograph of typical response curves obtained on standards and relatively pure materials. The response at the left of the photograph shows the stable base line obtained and the ability to match flow cell blanks with the cell positioner. The short spikes seen on the base line are the switch points of the flow cells. Peaks 1, 3, and 5 are standards and peaks 6, 8, and 10 are the blanks associated with the standard. Peaks 7 and 9 are the first sample and 12 and 14 are the associated blanks. It is apparent that the blanks are located about midway between sample peaks and are offset from their respective samples by 5 places due to the difference in transit times of the two channels. A time delay coil could be installed in the sample stream to provide adjacent sample and blank peaks if

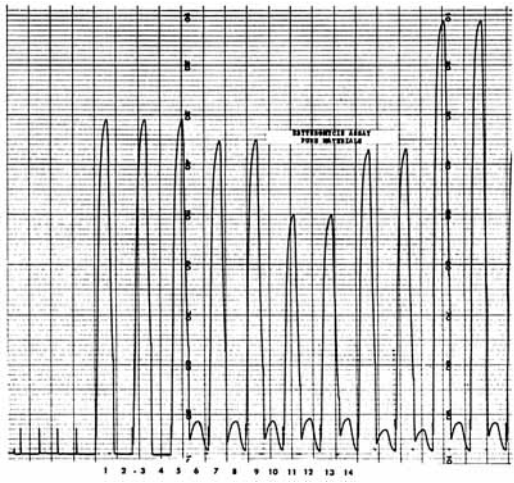


Fig. 2—Recording of replicate samples of erythromycin standards and samples of relatively pure materials illustrating the dual-channel recording technique.

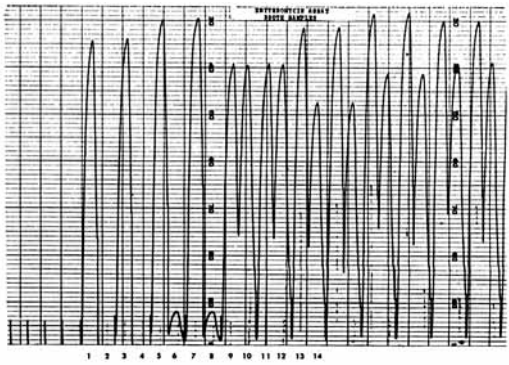


Fig. 3—Recording of replicate samples of erythromycin fermentation broth samples.

desired. The excellent reproducibility of the system is evident in the illustration. There is no sample interaction, although this is not clearly illustrated in Fig. 2 because of the necessity for cell switching. In practice, small changes in phasing occur from day to day and within days; but since only individual peaks need to be observed, rephasing is hardly ever re-

[3] Marketed as Brij 35 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE I—COMPARATIVE DATA ON ERYTHROMYCIN SAMPLES

Lot No.	Manual	Automatic	Microbiological
1	938 mg./Gm.	937	970
2	932 mg./Gm.	926	933
3	905 mg./Gm.	907	965
4	930 mg./Gm.	936	960
5	20,600 mcg./ml.	20,300	
6	44,900 mcg./ml.	44,700	
7	2,430 mcg./ml.	2,670	2,180
8	2,100 mcg./ml.	2,180	2,320
9	1,480 mcg./ml.	1,560	1,280

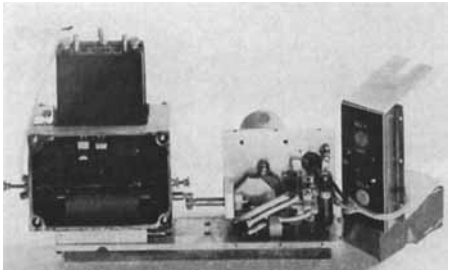


Fig. 4—Interior of the cell positioner.

quired. A water blank is inserted in the sample tray occasionally to assure that no base line drift has occurred. Relative standard deviations of the average of five replicates corrected for blank are typically less than 1%. Occasionally deviations greater than those indicated are observed in the sample readings, but when the associated blanks are subtracted, the relative standard deviations are normal. This may occur because of some contamination of the sample by the sample cup since sample cups are not prewashed in this laboratory.

Figure 3 illustrates typical response curves obtained on fermentation broth samples. Peaks 1 and 3 are standards and 6 and 8 are the respective blanks. Peaks 5 and 7 are the first broth sample and 10 and 12 are the blanks for the first sample. The blank absorbances in this case are much higher, and net absorbances are relatively small. The need for precision in the system is readily evident. This also illustrates the problems that would occur if a differential spectrophotometric method were employed since only slight phasing changes would result in gross errors.

Some comparative results are shown in Table I. Validity of the analysis is demonstrated by comparison with microbiological assays. The first four results are on relatively pure materials, the next two on process samples, and the last three on fermentation broths. The automatic and manual methods are in good agreement, and both closely follow the results obtained by microbiological analysis.

Previous experience in this laboratory with a large number of flow cells has resulted in a distinct preference for the design of the A. H. Thomas flow cell. This flow cell has excellent clearance characteristics, has a small internal volume, and any occasional air bubbles are readily cleared. The authors also wanted to use the Hitachi Perkin-Elmer model 139 spectrophotometer because of its excellent stability and proven performance. The only cell positioner available for this combination of components was the A. H. Thomas cell positioner but, unfortunately, it was not designed to position microflow cells with small cell windows, and large errors were observed in its use due to positioning problems alone. With no other cell positioner commercially available, the authors decided to design and build a precision cell positioner. Figure 4 is a photograph of the interior of the cell positioner that was constructed from this design. Only two positions were required for this application, so a unit was designed that would position between two adjustable stops. The stops can be seen mounted into the ends of the cell compartment. A new cell holder carriage was made to provide a firm grip on the cell holder. The manual cell positioner shaft was removed from the cell compartment and was replaced by a smooth shaft fitted at one end with a ball joint which engaged a pin fastened to the underside of the cell holder carriage. The other end of the shaft was connected by means of a partially threaded pin to the shaft of the cell positioner assembly. The cell holder carriage can be easily uncoupled from the cell positioner and moved manually to one of the other two cell positions, if desired. A Slo Syn motor (SS25 Superior Electric Co., Bristol, Conn.) operates a yoke fabricated from an acetyl resin[4] which in turn is mounted on the sliding shaft of the cell positioner. The yoke drives the shaft until one end of the cell holder carriage contacts one of the adjustable stops and the remainder of the stroke is taken up by a spring located within the arms of the yoke. A similar action occurs on the return stroke. The spring between the arms of the yoke is slidably mounted on the cell positioner shaft and is retained by small horseshoe-shaped keepers. In addition to providing facilities for overtravel of the yoke, the spring also assures a firm reproducible contact with the adjustable stops and reproducible cell positioning. The stops are adjusted from the outside of the cell compartment for proper cell alignment and matching of cell blanks. The Slo Syn motor used in this device caused an annoying vibration in the cell positioner, and the condition was corrected by installation of a 500 ohm 5-w. variable resistor in parallel with the motor. This dropped the voltage applied to the motor to about 50 v.

A cell positioner should be interlocked with the rest of the analytical train so that a slow cumulative drift cannot occur. The positioner just described is controlled by a special modification of the automatic sampler. Figure 5 shows a wiring diagram of the control circuit for the cell positioner. A microswitch was installed on an existing mounting post inside the sampler. This switch is labeled as Technicon sampler switch in the diagram and is actuated by a double rise split cam attached to the shaft of the sampler timing motor. The cam on the timing motor is split so that the ratio of dwell times in positions A and B of cell positioner can be varied, if necessary. As the sampler switch is moved to one position, it completes a circuit through the switch on the cell positioner and through the motor. The motor will drive until a single rise cam attached to the motor shaft opens the cell positioner switch and

[4] Marketed as Delrin by E. I. duPont de Nemours & Co., Wilmington, Del.

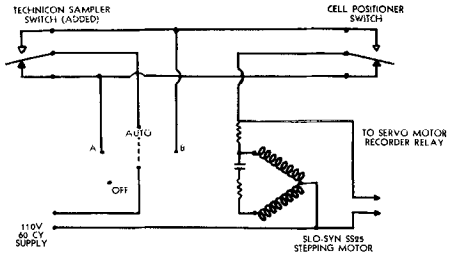


Fig. 5—Wiring diagram of the control circuit for the cell positioner.

thus opens the circuit. This action corresponds to one switching movement of the flow cells. The sampler timing motor continues to run until the double rise cam returns the sampler switch to its former position, whereupon the motor circuit is again closed and the motor drives to its former position, thus completing its switching cycle. Large spikes resulting from temporary blockage of the light path due to switching are prevented from being recorded on the recorder by insertion of an air damped relay into the servo motor circuit of the recorder. The relay is activated every time the motor is energized. The delay can be adjusted to essentially eliminate any spikes without significant loss of meaningful recorded data. The cell positioner can also be operated manually for set-up purposes by use of the A and B position manual switch. The cell positioner as described was designed for a sampling rate of 20 per hour. If a higher sampling rate is desired, the number of raised portions on the split cam mounted on the sampler timing motor must be increased to correspond to the number on the programming cam controlling the sampler.

Approximate phasing of the two channels was accomplished in the usual way by insertion of a time delay coil in one of the channels. The cell positioner must then be phased with the sampler so that cell positioning is achieved at the appropriate time. This was accomplished by designing a new programming cam for the sampler. A double rise split cam was fabricated from the acetyl resin and both halves of the split cam can be rotated with respect to the center hub of the cam which is driven by the timing motor. Adjustment of the split cam provides for a variable sample to rinse ratio. Rotation of the adjusted split cam on the hub provides for advance or delay of sampling with respect to the double rise cam controlling the cell positioner and in effect controls the time span between sampling and cell positioning.

The absorbance of the solutions in both channels is recorded on a Honeywell Electronic 15 strip chart recorder equipped with a 1 cycle log-characterized slide wire and having a range of 0 to 1 absorbance unit. This recorder provides a very precise and reliable recorder for spectrophotometric work at moderate cost. Linear potentiometric recording cannot be done on the instrument modified in this manner; but since this recorder was to be used for spectrophotometric work only, this limitation is not serious.

The lengths of the various heating coils, the temperatures of the baths, and the concentration of reagents were all experimentally determined to substitute for the conditions found in the manual method. A 9-min. acid inactivation of the erythromycin at 43° with 0.5 *N* sulfuric acid was found to be equivalent to the 1-hr. inactivation at room temperature with 0.25 *N* sulfuric acid as performed in the manual method. A plateau between 38 and 48° was found for the inactivation temperatures. Any temperatures above or below these figures produce high and invalid blanks.

Certain special precautions need to be observed in the use of this system. Because of the low wavelength, *i.e.*, 236 mμ, considerable and variable extraneous U.V. absorbance can be picked up from some lots of Tygon tubing especially in the presence of warm alkaline solutions. Teflon, polyethylene, or glass tubing was used throughout the system except for the pump tubings. The cell compartment was fitted with an A. H. Thomas high rise cell cover and the cells were connected to the debubbler fitting with thin wall flexible polyethylene tubing. Relative dilutions in the two channels are important in a dual-channel system, especially where one channel is a blanking channel having high absorbance. In an attempt to determine relative dilutions, erythromycin was run as a sample in both channels, but subsequently it was found that some erythromycin was destroyed in the extra heating step of the blank channel. A stable compound having U.V. absorbance at 236 mμ was needed, and sodium benzoate was found to work very well for this purpose. The pump tubings are adjusted until both channels provide the same dilution of the sample. The model 139 spectrophotometer has a very rapid response, and somewhat noisy tracings were initially obtained. By using the spectrophotometer in its slow response mode, very smooth response curves were obtained without any loss in precision.

ANALYTICAL POTENTIAL

Aside from the use of this method for the analysis of erythromycin, this paper describes a general method of monitoring two analytical streams for both U.V. and colorimetric methods while using only one spectrophotometer and one recorder. This was made possible by the design of a precise cell positioner obviating the need for differential spectrophotometry and its attendant phasing problems. The unique design of the cell positioner also permits the use of microflow cells in a cell positioner suitable for use with a Hitachi Perkin-Elmer spectrophotometer. The cell positioner design could easily be adapted for use with other spectrophotometers. The concept of a cell positioner controlled by the modified automatic sampler could be utilized in a number of other applications.

REFERENCE

(1) Kuzel, N. R., Woodside, J. M., Comer, J. P., and Kennedy, E. E., *Antibiot. Chemotherapy*, 4, 1234(1954).

Automated Determination of Rat Adrenal Ascorbic Acid in the Bioassay of Corticotropin

By WILLIAM F. BEYER

An automated procedure for the determination of rat adrenal ascorbic acid used in the U.S.P. XVII bioassay of corticotropin is described. Ascorbic acid analyses of manually filtered, adrenal gland homogenates are performed with buffered 2,6-dichloro-indophenol sodium. A sample–rinse ratio of 1:2 is used and sample carry-over is not detectable. AutoAnalyzer Sampler II, proportioning pump, colorimeter, and recorder are used. The coefficient of variation for the automatic procedure is approximately 1 per cent at an effective sampling rate of 60/hr. An IBM 1620 computer is used for calculations of ascorbic acid determinations and for the bioassay of corticotropin using a 3 × 3 statistical design. Assay results using automated and manual ascorbic acid values compare favorably.

THE DEPLETION of ascorbic acid of rat adrenal glands is used as the measure of response in the bioassay of corticotropin as directed by U.S.P. XVII (1). For the subcutaneous assay, hypophysectomized rats are injected in random order with varying doses of standard or test preparations using a 3 × 3 design. Three hours after injection, the paired adrenals are removed, freed of excess tissue, weighed, and homogenized in 2.5% metaphosphoric acid. The ascorbic acid content of each pair of adrenal glands is then determined using buffered 2,6-dichloro-indophenol sodium. Statistical analyses are applied to ascorbic acid values to determine potency, precision, and validity of the assay. This report describes an automated procedure for the determination of rat adrenal ascorbic acid used in the bioassay of corticotropin. The automated method, based on the U.S.P. XVII procedure, requires manual filtration of adrenal gland homogenates. Automatic sampler,[1] proportioning pump, colorimeter, and recorder are used, permitting the analysis of 60 samples per hour, including wash between samples.

MATERIALS AND METHODS

Instruments—Automatic sampler, proportioning pump, colorimeter with 524 mμ filters and 1.5-cm. flow cell, recorder, and chart reader.

Reagents—(*a*) 2,6-Dichloro-indophenol sodium, 48.0 mg./1000 ml. Filter and store in a brown bottle under refrigeration. (*b*) Sodium acetate trihydrate, 45.3 Gm./1000 ml. Adjust to pH 7 with acetic acid. Store in a brown bottle under refrigeration. (*c*) Color reagent, equal parts of 2,6-dichloro-indophenol sodium and sodium acetate trihydrate solutions are mixed when cold. Store in a brown bottle under refrigeration. (*d*) Metaphosphoric acid, 2.5%. (*e*) Rinse solution for automatic sampler, 4.0 ml. of polysorbate 80 is dissolved in 1000 ml. 2.5% metaphosphoric acid.

Standards—Accurately weighed quantities of U.S.P. ascorbic acid reference standard or house standard are dissolved in 2.5% metaphosphoric acid within the range of 3 mcg./ml. to 11 mcg./ml. for the standard curve. Prepare just prior to use.

Test Samples—Paired adrenal glands of test rats are freed of excess tissue, weighed, and homogenized in 12.0 ml. of 2.5% metaphosphoric acid using a tapered glass tissue homogenizer.[2] Clear filtrates are then obtained using Whatman No. 2 filter paper placed in collection funnels.[3]

Procedure—Using the manifold as shown in the flow diagram of Fig. 1, the rinse line of the automatic sampler is placed in rinse solution for automatic sampler, and all other lines in water. With all instruments operating, a 99% transmission base line is established. The color reagent line is placed in iced color reagent and the sample line with probe is properly positioned in the rinse compartment of automatic sampler. Following a stable transmittance recording, the sampling rate is set at 60/hr. with a 1:2 sample–rinse ratio. Standards are placed in duplicate cups for the standard curve and test samples in single cups.

Calculations—The recorded transmittance peaks for each level of ascorbic acid standard are plotted, and a chart reader is used to determine the microgram per milliliter concentration of each test sample. A computer program is written in Fortran (formula translation) to calculate ascorbic acid in terms of milligrams ascorbic acid per 100 Gm. of adrenal tissue. The program also transforms the random order of samples into proper dosage groups, checks for outliers, and carries out statistics for the bioassay.

RESULTS AND DISCUSSION

Initial experiments with various manifold systems and sampling rates produced unreliable results and unsatisfactory recordings. Most of the difficulties were due to sample carry-over, erratic movement of the air bubble between sample and rinse, and pulsation from the pump action. Carry-over was eliminated by severing the 0.090-in. sample line at

Received December 1, 1966, from the Control Analytical Research and Development Department, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication January 19, 1967.

Presented in part to the Technicon Symposium, "Automation in Analytical Chemistry," New York, N. Y., October 1966.

The cooperation of Dr. N. W. Dunham and J. T. Keene, Product Control and Control Research, The Upjohn Co., is acknowledged with appreciation. The computer program and calculations performed with the IBM 1620 by R. J. Cole and W. D. Frailing, Control Statistical Services, The Upjohn Co., are also acknowledged.

[1] AutoAnalyzer Sampler II, Technicon Controls, Chauncey, N. Y.

[2] Manufactured by Norman Erway, Oregon, Wis.

[3] Marketed as DiSPo by Scientific Products, Division of American Hospital Supply Corp., Evanston, Ill.

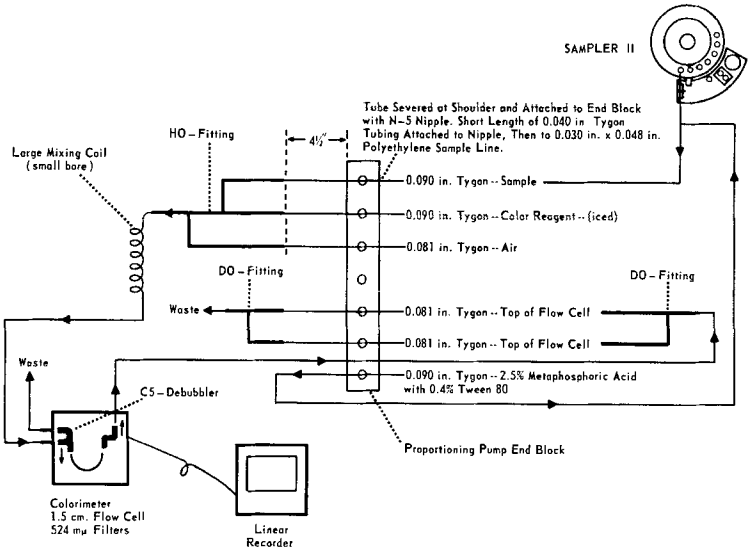


Fig. 1—Manifold flow diagram for the automated determination of ascorbic acid in rat adrenal gland homogenate. Analyses are made using Technicon AutoAnalyzer equipment: colorimeter, proportioning pump, recorder, and assorted tubing. Sampling rate is 60/hr. with a 1:2 sample–rinse ratio.

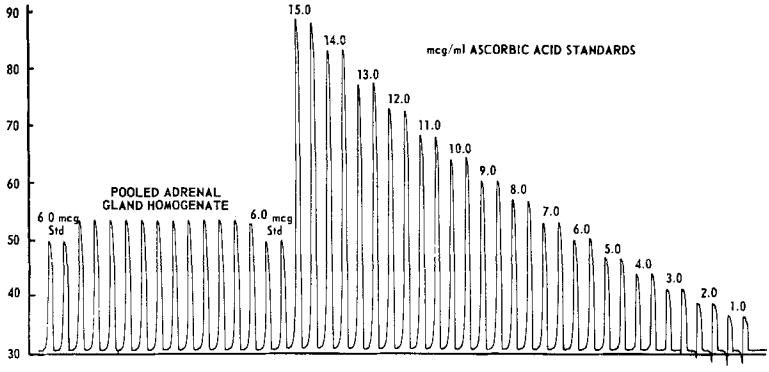


Fig. 2 — Transmittance-concentration recording of ascorbic acid standards and replicate sampling of pooled adrenal gland homogenate.

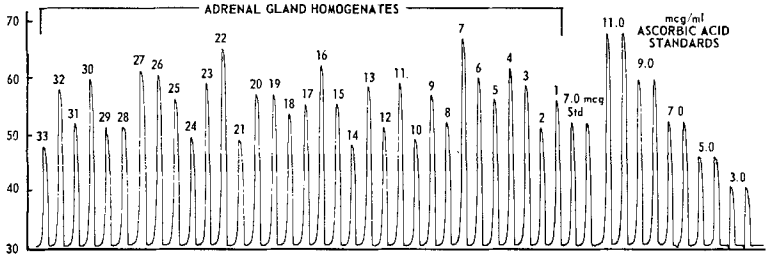


Fig. 3 — Representative transmittance recording of ascorbic acid standards and adrenal gland homogenates from a corticotropin bioassay.

the end block (Fig. 1) and attaching it to a 0.030 × 0.048-in. polyethylene sample line, and by using a 1:2 sample–rinse ratio. Satisfactory movement of the air bubble between samples and rinse occurred when polysorbate 80 was added to the metaphosphoric acid rinse. Polysorbate 80 also reduced the deposition of fatty material from adrenal gland homogenates on the walls of the sample line. The effect of pulsation was lessened by allowing approximately 4.5 in. of manifold tubing to extend beyond the exit end of the end block and by using an H-O cactus.

Twelve milliliters of 2.5% metaphosphoric acid was used in place of the 8.0 ml. as directed by U.S.P. XVII in order that adequate adrenal gland homogenate was available for both automated and manual ascorbic acid determinations. The concentration of 2,6-dichloro-indophenol was reduced to compensate for the greater volume of adrenal gland homogenate.

Attempts to filter the adrenal homogenate automatically with Technicon's continuous filter using various filter papers and a Millipore[4] nylon roll filter

[4] Millipore Filter Corp., Bedford, Mass.

TABLE I—PRECISION OF REPLICATE ASCORBIC ACID DETERMINATIONS

Ascorbic Acid Std., mcg./ml.						Adrenal Gland Homogenate, mcg./ml.
2.0	4.0	6.0	8.0	10.0	12.0	
2.00	3.90	6.00	7.90	9.85	12.05	7.10
2.00	4.00	5.95	7.95	10.00	12.00	7.10
2.00	4.10	6.00	8.00	10.00	12.00	7.15
2.00	4.05	5.95	8.05	10.00	12.25	7.15
2.05	4.00	6.00	8.05	9.90	12.00	7.15
2.05	4.00	6.05	8.05	10.00	12.00	7.15
2.00	4.00	6.05	8.05	9.90	12.05	7.10
2.00	3.95	6.00	8.00	10.00	12.00	7.10
						7.10
						7.15
						7.10
						7.10
			Coefficients of Variation, %			
1.15	1.49	0.60	0.67	0.62	0.72	0.36

TABLE II—RECOVERY OF ASCORBIC ACID FROM VARIOUS QUANTITIES OF ADRENAL GLAND HOMOGENATE ADDED TO ASCORBIC ACID STANDARD

Ascorbic Acid from Homogenate, mcg./ml.	Ascorbic Acid Std. Added, mcg./ml.	Theoretical Amt. of Ascorbic Acid, mcg./ml.	Ascorbic Acid Found, mcg./ml.	Recovery, %
0.45	4.85	5.30	5.25	99.1
0.95	4.85	5.80	5.80	100.0
1.90	4.85	6.75	6.75	100.0
2.85	4.85	7.70	7.65	99.4
3.80	4.85	8.65	8.65	100.0
4.75	4.85	9.60	9.70	101.0
				Av. 99.9

were unsatisfactory. Dialysis was not attempted in these studies; however, it is possible that newer membranes permitting more efficient dialysis and a flow cell with a longer light path would provide the necessary sensitivity.

Figure 2 gives a recorder tracing of ascorbic acid standards in the range of 1.0–15.0 mcg./ml. and replicated samples of pooled adrenal gland homogenates. The uniformity of replicate analyses and the return to base between samples can be noted. When the values for the standards of Fig. 2 are plotted, a linear relationship exists for the logarithm of transmittance and ascorbic acid concentration. Figure 3 gives transmittance recordings for standards and a portion of the recordings from adrenal gland homogenates in a typical corticotropin bioassay.

The precision of the automated method was determined by repetitively assaying 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mcg./ml. ascorbic acid standards and pooled adrenal gland homogenate. In Table I, it can be seen that the coefficients of variation range from 0.36% to 1.49%, with less precision occurring at lower ascorbic acid concentrations.

Recovery of ascorbic acid with the automated procedure was determined by adding standard to various quantities of adrenal gland homogenate. An average recovery of 99.9% was obtained, varying from 99.1% to 101.0% (Table II). Similar ascorbic acid values for adrenal gland homogenates were obtained for both automated and manual methods of assay. Table III shows a representative adrenal ascorbic acid depletion assay for corticotropin with ascorbic acid determined on parallel samples of homogenate. It can be noted that statistical data for the corticotropin bioassay of Table III are in close agreement. Numerous additional bioassays have been carried out using both manual and automated procedures for adrenal ascorbic acid with satisfactory results.

TABLE III—PROTOCOL OF AN ADRENAL ASCORBIC ACID DEPLETION BIOASSAY FOR CORTICOTROPIN[a]

mg. Ascorbic Acid/100 Gm. Gland											
U.S.P. Corticotropin Std.						Corticotropin Extract					
40 mu/Rat		100 mu/Rat		250 mu/Rat		40 mu/Rat		100 mu/Rat		250 mu/Rat	
M	A	M	A	M	A	M	A	M	A	M	A
345.8	356	308.6	329	290.8	293	330.7	333	309.5	320	282.1	286
414.9	411	361.8	372	272.3	282	359.0	362	310.8	324	284.5	286
461.9	465	323.8	326	232.5	242	484.4	478	290.7	298	270.3	281
437.2	444	310.8	320	241.0	247	479.6	483	313.2	324	235.7	257
395.9	396	335.9	344	273.2	284	367.7	374	295.9	304	307.2	318
400.8	405	262.3	289	274.4	283	382.6	384	333.3	340	253.1	261
402.1	411	292.9	299	226.9	243	399.3	410	323.2	342	293.8	308
				M		A					
Potency (units/ml.)				44		44					
Variance				1216		1094					
Test for parallelism of slopes (F)				0.8580		1.1415					
Log-confidence interval (L)				0.2576		0.2554					

[a] Ascorbic acid determinations were made on each pair of rat adrenals by both manual (M) and automated (A) procedures. Statistical analysis carried out as directed by U.S.P. XVII.

An alternate procedure has been developed for calculations of adrenal gland ascorbic acid based on absorbance readings. Absorbance paper is used in conjunction with a single 8.0-mcg./ml. level of ascorbic acid standard. A computer program is used that provides for the subtraction of recorded absorbance readings of standard and test samples from the absorbance of the color reagent +2.5% metaphosphoric acid base line. The program also provides for the computation of milligrams ascorbic acid per 100 Gm. of paired adrenal glands based on the relative absorbances of the 8-mcg./ml. standard and test samples and on the weights of paired adrenal glands. The computer program places ascorbic acid values in their proper dosage groups and provides for statistical calculations for the assay as directed by U.S.P. XVII.

SUMMARY

An automated procedure is described for the determination of ascorbic acid in rat adrenal gland homogenates in the bioassay of corticotropin. The procedure, based on that of the U.S.P. XVII, permits the analysis of 60 samples/hr. with a coefficient of variation of approximately 1%. Ascorbic acid was recovered satisfactorily from standard samples spiked with varying amounts of adrenal gland homogenate. Corticotropin bioassays using ascorbic acid results obtained with manual and automated procedures compare favorably.

The automated ascorbic acid procedure and the computer program should have application in the Parlow ovarian ascorbic acid depletion bioassay of luteinizing hormone, chorionic gonadotropin, and pregnant mare's serum (2).

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) Parlow, A. F., *Federation Proc.*, **17**, sect. 1587(1958).

Notes

Estimation of Volume of Distribution and Half-Life of a Compound After Rapid Intravenous Injection

By JOHN G. WAGNER and JACK I. NORTHAM

It is common practice to plot the plasma or serum concentrations of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant ($\hat{\beta}$) for loss of compound from the body or half-life ($0.693/\hat{\beta}$) from the slope of the terminal linear segment, extrapolate the linear segment to time zero, and divide the intercept into the dose to obtain the apparent volume of distribution ($\hat{V}_d$). Under certain conditions the estimates obtained by this procedure may be valid. However, this report shows that if the model which applies is a two compartment open system, then the $\hat{V}_d$ estimated by this method is always an overestimate of the true total volume ($V_1 + V_2$), and the error depends upon the relative values of V_1/V_2 and K_1/K_2 where K_1 is the first-order rate constant for distribution and K_2 is the first-order rate constant for loss from the inner compartment. The half-life estimates ($0.693/\hat{\beta}$) will always be greater than the true half-life ($0.693/K_2$), and $\hat{\beta}$ as an estimator of K_2 also depends on the ratios V_1/V_2 and K_1/K_2.

IT IS COMMON practice[1] (1–5) to plot the plasma or serum concentration of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant for loss of compound from the body ($\hat{\beta}$) or the half-life ($0.693/\hat{\beta}$) from the slope ($\hat{\beta}/2.303$) of the terminal linear segment; and extrapolate the linear segment to time zero and divide the intercept ($\hat{B}_1$) into the dose (D) to obtain the apparent volume of distribution, pool size, or "space" ($\hat{V}_d$). If the model which applies to the particular system involves a single compartment and the initial nonlinearity of the semilogarithmic plot is assumed to be due to mixing, then the estimates $\hat{\beta}$ and $\hat{V}_d$ would be very close to those expected on the basis of the appropriate mathematical expression.

Nelson (6), however, pointed out that the error in the estimated volume of distribution may be substantial if there are two compartments with volumes V_1 and V_2 and the volume of the outer compartment (V_2) is large, and rate of attainment of equilibrium is slow. This report will show that if the model which applies is a two compartment open system, then $\hat{V}_d$ always overestimates the true total volume

Received November 8, 1966, from the Research Division, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication December 22, 1966.

[1] The practice is so common that the authors considered it unnecessary to provide an extensive bibliography. However, *References 1–5* are a representative sample.

An alternate procedure has been developed for calculations of adrenal gland ascorbic acid based on absorbance readings. Absorbance paper is used in conjunction with a single 8.0-mcg./ml. level of ascorbic acid standard. A computer program is used that provides for the subtraction of recorded absorbance readings of standard and test samples from the absorbance of the color reagent +2.5% metaphosphoric acid base line. The program also provides for the computation of milligrams ascorbic acid per 100 Gm. of paired adrenal glands based on the relative absorbances of the 8-mcg./ml. standard and test samples and on the weights of paired adrenal glands. The computer program places ascorbic acid values in their proper dosage groups and provides for statistical calculations for the assay as directed by U.S.P. XVII.

SUMMARY

An automated procedure is described for the determination of ascorbic acid in rat adrenal gland homogenates in the bioassay of corticotropin. The procedure, based on that of the U.S.P. XVII, permits the analysis of 60 samples/hr. with a coefficient of variation of approximately 1%. Ascorbic acid was recovered satisfactorily from standard samples spiked with varying amounts of adrenal gland homogenate. Corticotropin bioassays using ascorbic acid results obtained with manual and automated procedures compare favorably.

The automated ascorbic acid procedure and the computer program should have application in the Parlow ovarian ascorbic acid depletion bioassay of luteinizing hormone, chorionic gonadotropin, and pregnant mare's serum (2).

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) Parlow, A. F., *Federation Proc.*, **17**, sect. 1587(1958).

Notes

Estimation of Volume of Distribution and Half-Life of a Compound After Rapid Intravenous Injection

By JOHN G. WAGNER and JACK I. NORTHAM

It is common practice to plot the plasma or serum concentrations of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant ($\hat{\beta}$) for loss of compound from the body or half-life ($0.693/\hat{\beta}$) from the slope of the terminal linear segment, extrapolate the linear segment to time zero, and divide the intercept into the dose to obtain the apparent volume of distribution ($\hat{V}_d$). Under certain conditions the estimates obtained by this procedure may be valid. However, this report shows that if the model which applies is a two compartment open system, then the $\hat{V}_d$ estimated by this method is always an overestimate of the true total volume ($V_1 + V_2$), and the error depends upon the relative values of V_1/V_2 and K_1/K_2 where K_1 is the first-order rate constant for distribution and K_2 is the first-order rate constant for loss from the inner compartment. The half-life estimates ($0.693/\hat{\beta}$) will always be greater than the true half-life ($0.693/K_2$), and $\hat{\beta}$ as an estimator of K_2 also depends on the ratios V_1/V_2 and K_1/K_2.

IT IS COMMON practice[1] (1–5) to plot the plasma or serum concentration of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant for loss of compound from the body ($\hat{\beta}$) or the half-life ($0.693/\hat{\beta}$) from the slope ($\hat{\beta}/2.303$) of the terminal linear segment; and extrapolate the linear segment to time zero and divide the intercept ($\hat{B}_1$) into the dose (D) to obtain the apparent volume of distribution, pool size, or "space" ($\hat{V}_d$). If the model which applies to the particular system involves a single compartment and the initial nonlinearity of the semilogarithmic plot is assumed to be due to mixing, then the estimates $\hat{\beta}$ and $\hat{V}_d$ would be very close to those expected on the basis of the appropriate mathematical expression.

Nelson (6), however, pointed out that the error in the estimated volume of distribution may be substantial if there are two compartments with volumes V_1 and V_2 and the volume of the outer compartment (V_2) is large, and rate of attainment of equilibrium is slow. This report will show that if the model which applies is a two compartment open system, then $\hat{V}_d$ always overestimates the true total volume

Received November 8, 1966, from the Research Division, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication December 22, 1966.

[1] The practice is so common that the authors considered it unnecessary to provide an extensive bibliography. However, *References 1–5* are a representative sample.

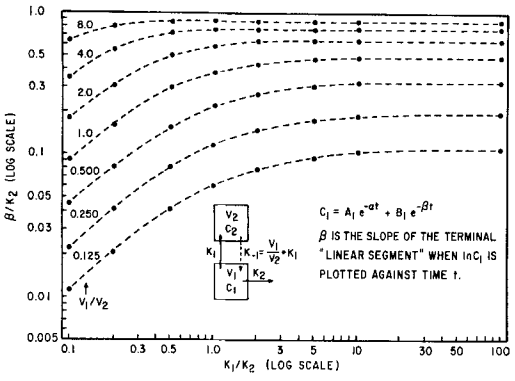


Fig. 1—Plot of the ratio β/K_2 versus *the ratio* K_1/K_2 *on a log-log scale.*

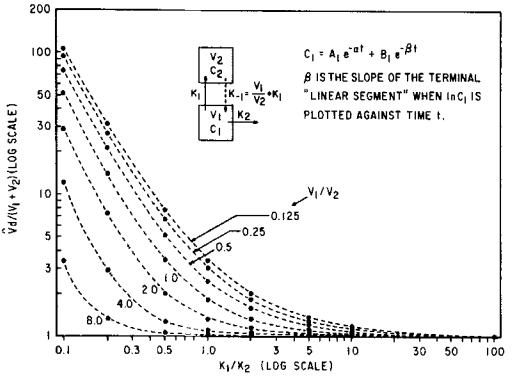


Fig. 2—Plot of the ratio $\hat{V}_d/(V_1 + V_2)$ versus *the ratio* K_1/K_2 *on a log-log scale.*

$(V_1 + V_2)$, and the error depends upon the relative values of V_1/V_2 and K_1/K_2, where K_1 is the first-order rate constant for distribution and K_2 is the first-order rate constant for loss from the inner compartment. That is, the errors involved in $\hat{V}_d$ as an estimator of $V_1 + V_2$ and $\hat{\beta}$ as estimator of K_2 depend on the ratios V_1/V_2 and K_1/K_2. If a compound is widely distributed in body water, then physiologically (7, 8) one might expect V_2 to be considerably greater than V_1; hence, $V_1/V_2 < 1$ and $\hat{V}_d$ will be a poor overestimator of $V_1 + V_2$ and $\hat{\beta}$ will be a poor underestimator of K_2.

The equations appropriate to the two compartment open system in which drug is lost only from the inner compartment are given under *Appendix* where all symbols are defined. Based on Eqs. 13 through 16 of the *Appendix*, a digital computer program was written which allowed print-out of the values β/K_2, α/K, and $\hat{V}_d/(V_1 + V_2)$ when the input data were V_1/V_2 and K_1/K_2. Values of β/K_2, α/K_1, and $\hat{V}_d/(V_1 + V_2)$ were obtained corresponding to $K_1/K_2 = 0.1$, 0.5, 1, 2, 5, 10, and 100 and $V_1/V_2 = 0.125$, 0.25, 0.5, 1, 2, 4, and 8. These data allowed preparation of Figs. 1 and 2.

Figure 1 is a plot of the ratio β/K_2 *versus* the ratio K_1/K_2 on a log-log scale. The figure illustrates that over the range considered β/K_2 ranges from 0.0113 when $K_1/K_2 = 0.1$ and $V_1/V_2 = 0.125$ to 0.889 when $K_1/K_2 = 100$ and $V_1/V_2 = 8$. As $V_1/V_2 \to \infty$, $\beta/K_2 \to 1$. Hence, half-life estimates $(0.693/\hat{\beta})$ will always be greater than the true half-life $(0.693/K_2)$.

Figure 2 is a plot of the ratio $\hat{V}_d/(V_1 + V_2)$ *versus* the ratio K_1/K_2 on a log-log scale. The figure illustrates that when $K_1/K_2 < 5$ and $V_1/V_2 < 1$ relatively large errors are made in the estimated volume. When $K_1/K_2 \gtrsim 100$, then V_d is within 1% of $(V_1 + V_2)$, provided V_1/V_2 is not extremely small.

When employing experimental data, estimates $\hat{B}_1$ and $\hat{\beta}$ of the intercept B_1 and the slope β, respectively, are usually obtained by the method of least squares using the terminal ln C_1 and time t values.[2] Table I gives an indication of the relatively small errors involved in this procedure in the absence of experimental error. In the case of some experimental data, however, the number of available points and the accuracy and precision of the assay would influence the estimates.

Additional observations about the two compartment open system may be of interest. During the interval $t < t'$, $C_1 > C_2$ but during the interval $t' < t < \infty$, $C_2 > C_1$ where $t' = \ln(\beta/\alpha)/(\beta - \alpha)$. When $10 < K_1/K_2 < 100$ and $t > t'$, C_2 is always only slightly greater than C_1 and for all practical purposes $C_1 \simeq C_2$. For a given ratio K_1/K_2 the smaller the ratio V_1/V_2 the more delayed is t'; for example, with $K_1/K_2 = 1$, $t' = 7.45$, 9.84, 11.4, and 13.4 hr. when $V_1/V_2 = 1$, 0.5, 0.33, and 0.2, respectively. For given values of V_1 and V_2 the smaller the ratio K_1/K_2, the more delayed is the time at which a semilogarithmic plot of C_1 *versus* time apparently becomes linear. Conversely, when $K_1/K_2 \geq 100$ a semilogarithmic plot of C_1 *versus* time becomes linear within an hour or less postadministration if the true half-life $(0.693/K_2)$ is 6 hr. or less.

It should be noted that if a compound is administered by any other route than intravenously (*e.g.*, orally, intramuscularly, *etc.*) and the terminal linear segment of the semilogarithmic plot of the serum or plasma concentration is extrapolated to zero time, the quotient obtained by dividing the intercept into the dose is not a "volume of distribution." The number obtained in such a case is a complex function of many variables among which are the following: (*a*) the true volume of distribution, (*b*) *all* the rate constants involved in the model which applies to the particular set of data, (*c*) the fraction of the dose absorbed, and (*d*) the lag time between time of administration and the time when absorption begins.

APPENDIX

The Two Compartment Open System with the Dose (D) Introduced into Compartment 1 at Zero Time.—Let V_1 be the volume and C_1 be the concentration of unchanged drug at time t in the inner compartment or pool and V_2 be the volume and C_2 be the concentration of unchanged drug at time t in the outer compartment or pool. Let K_1 be a first-order rate constant representing the instantaneous fraction of drug in compartment 1 being transferred to compartment 2 and K_2 be a first-order rate constant representing the instantaneous fraction of drug in compartment 1 which is being lost

[2] This approximation is valid since

$$-\beta = \lim_{t \to \infty} \left\{ \frac{d \ln C_1(t)}{dt} \right\}$$

TABLE I—INDICATION OF THE SMALL ERRORS INVOLVED WHEN THE INTERCEPT B_1 AND THE SLOPE β ARE ESTIMATED BY THE METHOD OF LEAST SQUARES USING THE TERMINAL ln C_1, t VALUES[a]

K_1/K_2	Time Range, hr.	B_1	$\hat{B}_1$[b]	β	$\hat{\beta}$	$\hat{V}_d$ $100/B_1$	$\hat{V}_d$ $100/\hat{B}_1$
1	17–24	.00553	.00609	.0441	.0482	18,100	16,400
2	13–24	.00758	.00760	.0506	.0508	13,200	13,150
5	11–24	.00901	.00899	.0549	.0548	11,100	11,120
10	9–24	.00950	.00950	.0563	.0563	10,530	10,530
100	1–24	.00995	.00995	.0576	.0576	10,050	10,050

[a] For all examples shown $V_1 = V_2 = 5{,}000$, $K_2 = 0.1155$, and a dose of 100 units was placed in compartment 1 at time zero. [b] $\hat{B}_1$ is the antilogarithm of the estimated intercept and $\hat{\beta}$ is the estimated slope obtained by the method of least squares for the regression of ln C_1 on time where $C_1 = A_1e^{-\alpha t} + B_1e^{-\beta t}$, and t was taken at 0.5-hr. intervals over the time range indicated.

from compartment 1. For convenience refer to the compartmental diagram shown in Fig. 1. Let D be the dose of drug introduced into the inner compartment at time zero.

The appropriate differential equations, which incorporate Fick's law, are:

$$V_1 \frac{dC_1}{dt} = -V_1K_1 (C_1 - C_2) - V_1K_2C_1 \quad \text{(Eq. 1)}$$

and

$$V_2 \frac{dC_2}{dt} = V_1K_1 (C_1 - C_2) \quad \text{(Eq. 2)}$$

The time-concentration curves resulting from the solution are:

$$C_1 = A_1e^{-\alpha t} + B_1e^{-\beta t} \quad \text{where } \beta < \alpha \quad \text{(Eq. 3)}$$

and

$$C_2 = A_2e^{-\alpha t} + B_2e^{-\beta t} \quad \text{(Eq. 4)}$$

A semilogarithmic (base e) plot of C_1 *versus* t is terminally linear with slope-β and intercept ln B_1 (by extrapolation). The residuals give a linear semilogarithmic plot with slope-α and intercept ln A_1. It follows that $(C_1)_{t=0} = A_1 + B_1$.

In Eq. 3,

$$A_1 = \frac{(K_1 + K_2 - \beta)(C_1)_{t=0}}{\alpha - \beta} \quad \text{(Eq. 5)}$$

and

$$B_1 = \frac{(K_1 + K_2 - \alpha)(C_1)_{t=0}}{\beta - \alpha} \quad \text{(Eq. 6)}$$

In Eq. 4,

$$A_2,B_2 = \pm \frac{K_1V_1(C_1)_{t=0}}{(\beta - \alpha)V_2} \quad \text{(Eq. 7)}$$

By appropriate algebraic manipulation we find

$$K_1 = \frac{A_1\alpha + B_1\beta}{A_1 + B_1} \quad \text{(Eq. 8)}$$

$$K_2 = \frac{\alpha\beta(A_1 + B_1)}{A_1\beta + B_1\alpha} \quad \text{(Eq. 9)}$$

$$V_1 = \frac{D}{A_1 + B_1} \quad \text{(Eq. 10)}$$

$$V_2 = V_1 \cdot \frac{K_1K_2}{\alpha \cdot \beta} \quad \text{(Eq. 11)}$$

$$C_1 = C_2 \text{ and } C_2 = \text{a maximum at } t' = \frac{\ln \beta - \ln \alpha}{\beta - \alpha} \quad \text{(Eq. 12)}$$

Equations 1 through 11 have been reported in similar or modified form by Teorell (9), Gaudino (10), Dominguez (11), Tait *et al.* (12), and Cornfield *et al.* (13).

In preparing Figs. 1 and 2 the following equations were employed:

$$\beta/K_2 = \left[1 + \frac{K}{f} - \sqrt{\left(1 - \frac{K}{f}\right)^2 + 4K}\right]/2 \quad \text{(Eq. 13)}$$

where $K = K_1/K_2$, $V = V_1/V_2$, and $f = V_2/(V_1 + V_2) = \frac{1}{V+1}$.

$$\frac{\hat{V}_d}{V_1 + V_2} = (1 - f) \cdot \left[\frac{\alpha/K_2 - \beta/K_2}{\alpha/K_2 - K - 1}\right] \quad \text{(Eq. 14)}$$

The latter is conveniently calculated since, when getting β/K_2, one can easily calculate:

$$\alpha/K_2 = \left[1 + \frac{K}{f} + \sqrt{\left(1 - \frac{K}{f}\right)^2 + 4K}\right]/2 \quad \text{(Eq. 15)}$$

In addition one can calculate α/K_1 as follows:

$$\alpha/K_1 = (V_1/V_2)/(\beta/K_2) = V/(\beta/K_2) \quad \text{(Eq. 16)}$$

REFERENCES

(1) Dominguez, R., Corcoran, A. C., and Page, I. H., *J. Lab. Clin. Med.*, **32**, 1192(1947).
(2) Soberman, R., Brodie, B. B., Levy, B. B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, **179**, 31(1949).
(3) Peterson, R. E., "Recent Progress in Hormone Research," vol. XV, Pincus, G., ed., Academic Press Inc., New York, N. Y., 1959, pp. 231–249.
(4) Czaczkes, J. W., and Kleeman, C. R., *J. Clin. Invest.*, **43**, 1649(1964).
(5) Silver, S., *New Engl. J. Med.*, **272**, 515(1965).
(6) Nelson, E., *Antibiot. Chemotherapia*, **12**, 29(1964).
(7) Krüger-Thiemer, E., *Arzneimittel-Forsch.*, **14**, 1332 (1964).
(8) DeLand, E. C., and Bradham, G. B., *Ann. N. Y. Acad. Sci.*, **128**, 795 (1966).
(9) Teorell, T., *Arch. Intern. Pharmacodyn.*, **57**, 226 (1937).
(10) Gaudino, M., *J. Biol. Chem.*, **70**, 672(1949).
(11) Dominguez, R., "Kinetics of Elimination, Absorption and Volume of Distribution in the Organism," Medical Physics II, Yearbook Publishers, Inc., Chicago, Ill., 1950, pp. 476–489.
(12) Tait, J. S., Tait, S. A. S., Little, B., and Laumas, K. R., *J. Clin. Invest.*, **40**, 72(1961).
(13) Cornfield, J., Steinfeld, J., and Greenhouse, S. W., *Biometrics*, **16**, 212(1960).

Isolation and Identification of Benzyl Ether in a Sample of Basil Oil

By DENNIS POGANY, CHARLES L. BELL, and ERNST R. KIRCH

A gas chromatographic fraction of a sample of basil oil was found to contain benzyl ether, a compound heretofore not reported to be present in this oil. Identification was established by comparison of various spectra of known samples of the ether. It was further observed that the published mass spectral data for benzyl ether was in error.

IN THE COURSE of an investigation dealing in part with the analysis of an essential oil obtained from basil, it became necessary to establish the reproducibility of the method of assay. For this purpose a commercial[1] sample of oil of basil was purchased and subsequently analyzed by gas chromatographic and spectroscopic methods. It was observed that among other known components (1, 2) an ether (benzyl ether) could be isolated from this particular sample of oil.

EXPERIMENTAL

The oil of basil was subjected to gas chromatographic separation and the fractions were examined spectroscopically.

Gas Chromatography—Instrument: Aerograph Autoprep, model A-700, gas chromatograph equipped with a thermal conductivity detector. Conditions: a 20 ft. × 3/8 in. o.d. aluminum column packed with 30% SE-30 on Chromosorb W (mesh 45/60) was used. The flow rate of the carrier gas, helium, was 200 ml./min. The oven temperature was programmed (nonlinear) between 100 and 250°. Sample: an 0.5-ml. portion of the oil was injected for each of five consecutive runs.

Nuclear Magnetic Resonance Spectroscopy—Instrument: Varian model A-60. Conditions: an approximately 50% solution of the fraction and a known sample of benzyl ether were prepared using $CDCl_3$ + 1% TMS[2] and the spectrum was taken in a glass microcell at probe temperature.

Mass Spectroscopy—Instrument: Hitachi-Perkin-Elmer model RMU-6D. Conditions: the sample was introduced through the reservoir and a heated glass inlet kept at a temperature of 200° and at a sample pressure of 2×10^{-7} mm. The ionizing electron beam was set at 75 ev.

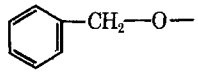
Infrared Spectroscopy—Instrument: Perkin-Elmer grating infrared spectrophotometer model 337. Conditions: the sample was introduced as a film between two sodium chloride plates.

RESULTS AND DISCUSSION

Fractions of oil of basil isolated by preparative gas chromatography were examined by mass spectroscopy and the *m/e* values obtained were those expected for terpenoids or sesquiterpenoids. Some of the compounds identified so far include the following: 1,8-cineole, linalool, anethole, methyl chavicol, *o*- or *p*-methoxy cinnamaldehyde, and methyl eugenol. The sesquiterpene fractions, all with an *m/e* of 204, have not as yet been completely identified.

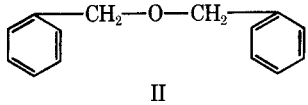
Upon examination of the last fraction of the oil which eluted approximately 1.5 hr. after injection in the gas chromatograph, it was found that its apparent parent ion in the mass spectrum was at *m/e* 107. This was unexpected for two reasons. First, the time of elution in the nonpolar liquid phase in general followed the order of increasing molecular weight, and second, this odd-numbered molecular weight indicated the presence of an odd number of nitrogens (3) in the molecule. Since the chemical analysis of this particular fraction did not show the presence of nitrogen, it was assumed that the apparent molecular ion, 107, was actually a fragment of a larger parent ion. Further examination of this fraction by nuclear magnetic resonance showed a very simple spectrum with only two sharp absorption signals, one at 4.54 δ and one at 7.33 δ downfield from the tetramethylsilane signal. Their integrated areas were in the ratio of 2:5, respectively. The signal at 7.33 δ indicated an unconjugated phenyl group resonance. The position of the less intensive signal at 4.54 δ is at a somewhat lower field than expected for a simple R—CH_2—O— group. This indicates that the methylene group is adjacent not only to an oxygen but also to another deshielding group.

From these data it was assumed that the compound has the structural element shown in I.



I

The absence of any other signal in the NMR spectrum and the lack of exchangeable protons as well as the mass spectral data lead to the postulation that the molecule is a symmetrical ether with the formula shown in II.



II

An infrared spectrum of the fraction was taken and compared with that of benzyl ether reported in the Sadtler standard spectra. These spectra were found to be identical (4). It was concluded, therefore, that the fraction as isolated was indeed benzyl ether.

As further verification, benzyl ether was not only prepared in our laboratory (5) but also purchased from the Eastman Kodak Co. Both samples were purified by gas chromatography and examined spectroscopically. The mass, infrared, and NMR

Received July 25, 1966, from the Department of Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680

Accepted for publication November 2, 1966.

[1] This sample was characterized by the supplier, Magnus Mabee and Reynard, Inc., to have been "received directly from Comores Islands."

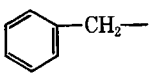
[2] Marketed as Silanar C by Merck Sharp & Dohme of Canada.

TABLE I—SIX MOST ABUNDANT PEAKS IN THE SPECTRA OF BENZYL ETHER IN DECREASING ORDER OF ABUNDANCE

Peak No.	Present Findings m/e	Present Findings % Rel. Abundance	Published Findings (6) m/e	Published Findings (6) % Rel. Abundance
1	92	100	77	100
2	91	83	105	75
3	79	20	106	74
4	65	18	51	57
5	77	17	50	33
6	107	15	78	22

spectra were found to be identical with those of the fraction obtained from the oil.

Six major peaks are shown in the mass spectrum of benzyl ether as published in a compilation of mass spectral data (6). In the comparison of our observations with these published data the great discrepancy shown in Table I was found. The spectrum as reported (6) is obviously in error since compounds having a moiety (III) will invariably give rise to a *m/e* 91 fragment (tropylium ion) in substantial abundance. It has been reported that benzyl ether slowly decomposes to benzaldehyde (7). A comparison of the six major peaks for benzaldehyde with those in the spectrum published for benzyl ether showed that they were coincidental and almost identical in relative abundances.



III

The correct mass spectrum of benzyl ether is shown in Fig. 1. The lack of the parent ion (*m/e* 198) is not entirely unexpected since it is a common occurrence with higher molecular weight ethers (8). In this case the parent ion is apparently much less stable than either the benzyloxy or the tropylium ions.

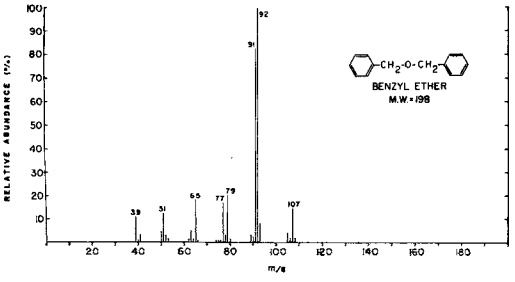
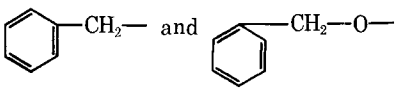


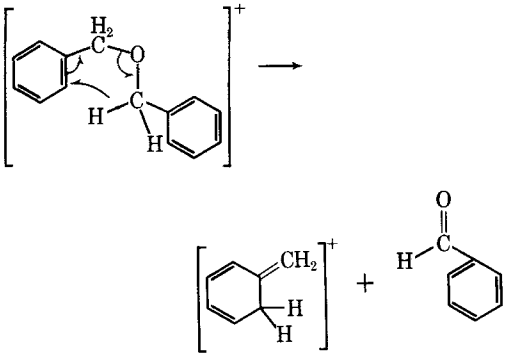
Fig. 1—Mass spectrum of benzyl ether.

The cleavage of the carbon–oxygen bond occurs readily, giving rise to IV and V with the positive charge being carried by either fragment (*m/e* 91 and *m/e* 107); however, the very stable tropylium ion (*m/e* 91) is favored and has the greater abundance.



IV V

The *m/e* 92 fragment is the base peak and somewhat unexpected; however, this occurrence has been reported for compounds with the general structure of AR—CH_2—O—RH (9). This can occur by the postulated rearrangement (10) shown in Scheme I.



m/e 92

Scheme I

Based on the comparison of the spectral data of the fraction isolated from oil of basil with those of the known samples of benzyl ether, the presence of the latter has been definitely established in a sample of oil of basil, in a concentration of about 4%. Another sample of oil of basil was obtained in this laboratory by steam distillation of *Ocimum basilicum* L., var. *glabratum* Bentham, sub-var. *vulgare* Alefeld, forme *thyrsiflorum* L.[3] The benzyl ether could not be detected in this particular oil. Studies reported on the chemical composition of *O. basilicum* as reviewed by the authors have not indicated the presence of this ether in the oil.

Benzyl ether was detected by MacLeod *et al.* as a component not natural to lemon in a commercial sample purported to be cold-pressed lemon oil (11). These investigations suggest that the ether had been added to the sample of lemon oil.

REFERENCES

(1) Guenther, E., "The Essential Oils," D. Van Nostrand, Co., Inc., New York, N. Y., 1949, vol. III, pp. 399–415.
(2) Hoerhammer, L., Hamidi, A. E., and Richter, G., *J. Pharm. Sci.*, **53**, 1033(1964).
(3) Bieman, K., "Mass Spectrometry, Organic Chemical Applications," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 54.
(4) The Sadtler Standard Spectra, No. 13,509, Sadtler Research Laboratories, Philadelphia, Pa.
(5) Senderens, J. B., *Compt. Rend.*, **188**, 1073(1929).
(6) "Index of Mass Spectra," American Society for Testing and Materials, Philadelphia, Pa., p. 37.
(7) "Merck Index of Chemicals and Drugs," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 139.
(8) McLafferty, F. W., *Anal. Chem.*, **29**, 1783(1957).
(9) McLafferty, F. W., "Mass Spectral Correlations," Advances in Chemistry Series No. 40, American Chemical Society, Washington, D. C., 1963, p. 39.
(10) Bieman, K., *op. cit.*, p. 123.
(11) MacLeod, W. D., Jr., Lundin, R. E., and Buigues, N. M., *J. Food Sci.*, **29**, 802(1964).

[3] The authors acknowledge with appreciation the work of Mr. Floyd Swink, Taxonomist, Morton Arboretum, Lisle, Ill., in classifying the plant.

Modification of Iron Toxicity and/or Absorption in Mice

By THEODORE H. EICKHOLT* and WALLACE F. WHITE

A study was made of the toxicity of iron administered orally in mice under various conditions. In a quantitative 6-group technique α-aminoacetic acid effected a statistically significant increase in mortality rate of iron. Conversely, sodium succinate reduced the mortality rate of iron with statistical significance. Comparisons of slopes of dosage response curves of mortality rates of iron administered orally with and without additives showed very close parallelism.

IRON COMPOUNDS have received considerable attention regarding methods of improving absorption and decreasing irritation. Dietary factors have been found to have a great influence upon the absorption of iron. Gastric acidity, ascorbic acid, SH groups of proteins, and other reductants in foods help convert ferric iron to the apparently more readily absorbable ferrous form.

Many different iron compounds alone or with other additive compounds have been employed in the attempt to increase iron absorption and decrease the usual side effects of orally administered iron. Iron choline citrate chelate (1, 2), ferrous fumarate (3–5), ferrous aminoacetosulfate (6–10), an iron carbohydrate complex (11), and combinations of iron with polyol monolaurate (12), polysorbate 20,[1] polysorbate 80[2] (13, 14), magnesium aluminum hydroxide (15), and D-sorbitol (16–18) are several common examples.

It was, therefore, the purpose of this study to demonstrate any influence which glycine and sodium succinate had on the mortality rate of orally administered iron, a greater mortality resulting from increased toxicity or absorption and a reduced mortality resulting from decreased toxicity or absorption at a given dose of iron.

EXPERIMENTAL

In the initial stages of this toxicity study it was observed that unfasted animals gave erratic results when fed iron compounds orally (19). Likewise, it was found that mice fasted 22 ± 2 hr. were much more uniformly sensitive to iron toxicity than were unfasted mice. Also, although mice fasted under conditions preventing their exposure to feces and sawdust have been shown to increase their susceptibility to iron toxicity (19), the Fairfield Webster female mice in this study were all fasted 22 ± 2 hr. with water *ad libitum*, and exposed to both of these elements to prevent the complication of cannibalism. The animals weighed approximately 25 Gm. The room temperature of 24° was kept as constant as possible. All medication was given by the oral route *via* stomach intubation. The doses of all compounds were calculated on a milligram per kilogram basis and the number of animals dead after 24 hr. was observed; the remaining animals were observed for a period of 30 days for any possible latent effects.

To limit variability, the following experimental design was employed in which 60 mice were divided at random into the following six groups for concurrent testing.

Group A—Controls; received 0.3 ml. distilled water.

Group B—Additive; received an additive compound without iron (at one or more subsequent dose levels).

Group C—Iron standard; a solution containing 125 mg. of ferrous sulfate (25 mg. of iron) per ml.,[3] referred to hereafter as ferrous sulfate solution, dose 160 mg. Fe/Kg.

Group D—Iron standard; ferrous sulfate solution, 240 mg. Fe/Kg.

Group E—Ferrous sulfate solution, 240 mg. Fe/Kg. and additive at dosage level of group *B*.

Group F—Ferrous sulfate solution, 240 mg. Fe/Kg., and additive at dosage level of group *B*.

All six groups received the solutions within a 1-hr. period. The experiment was repeated to give totals of 20 mice per group. The iron doses were chosen so as to provide a range for observation of possible protection as well as potentiation of the iron toxicity by the additive compounds.

The additives were employed as 20% solutions. Two doses of glycine were employed in the attempt to determine the effect of the additive on the toxicity of iron.

The data from the six-group design were analyzed to determine statistically significant differences between the iron administration alone and with the additive. The basis for the estimation of toxicity was a quantal response in which a probit analysis was employed [Finney (20), Burns (21), Fisher and Yates (22)]. The analyses, per cent mortality values, ρ values, and confidence limits were calculated on each individual group of ten animals as well as on the data from the combined groups giving 20 animals per group so as to observe any day-to-day variation, if present.

For confirmation of the analyses of the additives found to be significant, five 27-Gm. mice were fasted 22 ± 2 hr. with water *ad libitum*, and given orally 3.2 mg./Gm. α-aminoacetic acid and 48 mcg. Fe/Gm. Another group of five 27-Gm. mice were similarly fasted and given orally 3.2 mg./Gm. sodium succinate and 48 mcg. Fe/Gm. Both groups were allowed 1 hr. before sacrificing and subsequent separation of the gastrointestinal tract from the remainder of the carcass. The five carcasses and five gastrointestinal tracts of each group were combined and digested with sulfuric and nitric acids until only a clear solution in sulfuric acid with

Received October 13, 1966, from the Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455
Accepted for publication December 20, 1966.
Abstracted in part from a thesis submitted by Theodore H. Eickholt to the Graduate School, University of Minnesota, Minneapolis, in partial fulfillment of Doctor of Philosophy degree requirements.
The authors express their appreciation to Mead Johnson and Co. for supplying the Fer-In-Sol which was used as the iron standard in this study.

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[1] Marketed as Tween 20 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] Marketed as Fer-In-Sol by Mead Johnson and Co., Evansville, Ind.

a small amount of white precipitate remained. These four resulting solutions were cooled, made up to 100 ml. in a volumetric flask, and used as earlier described under the modified Fisher method for the determination of iron used in this laboratory (23). The colorimetric analysis was made after the iron was liberated and oxidized with sulfuric acid and potassium persulfate and treated with potassium thiocyanate.

Calculations of slopes of dosage response curves for iron administered orally with and without additives were performed to determine their parallelism.

RESULTS AND DISCUSSION

The data obtained using the six-group design are presented in Table I.

The results of the statistical analyses of the separate and combined data are summarized as follows. Glycine, 3.2 mg./Gm., potentiated the toxicity of orally administered iron and sodium succinate, 3.2 mg./Gm., decreased the toxicity of orally administered iron. Both of these results were shown to represent statistically significant differences.

The results of the chemical determinations of iron content in mice in which the additive agents produced statistical results are given in Table II.

The dose of 48 mcg. Fe/Gm. mouse for a 27-Gm. animal would amount to 1.296 mg. iron and added to the previously published average iron content per mouse of 1.457 mg. would be a calculated total of 2.753 mg. or 102 mcg. Fe/Gm., compared to the determined average figure of 2.270 mg. and 84 mcg. Fe/Gm. An average of 483 mcg. Fe or 18 mcg. Fe/Gm. mouse was unaccounted for, as noted previously, probably due to the inherent properties of the colorimetric method itself.

However, the averages of iron content in the respective glycine and succinate gut, 840 and 1000 mcg., represent iron absorption of 456 and 296 mg., respectively, on the basis of the calculated 1.296 mg. of iron administered orally. This serves to support the statistical significance arrived at in the statistical calculations, *i.e.*, glycine potentiates while sodium succinate inhibits iron absorption.

In these experiments, calculations of slopes of dosage response curves in which mortality rates of iron administered orally without additives were obtained. The mean slope was 7.038, compared with a mean slope of 7.577 for the average of groups in which iron and additives were administered together. This very close parallelism between slopes would appear to indicate that death was due to iron toxicity and that the effect of additives was merely to modify absorption.

The 60 mice in the control groups receiving oral intubations of distilled water were without deleterious effects and were alive after the 30-day observational period, indicating that the technique itself was not a contributing factor in this toxicity study.

SUMMARY AND CONCLUSIONS

A study was made of the absorption of iron administered orally to mice under various conditions.

Of the additive compounds tested using the quantitative 6-group method, glycine showed a statistically significant increase in the mortality of iron. Likewise, sodium succinate gave a statistically significant reduction in the mortality of iron. These significant results were verified by oral administration of the compounds with iron, subsequent digestion, and chemical determination of the iron content by the modified Fisher method reported earlier.

Calculation of slopes of dosage response curves, in which mortality rates of iron administered orally with and without additives were compared, showed close parallelism.

The technique of oral intubation was demmonstrated to be adequate for the study of absorption and safe, as exemplified by the lack of deleterious effects on the control groups of animals receiving only distilled water.

TABLE I—MODIFYING EFFECT OF ADDITIVES ON THE TOXICITY OF IRON ORALLY IN MICE USING THE STATISTICALLY DESIGNED SIX GROUP TECHNIQUE (PER CENT MORTALITY IN 24 hr.)[a]

Additive	Group B	Group C	Group E	Group D	Group F
		32 mcg. Fe/Gm.		48 mcg. Fe/Gm.	
	Additive Alone	Alone	Plus Additive	Alone	Plus Additive
Glycine					
1.6 mg./Gm.	0	10	30	50	60
3.2 mg./Gm.	0	5	15	20	55
Sodium succinate					
3.2 mg./Gm.	0	5	0	40	10

[a] Sixty mice in group *A* were given distilled water alone and all lived; therefore, group *A* is not included.

TABLE II—AVERAGES FROM MICE IN GROUPS OF FIVE OF IRON PRESENT PER MOUSE CARCASS AND GUT, SEPARATE AND COMBINED

Group	Av. Iron Content in Carcass, mg.	Av. Iron Content in Gut, mcg.	Av. Iron Content/ Mouse, mg.	Av. Iron Content/ Gm. Mouse, mcg.
3.2 mg./Gm. glycine and 48 mcg. Fe/Gm.	1.480	840	2.320	86
3.2 mg./Gm. Na succinate and 48 mcg. Fe/Gm.	1.220	1000	2.220	82

REFERENCES

(1) "New and Nonofficial Drugs," *J. Am. Med. Assoc.*, **171**, 891(1959).
(2) Franklin, M., Rohse, W. G., de la Huerga, J., and Kemp, C. R., *J. Am. Med. Assoc.*, **166** (14), 1685(1958).
(3) "New and Nonofficial Drugs," *J. Am. Med. Assoc.*, **171**, 1104(1959).
(4) Swan, H. T., *Brit. Med. J.*, **11**, 782(1959).
(5) Shapleigh, J. B., and Montgomery, A., *Am. Pract.*, **10**, 461(1959).
(6) Rummel, W., and Candon, B., *Intern. Rec. Med.*, **169**, 12(1956).
(7) Todd, R. M., *Practitioner*, **181**, 736(1958).
(8) Jennison, R. F., *ibid.*, **181**, 731(1958).
(9) Feldman, H., and Clancy, J., *Geriatrics*, **13**, 517(1958).
(10) Stone, M. L., Donnenfeld, A. M., Pomeranze, J., and Salerno, L. J., *Postgrad. Med.*, **25**, 761(1959).
(11) Weaver, L. C., Gardier, R. W., Robinson, V. B., and Bunde, C. A., *Am. J. Med. Sci.*, **241**, 295(1961).
(12) Ausman, D. C., *J. Am. Geriat. Soc.*, **7**, 268(1959).
(13) Wissler, R. W., Bethard, W. F., Barker, P., and Mori, H. D., *Proc. Soc. Exptl. Biol. Med.*, **86**, 1170(1954).
(14) Mori, H. D., Barker, P. A., Juias, D. S., and Wissler, R. W., *J. Clin. Invest.*, **6**, 421(1957).

(15) Price, A. H., Erf, L., and Bierly, J., *J. Am. Med. Assoc.*, **167**, 1612(1958).
(16) Gray, W. D., *Am. Pract.*, **10**, 660(1959).
(17) Herndon, J. F., Rice, E. G., Tucker, R. G., Van Loom, E. J., and Greenberg, S. M., *J. Nutr.*, **64**, 615(1958).
(18) Migden, J., *J. Am. Geriat. Soc.*, **7**, 928(1959).
(19) Eickholt, T. H., and White, W. F., *J. Pharm. Sci.*, **54**, 1211(1965).
(20) Finney, D. J., "Statistical Method in Biological Assay," Charles Griffin and Co. Ltd., London, Hafner Publishing Co., New York, N. Y., 1952.
(21) Burns, J. H., "Biological Standardization," Oxford University Press, London, England, 1937.
(22) Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Hafner Publishing Co., Inc., New York, N. Y., 1957.
(23) Eickholt, T. H., and White, W. F., *J. Pharm. Sci.* **53**, 1418(1964).

Vitamin C and Choline Content of *Chlorella vulgaris* and *C. pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

The choline content of *Chlorella vulgaris* and of *C. pyrenoidosa* (expressed as micrograms per milligram dry cell weight) remained relatively constant during a 3-week growth period. However, the concentration in the latter species exceeded slightly that of the former at all harvest times. In both species, after reaching a peak early in the culture period (5–7 days), the concentration of ascorbic acid decreased continuously. The total yield of both vitamins (micrograms per milliliter of culture) increased continuously because of the increment in cell mass.

THIS NOTE records data concerning yields of vitamin C (total ascorbic acid) and of choline from two species of unicellular green algae and is part of a continuing study of production of vitamins and accessory growth factors by *Chlorella vulgaris* and *C. pyrenoidosa* (1–5).

METHODS

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* employed, the method of maintaining stock cultures, the composition and inoculation of stock and experimental culture solutions, and the details of environmental conditions provided and of harvesting experimental cultures have been described previously (1). In summary, an inorganic medium consisting of minerals and micro-elements was inoculated with the algae and was continuously aerated with a CO_2–air mixture. Continuous illumination (600 f.c.) was provided from a Mazda source and the temperature was maintained at 20.5 ± 0.5°. Cells were harvested at intervals during the 3-week culture period and vitamin C and choline content were determined.

Assays—*Vitamin C Activity*—Since ascorbic and dehydroascorbic acids exhibit equal antiscorbutic activity, the method of Roe and Osterling (6) was applicable and was employed to determine "total ascorbic acid." This entailed extraction of cells with a metaphosphoric acid mixture and treatment with a decolorizing agent[1] which oxidized ascorbic to dehydroascorbic acid and clarified the solution. The oxidized form was then coupled with 2,4-dinitrophenylhydrazine and the resulting derivative, when treated with H_2SO_4, produced a red color which could be quantitated photometrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was assayed in duplicate for total ascorbic acid. The standard deviations of the assays were within ±5% of the mean; most were within ±2%.

Choline—Cells were autoclaved in 3% H_2SO_4 at 15 lb. pressure for 2 hr. and then were neutralized with BaOH, following the method of Horowitz and Beadle (7). After filtration, the extract was passed through a Permutit column to separate choline from methionine and to eliminate other substances which might interfere with the assay.

Choline content of the extract was determined using *Neurospora crassa* ATCC 9277 as the test organism in bacto-choline assay medium (8). Growth response of the organism was determined gravimetrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was then assayed in duplicate. For all assays the standard deviation was within the range ±10% of the mean, generally falling in the range ±5% or less.

RESULTS

The maximum ascorbic acid content detected in the cells of both organisms was about 1.1 to 1.2 mcg./mg. of cell dry weight. This peak occurred in the early period of growth (5 to 7 days) and then the concentration declined continuously throughout the remainder of the 21-day culture period (Table I, A). Choline appeared more constant in concentration. The apparent trend in the average values for choline in *C. vulgaris* is not supported by the data from individual runs. Variations were random. Neither compound was detectable in the harvested culture medium.

When results are expressed in terms of micrograms per milliliter of culture, both compounds are seen to have increased continuously throughout the culture period (Table I, B). This is a reflection of the continuous increase in cell mass as growth progressed.

The levels of choline and the peak levels of ascorbic acid (Table I, A) compare favorably with data reported by Morimura (9) for short-term (2–3 days) synchronized cultures of *C. ellipsoidea*. He also found that the choline content remained fairly constant, but reported that ascorbic acid "increased considerably during the stages . . . in which the photosynthetic process occurred most actively." Confirmatory evidence for this view is provided by the facts that (*a*) in the present

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This work was supported entirely by intramural funds, University of California.
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(15) Price, A. H., Erf, L., and Bierly, J., *J. Am. Med. Assoc.*, **167**, 1612(1958).
(16) Gray, W. D., *Am. Pract.*, **10**, 660(1959).
(17) Herndon, J. F., Rice, E. G., Tucker, R. G., Van Loom, E. J., and Greenberg, S. M., *J. Nutr.*, **64**, 615(1958).
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(20) Finney, D. J., "Statistical Method in Biological Assay," Charles Griffin and Co. Ltd., London, Hafner Publishing Co., New York, N. Y., 1952.
(21) Burns, J. H., "Biological Standardization," Oxford University Press, London, England, 1937.
(22) Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Hafner Publishing Co., Inc., New York, N. Y., 1957.
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TABLE I—VITAMIN C AND CHOLINE CONTENT OF *C. vulgaris* AND *C. pyrenoidosa*[a]

Vitamin	*C. vulgaris* 5 Days	7 Days	11 Days	14 Days	18–21 Days	*C. pyrenoidosa* 5 Days	7 Days	11 Days	14 Days	18–21 Days
A. Content Expressed as mcg./mg. Dry Wt.										
C	0.984	1.181	0.679	0.507	0.469	1.104	0.885	0.585	0.515	0.386
Choline	1.783	2.365	2.400	2.617	2.611	2.952	2.912	2.767	2.676	2.879
B. Content Expressed as mcg./ml. of Culture										
C	0.345	1.186	1.675	1.626	1.936	0.613	1.111	1.504	1.818	2.065
Choline	0.538	2.383	5.941	8.386	10.736	1.505	3.797	8.142	9.532	15.065

[a] Values recorded are averages from four separate experiments, except for 5-day results which are averaged from three experiments.

TABLE II—CHOLINE AND VITAMIN C CONTENT OF *Chlorella* AND OF SOME CONVENTIONAL FOODS (mg. VITAMIN/100 Gm. DRY WEIGHT)[a]

Item	Choline	Vitamin C
C. vulgaris (7-day)	236	118
C. vulgaris (21-day)	261	47
C. pyrenoidosa (7-day)	291	110
C. pyrenoidosa (21-day)	288	39
Beef (round steak)	325	0
Lamb (chop)	300	0
Liver (beef)	1883	31[b]
Pork (chop)	166	0
Eggs (yolk)	3018	0
Milk	105	1[b]
Wheat (whole)	93	0
Barley	143	0
Rice (polished)	93	0
Soybean	305	Tr.
Cabbage	267	50[b]
Carrots	103	4[b]
Oranges	...	49[b]
Parsley	...	193[b]
Pepper, bell	...	120[b]
Potato, white	121	17[b]
Spinach	253	59[b]

[a] Values for foods from "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962, and from Sebrell, W. H., and Harris, R. S., "The Vitamins," Academic Press Inc., New York, N. Y., vols. I and II, 1954. [b] Milligrams of vitamin/100 Gm. raw edible portion.

experiments of longer duration, ascorbic acid content of the cells declined continuously after achieving a peak level early in the culture period, and (*b*) the rate of photosynthesis continuously decreases with advancing age of the culture.

In the current study, the final concentration of ascorbic acid in the cells of *C. vulgaris* was approximately 40% of the early maximum. Previous work has shown that under similar conditions (differing only in light intensity) the rate of photosynthesis at day 21 is only about 43% of the rate determined on the fourth day (10). Calculations (10), based on data reported by Sargent (11), indicate that the decrease in rate of photosynthesis in cultures of *C. pyrenoidosa* may be somewhat greater than for *C. vulgaris*, and it may be significant that in the present experiments the ascorbic acid content in cells from 21-day-old cultures of *C. pyrenoidosa* was only about 35% of the early peak concentration.

The above observations and Morimura's report (9) seem to confirm in the thallophytes the conclusion, based on work with higher plants in 1928, that light has a beneficial effect on the production of ascorbic acid (12). Many of the numerous subsequent direct and indirect verifications of that report and of an association between ascorbic acid and photosynthesis in higher plants have been cited by Mapson (13).

Table II shows that *Chlorella* compares favorably with several conventional dietary sources of choline but less favorably with the better known sources of vitamin C.

In comparing vitamin C content of the *Chlorella* species with that of the dietary sources listed, allowance must be made for the fact that values for *Chlorella* are for dried material, whereas those for the other sources are for fresh material. Recent determination of fresh and dry weights of *Chlorella* revealed approximately 75% water content (70% in *C. vulgaris* and 78% in *C. pyrenoidosa*). Therefore, vitamin C content of the two algae, expressed in terms of fresh weight would be about 25% of the figures given in the table.

REFERENCES

(1) Pratt, R., and Johnson, E., *J. Pharm. Sci.*, **52**, 979 (1963).
(2) *Ibid.*, **53**, 151(1964).
(3) *Ibid.*, **53**, 1135(1964).
(4) *Ibid.*, **54**, 871(1965).
(5) *Ibid.*, **55**, 799(1966).
(6) Roe, J. H., and Osterling, M. J., *J. Biol. Chem.*, **152**, 511(1944).
(7) Horowitz, N. H., and Beadle, G. W., *ibid.*, **150**, 325 (1943).
(8) "Microbiological Assay of Vitamins and Amino Acids," Difco Laboratories, Detroit, Mich., 1963, No. 247, p. 29.
(9) Morimura, Y., *Plant Cell Physiol.*, **1**, 63(1959).
(10) Pratt, R., *Am. J. Botany*, **30**, 404(1943).
(11) Sargent, M. C., *Plant Physiol.*, **15**, 275(1940).
(12) Heller, V. G., *J. Biol. Chem.*, **76**, 499(1928).
(13) Mapson, L. W., in "The Vitamins," Sebrell, W. H., and Harris, R. S., eds., Academic Press Inc., New York, N. Y., 1954, vol. 1, p. 220.

Oxazepam Excretion by Chlordiazepoxide-^{14}C-Dosed Dogs

By HAZEL B. KIMMEL and SIDNEY S. WALKENSTEIN

The metabolism of chlordiazepoxide was studied in dogs using the ^{14}C-labeled drug. Following a single 26 mg./Kg. dose, oxazepam amounting to 1.1 per cent of the dose was found in urine and 1.3 per cent in feces. The oxazepam was identified by thin-layer chromatography. Chronic administration of chlordiazepoxide at a daily dose of 5 mg./Kg. for 9 weeks had no significant effect on the amount converted to oxazepam.

In previous reports on the metabolism in dogs of chlordiazepoxide (1) (7-chloro-2-methylamino-5-phenyl-3H-1, 4-benzodiazepine 4-oxide) and diazepam (2) (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), the authors concluded that the 3-position in the diazepine ring of chlordiazepoxide was not subject to metabolic attack. This would eliminate the possibility of oxazepam[1] (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one) formation from chlorodiazepoxide. However, evidence for the metabolic conversion of chlordiazepoxide to oxazepam in the dog in small but significant amounts has been obtained.

The conclusion is based on results of preliminary studies in which oxazepam was recovered from the urine of dogs fed "lactam" (7-chloro-1, 3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one 4-oxide), a major metabolite of chlordiazepoxide (1), and on chromatographic evidence obtained in studies in dogs fed chlordiazepoxide labeled with ^{14}C in the 2-position of the diazepine ring (specific activity 1.2 μc./mg.). The effect of chronic administration of chlordiazepoxide on the conversion to oxazepam was also investigated.

EXPERIMENTAL

Four dogs were given nonradioactive chlordiazepoxide in 5 mg./Kg. daily oral doses for 9 weeks followed by a final similar dose of chlordiazepoxide-^{14}C. In one phase of this study, two female dogs were given single 26 mg./Kg. oral doses of tagged chlordiazepoxide, and urine and feces were collected at intervals up to 48 hr. For thin-layer chromatography, the urine samples were exhaustively extracted with ethylene dichloride, and the aqueous residues were treated with β-glucuronidase at pH 5.5 for 48 hr. and then re-extracted with ethylene dichloride. Feces were also extracted with ethylene dichloride, but without glucuronidase treatment, since in a previous study (3) oxazepam was found to be excreted in feces unconjugated. The ethylene dichloride extracts were concentrated under vacuum to small volume and subjected to thin-layer and paper chromatography. In addition to the many solvent systems given in the references cited, the following were employed in thin-layer chromatography: (*a*) on Whatman Silica Gel SG 41: benzene–pyridine–acetic acid (80:5:2, v/v/v); chloroform–acetone–ethanol (8:5:5, v/v/v); and chloroform–acetone–ethanol (8:1:1, v/v/v); and (*b*) on MN-Kieselgel G-HR: *n*-butanol–*n*-butyl ether–acetic acid (4:8:1, v/v/v); and (*c*) on Silica Gel G (Merck, Darmstadt): heptane–chloroform–ethanol (5:5:1, v/v/v). After solvent development and air drying, the chromatograms were placed in contact with DuPont Cronex II medical X-ray film for 1–4 weeks, depending on the amount of radioactivity present. Radioactive areas on thin-layer plates as defined by radioautography were scraped into scintillation vials for assay on a Packard Tri-Carb liquid scintillation spectrometer using a dioxane "cocktail" (100 Gm. naphthalene, 10 Gm. POP, 250 mg. dimethyl POPOP, and 200 ml. methanol in 1 L. spectroquality 1,4-dioxane).

RESULTS AND DISCUSSION

Figure 1 is a radioautograph of a paper chromatogram of urine samples applied directly without prior treatment or extraction. The characteristic shape and location of oxazepam glucuronide can be seen duplicated in the urine samples from dogs dosed with diazepam and chlordiazepoxide, respectively. However, this area in the diazepam sample contains, in addition, the glucuronide of 3-hydroxy-diazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), and the same fraction in the chlordiazepoxide urine may also contain other glucuronides. Glucuronidase treatment of urine resulted in the disappearance of this glucuronide area, and gave rise to an ethylene dichloride extractable substance which when chromatogramed in all thin-layer systems cited under *Experimental* migrated identically with

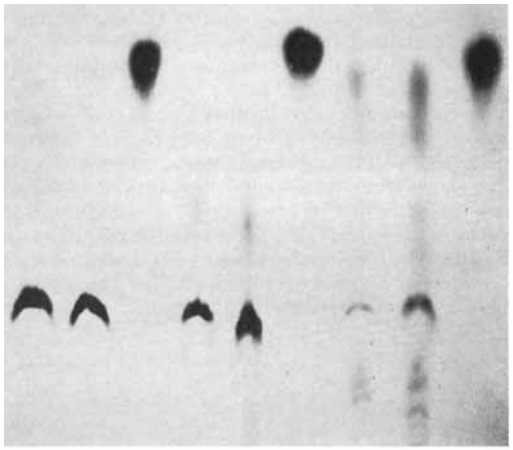

Fig. 1—Radioautograph of a paper chromatogram of urine samples applied directly without prior treatment or extraction.

Received October 28, 1966, from the Research and Development Department, Radiochemistry Section, Wyeth Laboratories, Philadelphia, PA 19101

Accepted for publication December 20, 1966.

Presented in part to the ASPET, Mexico City meeting, July 1966.

The authors thank Dr. C. H. Gudmundsen for the synthesis of ^{14}C-labeled compounds and Mr. M. O. King and Mrs. D. Infante for technical assistance.

[1] Marketed as Serax by Wyeth Laboratories, Inc., Philadelphia, Pa.

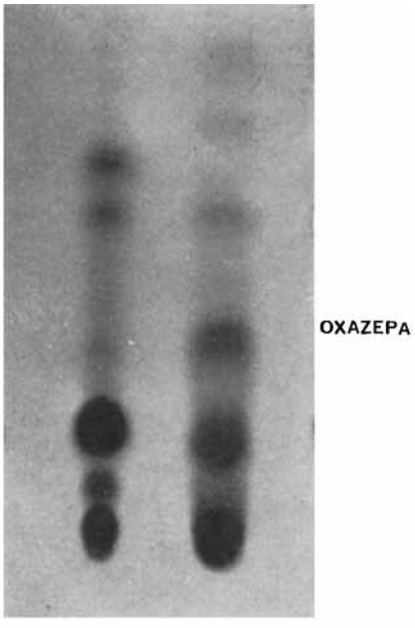

Fig. 2—Chromatogram made from urine collected between 24 and 48 hr. following a dose of ^{14}C-tagged drug to an animal on the chronic regimen.

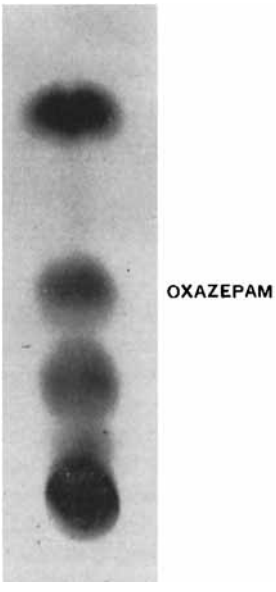

Fig. 3—Chromatogram made from feces collected between 24 and 48 hr. following a dose of ^{14}C-tagged drug to an animal on the chronic regimen.

authentic oxazepam. The chromatogram shown in Fig. 2 was made from urine collected between 24 and 48 hr. following a dose of ^{14}C-tagged drug to an animal on the chronic regimen. The oxazepam area accounts for about 16% of the radioactivity in this sample, as determined by scintillation counting. The corresponding area obtained from an extract of the 24–48 hr. feces collection (Fig. 3) of an acutely dosed animal contained 13%. The thin-layer systems which gave the best resolution of oxazepam were: (*a*) benzene–pyridine–acetic acid (80:5:2) on Whatman Silica Gel SG 41; (*b*) *n*-butanol–*n*-butyl ether–acetic acid (4:8:1) on MN-Kieselgel G-HR; (*c*) heptane–chloroform–ethanol (5:5:1) on Silica Gel G. Co-chromatography of extracts containing added nonradioactive oxazepam resulted in a single homogeneous spot at the R_f of oxazepam at which HCl-induced fluorescence corresponded exactly with the dark area on the radioautograph.

Prolonged administration of chlordiazepoxide appeared to have no effect on the extent of its conversion to oxazepam (Table I). Total amounts converted ranged from 2.4% in the acute study to 4.5% in the chronic study.

TABLE I—OXAZEPAM-^{14}C EXCRETION BY CHLORDIAZEPOXIDE-^{14}C-DOSED DOGS

	Values in % of Dose						
	Urine				Feces		
		$EtCl_2$ Extracts				$EtCl_2$ Extracts	
		Before β-Gluc.	After β-Gluc.				
	Total ^{14}C		Oxazepam	Residuum	Total ^{14}C	Oxazepam	Residuum
A[a]	25.7	12.4	1.1	6.5	49.3	1.3	16.9
C[a]	23.4	10.4	1.2	6.6	61.9	3.3	42.4

[a] A, acute; C, chronic.

REFERENCES

(1) Koechlin, B. A., Schwartz, M. A., Krol, G., and Oberhansli, W., *J. Pharmacol. Exptl. Therap.*, **148**, 399(1965).

(2) Schwartz, M. A., Koechlin, B. A., Postma, E., Palmer, S., and Krol, G., *ibid.*, **149**, 423(1965).

(3) Walkenstein, S. S., Wiser, R., Gudmundsen, C. H., Kimmel, H. B., and Corradino, R. A., *J. Pharm. Sci.*, **53**, 1181(1964).

Cardiotonic Activity in Callus Tissue of *Digitalis mertonensis*

Sir:

It is well known that the quantitative estimation of digitalis glycosides by chemical methods does not always indicate an equivalent biological effect. There are many glycosides that do not produce a biological effect that are measured in chemical assays. Bioassay is therefore still preferred to the chemical assay when estimating the activity of cardiotonic glycosides (1–3).

Static cultures of callus tissue of *D. mertonensis* Buxton and Darlington seedlings were grown in the dark at 27° ± 1° in White's (4) modified basic media to which ingredients suggested by Staba (5) were added. To induce formation of callus in 14-day-old seedlings, 2,4-dichlorophenoxyacetic acid and 2-benzothiazoleoxyacetic acid in 1 and 2 p.p.m., respectively, were used. Thirty-seven-day-old callus tissue was harvested and dried, and a tincture was prepared from it according to the U.S.P. (6) method for digitalis leaf.

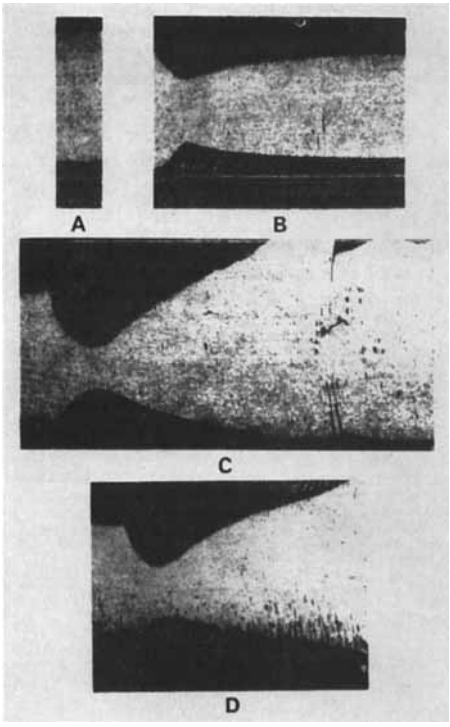

Fig. 1—Recordings of an isolated guinea pig heart preparation on administration of U.S.P. standard digitalis tincture and D. mertonensis *callus tincture* IB_{2c}. *Key:* A, *normal recording;* B, *effect of 0.25 ml. of 20% alcohol;* C, *effect of 25 mcg. total glycosides in diluted tincture of* IB_{2c} *injected in 3 graded doses over a period of 8 min. (note + inotropic response);* D, *effect on same preparation 1.5 min. following injection of 33 mcg. of total glycosides in diluted standard tincture.*

Isolated guinea pig heart preparations were used to study the cardiotonic activity of the callus. Guinea pigs of either sex weighing from 500 to 900 Gm. were used. The isolated mammalian heart technique was basically that of Langendorff (7). The perfusion fluid used was that suggested by Chenoweth and Koelle (8) slightly modified by using magnesium sulfate in place of magnesium chloride (9). Following cervical dislocation, the hearts were quickly removed and suspended in an Anderson-Craver heart perfusion apparatus (10). When the isolated heart, under a perfusion head pressure of 54 cm. of water and at a constant temperature of 37.5°, became stabilized, all extraneous tissues including the pericardium were dissected free. A stainless steel hook was inserted into the apex. The cardiac contractions were recorded on a slowly moving smoked kymograph by means of a cotton thread running from the hook, through a pulley system to a recording lever. All perfusate was discarded.

The tinctures were diluted sufficiently to reduce the concentration of alcohol to less than 25%. After recording the normal cardiac contractions, the effect of 0.25 ml. of 20% alcohol was recorded as a control. This was followed by equivalent doses of test sample. In each case the test sample was completely washed in by displacing the liquid in the injection spiral (1.5 ml.) with fresh perfusate. When the heart returned to normal, a diluted standard digitalis tincture was also introduced and its effect recorded. The results were then compared. (Fig. 1.)

It was found that the callus tincture produced a typical digitalis-like response in the isolated guinea pig heart. There was an increase in amplitude with a dose of 25 mcg. of total glycosides[1] over a period of 8 to 10 min. In four separate trials this effect was comparable to an increase in amplitude produced by 333 mcg. of total glycosides[1] in the standard digitalis tincture. However, in terms of dry callus 37.5 mg. was

[1] The total glycosides in the callus tincture and the standard digitalis tincture were calculated as digitoxin based on the Knudson and Dresbach colorimetric assay for total digitalis glycosides (11).

equivalent to the activity of 12 mg. of dried digitalis leaf.[2]

Digitalinum verum and verodoxin have been identified tentatively in *D. mertonensis* by thin-layer chromatography (12). *Digitalinum verum* has been shown to have cardiotonic activity in cats (13). Hence, it could be one of the active glycosides. From the data obtained thus far, it also seems that the glycosides present in the callus tissue are more active per gram weight of total glycosides as compared with those present in the leaf.

(1) Imai, S., Murase, H., Katori, M., Okada, M., and Shigei, T., *Japan. J. Pharmacol.*, **15**, 62(1965).
(2) Kohli, J. D., and Vohra, M. M., *Arch. Intern. Pharmacodyn.*, **126**, 412(1960).
(3) Vohra, M. M., Kohli, J. D., and De, N. N., *ibid.*, **133**, 265(1961).
(4) White, P. R., "The Cultivation of Animal and Plant Cells," 2nd ed., Ronald Press, New York, N. Y., 1963, pp. 60–61.
(5) Staba, E. J., *J. Pharm. Sci.*, **51**, 249(1962).
(6) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 189–190.
(7) Langendorff, O., *Arch. Ges. Physiol.*, **61**, 291(1895).
(8) Chenoweth, M. B., and Koelle, E. S., *J. Lab. Clin. Med.*, **31**, 600(1946).
(9) Smookler, H. H., and DeFeo, J. J., *J. Pharm. Sci.*, **51**, 736(1962).
(10) Anderson, F. F., and Craver, B. N., *J. Pharmacol. Exptl. Therap.*, **93**, 135(1948).
(11) Knudson, A., and Dresbach, M., *ibid.*, **20**, 205(1922).
(12) Medora, R. S., Ph.D. Thesis, University of Rhode Island, Kingston, R. I., 1965.
(13) Lendle, L., in "Handbuch der Experimentellen Pharmakologie," Heffter, Heubner, and Shuller, eds., Erganzungswerk, Springer, Berlin, Germany, 1935, vol. 1, pp. 11–265.

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Received November 17, 1966.
Accepted for publication February 16, 1967.

[2] U.S.P. digitalis reference standard (6).

Tumor Inhibitors XXII. Senecionine and Senecionine *N*-Oxide, the Active Principles of *Senecio triangularis*

Sir:

In the course of the continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Senecio triangularis* Hook. (*Compositae*)[1] was found to have reproducible activity against the Walker 256 carcinosarcoma tumor (intramuscular) in rats.[2] The fractionation of the active extract and the isolation and characterization of the active principles, senecionine (I) and senecionine *N*-oxide (II), are reported here. Senecionine has been isolated from *Senecio* species and other plants of the *Compositae* and *Leguminosae* (1, 2). While indirect evidence has been advanced for the occurrence of senecionine *N*-oxide in several plants (3–6), the isolation and characterization of the compound have not been reported previously.

The systematic fractionation of the alcoholic extract of *S. triangularis* is summarized in Scheme I; the *in vivo* assay data for the fractions obtained in a typical experiment are reported in Table I. The evaluation of assay results by CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less.[2] The absence of tumor inhibitory activity from fractions B and C, coupled with the high yields of senecionine and senecionine *N*-oxide isolated from the alkaloidal mixture, support the conclusion that the latter two compounds were principally—if not solely—responsible for the tumor inhibitory activity of the alcoholic extract of *S. triangularis*. The present report appears to be the first recorded observation of the inhibitory activity of pyrrolizidine alkaloids against the Walker carcinosarcoma 256 tumor (intramuscular), although the activity of monocrotaline against the adenocarcinoma 755 tumor has been noted earlier (7).

When the isolation procedure of Koekemoer and Warren (8) was used (*i.e.*, partition of total extract between chloroform and 15% citric acid solution, and reduction of *N*-oxides in the aqueous layer with zinc and hydrochloric acid), the yield of total alkaloid was 1.65% of weight of dry plant. Hence, *S. triangularis* appears to be one of the richest sources of pyrrolizidine alkaloids (*cf. References 1, 2*).

Characterization of senecionine (I) was effected by comparison of (*a*) the melting points of the alkaloid and its picrate and nitrate derivatives

[1] Rhizomes, roots, stems, leaves and flowers, gathered in Colorado, August 1961. The authors acknowledge with thanks the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Dept. of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health (CCNSC).

[2] Assays were performed by the Wisconsin Alumni Research Foundation and Hazleton Laboratories, Inc., under contract to the CCNSC. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

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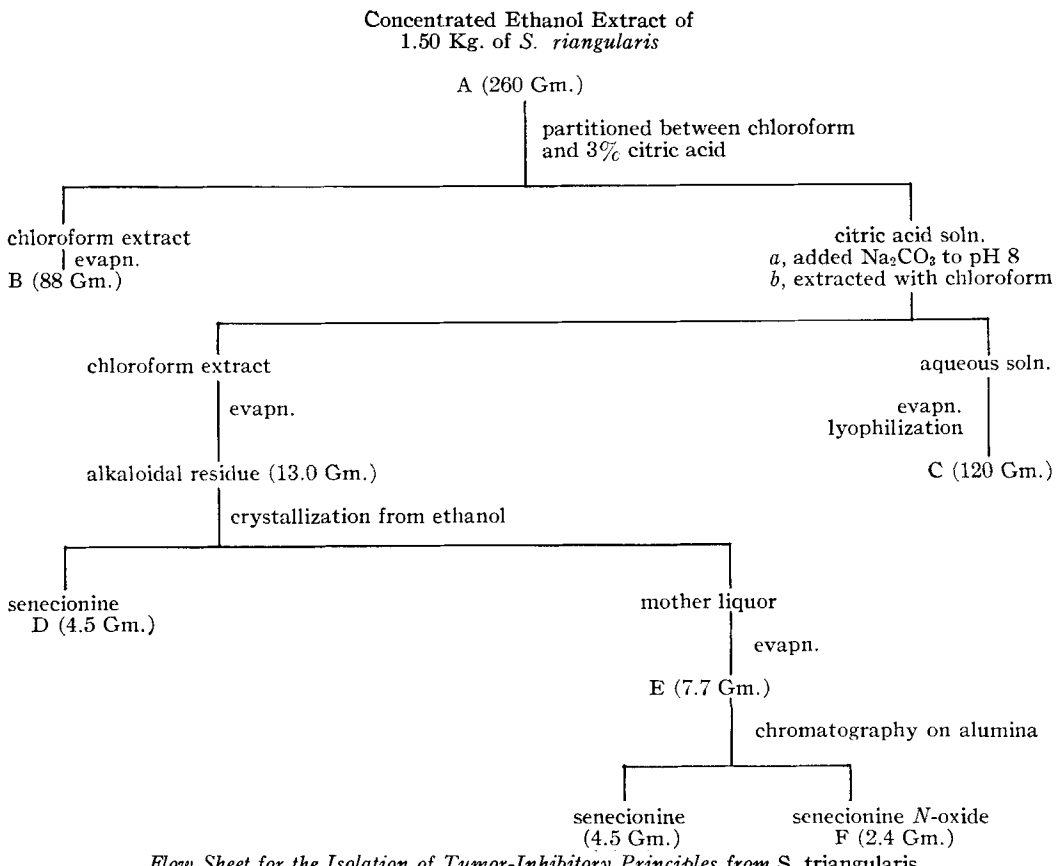


Flow Sheet for the Isolation of Tumor-Inhibitory Principles from S. triangularis
Scheme I

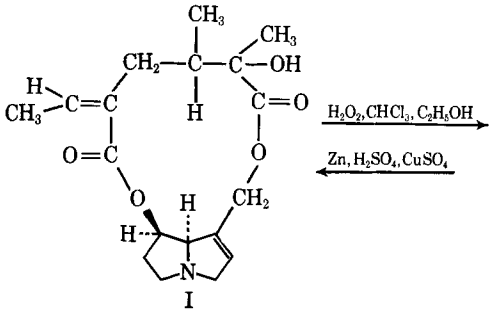
and (*b*) the specific rotation and infrared and NMR spectra with recorded data (9–12). In addition, hydrolysis with barium hydroxide solution afforded retronecine (III) (isolated as hydrochloride) and senecic acid (IV), characterized by comparing melting points and spectra with recorded data (9, 13).

Senecionine *N*-oxide (II) was crystallized from chloroform–petroleum ether as a chloroform solvate, m.p. 141–142° dec., $[\alpha]_D^{28}$ −22° (c 1.32, $CHCl_3$), $\lambda_{max.}^{Nujol}$ 5.73, 5.81, 6.04 μ.[3]

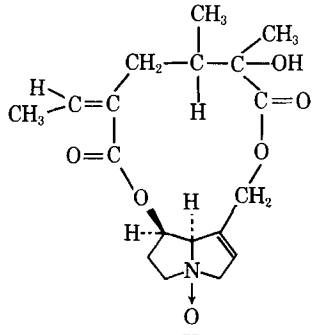
Anal.—Calcd. for $C_{18}H_{25}NO_6 \cdot {}^2/_3\ CHCl_3$: C, 52.02; H, 6.00; Cl, 16.45; N, 3.25. Found: C, 51.52; H, 6.24; Cl, 17.64; N, 3.55.

The compound was characterized by reduction of II with zinc, copper sulfate, and sulfuric acid to I, and by oxidation of I with hydrogen peroxide to II (14).

The NMR spectrum of senecionine *N*-oxide is similar to that of senecionine, except that the signals corresponding to the protons on carbon atoms adjacent to the nitrogen are shifted downfield by 0.6 to 1.2 τ units. Thus, the signal for the C-8 proton, which appears as a multiplet centered at 5.68 τ in the spectrum of senecionine (15), is shifted to 4.50 τ in the spectrum of the



I

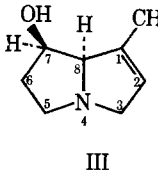


II

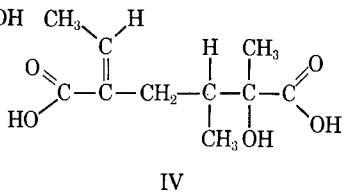
[3] Senecionine *N*-oxide is very hygroscopic; the crystals were filtered in a "dry box" and dried under reduced pressure at 60°.

TABLE I—ACTIVITY OF FRACTIONS OF *S. triangularis* IN WALKER CARCINOSARCOMA 256 (INTRAMUSCULAR)

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change Diff., Gm. (T-C)	Tumor Wt., Gm. (Test/Control)	T/C × 100
A	210	4/4	−11	1.5/6.6	22
	140	4/4	−6	3.0/6.6	45
	93	4/4	−5	3.9/6.6	59
B	360	4/4	+4	6.7/6.6	101
	180	4/4	−5	7.6/6.6	115
	90	4/4	0	6.7/6.6	101
C	500	4/4	+2	5.2/6.6	78
	250	4/4	−2	6.3/6.6	95
	125	4/4	−2	7.1/6.6	107
D	30	2/4	−10	1.8/4.4	...
	25	4/4	−3	1.3/7.8	16
	20	4/4	−7	1.7/7.8	21
	15	4/4	+6	4.5/7.8	57
	10	4/4	−7	5.8/6.6	87
	5	4/4	−3	4.4/6.6	77
E	80	0/4	...	...	...
	40	4/4	−10	2.7/5.7	47
	20	4/4	0	3.0/5.7	52
F	50	3/4	−4	1.8/7.0	25
	40	4/4	−4	1.9/9.0	21
	30	4/4	−2	3.5/7.0	50
	20	4/4	−4	5.7/7.8	73
	10	4/4	−2	6.8/7.8	87



III



IV

N-oxide. The signals for the C-3 protons [multiplets at 6.14 and 6.53 τ for senecionine (15)] appear as a strong (2-proton) multiplet at 5.45 τ in the spectrum of the *N*-oxide. The shifts for protons on carbons adjacent to the nitrogen in the *N*-oxide are noteworthy, and may be valuable in structural studies of amines and their corresponding *N*-oxides.

It is noteworthy that various species of *Senecio* have been used in the treatment of cancer in many countries (16–19).[4] Indeed, *Senecio vulgaris*, known to contain senecionine (2), has been used since the 4th century A.D. for "tumors of the feet and sinews" (18), and since the 10th century for cancer (19). Furthermore, several other genera known to contain pyrrolizidine alkaloids [*e.g.*, *Petasites*, *Cynoglossum*, *Echium*, *Heliotropium*, *Tournefortia*, and *Cytisus* (2)] have also been described in folklore as plant remedies for cancer (16).

(1) Leonard, N. J., in Manske, R. H. F., "The Alkaloids," Academic Press Inc., New York, N. Y., vol. VI, 1960.
(2) Boit, H.-G., "Ergebnisse der Alkaloid-Chemie bis 1960," Academie-Verlag, Berlin, Germany, 1961.
(3) Culvenor, C. C. J., and Smith, L. W., *Australian J. Chem.*, **8**, 556(1955).

[4] The authors thank Dr. Jonathan L. Hartwell, National Cancer Institute, cordially for the references on the folklore on plant remedies for cancer.

(4) Koekemoer, M. J., and Warren, F. L., *J. Chem. Soc.*, **1955**, 63.
(5) Kompís, I., Schröter, H. B., Potěšilová, H., and Sǎntavý, F., *Coll. Czech. Chem. Commun.*, **25**, 2449(1960).
(6) Trivedi, B., and Sǎntavý, F., *ibid.*, **28**, 3455(1963).
(7) Kupchan, S. M., Doskotch, R. W., and Vaneenhoven, P. W., *J. Pharm. Sci.*, **53**, 343(1964).
(8) Koekemoer, M. J., and Warren, F. L., *J. Chem. Soc.*, **1951**, 66.
(9) Adams, R., and Govindachari, T. R., *J. Am. Chem. Soc.*, **71**, 1953(1949).
(10) Massagetov, P. S., and Kuzovkov, A. D., *Zh. Obshchei Chim.*, **23**, 158(1953).
(11) Atal, C. K., and Kapur, K. K., *Tetrahedron Letters*, **1966**, 537.
(12) Barger, G., and Blackie, J. J., *J. Chem. Soc.*, **1936**, 743.
(13) Barger, G., Seshadri, T. R., Watt, H. E., and Yabuta, Y., *ibid.*, **1935**, 11.
(14) Christie, S. M. H., Kropman, M., Leisegang, E. C., and Warren, F. L., *ibid.*, **1949**, 1700.
(15) Culvenor, C. C. J., Heffernan, M. L., and Woods, W. G., *Australian J. Chem.*, **18**, 1625(1965).
(16) Culpepper, N., "The English Physitian Enlarged," George Sawbridge, London, England, 1681.
(17) Heckel, E., "Catalogue Alphabetique. Raisonne des Plantes Medicinales et Toxiques de Madagascar avec Leur Emploi Indigene," *Ann. l'Inst. Colon. Marseille*, (*Ser. 2*), **11**, 61(1903).
(18) Hunger, F. W. T., "The Herbal of Pseudo-Apuleius," E. J. Brill, Leyden, 1935.
(19) Cockayne, O., "Lech Book of Bald," in "Leechdoms, Wortcunnings, and Starcraft of Early England," Longmans, London, England, vol. 2, 1864–1866.

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Received December 23, 1966.
Accepted for publication February 13, 1967.

This investigation was supported by grant CA-04500 from the National Cancer Institute, grant T-275 from the American Cancer Society, and a contract PH 43-64-551 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
Previous paper: Kupchan, S. M., Hemingway, R. J., Knox, J. R., Barboutis, S. J., Werner, D., and Barboutis, M. A., *J. Pharm. Sci.*, to be published.

* National Institutes of Health Predoctoral Fellow, 1966–1967.

REVIEWS

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

May 1967 volume 56, number 5

Review Article

Tissue Culture in the Study of the Effects of Drugs

By M. DAWSON and W. F. DRYDEN

ALTHOUGH the techniques of cell, tissue, and organ culture have been known and developed over many years, surprisingly little use has been made of them until recently in pharmaceutical research for the direct examination of the effects, beneficial or adverse, on cells, healthy or abnormal, of substances of physiological or medicinal interest. Even now the full potential of the technique for this purpose is not being realized. The applications presently being made of tissue culture techniques are mainly in the fields of cytology, cell biochemistry and metabolism, cell differentiation, growing of viruses in cells, pathology (especially cancer), and the storage of viable material for transplantation. Thus, there is scope for considerable expansion in the application of tissue culture techniques to drug screening and action, where it can be a useful precursor or supplement to whole-animal tests in determining the effects of drugs at cellular level.

The method has several advantages, the most obvious being a reduction in the number of animal tests required, the relative cheapness of the technique, and the fact that less drug is used. Another advantage is that elimination of misleading results due to species difference may be brought about by the use of material of human origin. That species difference exists even at cellular level has been demonstrated (1, 2). Nevertheless, of the 1,461 references on the topic of the effects of drugs on cells retrieved by the Medlars organization recently, only 182 had made use of human cells. This organization is a computerized information retrieval system using tapes made from the references quoted in "Index Medicus." These 1,461 references cover the period mid-1963 to end-1966. Thus, it can be seen that even at the present time advantage is not being taken of the fact that tissue culture allows the use of human material on a scale not possible *in vivo*. There are, in fact, workers who deliberately choose amphibian material because it is poikilothermic. However, the present authors do not see that the provision of warmed surroundings, either in the form of a standard incubator or in the form of a warmed microscope stage, presents an insurmountable obstacle.

The use of cell culture also makes possible the setting up of replicate experiments and controls using identical material. Established cell lines are now easily available from many commercial suppliers. There is also a collection maintained by the American Type Culture Collection, and in Great Britain information on private collections is obtainable through the British Society for Cell Biology (formerly the British Tissue Culture Association).

Received from the Department of Pharmacy, University of Strathclyde. Glasgow, Scotland.
The authors thank Professor Elworthy for his interest and helpful advice.

Many cell lines are now stored deep-frozen to avoid the work of continuous subculturing and to keep down changes which may be introduced in new generations (3). It has, however, recently been observed that this does not guarantee freedom from variation in the course of time, and this point must be remembered if one is comparing current work with earlier work (4). Also even a few days' freezing and subsequent thawing have been observed (5) to cause cell changes, and even the dimethylsulfoxide usually used in the technique of deep freezing may *per se* produce cell changes (6, 7). Thus, although large quantities of uniform material may be used at any one time, caution must be observed in comparing results of experiments performed even on the same cell line if these experiments are long separated in time. Such changes in cells used may explain some of the contradictory findings periodically reported.

A further advantage of tissue culture is that the cells may be grown in a controlled environment. Many established lines will grow in chemically defined media. Also, if a solution of the test drug in nutrient medium is continuously perfused around the cells, the exact concentration of drug is known to which each cell is exposed for the duration of the experiment.

Surprisingly little use has been made too of cultures of diseased cells, other than cancer cells, most of the work so far having concerned either the killing of these, or freedom from toxicity on the part of the test drug toward healthy cells. There is also little work on mixtures of drugs to study synergism or antagonism, which could also be observed *in vitro*.

The tissue culture method has, however, the limitation that a drug metabolized to an active form *in vivo* could not be tested *in vitro* unless the active form were first prepared. A second disadvantage is that, although the cultured material may be structurally unchanged, it may have changed functionally (4, 8), *e.g.*, loss of neoplastic properties, loss of transplantability, respectively. Third, in systems which involve several cells, such as the central nervous system, the gross effect of a drug may not be demonstrable. Rather in this instance the often overlooked effects of the drug on the component cells of the system may be more easily elucidated by biochemical and cytophysiological means.

Finally, like the results of whole animal tests, those from tissue culture tests must of course be correlated with clinical findings. Once the need for such correlation and the limitations of the method are recognized, tissue culture may be seen to provide a useful means of assessing activity and toxicity of many drugs (9–12).

TISSUE CULTURE STUDIES ON DRUG ACTION

General—Perhaps the most complete list of publications on all aspects of tissue culture is that of Murray and Kopech (13) covering the years 1885–1950 and including some 15,000 references. There has since been a supplement to this. At present a bibliography by the same authors of more recent work is being published in parts, the first part having contained 1,213 references and the second part 1,244 references, this part coming out only 4 months later. This, taken in conjunction with the 1,461 Medlars references on drug action in tissue culture alone, is an indication of the vast increase in tissue work as a whole which has recently taken place. Obviously, a complete list of references even on this one aspect of tissue culture is beyond the scope of a single review article. Only the most important references since 1953 will be mentioned with in addition a few earlier works necessary for the explanation of the more recent work.

METHODS USED IN ASSESSING THE BENEFICIAL AND ADVERSE EFFECTS OF DRUGS ON CELLS

Many microscopic methods have been used for this purpose, varying from observation of cell death to observation of minor, reversible changes. When the end point of the experiment is death, vital staining has been used (14) to indicate the percentage killed. On the other hand, attention has been drawn (15) to the fact that increased cell permeability by a dye is not necessarily synonymous with loss of viability. Thus, some other index of cell death is preferable. Direct observations by microscope of hanging drop preparations of cells have been used (16, 17) either to follow slowing or stopping of growth, or to see changes within the cell. Chromosome examination is another measure sometimes used.

Usually phase-contrast microscopy is used to allow observation of live, unstained material, and this may be usefully allied to time-lapse filming to obtain a permanent continuous record of the experiment. The authors' design of such apparatus allows for controls being recorded simultaneously (18–22). Electron microscopy examinations are being undertaken with increasing frequency as will be seen from later sections of this paper.

In many experiments, use is made not of cell lines which have grown for many generations *in vitro*, but of primary explants of tissue fragments (23). These are sometimes preferred because of the changes, morphological, chromosomal, or metabolic, which may occur, apparently spontaneously, in cells after some time in culture.

A great amount of work on such explants of various tissues has been carried out by Pomerat and Leake (1), who measured the concentrations of many pharmaceutically important substances which would inhibit the outgrowth of cells from such explants. Others (24, 25) have also measured the area of explant growth, or the time of closing of a small hole punched in the explant (26). Such measures of area, however, take no account of the depth of the growth or of whether the cells in the halo of outgrowth are loosely or tightly packed.

One disadvantage of using fragments of tissue or small organs, as distinct from separated cells, is that the drug may not have access to the cells at the center of the piece or organ. This may lead to false results if the experiment is of the type where the material is exposed to the drug for a fixed time and then is returned to growth medium without drug. On the other hand, such conditions may be desirable for some experiments because they more nearly approximate to *in vivo* conditions where each cell, for example in a tumor, is not necessarily bathed in drug solution. Another point that must be kept in mind in examining outgrowths from explants is that the rate of growth of the different cell types in the explants will probably vary—for example, a small number of epithelial cells may first be seen and later be completely overgrown with a halo of fibroblasts. Adequate control experiments are necessary before any conclusions can be drawn about a drug having a differential toxicity for epithelial and fibroblast cells under such conditions.

Inhibition of macrophage migration from spleen explants has also been used as an index of toxicity (27), as has the rate of migration of white blood cells in cultures of these alone (28). An interesting technique (29) overlays monolayers of cells with semisolid agar, through which the drug diffuses from paper disks as in an antibiotic assay. The agar is then poured off, the cells incubated, and examined for death or other changes. A comparison can be made with control cells on the same monolayers since the agar localized the drug. The only criticism of the method would be that it is necessary to verify chemically, where the result is unchanged cells, that the drug did elute from the paper.

Other methods of measuring growth in tissue cultures have been fully described (30, 31) or reviewed (32). These methods include total cell counts, viable counts, nuclear counts (in hemocytometer chambers or electronic counters), cell size measurements, packed cell volume or dry weight measurements, estimation of cell constituents such as protein, DNA, RNA, measurements of glucose utilization, mitotic index or duration of mitosis, or of respiration (33–37). Among respiration techniques, the authors' experience shows that micro-techniques using Cartesian diver types of respirometer are particularly useful and give a relatively rapid result.

Thus there are many indices from which to choose. Those chosen should always in the authors' experience include looking for simple morphological change because this is usually more sensitive than other indices. However, a change capable of being expressed objectively is better than one that can be described only subjectively, *e.g.*, number of polynucleated cells produced would be better than simply change of shape of cell. In using the other indices, *i.e.*, cell number, weight, volume, or biochemical measurements, which can all be expressed mathematically, it is necessary to do sufficient experiments and simultaneous controls for results to be statistically sound. Regrettably, the extent of variation among normal, untreated, replicate tissue cultures is all too often not recognized. This is surprising, because in recent years the need for good design in conventional, whole animal, pharmacological experiments has become generally appreciated, and one would have thought the same principles would have been applied to tissue culture methods. It is impossible to evaluate work where insufficient replicates and no controls are done, nor under such circumstances can one know what relative "weight" to assign to the all too many papers showing contradictory results.

Finally, whatever method is adopted for assessing the effect of a drug on cells, fully detailed reporting of that method is absolutely essential. Even very minor experimental variations may affect the results. Items which should be included in reports are details of the cell type, strain, origin, duration and treatment between obtaining and growing, duration since explanting, temperature and atmosphere of growing, medium in which growing, and frequency and methods of feeding and subculture. Whether material is fresh or frozen/thawed may affect its appearance (5), and also the effect of a drug may vary with the growth phase of the cells when treated. If synchronized cultures are used, this should be stated. Growth can be affected even by whether the cultures are exposed to light and by whether centrifuging is a stage in the subculturing procedure (38). The converse effect, weightlessness, is still of course under active investigation!

Only if all these qualitative and quantitative aspects are planned for and reported is a paper of present or future value and able to be put in true perspective in relation to other papers.

GROUPS OF DRUGS WHICH HAVE BEEN EXAMINED BY TISSUE CULTURE METHODS

The various drugs which have been so examined fall into four main groups—anticancer agents, antimicrobial agents, hormones, and alkaloids. Although the latter is generally regarded as a chemical group, as opposed to the first three being pharmacological groups, from the point of view of change in cells, this too is a pharmacological group. There are fewer references to other drugs, and here these will be grouped under *Other Drugs*, including both chemical and biological substances.

There are few references to the substances, other than the active ingredient, in pharmaceutical formulations. Such items as vehicles, ointment bases, adjuvants, diluents, stabilizers, emulsifying agents, substances which increase viscosity (39), surface-active agents, tablet lubricants, disintegrants, coatings and polishes, flavorings and dyes, and materials used in surgical dressings and sutures—especially where the materials are new—are all items which could properly be examined by tissue culture. It is just as important that they be free from toxicity as it is for the active ingredient to be.

Anticancer Agents—It is extremely reasonable to use a cellular method for testing such drugs, since primarily a differential action on normal and malignant cells is being sought. Some of the material taken as "normal" in some experiments, however, scarcely merits this description, *e.g.*, benign growths which have been removed and varicose vein material (40).

The field of anticancer agent screening is in fact the place where the most active work is taking place using the tissue culture method. Such works may be said to have increased explosively since it was said (40) in 1961 that "the application of human cell cultures for the evaluation of cytotoxic activity of new carcinostatic agents has been reported by a few investigators."

The earlier papers in this field tended to emphasize the ease with which malignant cells could be grown *in vitro*, resulting in the availability of large amounts of uniform test material. This idea should be treated with reservation, however, from the viewpoints of both ease and uniformity. In considering ease of growth, although undoubtedly cells planted into a bottle or flask and grown to form a monolayer usually, in the case of normal cells, cease growing once intercellular contact is established—and in the case of malignant cells may not so cease—yet the proportion of explants of malignant tissue actually producing growth *in vitro* is far from 100% (41–44). Figures like 20–60% are more common. There has even been a report on a means of growing sarcoma cells free from healthy cells (45). Also, concerning uniformity of material produced, this has been taken for granted too much. Recent work has shown that variations do in fact occur over a period in cultured material. For example, cells have been shown to fail to maintain their blood groups in tissue culture (46), although this is not a unanimous finding, and chromosome variations have been observed (4). The heterogeneity of response of individual cells in neoplastic populations has also been discussed (47).

Clinical observations on the variation in response to the same drug in different patients with histologically indistinguishable tumors has resulted in the suggestion that it is useful to test drugs on primary explants of an individual patient's tumor at its particular stage of development (40, 42, 48–50). Also, in view of the current idea that an immune component may be concerned in some tumors, the concept of individual host specificity must be borne in mind (51). The idea of using the patient's own material may be very pertinent if the patient has been receiving chemotherapy because of the reported (52) difference between tumor materials from treated and untreated sources in terms of biochemistry, growth kinetics, and karyotype. On the other hand, it has been suggested (40) that prior *in vivo* chemotherapy made no difference to the tissue culture findings.

A further advantage of the tissue culture method for finding the best available drug for any one patient is that it saves involving the patient in the drugs, whose effects may be cumulative, synergistic, or antagonistic (51). In the authors' opinion it is erroneous to decry the tissue culture screening method (40) because of inconsistencies between clinical findings and tissue culture results with apparently identical samples. It would be more correct to realize that samples histologically the same might differ physiologically and functionally. A further advantage of using primary explant material from the patient is that it has not yet become overgrown with one dominant cell type at the expense of others. This is important in view of the numerous reports of many substances having different toxicities toward different cell types.

Whatever type of material, experimental method, and index of toxicity is used, it is necessary that all reports be presented in complete detail so that valid comparisons may be made. For example, results have varied (53) with the percentage of serum in the medium, and even undue chopping of the original material may

produce regenerative hyperplasia (54). Wilson and Barker (49) support the authors' opinion that one index of toxicity used should be morphology. They find it preferable to enzyme inhibition or ^3H uptake, and Morasca (55) finds it more sensitive than mitotic index change and ^3H uptake. Also George *et al.* (56) caution that antimitotic activity is not necessarily equivalent to antitumor activity, basing this conclusion on the fact that various antimitotic substances had very different antitumor activities.

The use of a tissue culture screening method has of late produced a flood of publications with the negative conclusion that "no differential effect was observed between this substance's action on normal and malignant cells." The greatest bulk of screening experiments is that carried out by and for the Cancer Chemotherapy National Service Center Screening Laboratories, the results of which are published in *Cancer Research* supplements. Both synthetic and natural substances are reported. For example, several of the monthly 1965 and 1966 issues contain reports on between 1,000 and 2,000 substances of either type. These reports cover only substances with which it was found not worth proceeding. There are now several years of experience behind the experiments of these workers, who have streamlined their test to use only one cell line (KB cells derived from a carcinoma of the nasopharynx). They did this after tests on more than 1,000 substances indicated less variation among different cell lines used simultaneously than with successive tests using one cell line. A note of caution must here be introduced, however, to mention the variations more recently found to occur in various KB lines with time (4). Only one index of toxicity is routinely used in these experiments: finding the dose inhibiting protein synthesis to 50% of the level found in controls. While this measure by no means exhausts the effects that these substances may produce in cells, it has been considered an adequate indication of whether further *in vivo* testing is likely to lead to any valuable findings. By mid-1966 these reports spoke of decreasing the amount of tissue culture work performed because it had not yet yielded any useful drug. In the authors' opinion decreasing is the wrong solution to this—a diversification of indices of toxicity used would be likely to lead to other results. Also, regrettably, tissue culture is not the only research method which has failed to yield useful anticancer drugs.

Other recent reports of anticancer screening are now numerous (44, 51, 54, 56–67). The gradual accumulation of contradictory findings among these reports further indicates the importance of detailed reporting of cells, media, cultural conditions, and index of toxicity used—for example, not simply referring to techniques as "standard" (44). The authors think, however, that this need for detail is very slow in being realized. Very few papers (68) draw attention to the advantages of using human rather than experimental animal material or to the good mathematical planning of experiments. For example, at least three dose levels separated in geometric progression (61, 64, 69–71), a principle long recognized (72) in whole animal techniques, are used.

There are many other publications dealing with the effects of drugs on malignant cells, but these were conducted to advance knowledge on the nature of the differences between these cells and normal cells, and thus the drugs were incidental to these experiments and not the main object. Such papers are therefore not within the scope of the present article. Often it is interesting to observe the comparison of malignant to embryonic cells in seeking factors affecting cell differentation—a large part currently played by tissue culture in cancer research.

However, clearly anticancer screening is a field in which much work remains to be done, both in seeking new substances and in choosing the best existing one for a particular patient. In the first case morphological change seems to be the most sensitive index to use, and in the second case, where time presses, the differential respirometer apparatus seems, in the authors' experience with melanomas, to form a suitable measure. Results may be obtained from this index more quickly than from simple growth or from such measures as dehydrogenase activity (51). The large number of replicate experiments possible *in vitro* facilitates the simultaneous examination of various modifications of a molecule found somewhat active in this field.

Antimicrobial Agents—Both antibacterial and antiviral drugs have been tested *in vitro* for freedom from toxicity toward cells. The method is particularly applicable to antiviral drugs where the viruses are growing intracellularly both *in vivo* and *in vitro*.

It is rather surprising to find some very early work (73) on antibacterials, including reports on *in vitro* tests for freedom from toxicity, and then to observe that there followed a long period when no such tests were performed. Only now is the method again being appreciated.

A notable early example of such work was its use by Sir Alexander Fleming in 1929 (28) in his original investigations of penicillin. He used the activity of leucocytes (species of origin not stated)

in broth as his criterion of toxicity, and reported that he could find no impairment of function in the presence of penicillin. Similar work with purer penicillin was later reported (74, 75). The latter work established the routine inclusion of penicillin in tissue culture media to prevent the growth of microorganisms, which multiply all too easily in any media supporting the growth of other cells. One group of organisms particularly troublesome in tissue culture is the *Mycoplasmataceae* (pleuro-pneumonia-like organisms). A by-product of work on their elimination is further information on the concentration of many antibiotics which are harmless to various cells, one report alone (76) mentioning 50 antibiotics. Other reports on relative toxicities of various antibiotics indicate (25) that chloramphenicol and chlortetracycline HCl[1] are more toxic in culture than penicillin, and actinomycin and xanthomycin (77, 78) more toxic than other antibiotics.

In the field of antimicrobial testing, nonhuman material is too often used, especially chick embryo material. This seems a needlessly indirect procedure. Early work by Sallé and co-workers (79–82) unfortunately used such material, but they made an advance in the field by reporting results in terms of "toxicity index," relating cell toxicity to antibacterial effect. This was based on the idea that a substance, although very active against bacteria, would have no clinical application if it were at the same time toxic to tissue. The work was extended (83, 84) to measure the maximum time of contact with a given concentration of drug compatible with cell growth, rather than the less realistic measure of maximum concentration allowing cell growth, and was later made quantitative (85) by measuring the number of tissue fragments out of 10 surviving in each part of the experiment and control.

Much of the work on antibacterials has involved ranking currently available substances in order of freedom from toxicity to cells, for example, the comparison of sulfonamides and proflavine (86, 87) on several cell types—the sulfonamides being found to be much less toxic than proflavine—or the comparison of several sulfonamides (88). More recent papers investigate in more detail how these substances affect cells, *e.g.*, proflavine has been reported to produce chromatid breaks (89) or the synthesis of an abnormal RNA (90), and recent work on sulfonamides (91) finds a contradiction in a non-increase in protein synthesis associated with an increase in ribosomes. Rightly, the conclusion is that much further work is required on the action of sulfonamides on cells. Several dyes have also been compared. For example, brilliant green, gentian violet, and acriflavine have been reported to be more toxic than proflavine (92).

Some experiments have been rather more complicated (93–97), being designed to study the effects of antibiotics on live bacteria once these were inside phagocytes. It was found that the phagocytes did, to a certain extent, prevent access of some antibiotics to the bacteria, *e.g.*, penicillin and streptomycin (93), penicillin, streptomycin, and erythromycin (94), streptomycin (95), ampicillin, erythromycin, and oleandomycin (96), but not benzylpenicillin (96), or chloramphenicol, or tetracycline (94). The other report (97) indicated that although the monocytes protected *B. abortus* against streptomycin alone, they did not protect if chlorpromazine were also present. The conclusion is that it helped the streptomycin to penetrate the cells. Similar work not confined to antibiotics, on ingested *M. tuberculosis* (98, 99), reported protection against streptomycin, viomycin, and neomycin B, but not oxytetracycline HCl[2] and isoniazid. The later work on antibiotics also deals with the more detailed effects of these on cells; *e.g.*, streptomycin has been reported to produce nonrandom chromosome breaks in leucocytes (100) and puromycin to inhibit RNA synthesis (101). Such work indicates that new antibiotics might well require careful screening *in vitro* before being marketed.

With all the work on antibacterial substances, only one paper (102) seems to have considered the toxic effects on skin cells of by-products of bacteria multiplying in wounds. This paper reported on pyocyanin from *Ps. pyocyanea* and concluded, from its effects on human skin cells and leucocytes *in vitro*, that it probably would not affect phagocytosis but that it might affect wound closure.

There is less available work on antiviral drugs, from which any useful conclusion can be drawn, than on antibacterial drugs, despite the large amount of work that has been carried out on antiviral chemotherapy. Most materials so far have unfortunately turned out to be too cytotoxic to use. It had been thought that the discovery of the existence of cell receptor sites for adsorbing viruses would have been helpful, but so far metabolic inhibitors once the virus is in the cell seem most useful (103). Many of the

[1] Marketed as Aureomycin by Lederle Laboratories, Pearl River, N. Y.

[2] Marketed as Terramycin by Chas. Pfizer and Co., New York, N. Y.

substances investigated are merely protein synthesis inhibitors, inhibiting virus only by inhibiting host cells. It seems unlikely (103) that there is any chance of inhibiting a pathway that occurs uniquely in infected cells, a problem analogous with that previously mentioned: finding a substance with a differential action on malignant and normal cells. In antiviral work, however, there is perhaps less scope because of greater species specificity (23), for using some of the exotic species of cells that have been chosen for antibacterial work simply because these cells happen to grow well in culture. Nevertheless, there are reports of the use of goldfish cells (104) and plant and insect cells (105) in the study of human pathogens.

Aside from the innumerable papers on various synthetic chemicals, there are also a few on natural products. Algal extracts (106) and bacterial extracts (107) have been screened, a few of each being reported as worth pursuing. Two other groups of workers (108, 109) have reported the interesting discovery of antiviral substances produced by apparently uninfected cells in culture, as distinct from the well-known production of interferon by virus-infected cells. The substances were effective against some enteroviruses (108) and influenza (109). The authors are cautious about interpreting their unexpected results. Tsilinsky's work (108), however, is extensively mathematical, and his conclusions seem entirely reasonable.

Thus, there is scope for much more work on antimicrobial substances for both systemic and topical use. One side effect of the large amount of tissue culture work done in connection with wound healing and skin grafting is that there are many papers on the growing of skin *in vitro*, either in fragments or reduced to cells (18, 26, 102, 103, 106, 107, 110–128). Also remnants of skin from grafts are readily available from surgical units. Thus, it is a practical proposition to grow skin *in vitro* in quantities suitable for testing ranges of potential topical antimicrobial substances for their effects on skin (129). In all reports on antimicrobial substances, as on anticancer substances, full details of materials and methods and statistics are required so that various papers may be compared. For example, the use of calcium-free medium to allow the cells to stay in suspension (130) could well create differences invalidating comparisons with other papers unless such points are kept in mind. The same authors remark on another important point: the differences of inhibition of respiration found, as between the use of tissue slices and individual cells. This again raises the problem of the drug's access to the cells, a point already noted in work on anticancer drugs. A further precaution to be observed in testing antiviral drugs for their effects on the morphology of cells is to distinguish these effects from the effects due to the viruses themselves. That such distinction is possible is seen from the work of Schauer and Likar (131). The principal advantages of the tissue culture method for antiviral screening of antimicrobial substances then appear to be: first, the number of replicates that can be set up; second, the overcoming of species differences, by the use of human material, in respect to both different species susceptibility to various viruses and different effectiveness of different drugs in different species. A further advantage is that quoted by Nemes (132) that one can find by histochemical techniques if the drug is being incorporated into the host cells.

Hormones—The use of tissue culture techniques in the study of the physiological and pharmacological properties of hormones is well established. As has been pointed out (133), the freedom from interference by nervous and other humoral influences prevents much confusion and conflicting conclusions. When one restricts the limit of perspective to those experiments which use pharmacological rather than physiological dose levels, one finds that the scene is dominated by the mass of work carried out on the steroids, natural or synthetic, although insulin and—to a lesser extent—thyroid hormone have received some detailed attention. These substances are used to replace natural deficiencies, and others are used in connection with hormone dependent or influenced tumors. They may therefore be classified as "drugs" in these circumstances. A recent review by LeDouarin (134) covers much recent work on the influence of most of the hormones and vitamins on the growth and differentiation of embryonic bone in culture.

Steroids—Considerable confusion existed in the literature concerning the early work on the adrenal steroids and their toxicity in tissue culture systems. Reports ranged from complete inhibition of growth at 100 mcg./ml. with slight inhibition at 50 mcg./ml. down to a marked restriction in growth at 0.3 mcg./ml. (135–140). The cells used in these experiments were largely fibroblasts derived from embryonic chick or mouse sources, although Geiger *et al.* (135) could only demonstrate inhibition using fibroblasts derived from adult sources. The variation in response obtained by the earlier workers could in part be attributed to the variation in cell type and culture systems used. It was subsequently shown that cells of epithelial origin were less af-

fected by steroids than were fibroblasts subjected to similar concentrations (138, 141, 142), although the closely related synthetic steroid, prednisolone, has been shown to increase the proportion of fibroblasts in cultures of bone marrow (143). This apparent anomaly is not substantiated by evidence from other sources, since prednisolone is regarded as more cytotoxic than cortisone (144, 145) and there must be some reserve until the complexities of a marrow culture system are better understood. There was also evidence that the observed effect was dependent upon growth vigor of the culture (146). Poor cultures were stimulated, while rapidly multiplying ones were inhibited. Whether the addition of steroid to the cultures returned suboptimal conditions to optimal and raised optimal to lethal, respectively, was not clarified, and the solution to the problem awaits further work. Pihl and Eker (142) obtained evidence for a tolerance period, after which a strain of liver cells was capable of multiplying even in the presence of a tenfold increase in prednisolone concentration.

Such dependence of results on the details of methods used in toxicity studies can only serve to emphasize the importance of standardizing the conditions of experiments before valid comparisons can be made.

The consensus of the reports would appear to point to a toxic or lethal concentration of 100 mcg./ml. below which the results are variable and also reversible (139). Below 10 mcg./ml. little or no effect was discernible by optical methods or by measuring cellular "growth" in one of its forms.

A similar series of studies was carried out on the related mineralocorticoid, deoxycorticosterone (DOCA), where doses of 35 and 70 mcg./ml. resulted in cellular hyperplasia and an accumulation of intracellular lipid. The changes were not reversible, but as with the glucocorticoids, fibroblasts showed much greater sensitivity than epithelium or endothelium (147, 148). When DOCA and cortisone acetate were added simultaneously to cultures, the effect of the former predominated (149). Whole thickness skin was used in these experiments, and its ability to serve as an autograft after a period in culture was used as a parameter in assessing the effect of these substances. It was found that the skin could be grafted back on the donor after 3 to 4 weeks of culture in a cortisone enriched medium as compared to the 10–14-day maximum time after which controls could not be regrafted. DOCA, on the other hand, while not prolonging the regrafting time, did maintain the structural integrity of the skin better.

This preservation effect of cortisone and related compounds in lower concentrations has been documented from other sources. Embryonic bones in organ culture (150) were maintained better in the presence of hydrocortisone than in its absence, while other reports, dealing with isolated cells in culture, have shown that at lower concentrations (0.1–10 mcg./ml.) the corticosteroids preserved the cells from the degenerative effects of culture exhibited by controls (151, 152). Others claim a positive stimulatory effect rather than simple preservation, using fibroblasts and low concentrations of drug (138, 153).

Not only fibroblasts but also glial cells in cultures of embryo rat cord ganglia (154) showed increased migration and better survival than controls, while in large pieces of tissue central necrosis, which is normally expected, was diminished. The production of myelin in this experiment was unaffected. It is of interest to note that the cells which show greatest sensitivity to the presence of steroid are those responsible for the structural framework and support of the tissues, a possible pointer to the mechanism of the anti-inflammatory properties of the steroids. It has been suggested that these cells may in normal culture be existing at a subphysiological level of steroid, and by adding low concentrations to the medium, the optimal level is attained. This suggestion has much to commend it in this instance where we are dealing with a drug which is also a normal constituent of the *in vivo* environment.

The fundamental reason for low concentrations being beneficial, however, is as obscure as ever. To answer the obvious question, attempts have been made to assess the influence of the corticosteroids on biochemical systems in cultivated cells.

The results of the simple toxicity tests are in part reflected in the data obtained at this level. High concentrations of cortisone were shown to depress the respiration of cells and increase glycolysis, while at lower concentrations aerobic respiration was stimulated (140). The level of alkaline phosphatase in epithelioid cells was raised by low concentration of prednisolone (155), while there was seen to be an increase in RNA synthesis which was not reflected in the RNA/DNA or protein/DNA ratios of the cultures (156). It must be assumed therefore that RNA turnover was increased. Such a fragmentary picture of the influence of the steroid on the molecular biology of the cell must be completed before any fundamental understanding of the influence of these drugs is possible.

The biochemical approach has been used by those interested in the use of steroids to alleviate inflammatory conditions. Mucopolysaccharide production by cells derived from articular or dermal tissue was found to be suppressed, despite a slightly increased rate of growth (153), and studies on the synthesis and subsequent sulfation of chondroitin in tissue culture (157) showed an inhibitory effect of steroids to be exerted at the latter stage.

Thus it becomes evident that while cells can be stimulated to reproduce more vigorously and maintain their morphology under the influence of the corticosteroids, the laying down by cells of a matrix for connective tissue is inhibited.

Kuchler and his co-workers have put forward an interesting theory on the mechanism whereby the corticosteroids achieve their effects (158, 159). The writers postulate, on the evidence obtained from experiments with synthetic steroids, such as estradiol and stilbestrol, that the drug molecules form a monolayer on the surface of the cell, thus preventing by occlusion of active sites, the transfer of ionized or highly polar substances across the cell membrane, as for example, amino acids. The work cited is rather small on which to base a theory so profound, but it is certainly worth pursuing. If this explanation is shown to be correct, then the lethal effects could be explained by a starvation of the cell, denied its nutrients by a blockade of steroid. However, the stimulating effect of lower concentrations awaits a rational explanation.

The chemical modifications which can be made to the steroid nucleus have led several workers to investigate the relationship between structure and activity. Substitution of a hydroxyl in the 11 position increased the toxicity markedly (160), while a keto group in the same position had little effect. Biswas and his colleagues (161) tested 99 different steroids in a simple system consisting of observing outgrowth of fibroblasts from explanted fragments of embryonic chick hearts, and of these substances 36 showed some degree of toxicity which could not be related to the chemical structure of the molecules. The stereochemical configurations were, however, not considered, and it is feasible that some evidence may be obtained to indicate a common mode of action. The addition of a fluorine atom to the molecule increased its toxicity despite the presence of other chemical groupings at other sites (162).

Berliner (163, 164) has observed that different types of cells vary in their metabolic substitution of the basic steroid molecule. On this basis, he postulates that the varying susceptibilities of cells may stem from the different cellular metabolites, which will vary in their toxicity. This work also deserves closer attention, and it is to be hoped that attempts will be made to place the suggestion on a firmer foundation than that upon which it presently rests.

The role of hydrocortisone in suppressing the immune mechanism of the body has been a source of considerable speculation and investigation resulting *inter alia*, in the discovery that cells pretreated for 1 hr. with hydrocortisone are more susceptible to infection by polio virus (165), although simultaneous addition of virus and steroid depressed the infectivity of the virus.

This latter observation received support in a series of experiments (166, 167) which showed that cortisone protected cells from the cytopathic effect of the virus normally caused possibly by lysis of lysosomal membranes, while Holowczak (168) showed that hydrocortisone and actinomycin D together substantially reduced the number of virus particles entering the cells.

The production of interferon by cells in culture is under intensive study, and the production of an antiviral substance by cells growing in the presence of hydrocortisone and virus (169) has been noted, although others claim that the presence of subtoxic concentrations of steroid depress the production of interferon by cells when in contact with pox or arbor viruses (170). Such an observation is in line with clinical observations, but the work is in its early stages and the whole question of interferon production and viral infectivity must be better understood before a rational explanation of the influence of steroids or any other drug is forthcoming.

The corticosteroids have not been the sole group of steroids to be examined for toxicity or activity in tissue culture systems. Of the sex steroids, estrone is unusual in that no toxic properties could be shown under the conditions of a simple outgrowth experiment (161). On the contrary, it was responsible for stimulating normal cell division in hamster ascites cells which would otherwise form multinucleate giant cells (171). This activity was suppressed by the incorporation, along with estrone, of progesterone or a chemical derivative. The degree of suppression could not be related to either chemical structure or progestational activity. Progesterone itself, and also the synthetic estrogen, estradiol, show varying toxicity to different cell types ranging from toxic to chick fibroblasts to growth stimulatory to other lines at similar concentrations (172, 173). Of the steroids tested on mouse mammary tumors, progesterone was the most toxic (174), although such a tissue, which is

normally influenced by the sex hormones, cannot be directly compared with the tissues used in other work. Testosterone and androsterone have also been shown to be toxic to most cells at low concentrations compared to the corticosteroids (151 ,173), although Lasnitzki (175), using organ culture, has maintained the differentiation of young rat prostate glands. Older glands display edepithelial hypertrophy and abnormal mitoses which raised the question of testosterone acting as a carcinogen to senile glands *in vivo*, a point which might be remembered when elderly patients are under treatment with the drug.

Such work, however, borders on the mass of study which has been undertaken, using cell and organ culture, on the physiological actions and relationships of the steroids and associated hormones. The line of demarcation between pharmacology and physiology is at best tenuous and ill-defined. Nevertheless, an account of this work must fall outside the scope of this review.

Thyroid—As in the case of steroids, there are conflicting reports here too, some finding stimulation and some finding inhibition. This difficulty has been mentioned in a general review on the mechanism of action of thyroid hormone by Barker (176). Much of the work involving thyroid has dealt with the development of embryonic bone in culture. Although originally undertaken as work on embryology, this type of work could serve as a basis for assay or as a means of comparing related substances. One early established fact (177) in work resulting from the observation of bone changes in hyper- and hypothyroidism was that different embryonic chick bones, in the same experimental conditions, showed different susceptibilities. For example, radii and humeri responded to the growth promoting activity of thyroxine, while to femora and tibiae a harmful effect was observed. This could well be one factor accounting for conflicting reports. An interesting later work by the same authors (178) compared the effects of L-triiodothyronine and of thyroxine on the same tissue, reporting that both substances inhibited growth, but that the former was about four times as potent as the latter. Both, however, showed more effect on the more rapidly growing bones. A further factor reported to affect the result is the age of the chick embryo, stimulation changing to retardation with increasing age, considering radii and ulnae. Different effects on different bones or on the same bones at different stages in development are noted also by Dieterlin-Lievre (133), who mentions that increase in dose can even change stimulation to inhibition. Thus, in any quantitative experiments investigating the effects of thyroid on bone, the bone used, its age, and its growth rate would have to be considered.

The effect of thyroid on tissues other than bone has been investigated less, although stimulation of nerve tissue has been reported (179) and also retardation of embryonic lung and kidney tissue (180). This report, however, mentions that no retardation was observed in lines of cells that had been long in culture, indicating again that these may change in metabolism and therefore be of less value in observing and measuring drug effect than are primary explants.

Parathyroid—There are reports too of bone resorption experiments in connection with parathyroid. One comprehensive and mathematically sound paper (181) lists the various factors affecting the result, which would have to be kept constant in a quantitative assay. The tissue culture method is compared with the rat assay method and found to be more sensitive and to require less material, but to be less precise and to take longer. However, the author cautions that even fuller investigations into the factors affecting the result by tissue culture are required since changes in sera in the media affected it, with no reason for this being evident. Another report (182) suggests that constituents in the medium which bind protein may affect the results, and a further report (183) indicates that sulfated polysaccharides may stimulate bone resorption by parathyroid—though it could not be determined whether it was by virtue of the sulfate *per se*.

Insulin—A hormone which has received much attention from a tissue culture point of view is insulin. It is in fact fairly often included in tissue culture media for a growth-promoting effect. As will be seen, however, many cell types have been used in investigations of insulin, and again contradictory results of stimulation are found in these reports, some of which are difficult to evaluate for want of adequate information.

Organ culture of chick bones has been used (184) to demonstrate the ability of insulin to inhibit longitudinal growth of the bones while the epiphyses became abnormally large. A curious phenomenon was described whereby a second shaft formed alongside the original bone shaft. The histological differentiation and maturation of the bones was retarded, with poorer staining of cartilage. The level of 0.16 I.U. insulin per ml. of medium is however far in excess of any therapeutic dose which tissues *in vivo*, other than the subcutaneous tissues at the site of injection, are likely to encounter. Further work on bone (133) indicated that previously

reported micromelia was in fact directly due to insulin and not an indirect effect of hypoglycemia.

The same organ culture technique has been used (185) to study the effects of insulin on brown fat. Both glycogen and lipid contents of the cells showed an increase, although the glycogen fell after 3 days while the level of intracellular lipid remained high. The response was most marked in a medium of serum, but such results could be explained by the presence of insulin in the serum itself in addition to the added hormone, or to selective adsorption as suggested by Waymouth (186). She concluded from her own work and the examination of that of numerous others that, since insulin is adsorbed onto glass especially in the absence of other protein, in media containing protein the effect of insulin would probably be seen at lower levels.

An attempt to observe cellular uptake of fluorescein-labeled insulin (187) was unsuccessful. Failure was attributed to differing sensitivities of cell types (those used here being hamster kidney, both normal and tumor and Landschütz ascites cells) since Paul (188) had noted a greatly increased rate of pinocytosis (*i.e.*, uptake) in HeLa cells. This he conjectured to be due to either more energy made available to the cell by insulin for this kind of activity or a direct action of insulin on the cell membrane. On the other hand, Danes and Freshney (189) did not find insulin increasing pinocytosis, their measure of pinocytosis being not vacuole counts but uptake of iron–dextran.

This sensitivity difference of different cell types toward insulin and variation with media used was again recorded (190), insulin being found to promote growth of human fetal skin fibroblasts in maintenance medium but having no effect on their growth rate in growth-promoting medium. HeLa cells, on the other hand, increased their cell material but not cell number under the influence of insulin in maintenance medium, and their growth rate in growth-promoting medium was also significantly increased in the presence of the hormone. Insulin has also been found to have a significant effect on cell numbers in chick heart explants. The increase in the rate of cell division was coupled with a rise in RNA phosphate per cell (191). Waymouth (186) reported also an elongating effect of insulin on L cells. Since this took place after a few hours, it is considered a direct effect on existing cells and not selective survival of long cells.

The metabolic effects of insulin have been studied, using fresh chick embryo and L cells (188). Carbohydrate metabolism was stimulated, with a rise in glucose utilization and lactate production, while pyruvate levels in the medium fell. Stimulation of synthetic processes was found to be erratic, especially with fresh explants. The explanation of this unpredictable reaction was not forthcoming, but the suggestion was made that it might be associated with the ability of the cells to produce high energy compounds dependent on the previous treatment of the cells. Glucose utilization increase by HeLa cells in growth-promoting medium, was reported by Leslie *et al.* (190), and the rate of incorporation of various amino acids into protein has also been reported (192).

Briefly, the effect of insulin is most marked at a metabolic level, stimulating carbohydrate, lipid, and protein metabolism. The effects of insulin are due solely to its hormonal action since the concentrations of the hormone were normally too low for it to be utilized by the cells as a normal protein.

Gonadotropic Hormones—Much work has been done on these also in tissue culture (193). Human chorionic gonadotropin or prolactin separately were almost inactive in incorporating acetate-^{14}C into cholesterol in normal rat corpus luteum *in vitro*, but together they increased the cholesterol labeling by 90%. Such synergism would need to be taken into account if either was being assayed *in vitro*. Follicles and stromal cells of rat ovary are also reported (194) to be stimulated by gonadotropic hormone in culture, but this work comments that species of origin of tissue may be important in assessing these substances, different results having been obtained with mouse tissue.

Alkaloids—These substances and other weak bases, although differing widely in their pharmacological action *in vivo*, mostly seem to produce similar morphological change, vacuolization, in cells in culture. Tissue culture could not so far be used to distinguish many alkaloids from one another, although it could distinguish them from toxins or metabolic inhibitors whose effect is irreversible. There is one extremely comprehensive article on substances producing vacuolization (195) showing that this is an easily observed cell change and the extent of it varied with concentration. The article goes on to show that changes with concentration are in fact caused by this and not by osmotic pressure changes. From the point of view of making a quantitative assay the occurrence or nonoccurrence of vacuolization would be preferable as an end point to a rating system for amount of vacuolization.

Alkaloids and other weak bases producing vacuolization include procaine hydrochloride and amide, cocaine, atropine, ephedrine, pilo-

carpine, and ammonium chloride. However, dibucaine and quinidine, in similar concentrations, did not produce vacuoles in similar cells, although the cells did die (195). A further report of Buchsbaum and Kuntz (196) also refers to vacuolization, and makes the very useful contribution of an excellent design of equipment for such study. Another useful article on drug-induced vacuolization (197) describes similar reversible effects produced by various autonomic drugs in malignant but not in normal cells, and only if the cells were initially undamaged. Structure of the drug molecule could not be correlated with production of vacuoles, and some drugs antagonistic *in vivo* had additive effects in respect of vacuolization.

The present authors have never observed, or seen illustrations of, drug-induced vacuolization as extensive as the state produced by vacuolating virus. The actual cell size does not seem to enlarge in the former. It may be as Yang, Strasser, and Pomerat (195) reported that the vacuole is an attempt to dilute out the drug, and if this is so, it may be that the cell is overcome ultimately by lethal effects of the drug setting in before vacuolization becomes as pronounced as with virus. None of the above experiments, however, was continued to cell death, since the reversibility of this effect was one of the objectives of this investigation. Vacuole production is not specific to alkaloids and chemically similar substances, having been observed also from plant polysaccharides (197). Nevertheless, its nonspecificity does not prevent its being a useful index of toxicity. A more fundamental investigation of precise causes of vacuolization and nature of vacuole contents is difficult owing to the intangible nature of the vacuoles. Morphine sulfate also produced vacuoles (198) and in fact the cells developed a tolerance to it, becoming able to withstand doses which would have been lethal to fresh cells. In addition, the cells exhibited withdrawal symptoms, adopting morphological characteristics of unhealthy cells. Another alkaloid inducing vacuolization is nicotine (199, 200), but unlike the experiments with morphine, those with nicotine showed neither dependence nor withdrawal symptoms. Another effect of nicotine on cells has also been reported (201). It depressed ciliary activity in ciliated cells, doing this to a greater extent in the presence of influenza infection than when alone. Nevertheless, these works show that ciliary activity is a useful change to observe in cells, and that it can be observed with normal cinemicrographic apparatus, high speed photography of the cilia not being necessary. Another work (202) on inhibition of cilia by nicotine unfortunately used amphibian cells growing at room temperature. The effect of berberine has also been observed (17). The cells rippled, frothed, and bubbled violently, similar to the effects produced by the dibucaine and quinidine mentioned above.

One alkaloid with a very characteristic effect on cells is, of course, colchicine, which arrests mitosis in the metaphase. This effect and its inhibition have been studied (17) using phase contrast cinemicrography, and a method has been developed (203) for measuring movements of isolated cells under the influence of colchicine or other substances. Experiments on combinations of colchicine and other substances showed that narcotine, bulbocarpine, and some apomorphine derivatives exerted a synergistic effect on the action of colchicine and related compounds (204), while tropolone and myo-inositol specifically antagonized its action (205). Such results with mixtures would have to be borne in mind in assays for any of these substances, just as in the case of chorionic gonadotropic hormone and prolactin mentioned above.

Other Drugs—No other drug or group of drugs has been studied nearly so extensively as the above in respect to its effects on cells.

Some antihistamine drugs have been studied (206) since they may be applied directly to nasal mucosa cells. These cells unfortunately were not used in the experiments, but in all the types of cells that were used (heart, intestine, and lung fibroblasts) clinical concentrations of the drugs produced no inhibition of growth, although the cells were vacuolated, however. On the other hand, the same workers (1) comment on the high toxicity of various antihistamines toward outgrowth from primary explants of normal adult human skin. Inhibition of tumor cells *in vitro* has also been reported (207), the authors speculating on the relation of this to reduced histamine found in cancer tissue.

One drug, sodium fluoride, has been used in experiments which have received more publicity than was perhaps expected. While the effects of sodium fluoride on cells are of interest to cytologists, they are of interest also to those concerned with adding the substance to water supplies deficient in it, to prevent dental decay in young children. There has been controversial correspondence on whether growth was slowed by the concentrations advocated for drinking water or only by higher concentrations (208–213). A further report (214) deals with inhibition of oxygen uptake and glycolysis but not inhibition of phagocytosis by sodium fluoride. So far it

can be said that inhibition does occur at the various concentrations reported on the cell types used, but it is too early to correlate this with any effect *in vivo*.

Salicylate analogs, cinchophen and chloroquine derivatives, and phenylbutazone and related compounds were examined by Whitehouse (215) using his technique of culturing and assaying cartilage. Anomalies between his results and *in vivo* findings were encountered and probably point to intermediate metabolism of the drugs into active forms by other organs. Sodium aurothiomalate and saligenin, for example, were inactive, but thiomalic acid and salicylate were effective.

Chloroquine has been further investigated for cell-inhibiting properties (62, 216) using chick embryo cells. Vacuolization and decreased growth were observed at 1/40,000, although lower concentrations stimulated growth. The cells also disaggregated to individual cells, like trypsinized cells. A detailed work on chloroquine (217) was undertaken, in view of its use in some conditions with abnormalities of the immune response, to find exactly what its mode of action in such cases might be. It was concluded that it "stabilized" lysosome membrane preventing enzyme release which would start lymphocyte derepression. It appeared to act only on lymphocytes responding to a stimulus. This paper throughout seems logically reasoned and mathematically sound, and for these reasons, as well as its topic, it is of interest.

Organophosphorus substances have been studied (218), their amount being estimated by the effect on ciliary activity of cells *in vitro*. Decrease of ciliary activity is not of course a specific effect of these substances, having been observed also in connection with local anesthetics (219) and nicotine (201).

An interesting use has been made of isolated, beating, chick embryo heart cells to compare digoxin, quinidine, and procaine amide (220). The action of digoxin was to produce arrhythmias, and this is in keeping with the view that digitalis preparations have an intracellular action. Quinidine and procaine amide had a depressant action on beating, and the potentiating effect of quinidine on digitalis recorded *in vivo* was reflected in tissue culture, indicating the potential usefulness of tissue culture model in studying cardiac drugs. Very little use has been made of this so far. In fact, the comment made in 1954 (1) is still applicable: "Similar (i.e. tissue culture) screening may be made of the action of cardiac glucosides on beating heart fragments. This possibility has not yet been adequately explored, but it offers many promising opportunities for fruitful observations."

Acetylcholine has also been used (221) in a study of the sensitivity to the drug of rat diaphragm muscle fibers when grown by organ culture methods. It was concluded that muscle fibers themselves produced changes in the fiber membrane, once nervous connection had been lost, to enable it to react to cholinergic drugs, thus confirming previous *in vivo* findings.

The irritant action of a number of substances, including podophyllin, croton oil, turpentine, and peptone was studied (222), and their effect on the motility of leucocytes was observed.

Anticoagulants have not been widely studied (223, 224). The former studies compared the effect of clinical concentrations of heparin, polyanetholsulfonic acid, and sodium citrate on leucocyte migration and phagocytosis. Only the heparin did not affect these. However, the author found no correlation between migration and phagocytosis, though later work usually describes a cessation of movement during pinocytosis. The other work involving heparin was with hamster sarcoma cells where effects were seen on the morphology, contents, mitotic index, and chromosomes, leading the authors to wonder if these were caused by a combination of sodium citrate with the proteins of the mitotic spindle. Further evidence on a relation between heparin and cell division was described by Lippman (225), who considered that, if mucopolysaccharides were removed from the cell surface, Ca^{2+} could then penetrate, increase cytoplasmic viscosity, and initiate chromosome condensation, and thence cell division. This paper includes a list of many sulfated polysaccharides reported to block cell division in many types of cells.

The tissue culture method offers scope for the development of assay methods for some immunological preparations. As is well known (23), there are still several such products for which there is no assay which correlates well with clinical results. The group of immunological products most obviously and directly lending itself to this assay method is the antisera, whose protective capacity against the relevant toxin may be assessed on cells. There are of course already some officially recognized tests of this nature, *e.g.*, the Biological Assay of Poliomyelitis Neutralising Antibody of the "British Pharmacopoeia," where the content of this antibody in a sample of serum is determined by comparing the dose of serum necessary to protect tissue cultures against infection from a test dose of poliomyelitis virus with the dose of a standard serum necessary to give the same protection (226). The cells

used are usually monkey kidney and the index of toxicity is cell damage as seen by light microscope. Tissue culture work has included also work on antibody production *in vitro* (227) performed with a view to gathering further knowledge on the mechanisms of antibody production and therefore irrelevant to this paper.

The application of tissue culture in active immunity preparations would be in testing toxoids and vaccines for freedom from toxicity and, where appropriate, freedom from live viruses. There are five recent works on a tissue culture assay for diphtheria preparations. The indices of toxicity investigated have been demyelination of fetal rat cord ganglion cells (154) or inhibition of protein synthesis in KB cells (228), a result being obtainable here in 90 min., which is usefully quick. Also the cellular method has been reported (229) to be quicker and cheaper than the use of guinea pigs. However, one important point still to be cleared up is whether different cell types affect the result. There are reports both for and against this (*References 230* and *231*, respectively).

Staphylococcal toxins have also been investigated in tissue culture (232, 233) on various cell lines, using both morphological and biochemical indices of toxicity. Although the work was done to gain knowledge on the relation between pathogenicity and the production of various toxins, the method is equally applicable to assay, the basis being there already in the mathematical detail quoted in these papers.

In connection with diagnostic immunological preparations, a proliferative effect of tuberculin purified protein derivative on human peripheral macrophages has been reported (234), but no correlation was established between this effect and the donor's skin sensitivity state toward ordinary tuberculin testing.

In the field of nonmicrobiological immunologicals, spider venom has been reported (235) to produce degenerative changes in HeLa and L cells within a few hours, the changes not occurring if the venom was neutralized with antiserum. This again could serve as a basis for assay of the antivenom.

It seems then that the most promising "growth points" of tissue culture as applied to immunological preparations are the quantitative assays of antisera and the testing for freedom from toxicity of viral vaccines and bacterial toxoids.

Drugs acting on nerve cells are probably the most difficult to investigate by tissue culture (236), since these cells in culture, if normal, probably move and divide less than most other cell types. A biochemical rather than a microscopical index of effect is therefore indicated. However, other nervous system cells have been used—for example, oligodendrocytes (237)—to study the effect of chlorpromazine, an increase in contraction rate being the effect observed. The toxicity of barbiturates toward outgrowths from embryonic chick spinal cord and heart tissues has also been measured (238). It was noted that heart tissue was more resistant to the range of barbiturates tested. The toxicity of convulsant barbiturates was in keeping with their *in vivo* toxicity, but anomalies were blatant in the case of hypnotic barbiturates. Such anomalies would probably indicate an *in vivo* metabolic pathway synthesizing a more or a less toxic molecule.

When the discovery was being investigated that the antiepileptic drug phenytoin (B.P.) aided wound healing, it was found (239) that it stimulated the growth of a line of human gingival fibroblasts, although experiments with analogs failed to clarify which part of the molecule was responsible. The mode of action was not cleared up by a later comprehensive work (240), using primary explants from dental surgery from 15 patients to set up 596 test experiments and 533 controls.

Finally, the tragedy of thalidomide stimulated work into its effects on various tissues grown *in vitro*, resulting in the report (241) that the soluble hydroxy derivative of the drug was capable of inhibiting the synthesis of protein by embryonic cells. Other work (242) has endeavored to find the mechanism of action whereby thalidomide inhibited small lymphocytes in culture using the two racemates and various degradation products, all in concentrations similar to those teratogenic in animals. It was hoped that this might give a clue to the teratogenic mechanism. However, more work is required for any proof, and only a theory could be put forward, that thalidomide might be antagonistic to glutamic acid or glutamine.

CONCLUSION

The use of tissue culture techniques in the study of drug action and toxicity is as yet in its infancy. The bulk of the work presented in this publication has dealt with the toxicity of materials toward explants of tissues, often embryonic avian tissues whose metabolism cannot be comparable in every respect to the metabolism of human tissues.

Generally, toxicity is recognized by failure of a tissue or cell culture to grow. However, growth in tissue culture systems is difficult to define. It may be taken to mean an increase in cell number,

increase in cell size, or increase in metabolic rate. These criteria, however, may be completely independent and affected by unrelated factors. It has been repeatedly emphasized that the different indices of growth are not necessarily synonymous. Cunningham and Kirk (32) found area of explant, cell size, and cell number may vary independently, and Ritter (243) found that protein and cell counts could also vary independently. The authors feel confident that, as the effect of more drugs in tissue culture are investigated, more, and more specific, effects will be observed. For example, in their own work, a curious arcing of H.Ep. 2 cells has been observed with spermine (22). Effects on stages of cell division are the group of effects investigated in greatest detail as yet, leaving much to be done on all the other aspects of cell biology.

The advent of time-lapse cinemicrography and the sophistication of many techniques in cytology and biochemistry have led to the possibilities of investigation into the detailed action of medicinal substances on tissues and on cells, and of the metabolism of the drugs by the cells. For too long, this has been a subject where isolated workers scratch at the outside of a particular problem when the possibilities of reaching a fundamental solution have been numerous. Fortunately, in the laboratories of the world, attention is now being paid to those basic phenomena essential to the life of the cell. Ultimately there will be increased knowledge of the effect of drugs in their widest sense on those phenomena, a logical sequence to the more elementary gross toxicity tests which have been hitherto carried out. The rewards of such a study are potentially great, and tissue culture techniques have clearly an increasing part to play in the study of the interaction between the organism and pharmacologically active substances.

SUMMARY

This review investigates the effects of drugs on animal cells grown *in vitro*. The cells include separated individual cells, tissues, and small organs, both primary explant material and serially propagated lines, and also material from various species. The techniques used to examine the effects of the drugs on the cells include simple microscopy, time-lapse cinemicrography, electron microscopy, and many biochemical estimations.

It is shown that only a small fraction of available drugs have as yet been examined for direct cellular effect. Also it is shown that the method can, with value, be included in routine examination of new drugs. The advantages and limitations of this technique are listed and compared with other pharmacological techniques.

REFERENCES

(1) Pomerat, C. M., and Leake, C. D., *Ann. N. Y. Acad. Sci.*, **58**, 1110(1954).
(2) Diamond, L., and Koprowski, H., *Am. Assoc. Cancer Res. Proc.*, **6**, 15(1965).
(3) Coriell, L. L., Greene, A. E., and Silver, R. K., *Cryobiology*, **1**, 72(1964).
(4) Foley, G. E., Handler, A. H., Lynch, P. M., Wolman, S. R., Stulberg, C. S., and Eagle, H., *Cancer Res.*, **25**, 1254 (1965).
(5) Malinin, T. I., and Perry, V. P., *Cryobiology*, **2**, 18 (1965).
(6) Malinin, T. I., Perry, V. P., and Dolan, N. F., *ibid.*, **2**, 19(1965).
(7) Narula, P. N., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19**, No. 105 (1965).
(8) Gillette, R. W., Goulian, D., Jr., and Conway, H., *J. Natl. Cancer Inst.*, **34**, 643(1965).
(9) Pomerat, C. M., *Methods Med. Res.*, **4**, 198(1952).
(10) Pomerat, C. M., *J. Am. Vet. Med. Assoc.*, **129**, 458(1956).
(11) Fell, H. B., *J. Clin. Pathol.*, **11**, 489(1958).
(12) Osgood, E. E., *J. Lab. Clin. Med.*, **24**, 954(1939).
(13) Murray, M. R., and Kopech, G., "A Bibliography of the Research in Tissue Culture," Academic Press Inc., New York, N. Y., 1953.
(14) Phillips, H. J., and Andrews, R. V., *Exptl. Cell Res.*, **16**, 678(1959).
(15) Kay, E. R. M., *Cancer Res.*, **25**, 764(1965).
(16) Verne, J., *Ann. N. Y. Acad. Sci.*, **58**, 1195(1954).
(17) Lettré, R., *ibid.*, **58**, 1085(1954).
(18) Dawson, M., *J. Roy. Microscop. Soc.*, **82**, 1(1963).
(19) Dawson, M., Dryden, W. F., and Matthews, J. E., *ibid.*, **83**, 391(1964).
(20) Dawson, M., Dryden, W. F., and Matthews, J. E., *Proc. Roy. Microscop. Soc.*, **1**, 156(1966).
(21) Dawson, M., and Dryden, W. F., "Toxicity Study of Phagocytin on Human Skin Cells," film shown at annual meeting of British Association for the Advancement of Science, Cambridge, England, September 1965.
(22) Dawson, M., and Dryden, W. F. "Toxicity Testing in Tissue Culture," film shown at meeting of Research Film Section of International Scientific Film Association, Roscoff, France, September 1966.
(23) Dawson, M., "Immunological and Blood Products," Heinemann, London, England, to be published.
(24) Hsu, T. C., *Texas Rept. Biol. Med.*, **10**, 336(1952).
(25) Lépine, P., Barski, G., and Maurin, J., *Proc. Soc. Exptl. Biol. Med.*, **73**, 252(1950).
(26) Ulloa-Gregori, O., Blocker, T. G., Nowinski, W. W. and Pomerat, C. M., *Texas Rept. Biol. Med.*, **8**, 400(1950).
(27) Heilman, D. H., *Bacteriol. Proc.*, Abstr. No. M.8, 1965, p. 41.
(28) Fleming, A., *Brit. J. Exptl. Pathol.*, **10**, 226(1929).
(29) Chabbert, Y. A., and Vial, H., *Exptl. Cell Res.*, **22**, 264(1961).
(30) Paul, J., "Cell and Tissue Culture," 3rd ed., Livingstone, Edinburgh, Scotland, 1965, pp. 304–343.
(31) Merchant, D. J., Kahn, R. H., and Murphy, W. H., Jr., "Handbook of Cell and Organ Culture," 2nd ed., Burgess Publishing Co., Minneapolis, Minn., 1964.
(32) Cunningham, B., and Kirk, P. L., *J. Cellular Comp. Physiol.*, **20**, 343(1942).
(33) Cruickshank, C. N. D., "Report of the Medical Research Council for the Year 1959–1960," Her Majesty's Stationery Office, London, England, 1961, p. 40.
(34) Cruickshank, C. N. D., *Exptl. Cell Res.*, **7**, 374 (1954).
(35) Glick, D., "Quantitative Chemical Techniques of Histo- and Cytochemistry," Interscience, vol. 1, London, England, 1964.
(36) Danes, B. S., Christiansen, G. S., and Leinfelder, P. J., *Exptl. Cell Res.*, **5**, 234(1953).
(37) Danes, B. S., Broadfoot, M. M., and Paul J., *ibid.*, **30**, 369(1963).
(38) Edwards, B. F., and Lee, J. B., "Tissue Culture Association Annual Meeting Abstract Papers, in *Excerpta Med.*, **19**, No. 63 (1965).
(39) Holmes, R., and Eliassen, B. P., *ibid.*, **19**, No. 61 (1965).
(40) Cobb, J. P., Walker, D. G., and Wright, J. C., *Cancer Res.*, **21**, 583(1961).
(41) Paul, J., *ibid.*, **22**, 431(1962).
(42) Hurley, J. D., and Yount, L. J., *Am. J. Surg.*, **109**, 39(1965).
(43) Wojewski, A., and Przeworska-Kaniewicz, D., *J. Urol.*, **93**, 721(1965).
(44) Wright, J. C., Cobb, J. P., Gumport, S. L., Golomb, F. M., and Safadi, D., *New Engl. J. Med.*, **257**, 1207(1957).
(45) Chýle, P., *Folia Biol. (Prague)*, **9**, 312(1963).
(46) Kuhns, W. J., Bramson, S., Chessin, L., and Hirschhorn, K., *Federation Proc.*, **24**, 368(1965).
(47) Caspersson, T., Farber, S., Foley, G. E., Killander D., and Zetterberg, A., *Exptl. Cell Res.*, **39**, 365(1965).

(48) Di Paolo, J. A., *Cancer Chemotherapy Rept.*, **44**, 19(1965).
(49) Wilson, C. B., and Barker, M., *ibid.*, **44**, 9(1965).
(50) McDonald, G. O., and Cole, W. H., *Surgical Forum*, **X**, 67(1959).
(51) Di Paolo, J. A., and Dowd, J. E., *J. Natl. Cancer Inst.*, **27**, 807(1961).
(52) Johnson, R. E., Zelen, M., and Kemp, N. H., *ibid.*, **34**, 265(1965).
(53) Kupiecki, F. P., *Cancer Res.*, **25**, 417(1965).
(54) Lasnitzki, I., *J. Natl. Cancer Inst.*, **35**, 339(1965).
(55) Morasca, L., *European J. Cancer*, **1**, 141(1965).
(56) George, P., Journey, L. J., and Goldstein, M. N., *J. Natl. Cancer Inst.*, **35**, 355(1965).
(57) Eagle, H., and Foley, G. E., *Am. J. Med.*, **21**, 739 (1956).
(58) Limbosch-Rolin, S., *Ann. Biol.*, **74**, 63(1963).
(59) Cobb, J. P., *Ann. N. Y. Acad. Sci.*, **84**, 513(1960).
(60) Galbraith, W., Mayhew, E., Roe, E., Handel, J., and Sugár, J., British Empire Cancer Campaign for Research, 41st Annual Report, 1963, p. 126.
(61) Yarnell, M., Ambrose, E. J., Shepley, K., and Tchao, R., *Brit. Med. J.*, ii, 490(1964).
(62) Hiraki, K., and Kimura, I., *Acta Med. Okayama*, **17**, 231(1963).
(63) Toplin, I., *Cancer Res.*, **19**, 959(1959).
(64) McBride, T. J., Schmitter, R. W., Oleson, J. J., and Toplin, I., *ibid., Suppl.*, **23**, 1(1963).
(65) Kim, J. H., and Eidenoff, M. L., *Federation Proc.*, **24**, 331(1965).
(66) Rich, M. A., Meyers, P., Weinbaum, G., Cory, G. C., and Suhadolnik, R. J., *Biochim. Biophys. Acta*, **95**, 194(1965).
(67) Antikajian, G., Wright, L. T., Plummer, J. I., and Weintraub, S., *J. Natl. Cancer Inst.*, **12**, 269(1952).
(68) Talyzina, V. A., Timofeevskaya, E. A., Urazova, A. P., and Firsova, G. A., *Antibiotiki*, **X**, 722(1965).
(69) Mungyerova, G., Babusikova, O., Kalafut, F., and Jacz, K., *Neoplasma*, **12**, 289(1965).
(70) Umeda, M., *Acta Pathol. Japon.*, **14**, 373(1964).
(71) Toplin, I., *Cancer Res.*, **21**, 1042(1961).
(72) Perry, W. L. M., Design of Toxicity Tests, Medical Research Council Special Report Series, No. 270, His Majesty's Stationery Office, London, England, 1950.
(73) Fleming, A., *Proc. Roy. Soc. Sci., Ser. B*, **96**, 171 (1924).
(74) Abraham, E. P., Chain, E., Fletcher, C. M., Gardiner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, ii, 177(1941).
(75) Gey, G. O., Gey, M. K., Inui, F., and Vedder, H., *Bull. Johns Hopkins Hosp.*, **77**, 116(1945).
(76) Perlman, D., and Brindle, S. A., *Bacteriol. Proc.*, Abstr. No. V136, 1965.
(77) Brotzu, G., *Minerva Chir.*, **17**, 1254(1962).
(78) Perlman, D., Guiffre, N. A., Jackson, P. W., and Giardinello, F. E., *Proc. Soc. Exptl. Biol. Med.*, **102**, 290(1959).
(79) Sallé, A. J., and Lazarus, A. S., *ibid.*, **34**, 371(1936).
(80) Sallé, A. J., McOmie, W. A., and Schechmeister, I. L., *J. Bacteriol.*, **34**, 267(1937).
(81) Sallé, A. J., McOmie, W. A., Schechmeister, I. L., and Foord, D. C., *ibid.*, **37**, 639(1939).
(82) *Ibid.*, **37**, 694(1937–1938).
(83) Witlin, B., *Am. J. Pharm.*, **113**, 203(1941).
(84) *Ibid.*, **113**, 353(1941).
(85) Paff, G. H., Lehman, R. A., and Halperin, W. H., *Proc. Soc. Exptl. Biol. Med.*, **58**, 323(1945).
(86) Jacoby, F., *Brit. J. Exptl. Pathol.*, **26**, 137(1945).
(87) Jacoby, F., Medawar, P. B., and Willmer, E. N., *Brit. Med. J.*, ii, 149(1941).
(88) Pomerat, C. M., Capouya, M., and Greenleaf, F. I., *Texas Rept., Biol. Med.*, **2**, 97(1944).
(89) Ostertag, W., and Kersten, W., *Exptl. Cell Res.*, **39**, 296(1965).
(90) Scholtissek, C., *Biochim. Biophys. Acta*, **103**, 146 (1965).
(91) Kutzsche, A., Merker, H.-J., and Schwarz, W., *Naturwissenschaften*, **52**, 118(1965).
(92) Robson, J. M., *J. Physiol.*, **102**, 7P(1943–1944).
(93) Baker, H. J., *Ann. N. Y. Acad. Sci.*, **58**, 1232(1954).
(94) Shaffer, J. M., Kucera, C. J., and Spink, W. W., *J. Exptl. Med.*, **97**, 77(1953).
(95) Zhukov, V. G., and Navashin, S. M., *Antibiotiki*, **X**, 318(1965).
(96) Zak, A. F., *ibid.*, **X**, 733(1965).
(97) Goldenbaum, E. G., and Fukui, G. M., *Bacteriol. Proc.*, Abstr. No. M. 176, 1965, p. 69.
(98) Mackaness, G. B., *J. Pathol. Bacteriol.*, **64**, 429 (1952).
(99) Mackaness, G. B., and Smith, N., *Ann. Rev. Tuberculosis*, **66**, 125(1952).
(100) Cohen, M. M., Shaw, M. W., and Craig, A. P., *Proc. Natl. Acad. Sci. U. S.*, **50**, 330(1963).
(101) Holland, J. J., *ibid.*, **50**, 436(1963).
(102) Cruickshank, C. N. D., and Lowbury, E. J. L., *Brit. J. Exptl. Pathol.*, **34**, 583(1953).
(103) Kaufman, H. E., *Progr. Med. Virol.*, **7**, 116(1965).
(104) Kroeker, H. M., and Bird, T. J., *Bacteriol. Proc.*, Abstr. No. V137, 1965.
(105) Maramorosch, K., Mitsuhashi, J., Streissle, G., and Hirumi, H., *ibid.*, Abstr. No. V138, 1965.
(106) Starr, T. J., Deig, E. F., Church, K. K., and Allen, M. B., *Texas Rept. Biol. Med.*, **20**, 271(1962).
(107) Carver, D. H., and Naficy, K., *Proc. Soc. Exptl. Biol. Med.*, **116**, 548(1964).
(108) Tsilinsky, Ya. Ya., *Acta Virol. (Prague) (Engl. Ed.)*, **7**, 350(1963).
(109) Pumper, R. W., Yamashiroya, H. M., and Alfred, L. J., *Nature*, **192**, 1101(1961).
(110) Billingham, R. E., and Reynolds, J., *Brit. J. Plastic Surg.*, **5**, 25(1952–1953).
(111) Medawar, P. B., *Nature*, **148**, 783(1941).
(112) Bassett, C. A. L., Evans, V. J., and Earle, W. R., *Plastic Reconstr. Surg.*, **17**, 421(1956).
(113) Fell, H. B., *Ann. N. Y. Acad. Sci.*, **58**, 1183(1954).
(114) Fell, H. B., *Brit. Med. Bull.*, **12**, 35(1956).
(115) McGowan, T. R., and Bang, F. B., *Bull. Johns Hopkins Hosp.*, **107**, 63(1960).
(116) Trowell, O. A., *Exptl. Cell Res.*, **6**, 246(1954).
(117) *Ibid.*, **16**, 118(1959).
(118) McGowan, T. R., *Natl. Cancer Inst. Monograph*, **11**, 95(1963).
(119) Boök, J. A., Fraccaro, M., and Lindsten, J., *Acta Paediat.*, **48**, 453(1959).
(120) Harnden, W. G., *Brit. J. Exptl. Pathol.*, **XLI**, 31 (1960).
(121) Gilbert, D., Mier, P. D., and Jones, T. E., *J. Invest. Dermatol.*, **40**, 165(1963).
(122) Medawar, P. B., *Quart. J. Microscop. Sci.*, **89**, 187 (1948).
(123) Parshley, M. S., and Simms, H. R., *Am. J. Anat.*, **86**, 163(1950).
(124) Söltz-Szöts, J., *Arch. Klin. Exptl. Dermatol.*, **214**, 594(1962).
(125) Lewis, S. R., Pomerat, C. M., and Ezell, D., *Anat. Rec.*, **104**, 487(1949).
(126) Penttinen, K., and Saxén, E., *Nature*, **184**, 1570 (1959).
(127) Terasaki, P. I., and Chamberlain, C. C., *J. Exptl. Med.*, **115**, 439(1962).
(128) Bakken, P. C., Evans, V. J., Earle, W. R., and Stevenson, R. E., *Am. J. Hyg.*, **73**, 96(1961).
(129) Dawson, M., Dryden, W. F., and Matthews, J. E., paper presented to the British Tissue Culture Association, Glasgow, Scotland, 1964.
(130) Currie, W. D., and Gregg, C. T., *Biochim. Biophys. Res. Commun.*, **21**, 9(1965).
(131) Schauer, P., and Likar, M., *Pathol. Microbiol.*, **28**, 371(1965).
(132) Nemes, M. M., and Hilleman, M. R., *Proc. Soc. Exptl. Biol. Med.*, **119**, 515(1965).
(133) Dieterlin-Lièvre, F., "Les Cultures Organotypiques," Masson & Cie, Paris, France, 1965, p. 135.
(134) LeDouarin, G., *Année Biol.*, **3**, 233(1964).
(135) Geiger, R. S., Dingwall, J. A., and Andrus, W. de W., *Am. J. Med. Sci.*, **231**, 427(1956).
(136) Kaufman, N., Masson, E. J., and Kinney, T. D., *Am. J. Pathol.*, **29**, 761(1953).
(137) Rozen, V. B., and Chernin, L. S., *Federation Proc. Transl. Suppl.*, **24**, T861(1965).
(138) Grossfield, H., and Ragan, C., *Proc. Soc. Exptl. Biol. Med.*, **86**, 63(1954).
(139) Holden, M., and Adams, L. B., *ibid.*, **95**, 364(1957).
(140) Grossfield, H., *Endocrinology*, **65**, 777(1958).
(141) Kline, I., Leighton, J., Belkin, M., and Orr, H. C., *Cancer Res.*, **17**, 780(1957).
(142) Pihl, A., and Eker, P., *Biochem. Pharmacol.*, **14**, 1065(1965).
(143) Farnes, P., and Barker, B. E., *Metabolism*, **14**, 75 (1965).
(144) Nowell, P. C., *Cancer Res.*, **21**, 1518(1961).
(145) Elrod, L. M., and Schrek, R., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19**, No. T.53 (1965).
(146) Sujard, C., and Chany, E., *Compt. Rend.*, **257**, 973 (1963).
(147) Cornman, I., *Science*, **113**, 37(1951).
(148) Gillette, R. W., and Buchsbaum, R., *Proc. Soc. Exptl. Biol. Med.*, **89**, 146(1955).
(149) Gillette, R. W., Goulian, D., and Conway, H., *J. Natl. Cancer Inst.*, **34**, 643(1965).
(150) Schryver, H. F., *Exptl. Cell Res.*, **37**, 327(1965).
(151) Arpels, C., Babcock, V. I., and Southam, C. M., *Proc. Soc. Exptl. Biol. Med.*, **115**, 102(1964).
(152) Connor, J., and Marti, A., *Federation Proc.*, **24**, 199(1965).
(153) Castor, C. W., *J. Lab. Clin. Med.*, **60**, 788(1962).
(154) Murray, M. R., and Peterson, E. R., *Excerpta Med., Intern. Congr. Ser.*, **94**, E221(1965).
(155) Melnykovych, G., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19**, No. T14 (1965).
(156) Hijmans, J. C., and McCarty, K. S., *ibid.*, No. T48 (1965).
(157) Whitehouse, M. W., and Lash, J. W., *Nature*, **189**, 37(1961).
(158) Kuchler, R. J., Arnold, N. J., and Graver, R. C., *Proc. Soc. Exptl. Biol. Med.*, **111**, 798(1962).
(159) Campbell, G. C., and Kuchler, R. J., *Bacteriol. Proc.*, **1965**, 120.
(160) Ruhman, A. G., and Berliner, D. L., *Federation Proc.*, **24**, 575(1965).
(161) Biswas, S., MacDougall, J. D. B., and Cook, R. P. *Brit. J. Exptl. Pathol.*, **45**, 13(1964).
(162) Perlman, D., Guiffre, N. A., Brindle, S. A., and Pan, S. C., *Proc. Soc. Expil. Biol. Med.*, **111**, 623(1962).
(163) Berliner, D. L., *Cancer Res.*, **25**, 1085(1965).
(164) Berliner, D. L., *Ann. N. Y. Acad. Sci.*, **116**, 1078 (1964).

(165) Hellman, A., Kalter, S. S., and Daye, G. T., *Acta Virol. (Prague)*, **9**, 224(1965).
(166) Macieira-Coelho, A., Fernandes, M. V., and Mellman, W. J., *Proc. Soc. Exptl. Biol. Med.*, **119**, 631(1965).
(167) Wheeler, C. E., Harvie, E. A., and Canby, C. M., *J. Invest. Dermatol.*, **36**, 89(1961).
(168) Holowczak, J. A., *Bacteriol. Proc.*, **1965**, 119.
(169) Hannoun, C., Fernandes, M. V., and Macieira-Coelho, A., *Proc. Soc. Exptl. Biol. Med.*, **119**, 153(1965).
(170) Mendelson, J., and Glasgow, L. A., *Bacteriol. Proc.*, **1965**, 98.
(171) Pincus, G., and Gordon, H. L., *Steroids Suppl.*, **1**, 193(1965).
(172) Kawada, I., *Japan. J. Exptl. Med.*, **29**, 615(1959).
(173) von Haam, E., and Cappel, L., *Am. J. Cancer*, **39**, 350(1940).
(174) Rivers, E. M., Elias, J. J., Bern, H. A., Napalkov, N. P., and Pitelka, D. R., *J. Natl. Cancer Inst.*, **31**, 671(1963).
(175) Lasnitzki, I., *J. Endocrinol.*, **12**, 236(1955).
(176) Barker, S. B., *Physiol. Rev.*, **13**, 205(1951).
(177) Fell, H. B., and Mellanby, E., *J. Physiol.*, **127**, 427(1955).
(178) *Ibid.*, **133**, 89(1956).
(179) Hamburgh, M., *Anat. Rec.*, **151**, 357(1965).
(180) Leslie, I., and Sinclair, R., *Exptl. Cell Res.*, **17**, 272 (1959).
(181) Raisz, L. G., *J. Clin. Invest.*, **44**, 103(1965).
(182) Gaillard, P. J., *Develop. Biol.*, **7**, 103(1963).
(183) Goldhaber, P., "Tissue Culture Association Annual Meeting, Abstract Papers," in *Excerpta Med.*, **19**, No. 47 (1965).
(184) Chen, J. M., *J. Physiol.*, **125**, 148(1954).
(185) Sidman, R. L., *Anat. Rec.*, **124**, 723(1956).
(186) Waymouth, C., and Reed, W. E., *Texas Rept. Biol. Med.*, **23**, (Suppl. 1) 413(1965).
(187) Cormack, W. H., and Ambrose, E. J., *J. Roy. Microscop. Soc.*, **81**, 11(1962).
(188) Paul, J., *J. Exptl. Zool.*, **142**, 475(1959).
(189) Danes, B. S., and Freshney, M. S., *Texas Rept. Biol. Med.*, **23**, (Suppl. 1), 221(1965).
(190) Leslie, I., Fulton, W. C., and Sinclair, R., *Biochem. J.*, **63**, 18p(1956).
(191) Leslie, I., and Davidson, J. N., *Biochem. J.*, **49**, XLI (1951).
(192) Singh, V. N., Nataf, B. M., and Chaikoff, I. L., *Life Sci.*, **4**, 1603(1965).
(193) Gospodarowicz, D., and Legault-Demare, J., *Acta Endocrinol.*, **42**, 509(1963).
(194) Pavic, D., *J. Endocrinol.*, **26**, 531(1963).
(195) Yang, W. C. T., Strasser, F. F., and Pomerat, C. M., *Exptl. Cell Res.*, **38**, 495(1965).
(196) Buchsbaum, R., and Kuntz, J. A., *Ann. N. Y. Acad. Sci.*, **58**, 1303(1954).
(197) Belkin, M., Hardy, W. C., Orr, H. C., and Lachman, A. B., *J. Natl. Cancer Inst.*, **28**, 187(1962).
(198) Corssen, G., and Skora, I. A., *J. Am. Med. Assoc.*, **187**, 328(1964).
(199) Bucher, O., *Bull. Schweiz. Akad. Med. Wiss.*, **1**, 252 (1945).
(200) Kokubu, T., and Pollak, O. J., *Exptl. Mol. Pathol.*, **1**, 293(1962).
(201) Dawson, F. W., Harding, H. B., Ballenger, J. J., De Ruyter, M., and Moore, J., *Federation Proc.*, **24**, 302 (1965).
(202) Amenta, P. S., Imbriglia, F. X., and Ferreira, R. C., *Anat. Rec.*, **151**, 481(1965).
(203) Schaerer, P., *Acta Haematol.*, **1**, 178(1948).
(204) Lettré, H., *Ann. N. Y. Acad. Sci.*, **58**, 1264(1954).
(205) Benitez, H. H., Murray, M. R., and Chargaff, E., *ibid.*, **58**, 1288(1954).
(206) Pomerat, C. M., Lay, M., and Emerson, G. A., *Texas Rept. Biol. Med.*, **2**, 316(1944).
(207) Honti, G., and Putnoky, Gy., *Acta Morphol., Suppl. XIII*, **1965**, 70.
(208) Armstrong, W. D., Pollock, M. E., and Singer, L., *Brit. Med. J.*, **i**, 1435(1965).
(209) Berry, R. J., and Trillwood, W., *ibid.*, ii, 1064(1963).
(210) *Ibid.*, **i**, 793(1965).
(211) Nias, A. H. W., *ibid.*, **i**, 1672(1965).
(212) Armstrong, W. D., Blomquist, C. H., Singer, L., Pollock, M. E., and McLaren, L. C., *ibid.*, **i**, 486(1965).
(213) Giancotti, M., and Gruddi, A., *Ann. Stomatol.*, **14**, 139(1965).
(214) Selvaraj, R. J., Ouchi, E., McRipley, R. J., and Sbarra, A. J., *Bacteriol. Proc.*, Abstr. No. M173, 1965, p. 68.
(215) Whitehouse, M. W., *Nature*, **194**, 986(1962).
(216) Lipp, R., *Arch. Klin. Exptl. Dermatol.*, **218**, 228 (1964).
(217) Hurvitz, D., and Hirschhorn, K., *New Engl. J. Med.*, **273**, 23(1965).
(218) di Mattei, P., and Melchiorri, P., *Arch. Intern. Pharmacodyn.*, **153**, 339(1965).
(219) Corssen, G., and Allen, C. R., *Anesthesiology*, **21**, 237 (1960).
(220) Mercer, E. N., and Dower, G. E., *J. Pharmacol. Exptl. Therap.*, **153**, 203(1966).
(221) Miledi, R., and Trowell, O. A., *Nature*, **194**, 981 (1962).
(222) Wallbach, G., *Arch. Anat. Microscop.*, **33**, 251(1937).
(223) Allgöwer, M., *Schweiz. Med. Wochschr.*, **77**, 40(1947).
(224) Coatăchel, Q., Fadei, R., and Nachtigal, M., *Exptl. Cell Res.*, **34**, 542(1964).
(225) Lippman, M., *Trans. N. Y. Acad. Sci., Ser. II*, **27**, 342(1965).
(226) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 1123.
(227) Ambrose, C. T., *Federation Proc.*, **24**, 377(1965).
(228) Moehring, T. J., Kuchler, R. J., and Solotorovsky, M., *Bacteriol. Proc.*, Abstr. No. M52, 1965, p. 48.
(229) Kyrchikov, B. A., Isupov, F. G., and Egorov, P. A., *J. Microbiol. Epidemiol. Immunobiol.*, **42**, 85(1965).
(230) Gabliks, J., *Bacteriol. Proc.*, Abstr. No. M53, 1965.
(231) Kamzolkina, N. B., Lukashova, N. I., and Borisova, L. V., *J. Microbiol. Epidemiol. Immunobiol.*, **42**, 80(1965).)
(232) Korbecki, M., and Jeljaszewicz, J., *J. Infect. Diseases*, **115**, 205(1965).
(233) Jeljaszewicz, J., Szmigielski, S., Korbecki, M., and Zak, C., *ibid.*, **115**, 421(1965).
(234) Gump, D. W., *Federation Proc.*, **24**, 368(1965).
(235) Morgan, P., and Felton, W. W., *Bacteriol. Proc.*, Abstr., No. V133, 1965, p. 120.
(236) Sato, S., *Tohoku J. Exptl. Med.*, **85**, 346(1965).
(237) Nakazawa, T., *Texas Rept. Biol. Med.*, **18**, 52(1960).
(238) Pomerat, C. M., Drager, G. A., and Painter, J. T., *Proc. Soc. Exptl. Biol. Med.*, **63**, 322(1946).
(239) Shafer, W. G., *ibid.*, **106**, 205(1961).
(240) Nease, W. J., *J. Periodontol.*, **36**, 22(1965).
(241) Miller, Z. B., *Lancet*, **ii**, 1068(1963).
(242) Roath, S., Elves, M. W., and Israëls, M. C. G., *ibid.*, **i**, 249(1963).
(243) Ritter, D., *Pathol. Microbiol.*, **26**, 303(1963).

Comparative Hydrolytic Rates of *N*-Substituted 6-Amino-thiouracils

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A study has been carried out on the acid and base-catalyzed hydrolysis of *N*-substituted 6-amino-thiouracils. Two series, aliphatic and halogenated aromatic *N*-substituted 6-amino-thiouracils, have been synthesized and investigated. The experimental results, obtained in this hydrolytic study, indicate that a number of factors may be involved and are rationalized in terms of steric and electronic effects.

INTEREST IN *N*-substituted 6-amino-thiouracils relative to their antitumor activity prompted this preliminary investigation of the influence of *N*-substitution on the stability of the C=S function. Although a survey of the literature indicates that a number of such compounds have been prepared (1, 2), their stability aspects are less known (3), although it would be expected that the *N*-substituted compound should be less stable than the parent unsubstituted thiouracils. Therefore, a systematic study concerning the effect of substituents on 6-amino-thiouracil upon the relationship of hydrolysis in acid and in base would be of value in the design and synthesis of sufficiently stable thiouracils for biological evaluation.

EXPERIMENTAL

Synthesis—Two series, aliphatic and halogenated aromatic *N*-substituted 6-amino-thiouracils, have been prepared by a modification of the condensation method of Carstens (4). The *N*-substituted thiourea and ethyl-cyanoacetate were added to absolute ethanol containing sodium ethoxide. The solution was refluxed with constant stirring for 4–5 hr., and then diluted with water and neutralized with acetic acid. On cooling a 60–80% yield was obtained. It was then purified by recrystallization and chromatographic techniques. The final products were identified by I.R. spectra and elemental analysis. Physical constants and analyses for all thiouracils synthesized are listed in Table I.

Kinetic Studies—Spectra of each thiouracil were taken in 0.3 *N* HCl and 0.3 *N* NaOH solution using a Beckman DB spectrophotometer equipped with a Sargent recorder. The $\lambda_{max.}$ and ϵ and hydrolytic rate constants are listed in Table II. The hydrolysis was carried out at 70° ± 0.1 in a 6 *N* HCl or 6 *N* NaOH solution, and the reaction course was followed by measuring the decrease in absorbance at $\lambda_{max.}$ utilizing a Beckman DU spectrophotometer. An accurate weight of the thiouracil was placed in a 100-ml. volumetric flask and brought to the volume with 6 *N* HCl or 6 *N* NaOH solution (previously warmed to 70°) to obtain an approximate concentration of 3×10^{-5} mole/L. The hydrolysis was carried out in a 100-ml. glass-stoppered volumetric flask which was immersed in a "Haake-Thermostate Unitherm" water bath, regulated at 70° ± 0.1. Aliquots were withdrawn periodically and diluted with water to 100 ml. and the absorbance read at $\lambda_{max.}$ In the same manner, hydrolysis at three temperatures, 70° ± 0.1, 60° ± 0.1, 50° ± 0.1, was carried out for the *N*-phenyl 6-amino-thiouracil in acid and base in order that additional data be obtained concerning the degradative pathway of these compounds.

RESULTS AND DISCUSSION

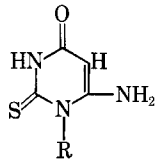
Prior to the hydrolytic study, ultraviolet absorption spectra of each thiouracil was obtained. Since the spectrum of the corresponding uracil exhibits a $\lambda_{max.}$ at approximately 260 mμ and shows little or no absorption at $\lambda_{max.}$ of the thiouracil, as shown in Fig. 1, all measurements were made at $\lambda_{max.}$ of the thiouracil. Uracil absorption causes no interference in thiouracil systems when the concentration of these thiouracils undergoing degradation is 3×10^{-5} mole/L. or less. In cases where this uracil interference is significant, particularly at higher concentration of the thiouracil, the use of two component analysis can be satisfactorily applied.

The degradation product of the *N*-substituted 6-amino-thiouracil in 6 *N* HCl or 6 *N* NaOH at 70° has been identified as the corresponding 6-amino-uracil using the thin-layer chromatographic technique. No ring cleavage was indicated in this case under the experimental condition employed.

Data obtained in this preliminary investigation indicate that the hydrolysis of these thiouracils follows a pseudo first-order rate law as shown in Figs. 2 and 3. A straight line relationship is obtained of a plot of log concentration against time. Kinetic data (Fig. 2) indicate that the aliphatic *N*-substituted 6-amino-thiouracils hydrolyze at a substantially faster rate than the corresponding

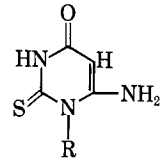
Received November 28, 1966, from the College of Pharmacy, University of Iowa, Iowa City, IA 52240
Accepted for publication January 10, 1967.
This work was supported by the American Cancer Society, Iowa Division.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

TABLE I—PHYSICAL CONSTANTS AND ELEMENTAL ANALYSIS FOR *N*-SUBSTITUTED 6-AMINO-THIOURACILS



R =	Formula	M.p., °C.	Anal. C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
H	$C_4H_5N_3OS$	>360	33.55	33.54	3.52	3.64	29.34	29.06
CH_3	$C_5H_7N_3OS$	255–256	38.20	28.30	4.48	4.66	26.73	26.47
C_2H_5	$C_6H_9N_3OS$	224–225	42.09	42.08	5.29	5.02	24.54	24.30
C_3H_7	$C_7H_{11}N_3OS$	221–222	45.39	45.37	5.98	6.21	22.69	22.52
C_4H_9	$C_8N_{13}N_3OS$	219.5–220.5	48.20	48.83	6.57	6.31	21.08	21.58
ϕH	$C_{10}H_9N_3OS$	221–222	54.80	54.66	4.14	3.95	19.20	19.05
ϕCl	$C_{10}H_8ClN_3OS$	230–232	47.33	47.05	3.71	3.48	16.56	16.38
ϕBr	$C_{10}H_8BrN_3OS$	233–233.5	40.27	40.46	2.70	2.82	14.09	13.98
ϕI	$C_{10}H_8IN_3OS$	234–235.5	34.79	34.90	2.33	2.37	12.17	12.30

TABLE II—KINETIC DATA OF *N*-SUBSTITUTED 6-AMINO-THIOURACILS



R =	$\lambda_{max.}$ (mμ) in HCl	$\lambda_{max.}$ (mμ) in NaOH	ε in HCl	ε in NaOH	Rate Constant × 10^{-1}, hr.$^{-1}$, 70° in HCl	Rate Constant × 10^{-1}, hr.$^{-1}$, 70° in NaOH	Half-Life, hr.$^{-1}$, 70° in HCl	Half-Life, hr.$^{-1}$, 70° in NaOH
H	274	284	16159	10296	0.575	0.086	12.05	80.58
CH_3	278	282	17427	14287	4.830	1.440	1.43	4.81
C_2H_5	278	284	17100	13338	3.910	1.090	1.77	6.36
C_3H_7	278	284	16835	12765	3.570	0.860	1.94	8.06
C_4H_9	278	282	17114	13134	3.1220	0.69	2.15	10.04
ϕH	276	286	18396	12483	8.492	1.320	0.81	5.25
ϕCl	276	286	18288	13208	5.410	1.210	1.28	5.73
ϕBr	276	286	18178	13112	4.600	1.090	1.51	6.36
ϕI	274	284	18285	14145	2.650	0.860	2.62	8.06

parent unsubstituted 6-amino-thiouracils, as was expected. This greater stability of the unsubstituted 6-amino-thiouracils can be attributed to a resonance structure effect since this resonance structure no longer exists after *N*-substitution. It is also interesting to note (Fig. 2) that an increase in the carbon number of the aliphatic substituent increases the stability of these compounds, probably due to steric hindrance. A bulky molecule in the *N*-position would certainly hinder the nucleophilic attack (5) of water on the partially positive carbon of the C=S reaction center resulting in a slight increase in stability. Although this aliphatic effect was not studied beyond C_4, it is reasonable to believe that extension of the chain length or branching of the chain would further increase the stability of these thiouracils.

As shown in Fig. 3, the introduction of a phenyl substituent at this *N*-position also results in a significant decrease in the stability. This increase in hydrolytic rate is due probably to a diminished resonance structure of the thiouracil and an electron delocalization to the phenyl ring. The electron pair on the *N*-nitrogen can be delocalized to the benzene ring system making the reaction center more positive, increasing the rate of water molecule attack in the rate-determining step. In the absence of phenyl *N*-substitution, this electron pair can be delocalized to the reaction center making it less positive, resulting in an increase in the stability of the compound as discussed in the aliphatic series. Introduction of a halogen in the *para*-position of the phenyl group increases the stability of the thiouracils somewhat. This slight increase in stability may be attributed to a decrease of the positive charge of the reaction center by a resonance contribution of the halogenated phenyl, resulting in a push of the electron pair on the nitrogen toward the reaction center.

The kinetic data (Table II) show that rate of hydrolysis of these *N*-substituted thiouracils is considerably greater in acid than in base. This suggests that the reaction centers of the 6-amino-thiouracils in the protonated form are more positive and favor this water molecule attack in the mechanism involved. Calculation of the apparent energy of

activation, E_a, from the slope of the Arrhenius plot (Fig. 4) for *N*-phenyl-6-amino-thiouracil in acid and in base yielded 23.5 Kcal./mole and 29.7 Kcal./mole, respectively. Since this apparent energy of activation includes the heat of ionization of water (approximately 11.5 Kcal./mole), the energy of activation for the acid and base hydrolysis is 13.1 Kcal./mole and 18.2 Kcal./mole, respectively, which is a reasonable value for hydrolytic reactions of this type (6).

As previously pointed out, the protonated thiouracil species undergo a faster rate of hydrolysis suggesting that specific acid catalysis is involved. This aspect was studied by an investigation of the hydrolytic rates of *N*-phenyl-thiouracil at various acid and base concentrations. Data for acid hydrolysis are shown in Fig. 5, A. Scheme I is a possible mechanism for this acid catalyzed hydro-

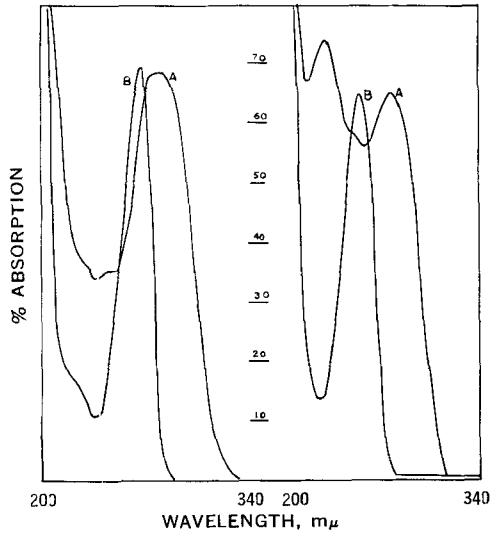


Fig. 1—U. V. spectra of 6-amino-thiouracil and 6-amino-uracil in 0.3 N HCl (lefthand curves) and 0.3 N NaOH (righthand curves). Key: A, 6-amino-thiouracil; B, 6-amino-uracil.

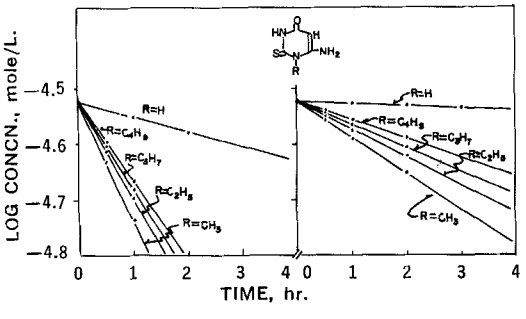


Fig. 2—Rates of hydrolysis of aliphatic N-substituted 6-amino-thiouracil in 6 N HCl (lefthand curves) and 6 N NaOH (righthand curves).

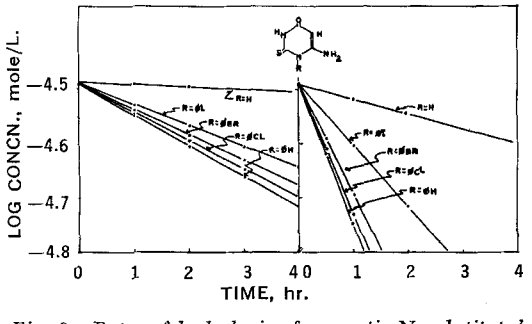


Fig. 3—Rates of hydrolysis of aromatic N-substituted 6-amino-thiouracil in 6 N NaOH (lefthand curves) and 6 N HCl (righthand curves).

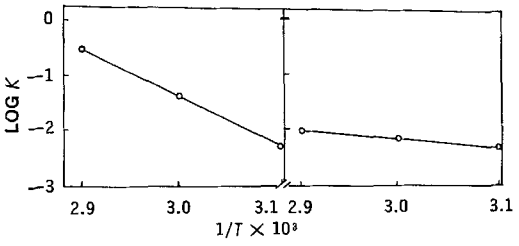


Fig. 4—Arrhenius plot of hydrolysis of N-phenyl-6-amino-thiouracil in 6 N HCl (lefthand curve) and 6 N NaOH (righthand curve).

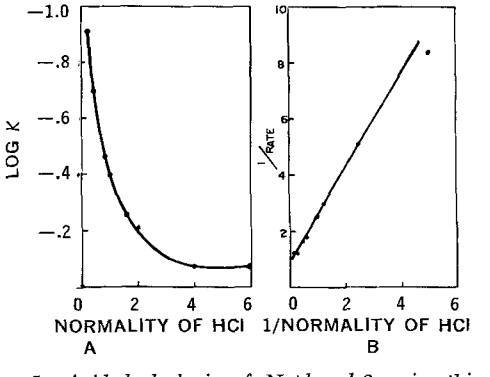
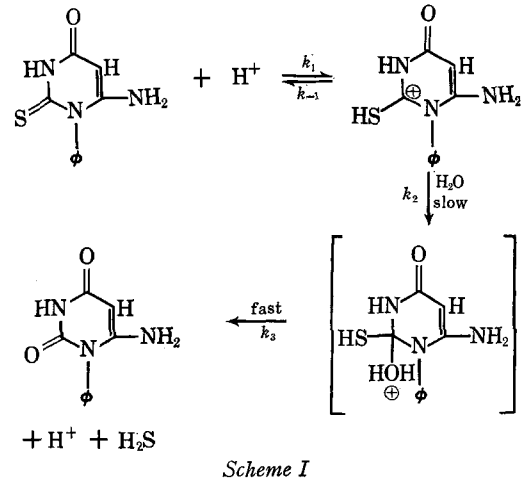


Fig. 5—Acid hydrolysis of N-phenyl-6-amino-thiouracil.



Scheme I

lytic reaction. This mechanism suggests that the thiouracils readily pick up a proton to form an intermediate which undergoes water attack and rapidly collapses to form the corresponding uracils.

Since the kinetic and mathematical aspects of such a system are adequately covered by Laidler (7), the final rate equation is listed here only for discussion purposes.

$$\text{rate} = \frac{k_1 k_2\,(\text{thiouracil})\,(H^+)}{k_1(H^+) + k_2 + k_{-1}} \qquad \text{(Eq. 1)}$$

An examination of this equation indicates that at high acid concentration the rate of hydrolysis reaches a limiting value and the rate equation becomes,

$$\text{rate} = K\,(\text{thiouracil}) \qquad \text{(Eq. 2)}$$

This is verified in Fig. 5, A, and illustrates specific hydrogen ion catalysis. At low (H^+) the rate is dependent on the equilibrium concentration of the protonated and nonprotonated thiouracil species in solution. Although only the acid hydrolysis of *N*-phenyl-thiouracil was studied, it is reasonable to assume that the other *N*-substituted derivatives would undergo a similar behavior.

At low acid concentration k_2 is small, and the rate equation then can be expressed as:

$$\text{rate} = \frac{k_2 k_1\,(\text{thiouracil})\,(H^+)}{k_1(H^+) + k_{-1}} \qquad \text{(Eq. 3)}$$

Rearrangement gives the following relationship:

$$\text{rate} = \frac{k_2\,(\text{thiouracil})}{1 + \dfrac{k_{-1}}{k_1(H^+)}} = \frac{k_2\,(\text{thiouracil})}{1 + \dfrac{K}{(H^+)}}$$

$$1 + \frac{k}{(H^+)} = \frac{k_2\,(\text{thiouracil})}{\text{rate}} \qquad \text{(Eq. 4)}$$

From Eq. 4 a straight line relationship would be obtained by plotting $1/(H^+)$ against $1/(\text{rate})$. This is shown in Fig. 5, B, suggesting specific acid catalysis.

Data for base catalysis of the *N*-phenyl-thiouracil are given in Fig. 6. No limiting of the hydrolytic rate was observed at high hydroxyl-ion concentration (6 *N*) as was seen in acid hydrolysis, suggesting that the rate is proportional to hydroxyl-ion concentration. Although the limited data available do not permit the calculation of exact rate equations, a possible mechanism for this degradation is listed in Scheme II based on the information available.

Schemes I and II are mechanisms for both acid and base hydrolysis involving a rapid polarization of the C=S or thio-group followed by the rate-determining step (8) of a water molecule attack to form a tetrahedral intermediate which rapidly collapses to form the corresponding uracil. Since water attack is postulated to be the rate-determining step, the greater the positive character of the reaction center or C=S function the faster should be the rate of hydrolysis. No bonded electrons on the *N*-nitrogen should be capable of localization toward the reaction center, such as in halogenated phenyl-*N*-substitution, which decreases the positive charge of the carbon in C=S resulting in an increase in stability.

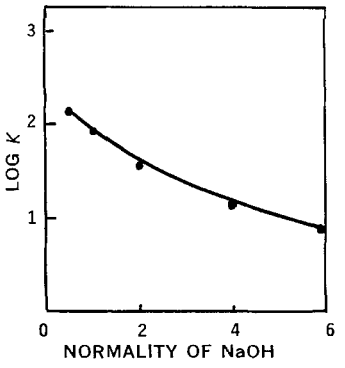
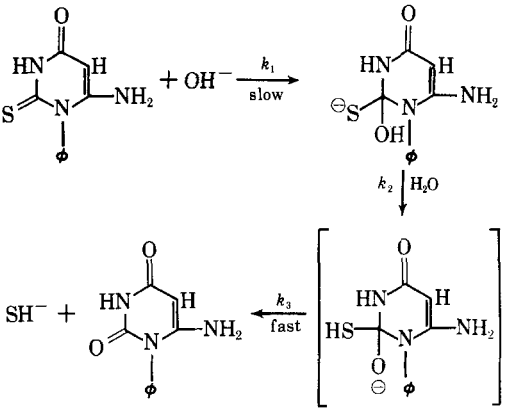


Fig. 6—Base hydrolysis of N-phenyl-6-amino-thiouracil.



Scheme II

REFERENCES

(1) Shvachkin, Y. P., Shprunka, I. K., and Kazakova, G. U., *Zh. Obshch. Khim.*, **34**, 3846(1964).
(2) Warrener, R. N., and Cain, E. N., *Chem. Ind. (London)*, **48**, 1989(1964).
(3) Moraes, W. B. C., and Meneghini, M., *Arquiv. Inst. Biol.*, **28**, 13(1961).
(4) Carstens, E., East Ger. pat. 23546 (August 1962).
(5) Gould, E. S., "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, New York, N. Y., 1962, p. 274.
(6) Hurd, R. N., and DeLaMater, G., *Chem. Rev.*, **61**, 45(1961).
(7) Laidler, K. J., "Chemical Kinetics," McGraw-Hill Book Co., New York, N. Y., 1965, p. 461.
(8) Witiak, D. T., Chin, T.-F., and Lach, J. L., *J. Org. Chem.*, **30**, 3721(1965).

Irreversible Enzyme Inhibitors LXXXII

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase VII. Derivatives of 2,4-Diaminopyrimidine I

By B. R. BAKER, GRAHAM D. F. JACKSON, and RICH B. MEYER, JR.

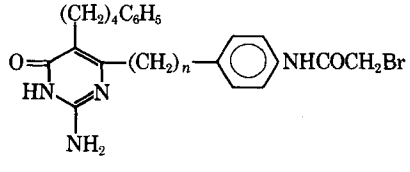
Fusion of α-benzoyl-α-phenylazoacetonitrile (V) with guanidine carbonate gave 2,4-diamino-6-phenyl-5-phenylazopyrimidine (VII) in 52 per cent yield; catalytic reduction of VII to 6-phenyl-2,4,5-triaminopyrimidine (VI) proceeded smoothly. Condensation of the triaminopyrimidine (VI) with *p*-nitrocinnamaldehyde to an anil followed by a two-stage reduction afforded 5-(*p*-aminophenylpropylamino)-2,4-diamino-6-phenylpyrimidine (XI); selective bromoacetylation on the *p*-amino group gave the candidate irreversible inhibitor, 5-(*p*-bromoacetamidophenylpropylamino)-2,4-diamino-6-phenylpyrimidine (XII). When XII was incubated with dihydrofolic reductase in the presence or absence of TPNH, no inactivation occurred; the possible conformational differences in binding to dihydrofolic reductase between XII and other successful active-site-directed irreversible inhibitors are discussed.

TWO TYPES of active-site-directed irreversible inhibitors (1, 2) derived from 2-amino-4-pyrimidinol have been found for dihydrofolic reductase; once the hydrophobic bonding region of dihydrofolic reductase had been discovered and explored (3), irreversible inhibitors could be designed. For example, the 5-phenylbutyl group of I and II is complexed in the hydrophobic region; the 6-side-chain is then projected into a hydrophilic region of the enzyme where covalent bond formation occurs that leads to irreversible inhibition of the enzyme (4). Similarly, the 6-phenyl group of III is complexed in the hydrophobic region which allows the alkylating function on the 5-side-chain to attack a nucleophilic group on the enzyme surface (5).

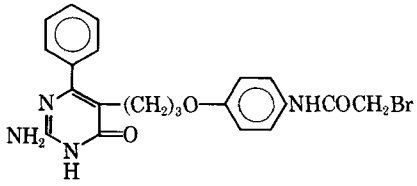
Where 50% of the enzyme was in the form of a reversible complex with the inhibitor, *I*, dihydrofolic reductase was inactivated with a half-life of about 12 min. at 37°. The concentration of the inhibitor, *I*, necessary to form 50% reversible complex (4×10^{-5} *M*) was considered to be too high to be of practical use in an intact animal (4). Therefore, the design of candidate irreversible inhibitors of dihydrofolic reductase that could form 50% reversible complex at a concentration of 10^{-6}–10^{-8} *M* was initiated. Since it is well known that 2,4-diaminopyrimidines are 300 to 3000-fold more effective as inhibitors of dihydrofolic reductase than the corresponding 2-amino-4-pyrimidinols (3, 6–8), this design centered on finding active-site-directed irreversible inhibitors derived from 2,4-diaminopyrimidines; that this design would not necessarily be a simple problem was indicated by the possibility that a 2,4-diaminopyrimidine and the corresponding 2-amino-4-pyrimidinol might not bind to the active site of dihydrofolic reductase in the same rotomeric configuration (7, 9). The first such attempt to find an active-site-directed irreversible inhibitor of dihydrofolic reductase of the 2,4-diaminopyrimidine type—that took into consideration the hydrophobic bonding region—is the subject of this paper; earlier attempts, prior to knowledge of the complications created by the hydrophobic bonding region, have been described (10, 11).

DISCUSSION

6-Phenyl-2,4,5-triaminopyrimidine (VI) was selected as the carrier for the covalent forming group, since VI could be readily synthesized in three steps

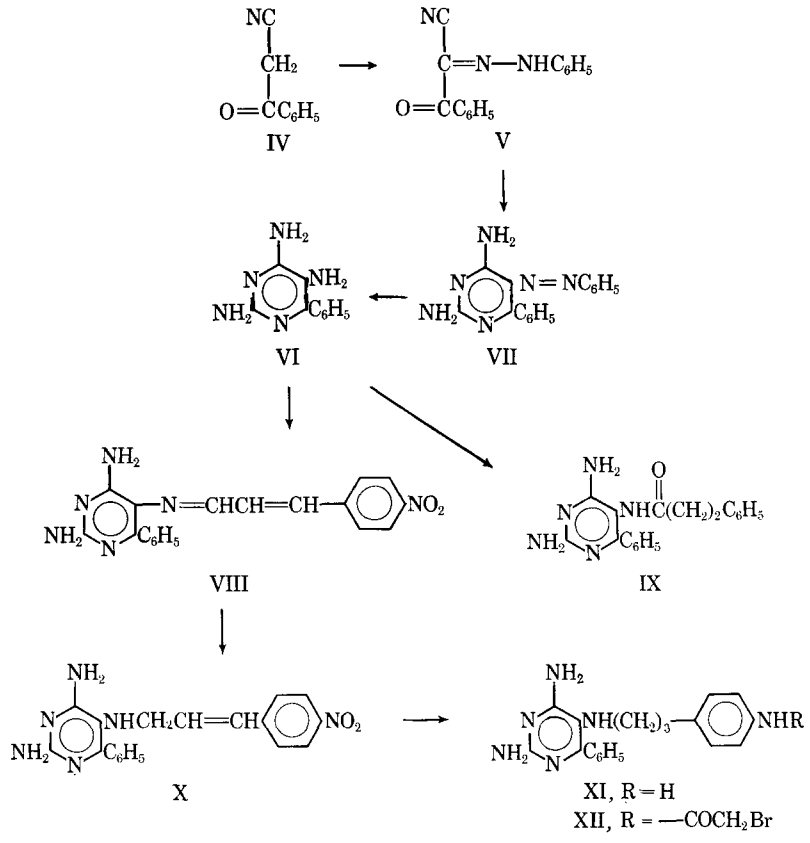


I, $n = 2$
II, $n = 4$



III

Received November 28, 1966, from the Department of Chemistry, University of California, Santa Barbara, CA 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication January 12, 1967.
This work was supported in part by grants CA-05867 and CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md., and training grant GE-7864.1 from the National Science Foundation.
Previous paper: Baker, B. R., and Novotny, J., *J. Heterocyclic Chem.*, **4**, 23(1967).



Scheme I

from benzoylacetonitrile (IV) (Scheme I). The latter smoothly coupled with diazotized aniline to the phenylhydrazone (V). When the ketonitrile (V) was fused with an excess of guanidine carbonate at 180°, the phenylazopyrimidine (VII) was obtained in 52% yield. Hydrogenation of VII with a Raney nickel catalyst afforded the desired triaminopyrimidine (VI)

2,4,5-Triamino-6-phenylpyrimidine (VI) was only one-seventh as effective a reversible inhibitor as the corresponding pyrimidine without the 5-amino group, namely, XIII (Table I); since the 5-amino group should not decrease the basicity of the 2,4-diaminopyrimidine (12), the poorer inhibition by VI than XIII may be due to repulsion of the 5-amino group from a hydrophobic area on the enzyme.

Conversion of the triaminopyrimidine (VI) to the 5-phenylpropionyl derivative (IX) with phenylpropionyl chloride resulted in a slight increase in inhibition (Table I); however, the effectiveness of IX was far less than the previously reported (9, 13) 5-anilinopropylpyrimidine (XIV). Thus, a 5-acylamido group on the pyrimidine is not well tolerated, as previously noted in the 2-amino-6-methyl-4-pyrimidinol series (14). Therefore, the triaminopyrimidine (VI) was converted to the 5-(*p*-aminophenylpropyl) derivative (XI), since 2-amino-6-methyl-5-phenylpropylamino-4-pyrimidinol was previously observed to be as good a reversible inhibitor as 2-amino-6-methyl-5-anilinopropyl-4-pyrimidinol (15).

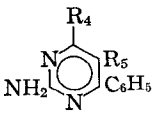
Condensation of the triaminopyrimidine (VI) with *p*-nitrocinnamaldehyde gave the anil (VIII) in 93% yield. Reduction of the anil with sodium borohydride in methanol afforded a 76% yield of X; further reduction of X catalytically afforded the desired 5-(*p*-aminophenylpropyl)pyrimidine (XI). Attempts to reduce VIII directly to XI by catalytic methods were unsuccessful. As anticipated, XI was as good a reversible inhibitor of dihydrofolic reductase as the corresponding 5-anilinopropyl pyrimidine (XIV).

Treatment of XI with bromoacetic anhydride in acetone, containing acetic acid to protonate the diaminopyrimidine system (16), gave the desired candidate active-site-directed irreversible inhibitor (XII); XII could be isolated as a crystalline picrate or as an amorphous hydrobromide.

The candidate irreversible inhibitor (XII) was an excellent reversible inhibitor of dihydrofolic reductase. When XII was incubated with dihydrofolic reductase in the presence of 12 μM TPNH, no inactivation of the enzyme occurred with a concentration of XII sufficient to complex 90% of the available enzyme; similar results were obtained in the absence of TPNH. In contrast, it was previously reported (5) that the corresponding 4-pyrimidinol (III) gave 50% inactivation of the enzyme in 50 min. when a concentration of III was employed that complexed 25% of the available enzyme, but in the absence of TPNH.

These contrasting results between III and XII

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_4	R_5	Reversible[a] μM Concn. for 50% Inhibition	Reversible[a] Est. $K_i \times 10^6 M$ [c]	Irreversible[b] μM Concn.	Irreversible[b] % $E \cdot I$ [d]	Irreversible[b] Time, min.	Irreversible[b] % Inactivation
XIII	NH_2	H	160[e]	...	...	...	...	...
VI	NH_2	NH_2	1100	...	...	...	...	...
IX	NH_2	$-NHCO(CH_2)_2C_6H_5$	280	...	...	...	...	...
XIV	NH_2	$-(CH_2)_3NHC_6H_5$	0.88[e]	...	...	...	...	...
XI	NH_2	$-NH(CH_2)_3C_6H_4NH_2$-*p*	0.42	...	...	...	...	...
XII	NH_2	$-NH(CH_2)_3C_6H_4NHCCH_2Br$-*p*	0.33	0.05	0.40	91[f]	120	90[f]
III[g]	OH	$-(CH_2)_3OC_6H_4NHCCH_2Br$-*p*	800	100	40	25	50	50

The technical assistance of Maureen Baker, Susan Lakatos, and Karen Smith is acknowledged. [a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH at pH 7.4 as previously described (18). [b] Dihydrofolic reductase was incubated with the inhibitor at 37° in 0.05 *M* Tris buffer (pH 7.4) containing no mercaptoethanol and no TPNH as previously described (19) unless otherwise indicated; in each case an enzyme control showed 0–4% inactivation. [c] Estimated from $K_i = I \times K_m/S$, where I = inhibitor concentration giving 50% inhibition (20); this equation is usually valid when $S > 4K_m$ and in this case $S = 6K_m$. [d] Calculated from $[EI] = E_t/(1 + K_i/I)$, where E_t = the total concentration of active enzyme and $[EI]$ = fraction of E_t reversibly complexed by I (1,21). [e] Data from *Reference 9*. [f] The same results were obtained in the presence of 12 μM TPNH in the incubation mixture. [g] Data from *Reference 5*.

clearly show that the terminal bromoacetamido group of the two compounds is positioned differently within the enzyme–inhibitor reversible complex. This different positioning of the bromoacetamido group can be rationalized in at least two different ways: (*a*) the pyrimidines may be complexed in the same way, but the allowable ground-state conformations of the 5-side-chains of III and XII are different, or (*b*) the 2,4-diaminopyrimidine (XII) may be complexed as a rotomer (7) different from the 2-amino-4-pyrimidinol (III), as previously suggested (9). Explanation (*a*) has been eliminated by synthesis of the analog of III where the 4-hydroxyl group has been replaced by 4-amino and both have identical side-chains; this diamino analog of III also failed to show irreversible inhibition of dihydrofolic reductase (17).

EXPERIMENTAL

Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on silica gel GF and spots were detected by visual examination under ultraviolet light.

α-Benzoyl-α-phenylazoacetonitrile (V)—A solution of 5.6 Gm. (60 mmoles) of aniline in 20 ml. of concentrated hydrochloric acid and 67 ml. of water and was treated at 0° with 4.2 Gm. of sodium nitrite in 8 ml. of water in the usual manner. The solution was carefully neutralized at −5° with a solution of 7.2 Gm. of anhydrous sodium carbonate in 72 ml. of water. To a solution of 6.05 Gm. (41.8 mmoles) of benzoylacetonitrile in 500 ml. of ethanol was added 40 ml. of 50% aqueous sodium acetate and 25 Gm. of solid sodium acetate; to this stirred suspension at 10° was added 150 ml. of above diazonium solution over a period of about 2 min. The product precipitated within a few minutes. The thick suspension was stirred at ambient temperature for 30 min., then cooled to 0° in an ice bath. After addition of 40 ml. of ether, the mixture was stirred an additional 30 min. at 0°, then filtered. The product was washed with 60% aqueous ethanol; yield, 8.28 Gm. (80%) of yellow needles, m.p. 137–138°. Recrystallization from petroleum ether gave yellow crystals, m.p. 137–138°; $\nu_{max.}$ 2200 (C≡N); 1640 (C=O), 1590, 1540 (C=C, C=N); 750, 718, 685, 670 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{15}H_{11}N_3O$: C, 72.3; H, 4.44; N, 16.9. Found: C, 72.5; H, 4.46; N, 16.9.

2,4-Diamino-6-phenyl-5-phenylazopyrimidine (VII)—A well-ground mixture of 2.49 Gm. (10 mmoles) of V and 1.80 Gm. (10 mmoles) of guanidine carbonate was heated in an oil bath; at 130° gas evolution began and the bath was brought to 170–180° over a period of 30 min. with occasional manual stirring. After 30 min. at 170–180°, gas evolution subsided and the mixture solidified. The cooled residue was digested with 30 ml. of boiling petroleum ether (b.p. 60–110°) and the solvent decanted. The residue was extracted with five 50-ml. portions of boiling ethanol. The combined extracts were heated to boiling, then filtered. The filtrate was heated to boiling and water was added to turbidity. After several hours at 0°, the mixture was filtered and the product was washed with 50% aqueous ethanol; yield, 1.51 Gm. (52%) of orange crystals, m.p. 236–237°. Recrystallization of a sample from aqueous ethanol afforded orange needles, m.p. 238–239°; $\nu_{max.}$ 3250 (NH); 1610, 1590, 1520 (NH, N=N, C=C, C=N); 760, 695, 680 cm.$^{-1}$ (C_6H_5);

$\lambda_{max.}$ (pH 1): 238, 285, 357; (pH 7): 230, 280, 379; (pH 13): 280, 378 mμ.

Anal.—Calcd. for $C_{16}H_{14}N_6 \cdot 1/2\ H_2O$: C, 64.2; H, 5.02; N, 28.1. Found: C, 64.6; H, 5.10; N, 28.1.

2,4-Diamino-6-phenylpyrimidine—Fusion of 725 mg. (5 mmoles) of IV with 900 mg. (5 mmoles) of guanidine carbonate was performed as described for the preparation of VII. The cooled melt was extracted with five 20-ml. portions of boiling petroleum ether (b.p. 60–110°). The combined decantates deposited 132 mg. (13%) of white crystals, m.p. 162–163°. This compound was identical with a sample prepared from 6-phenylisocytosine (22, 23).

6-Phenyl-2,4,5-triaminopyrimidine (VI)—A solution of 600 mg. (2 mmoles) of VII in 100 ml. of 2-methoxyethanol was shaken with hydrogen at 2–3 Atm. in the presence of 0.5 Gm. of No. 28 Raney nickel (W. R. Grace Co.) for 3 hr. When 4 mmoles of hydrogen was absorbed, the solution had changed from orange to pale yellow. The mixture was filtered through a pad of diatomaceous earth.[1] The filtrate was spin-evaporated *in vacuo*. Trituration of the residual oil with benzene gave nearly white crystals, m.p. 196–198°; yield, 320 mg. (80%). A portion was recrystallized from ethanol–benzene to give white crystals, m.p. 197–198°; $\nu_{max.}$ 3400, 3300 (NH); 1650–1550 (multiple peaks of NH, C=C, C=N); 760, 695 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1): 315; (pH 13): 322 mμ.

Anal.—Calcd. for $C_{10}H_{11}N_5$: C, 59.7; H, 5.51; N, 34.8. Found: C, 60.0; H, 5.38; N, 34.5.

2,4-Diamino-6-phenyl-5-phenylpropionamidopyrimidine (IX) Sulfate—To a stirred solution of 201 mg. (1 mmole) of VI in 10 ml. of reagent pyridine cooled in an ice bath was added 168 mg. (1 mmole) of hydrocinnamoyl chloride. After 30 min. at 0°, the solution was spin-evaporated *in vacuo* and the residue triturated with ethyl acetate. Recrystallization of the solid from 5 ml. of water gave 182 mg. (55%) of VII free base, m.p. 232–233°. A hot solution of 80 mg. of the free base in 4 ml. of water was quickly cooled to about 25°, then 0.2 ml. of 96% sulfuric acid was added with stirring. The sulfate salt was collected; yield, 80 mg. (84% conversion) of white crystals, m.p. 208–213°. Recrystallization from water gave the analytical sample, m.p. 204–206°.

Anal.—Calcd. for $C_{19}H_{19}N_5O \cdot 1/2H_2SO_4 \cdot H_2O$: C, 57.0; H, 5.49; N, 17.5. Found: C, 56.9; H, 5.18; N, 17.5.

2,4-Diamino-5-(p-nitrocinnamalamino)-6-phenylpyrimidine (VIII)—To a solution of 100 mg. (0.50 mmole) of VI in 5 ml. of hot ethanol was added a hot solution of 89 mg. (0.50 mmole) of *p*-nitrocinnamaldehyde in 5 ml. of hot ethanol. After the addition of 2 drops of glacial acetic acid, the mixture was heated 10 min. on a steam bath, then cooled to 0°. The product was collected on a filter and washed with cold ethanol; yield, 168 mg. (93%), m.p. 205–207°. Recrystallization of a portion from 2-methoxyethanol–ethanol gave red crystals, m.p. 206–207°; $\nu_{max.}$ 3500, 3400–3350 (NH); 1610, 1590, 1540 (NH, C=C, C=N); 1500, 1330 (NO_2); 830 (*p*-C_6H_4); 745, 695 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{16}N_6O_2$: C, 63.3; H, 4.48; N, 23.3. Found: C, 63.4; H, 4.60; N, 23.4.

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

2,4-Diamino-5-(p-nitrocinnamylamino)-6-phenylpyrimidine (X)—A hot solution of 540 mg. (1.5 mmoles) of VIII in 250 ml. of methanol was cooled to 25°, then 0.1 ml. of glacial acetic acid was added. Over a period of 30 min., the stirred solution was treated with 650 mg. of sodium borohydride. After a further 30 min., TLC showed only one component was present. The solution was concentrated to about 25 ml. *in vacuo*, then cooled. The product was collected on a filter and washed with cold methanol, then water; yield, 412 mg. (76%), m.p. 214–215°. Recrystallization from ethanol–ethyl acetate gave orange crystals, m.p. 214–215°; $\nu_{max.}$ 3500, 3400 (NH); 1610, 1590, 1555 (NH, C=C, C=N); 1500, 1330 (NO_2); 770, 742, 695 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{18}N_6O_2$: C, 63.0; H, 5.01; N, 23.2. Found: C, 63.0; H, 5.20; N, 23.0.

5-(p-Aminophenylpropylamino)-2,4-diamino-6-phenylpyrimidine (XI)—A hot solution of 362 mg. (1 mmole) of X in 150 ml. of boiling ethanol was stirred with 250 mg. of decolorizing carbon for about 20 min. The filtered solution was shaken with hydrogen at 2–3 Atm. in the presence of 20 mg. of platinum oxide until 4 mmoles of hydrogen had been absorbed. The filtered solution was spin-evaporated *in vacuo*. The residue was crystallized by solution in 2 ml. of ethyl acetate, addition of 15 ml. of benzene, then finally petroleum ether to turbidity; yield, 296 mg., m.p. 72–80°, which showed two spots on TLC. Several recrystallizations from benzene gave 102 mg. (31%) of white crystals, m.p. 91–94°, that were uniform on TLC and had $\nu_{max.}$ 3500, 3300, 3200, 3100 (NH); 1620, 1550 (NH, C=C, C=N); 768, 700 (C_6H_5); no NO_2 near 1500 or 1330 cm.$^{-1}$.

Anal.—Calcd. for $C_{19}H_{22}N_6$: C, 68.2; H, 6.63; N, 25.1. Found: C, 68.4; H, 6.80; N, 24.9.

5-(p-Bromoacetamidophenylpropylamino)-2,4-diamino-6-phenylpyrimidine (XII) Picrate—To a solution of 100 mg. (0.30 mmole) of XI in 2 ml. of acetone and 0.55 ml. of 3.6% aqueous acetic acid (0.66 mmole) at 0° was added 90 mg. (0.34 mmole) of bromoacetic anhydride in 0.5 ml. of acetone. After being stirred for 30 min. in an ice bath, the solution was treated with 0.5 ml. of saturated ethanolic picric acid; a picrate immediately separated. The mixture was warmed to complete solution, then allowed to cool slowly. The picrate was collected and washed with acetone; TLC showed the presence of one major component with two minor impurities. Four recrystallizations from aqueous 2-methoxyethanol and drying at room temperature in high vacuum gave 50 mg. (25%) of yellow crystals, m.p. 150–151° dec. TLC in 1:4 ethanol–chloroform showed one moving spot (viewed at 350 mμ) with the picric acid staying at the origin; the moving spot gave a positive 4-nitrobenzylpyridine test (16) for active halogen. The compound had $\nu_{max.}$ 3330 (NH); 1640 (C=O); 1530, 1420, 1330–1310 (NO_2); 1270, 1075 cm.$^{-1}$ (C—O—C).

Anal.—Calcd. for $C_{27}H_{28}BrN_9O_9 \cdot H_2O$: C, 47.2; H, 4.11; N, 18.4. Found: C, 47.3; H, 4.05; N, 18.1.

An amorphous hydrobromide was also prepared by adding 30% hydrogen bromide in acetic acid, then ether, to the reaction solution. The hydrobromide moved as a single spot on TLC, the spot giving a positive 4-nitrobenzylpyridine test; this hydrobromide was used for enzyme assays.

REFERENCES

(1) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site," John Wiley & Sons, Inc., New York, N.Y., 1967.
(2) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(3) *Reference 1*, chap. X.
(4) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **55**, 1417(1966).
(5) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422(1966).
(6) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).
(7) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
(8) Bertino, J. R., Booth, V. A., Bieber, A. L., Cashmore, A., and Sartorelli, A. C., *J. Biol. Chem.*, **239**, 479(1964).
(9) Baker, B. R., and Shapiro, H. S., *J. Pharm. Sci.*, **55**, 308(1966).
(10) Baker, B. R., and Santi, D. V., *ibid.*, **56**, 380(1967).
(11) Baker, B. R., and Ho, B.-T., *ibid.*, **56**, 28(1967).
(12) Brown, D. J., "The Pyrimidines," John Wiley & Sons, Inc., New York, N.Y., 1962, pp. 473, 476.
(13) Baker, B. R., Shapiro, H. S., and Werkheiser, W. C., *J. Med. Chem.*, **8**, 283(1965).
(14) Baker, B. R., and Sachdev, K., *J. Pharm. Sci.*, **53**, 1020(1964).
(15) Baker, B. R., and Santi, D. V., *ibid.*, **54**, 1252(1965).
(16) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(17) Baker, B. R., and Meyer, R. B., Jr., *J. Pharm. Sci.*, **56**, 570(1967).
(18) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(19) Baker, B. R., and Jordaan, J. H., *ibid.*, **2**, 21(1965).
(20) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(21) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(22) Russell, P. B., *J. Chem. Soc.*, **1954**, 2951.
(23) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 340(1965).

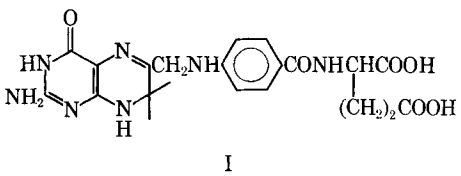
Irreversible Enzyme Inhibitors LXXXIV

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase IX. Derivatives of 2,4-Diaminopyrimidine III

By B. R. BAKER and RICH B. MEYER, JR.

The candidate active-site-directed irreversible inhibitor, 5-(*p*-bromoacetamidophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (VI) has been synthesized *via* the key intermediate, 2-amino-5-(*p*-nitrophenoxypropyl)-6-phenyl-4-pyrimidinol (XI*c*). Although VI was a better reversible inhibitor than the corresponding 4-pyrimidinol, 2-amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol (IV), VI failed to inactivate dihydrofolic reductase when incubated in the presence or absence of TPNH. The fact that IV in the absence of TPNH could inactivate the enzyme, but VI at the same concentration showed no inactivation of the enzyme, clearly demonstrated that IV and VI had different conformations when complexed to dihydrofolic reductase. Evidence for the possible binding conformations of IV and VI is presented.

THE SUCCESS of the 5-phenylbutyl-4-pyrimidinol (II) (1) and the 6-phenyl-4-pyrimidinol (IV) (2) as active-site-directed irreversible inhibitors (3, 4) of dihydrofolic reductase was predicated on a knowledge of (*a*) the strength and location of the hydrophobic bonding region on the enzyme (5–10), and (*b*) the probability that one of several possible rotomers of the pyrimidine ring could be complexed in the active-site (7) which was determined by the position of the hydrophobic group on the pyrimidine carrier (10). The substrate, dihydrofolate (I), must have some given conformation of the pteridine ring when complexed to the enzyme, which is arbitrarily depicted as indicated in I; other rotomeric conformations of the pyrimidines to be discussed below are in relation to the conformation depicted in I. Thus, the active-site-directed irreversible inhibitor of dihydrofolic reductase derived from a 4-pyrimidinol with a hydrophobic 5-phenylbutyl group (II) probably has its hydrophobic group projected vertically as indicated; the 4-pyrimidinol (II) is believed to complex in configuration II*A* which projected the 6-group into a hydrophilic region where covalent bond formation to the enzyme rapidly occurred within the enzyme-inhibitor complex with a half-life of about 12 min. (1). Similarly, the 6-phenyl-4-pyrimidinol (IV) is believed to be complexed in the configuration indicated in IV which projects the 5-group into the same area of the enzyme as the 6-group of II*A* is projected (2).



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Received December 8, 1966, from the Department of Chemistry, University of California, Santa Barbara, CA 93106
Accepted for publication January 30, 1967.
This work was partially supported by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md., and training grant GE-7864.1 from the National Science Foundation.
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REFERENCES

(1) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site," John Wiley & Sons, Inc., New York, N.Y., 1967.
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(8) Bertino, J. R., Booth, V. A., Bieber, A. L., Cashmore, A., and Sartorelli, A. C., *J. Biol. Chem.*, **239**, 479(1964).
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Irreversible Enzyme Inhibitors LXXXIV

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By B. R. BAKER and RICH B. MEYER, JR.

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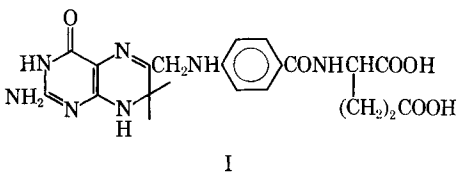

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Received December 8, 1966, from the Department of Chemistry, University of California, Santa Barbara, CA 93106
Accepted for publication January 30, 1967.
This work was partially supported by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md., and training grant GE-7864.1 from the National Science Foundation.
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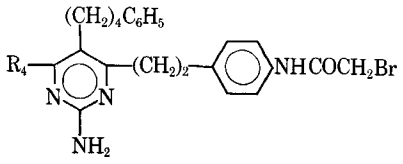
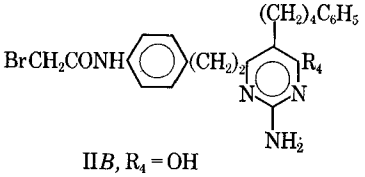
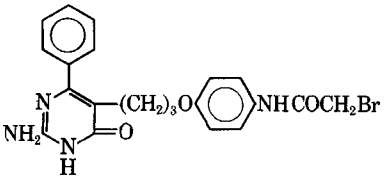


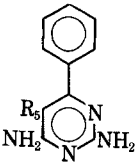
IIA, $R_4 = OH$
IIIA, $R_4 = NH_2$

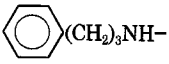


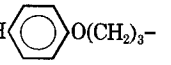
IIB, $R_4 = OH$
IIIB, $R_4 = NH_2$



IV



V, $R_5 = BrCH_2CONH$  $(CH_2)_3NH-$

VI, $R_5 = BrCH_2CONH$  $O(CH_2)_3-$

Although success with these two active-site-directed irreversible inhibitors of dihydrofolic reductase was greatly encouraging in the chemotherapeutic approach, these two compounds suffered from the drawback that too high a concentration was needed for reversible complexing of the enzyme, since the rate of the inactivation is dependent upon the fraction of the enzyme being in a reversible complex, which in turn is dependent upon the dissociation constant, K_i (11, 12).

Since 2,4-diaminopyrimidines are complexed as much as 3000 times more strongly to dihydrofolic reductase than 4-pyrimidinols (5, 13, 14), the synthesis and evaluation of candidate active-site-directed irreversible inhibitors from this system was undertaken. That such an undertaking might be more difficult than merely converting a successful 4-pyrimidinol-type irreversible inhibitor such as II or IV to III or VI, respectively, was indicated by earlier studies (10) on the mode of reversible binding by pyrimidines; 4-pyrimidinols were believed to have conformations II*A* or IV, whereas their 2,4-diamino counterparts would probably have conformation III*B* and VI, which would project the alkylating function in the opposite direction. Unfortunately, these earlier fears were borne out with the candidate 2,4-diaminopyrimidine irreversible inhibitors, III*B* (15) and V (16), which failed to inactivate dihydrofolic reductase, although the two compounds were much better reversible inhibitors of dihydrofolic reductase than II or IV. Since V had a slightly different side-chain than IV which may have made V ineffective as an irreversible inhibitor due to differences in allowable ground-state conformations of the 5-side-chain, the synthesis and evaluation of the exact 2,4-diamino analog (VI) of the 4-pyrimidinol (IV) was undertaken; the results are the subject of this paper.

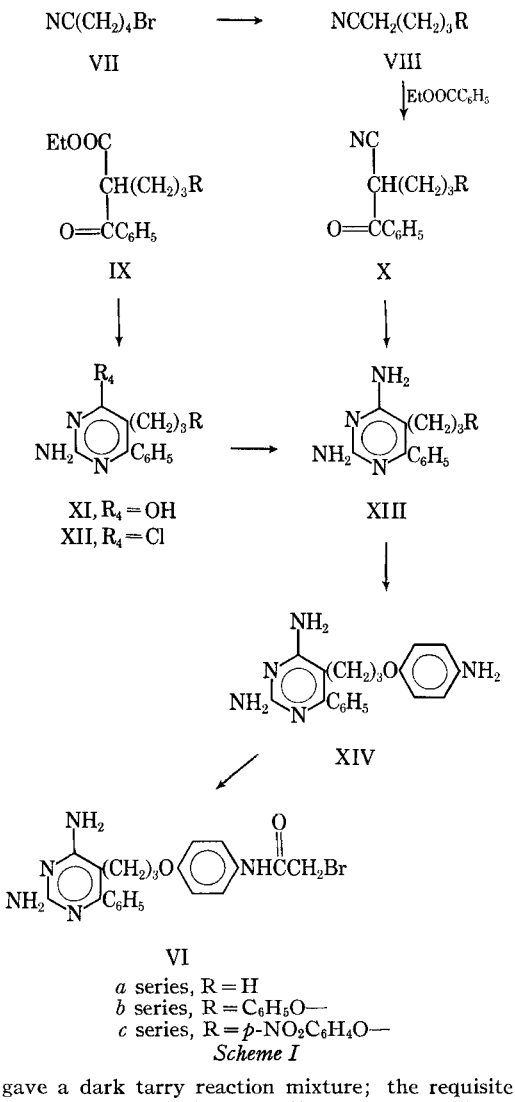
CHEMISTRY

Methods

Several routes to the key 2,4-diamino-5-(*p*-nitrophenoxypropyl)-6-phenylpyrimidine (XIII*c*) were investigated (Scheme I); the successful route will be discussed first. The previously described 5-(*p*-nitrophenoxypropyl)-4-pyrimidinol (XI*c*) (2) is readily synthesized by alkylation of ethyl benzoylacetate with bromopropyl *p*-nitrophenyl ether to IX*c*, followed by reaction with guanidine. Considerable difficulty was initially encountered in conversion of XI*c* to the 4-chloropyrimidine (XII*c*), then further amination to XIII*c*; the intermediate chloropyrimidine (XII*c*) could not be crystallized, and the diaminopyrimidine (XIII*c*) was less soluble in 10% aqueous acetic acid than previously encountered with 2,4-diamino-6-phenylpyrimidines (10, 17). By suitable modifications of the general procedures, as described under *Experimental*, XIII*c* was obtained pure as its hemisulfate salt in 15% yield for the two steps.

Hydrogenation of the nitro group of XIII*c* to give XIV in the presence of platinum oxide catalyst proceeded satisfactorily in aqueous ethanol only if excess hydrochloric acid was present; without the added acid, gummy products were obtained. Reaction of XIV with excess bromoacetic anhydride in acetone—in the presence of acetic acid to protonate the 2,4-diaminopyrimidine ring system (18)—afforded the candidate active-site-directed irreversible inhibitor (VI) isolated as its hemisulfate salt.

The 2,4-diamino-5-(phenoxypropyl)-6-phenylpyrimidine (XIII*b*), which was needed for reversible binding studies with dihydrofolic reductase, was synthesized by two routes previously used for related compounds (10, 15, 16). Reaction of 5-bromovaleronitrile (VII) with phenol and potassium carbonate in boiling acetone afforded the phenyl ether (VIII*b*) as an oil. Claisen condensation of VIII*b* with ethyl benzoate and sodium hydride in xylene afforded the keto nitrile (X*b*) as an oil that was estimated to be about 60% pure by thin-layer chromatography. Fusion of the crude keto ester (X*b*) with guanidine carbonate at 180° (10, 16)



Scheme I

gave a dark tarry reaction mixture; the requisite 2,4-diaminopyrimidine (XIII*b*) was readily isolated by extraction with 10% aqueous acetic acid, albeit in low over-all yield.

The second route to XIII*b* started with ethyl benzoylacetate which was alkylated with 3-bromopropyl phenyl ether to IX*b*; further reaction with guanidine carbonate in *tert*-butyl alcohol (19) afforded the 4-pyrimidinol (XI*b*) in 17% over-all yield for the two steps. Reaction of XI*b* with boiling phosphorus oxychloride gave the chloropyrimidine (XII*b*) as a gum which could not be crystallized; further reaction of the crude XII*b* with methanolic ammonia at 155° afforded the 2,4-diaminopyrimidine (XIII*b*).

Numerous attempts to prepare X*c* by condensation of 5-(*p*-nitrophenoxy)-valeronitrile (VIII*c*) with ethyl benzoate and sodium hydride in dimethylsulfoxide, benzene, or xylene gave dark tars; apparently the nitrophenyl group is incompatible with these Claisen conditions, as previously observed with *p*-nitrophenylacetonitrile (20). Although conversion of XIII*b* to XIII*c* by nitration might be a feasible reaction, it was not studied since the synthesis of XIII*c* *via* XI*c* was successful.

Synthesis

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% alcohol, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms were run on Brinkmann silica gel GF and spots were detected by visual examination under ultraviolet light.

2-Amino-5-phenoxypropyl-6-phenyl-4-pyrimidinol (XI*b*)—To a stirred solution of 15.5 Gm. (81 mmoles) of ethyl benzoylacetate and 17.5 Gm. (81 mmoles) of 3-bromopropyl phenyl ether in 75 ml. of reagent dimethylsulfoxide was added 4.0 Gm. (81 mmoles) of sodium hydride (as a 50% dispersion in mineral oil) over a period of 45 min. (21). After being stirred for 24 hr., the mixture was acidified with glacial acetic acid, then poured into a stirred mixture of 150 ml. of benzene and 200 ml. of water. The separated aqueous layer was extracted with two 50-ml. portions of benzene. The combined benzene solutions were washed with two 100-ml. portions of water, dried with magnesium sulfate, then evaporated *in vacuo;* yield, 28 Gm. of crude IX*b* which showed two spots on TLC in 5:1 petroleum ether (b.p. 60–110°)–ethyl acetate, neither of which were starting materials.

To a solution of this crude IX*b* in 100 ml. of *tert*-butyl alcohol was added 6.5 Gm. (36 mmoles) of guanidine carbonate. After being refluxed with magnetic stirring for 72 hr., during which time the product separated, the mixture was cooled and filtered (21). The product was washed with ethanol; yield, 4.0 Gm. (17%), m.p. 264–267°, suitable for further transformation. A sample was recrystallized three times from aqueous 2-methoxyethanol: white crystals, m.p. 270–274°, that showed one spot on TLC in 4:1 ethanol–chloroform. The compound had $\lambda_{max.}$ (pH 1, 7): 279 mμ; (pH 13): 286 mμ; $\nu_{max.}$ 3350, 3100 (NH); 1640, 1580, 1480 (C=O, C=N, C=C); 755, 697, 688 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{19}N_3O_2$: C, 71.0; H, 5.96; N, 13.1. Found: C, 70.9; H, 5.88; N, 12.9.

2,4-Diamino-5-phenoxypropyl-6-phenylpyrimidine (XIII*b*) Hemisulfate—*Preparation A*—A mixture of 2.4 Gm. (15 mmoles) of 5-bromovaleronitrile (VII), 1.40 Gm. (15 mmoles) of phenol, 2.0 Gm. (15 mmoles) of anhydrous potassium carbonate, and 5 ml. of reagent acetone was refluxed with magnetic stirring for 8 hr. The mixture was spin-evaporated *in vacuo*, then the residue was extracted with 20 ml. of benzene. The benzene solution was washed with two 20-ml. portions of 0.1 *N* aqueous sodium hydroxide, then three 20-ml. portions of water. Dried with magnesium sulfate, the solution was evaporated to residue *in vacuo;* yield, 2.40 Gm. (89%) of crude VIII*b;* $\nu^{film}_{max.}$ 2260 (C≡N); 1600, 1490 (C=C); 1240 (C—O—C); 755, 690 cm.$^{-1}$ (C_6H_5).

To a magnetically stirred solution of 4.8 Gm. (27 mmoles) of similarly prepared crude VIII*b* in 50 ml. of xylene was added 1.20 Gm. (26 mmoles) of sodium hydride (as a 50% dispersion in mineral oil) followed by 3.75 Gm. (25 mmoles) of ethyl benzoate. The mixture, protected from moisture, was care-

fully brought to the b.p., then refluxed for 24 hr. The mixture, acidified with glacial acetic acid, was poured into a mixture of 100 ml. of benzene and 150 ml. of water. The separated organic layer was washed with three 100-ml. portions of water, then spin-evaporated *in vacuo;* yield, 9.0 Gm. of crude X*b* which showed one major spot and several minor spots on TLC in 1:10 ethyl acetate–petroleum ether (b.p. 60–110°).

A mixture of the above crude X*b* and 4.5 Gm. (25 mmoles) of guanidine carbonate was placed in a bath preheated to 180°; after 30 min. at 180°, gas evolution was essentially complete. The cooled melt was stirred with 100 ml. of 10% aqueous acetic acid for about 30 min., then the mixture was filtered through a diatomaceous earth[1] pad. To the filtrate was added 10 ml. of 20% aqueous sulfuric acid. After several hours, the mixture was filtered and the product was recrystallized from aqueous 2-methoxyethanol; yield, 0.68 Gm. (5.9% over-all based on VII), m.p. 235–240°; $\lambda_{max.}$ (pH 1): 287 mμ; (pH 13): 297 mμ; $\nu_{max.}$ 3350, 3200 (NH); 1630, 1490 (NH, C=C, C=N); 1240 (C—O—C); 755, 685 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{20}N_4O \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 59.2; H, 5.97; N, 14.6. Found: C, 59.2; H, 6.11; N, 14.6.

Preparation B—A magnetically stirred mixture of 1.00 Gm. (3 mmoles) of XI*b* and 10 ml. of phosphorus oxychloride under a reflux condenser and protected from moisture was placed in a bath preheated to 110° and maintained at 110° for 1 hr. The cooled solution was poured into 60 ml. of petroleum ether (b.p. 60–110°), then allowed to stand until the supernatant liquid had cleared (1 hr.). The supernatant was then decanted from the gum. The gum was dissolved in chloroform and the solution washed with two 50-ml. portions of ice-cold 10% aqueous sodium acetate, then two 50-ml. portions of water; the layers readily emulsified, and separation was slow. Dried with magnesium sulfate, the chloroform solution was spin-evaporated *in vacuo* leaving about 1 Gm. of crude XII*b* which had $\lambda_{max.}$ (pH 1): 323 mμ; (pH 13): 308 mμ.

The crude XII*b* was heated with 40 ml. of methanol previously saturated with ammonia in a Parr bomb at 155° for 10 hr. The cooled mixture was spin-evaporated *in vacuo*. The residue was extracted with 50 ml. of 10% aqueous acetic acid on a steam bath. The decanted solution was clarified with carbon, then diluted with 20 ml. of 20% aqueous sulfuric acid. The cooled mixture was filtered and the product washed with water; yield, 0.223 Gm. (18%), m.p. 200–210°, that was identical with *Preparation A* as shown by U.V., I.R., and TLC in 5:1 chloroform–ethanol.

2,4-Diamino-5-(p-nitrophenoxypropyl)-6-phenylpyrimidine (XIIIc) Hemisulfate—The crude chloropyrimidine (XII*c*), prepared from 1.92 Gm. (5.11 mmoles) of XI*c* (10), as described for the preparation of XII*b*, was dissolved in 40 ml. of methanol previously saturated with ammonia at −10°. The solution was heated in a Parr bomb at 150–155° for 20 hr., then spin-evaporated *in vacuo*. The residue was extracted with 300 ml. of 10% aqueous acetic acid on a steam bath. The solution was clarified with decolorizing carbon by filtration through a diatomaceous earth pad. To the filtrate was added 20 ml. of 20% sulfuric acid. The cooled mixture was filtered and the product was washed with water; yield 1.15 Gm. (52%) of crystalline product, m.p. 239–256°, which showed two spots on TLC in 4:1 chloroform–ethanol. Six recrystallizations from aqueous 2-methoxyethanol gave pure product in 30% recovery, m.p. 261–263°; $\lambda_{max.}$ (pH 1): 293 mμ; (pH 13): 305 mμ; $\nu_{max.}$ 3300, 3100 (NH); 1660, 1620, 1500 (NH, C=C, C=N); 1250 (C—O—C); 1105 ($SO_4^=$); 890 (*p*-C_6H_4); 750, 695 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{19}N_5O_3 \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 52.8; H, 5.15; N, 16.2. Found: C, 52.7; H, 5.47; N, 16.0.

5-(p-Aminophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (XIV)—A solution of 300 mg. (0.70 mmole) of XIII*c* in 100 ml. of 85% aqueous ethanol and 0.20 ml. of 12 *N* hydrochloric acid was shaken with hydrogen at 2–3 Atm. in the presence of 50 mg. of platinum oxide for 30 min. when hydrogen uptake was complete. The solution was filtered through a diatomaceous earth pad, then evaporated *in vacuo*. The residual amine salt was dissolved in 20 ml. of boiling 50% aqueous ethanol; the solution was made alkaline with 2 ml. of 1 *N* aqueous sodium hydroxide, then water was added to turbidity. On cooling, the solution deposited 190 mg. (78%) of crystals that analyzed for a monohydrate, m.p. 158–159° dec.; $\nu_{max.}$ 3400 (H_2O); 3300, 3180–3050 (NH); 1620, 1550 (NH, C=C, C=N); 1245 (C—O—C); 820 (*p*-C_6H_4); 710 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1): 283 mμ; (pH 13): 297 mμ. After being dried at 80° for 1 day in high vacuum, the compound was still partially hydrated.

Anal.—Calcd. for $C_{19}H_{21}N_5O \cdot {}^1/_2H_2O$: C, 66.3; H, 6.44; N, 20.3. Found: C, 66.3; H, 6.65; N, 20.0.

3-(p-Bromoacetamidophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (VI) Hemisulfate—To a magnetically stirred mixture of 17 mg. (0.050 mmole) of XIV, 2 ml. of acetone, and 0.165 ml. (0.055 mmole) of 3.3 m*M* aqueous acetic acid cooled in an ice bath was added a solution of 16 mg. (0.06 mmole) of bromoacetic anhydride in 0.5 ml. of acetone. After 30 min., the solution was treated with 0.5 ml. of 0.2 *N* aqueous sulfuric acid. The solution was concentrated to about 1 ml. *in vacuo*. The mixture was warmed on a steam bath, then just sufficient alcohol was added to give a clear solution. Water was added to turbidity, then the solution was allowed to cool; yield 15 mg. (58%), that was uniform on TLC in 5:1 chloroform–ethanol. Recrystallization from aqueous 2-methoxyethanol gave the analytical sample, m.p. 171–172° dec.; $\lambda_{max.}$ (pH 1, 7): 280 mμ; (pH 13): 291 mμ.

Anal.—Calcd. for $C_{21}H_{22}BrN_5O_2 \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 48.1; H, 4.81; N, 13.4. Found: C, 48.2; H, 4.85; N, 13.3.

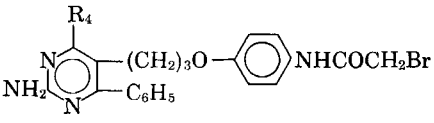
The compound gave a positive test for a reactive halogen with *p*-nitrobenzylpyridine (18) and negative Bratton-Marshall test for aromatic amine (18).

RESULTS AND DISCUSSION

Table I presents the results of the candidate 2,4-diamino active-site-directed irreversible inhibitor (VI) on inactivation of dihydrofolic reductase; the previous results with the corresponding 2-amino-4-pyrimidinol (IV) (2) are also presented. Note that

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_4	Estimated $K_i \times 10^8$ M[a]	Irreversible[b] Expt.	μM Concn. Inhibitor	μM Concn. TPNH	% $E \cdot \cdot \cdot I$[c]	Time, min.	% Inactivation
IV[d]	OH	130	*A*	40	0	25	50	50
VI	NH_2	1.7	*B*	2	12	54	60	0
IV	OH	130	*C*	20	0	13	120	37
VI	NH_2	1.7		20	0	>13[e]	120	0

The technical assistance of Barbara Baine, Maureen Baker, and Susan Lakatos with the assays of Tables I and II is acknowledged. [a] The dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol, as previously described (22). The K_i was estimated from $K_i = I \times K_m/S$, where I = inhibitor concentration giving 50% inhibition, $K_m = 1 \times 10^{-6}$ M, and $S = 6 \times 10^{-6}$ M; this equation is usually valid when $S > 4\ K_m$ (6, 23). [b] Dihydrofolic reductase was incubated with inhibitor at 37° in 0.05 M Tris buffer (pH 7.4) containing *no* mercaptoethanol as previously described (24); in each case an enzyme control was run and the per cent inactivation by inhibitor is corrected for the small amount of thermal inactivation in the enzyme control. [c] Calculated from $[EI] = E_t/[1 + K_i/I]$, where E_t = the concentration of total active enzyme, $[EI]$ = the fraction of E_t reversibly complexed by I, and I = the inhibitor concentration (3, 12). [d] Data from *Reference 2*. [e] The $[EI]$ concentration is unknown in the absence of TPNH, due to the ternary binding requirement for 2,4-diaminopyrimidines; see *Results and Discussion*.

VI gives no inactivation of the enzyme in the absence of TPNH or in the presence of 12 μM TPNH; in contrast, IV shows inactivation of the enzyme even at a concentration of 20 μM where IV reversibly complexes with only 13% of the total available dihydrofolic reductase.

Before discussion of these results, the recent experiments by Bertino *et al.* (25, 26) on measurement of binding to dihydrofolic reductase by tryptophan fluorescence spectra should be considered. They observed (25) that the 2,4-diamino-type inhibitor—2,4,7-triamino-6-phenylpteridine—could complex to dihydrofolic reductase in the absence of TPNH (binary binding), but binding was sixtyfold tighter in the presence of TPNH (ternary binding). In Table II are presented some of their results (26) on our 6-phenylpyrimidines.

Note that the 2,4-diamino-6-phenylpyrimidines with a 5-phenylbutyl (XXII) or 5-anilinopropyl (XXIII) show tighter binding in the presence of TPNH (ternary binding) than in the absence of TPNH (binary binding); the extent of this increased binding could not be measured due to the limit of detection of the ternary K_D, but the increase in binding was >fortyfold with XXII and >100-fold with XXIII. In contrast, a much less dramatic effect by TPNH was seen with the 2-amino-6-phenyl-4-pyrimidinols; with the 5-anilinopropyl (XXVII) and the 5-phenylbutyl side-chain (XXVI), only a 2.5-fold and twelvefold increase in ternary binding over binary binding being observed. Also note that the 2,4-diaminopyrimidines gave stronger binary binding than the corresponding 2-amino-4-pyrimidinols; with the 5-phenylbutyl side-chain, XXII was complexed sixtyfold better than XXVI and with the 5-anilinopropyl side-chain, XXIII was complexed thirtyfold better than XXVII.

Now let us return to experiment *C* in Table I. At a concentration of 20 μM, the diaminopyrimidine (VI) should complex more of the enzyme than the 2-amino-4-hydroxypyrimidine (IV) when TPNH is absent—although this exact number cannot be determined kinetically since TPNH is required for a kinetic experiment (25). The fact that 20 μM IV still shows inactivation of the enzyme, but 20 μM VI shows none, demonstrates unequivocally that IV and VI form complexes with dihydrofolic reductase that are conformationally different, since the terminal alkylating function is juxtaposed to a nucleophilic group on the enzyme in the case of IV, but not in the case of VI.

How can these conformations within the enzyme–inhibitor reversible complex be different? In Table II are listed reversible binding constants for five classes of compounds—determined kinetically in the presence of TPNH. There are at least three distinct modes of binding with respect to the side-chains.

In class *A* are listed the 5-(*p*-chlorophenyl)-2,4-diaminopyrimidines with varying substituents at the 6-position. It was previously noted that the *p*-chlorophenyl group gave a 5500-fold increment in binding due to hydrophobic bonding (6); this tremendous increase in binding was believed to be determinant (3, 10) on which rotomer (7) of the pyrimidine would complex to the enzyme. Note that there is less than a threefold difference in binding with small and large chains at the 6-position in class *A;* these results are best interpreted as indicating that the 6-side-chain is not in contact with the enzyme, but is projected into space.

Similar observations were made with the class *B* compounds containing a 5-phenylbutyl side-chain (except XX); the 5-phenylbutyl side-chain gives a 40,000-fold increment in binding (6), and presumably determines which rotomer (7) will complex to the enzyme (3,10); note again that small or large 6-side-chains have no more than a two fold effect on binding.

In class *E* are listed some 2-amino-4-pyrimidinols; note that with the 5-phenylbutyl side-chain a loss in binding occurs in proceeding from a 6-methyl (XXVIII) to a 6-*n*-propyl side-chain (XXIX); this result has been attributed to a repulsion of the propyl group from a hydrophilic area on the enzyme (10). Thus, the propyl group of XXIX would have to be in contact with the enzyme in order for this repulsion to occur, in contrast to the noncontact of the 6-group in series *A* and *B*.

A third type of binding is observed with the 2,4-diamino-6-phenylpyrimidines of class *C*. First, it should be noted that the 6-phenyl group of XXI gives only a sevenfold increment in binding (10) compared to the 6-methyl group of 2,4-diamino-6-methylpyrimidine (6)—much smaller than the 5500-fold increment observed with XV; thus the 6-phenyl group may not necessarily be determinant on which rotomer will bind even though most of the earlier data with 6-phenylpyrimidines could be rationalized on this basis (10). That the terminal phenyl group of a 5-side-chain in class *C* may not be complexed to the enzyme in the same place as a terminal phenyl group of class *B* is indicated by the following comparison; note that in class *B*, the phenylbutyl group of XVIII gives 85-fold tighter binding than the anilinopropyl group of XX, attributed to the terminal phenyl group being complexed in a hydrophobic region; in contrast, note in class *C* that the 5-phenylbutyl (XXII), 5-phenoxypropyl (XIII*b*), and 5-anilinopropyl (XXIII) side chains differ by no more than a factor of four in binding, indicating that the terminal phenyl group is in a more polar region. Similar results were previously observed in class *D* with XXVI and XXVII which should be compared with XXVIII, XXX, and XXXI in class *E*.

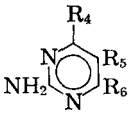
These reversible binding studies could indicate that the two candidate irreversible inhibitors, IV and VI (Table I), bind in a similar conformation; however, experiment *C* (Table I) indicates clearly that their conformations are different. A possible working hypothesis useful for further studies is the following.

The active-site-directed irreversible inhibitors of the 2-amino-4-pyrimidinol type such as II and XXXII are believed to have conformation *B* (Table III). This assignment is based on the decrease in binding when the 6-methyl group of class *E* compounds (XXVIII) (Table II) is extended to *n*-propyl (10), but the terminal phenyl group of XXXII gives a fourfold increase in binding (1); such a repulsion of an alkyl group by the enzyme is likely to take place if this alkyl group is complexed in the polar region that normally complexes the substrate, dihydrofolate, with assigned conformation I (10). Compound IV is believed to have conformation *A* (2, 10).

Since IV can inactivate dihydrofolic reductase, but the diaminopyrimidine (VI) does not, it follows that IV and VI must have different conformations when complexed to dihydrofolic reductase. That VI does not have a conformation *C* like that assigned to XVI is indicated by extra binding exerted by the phenoxyalkyl group of VI, whereas XVI shows no appreciable addition or loss of binding as the phenylalkyl group is varied (see class *A*, Table II).

A logical conformation for V and VI is *D* with the phenylalkyl group staggered to the right; it was pointed out above that the terminal phenyl group of class *B* compounds was complexed in a different area on the enzyme than class *C* compounds (Table II) since the side-chain binding of class *B* compounds such as XVIII and XX was considerably influenced when an NH group adjacent to the

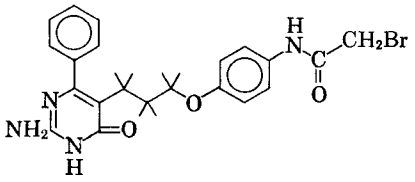
TABLE II—REVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY



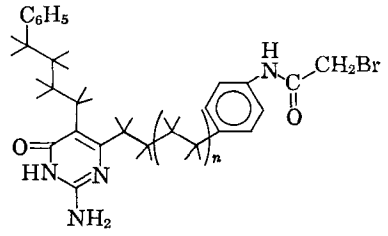
Compd.	Class	R_4	R_5	R_6	μM Concn. for 50% Inhibition[a]	$-K_D \times 10^9\ M$[b] Binary	Ternary
XV	*A*	NH_2	$-C_6H_4Cl$-*p*	CH_3	0.20[c]	...	...
XVI	*A*	NH_2	$-C_6H_4Cl$-*p*	$-(CH_2)_2C_6H_4NHCOCH_2Br$-*p*	0.53[d]	...	...
XVII	*A*	NH_2	$-C_6H_4Cl$-*p*	$-CH_2Br$	0.26[e]	...	...
XVIII	*B*	NH_2	$-(CH_2)_4C_6H_5$	CH_3	0.027[f]	...	...
XIX	*B*	NH_2	$-(CH_2)_4C_6H_5$	$-C_3H_7$-*n*	0.021[g]	...	...
III	*B*	NH_2	$-(CH_2)_4C_6H_5$	$-(CH_2)_2C_6H_4NHCOCH_2Br$-*p*	0.040[d]	...	...
XX	*B*	NH_2	$-(CH_2)_3NHC_6H_5$	CH_3	2.2[f]	...	...
XXI	*C*	NH_2	H	$-C_6H_5$	160[g]	...	...
XIII*a*	*C*	NH_2	$-C_4H_9$-*n*	$-C_6H_5$	29[g]	...	...
XXII	*C*	NH_2	$-(CH_2)_4C_6H_5$	$-C_6H_5$	1.1[g]	40	<1
XXIII	*C*	NH_2	$-(CH_2)_3NHC_6H_5$	$-C_6H_5$	0.88[g]	100	<1
XIII*b*	*C*	NH_2	$-(CH_2)_3OC_6H_5$	$-C_6H_5$	3.5	...	...
XIII*c*	*C*	NH_2	$-(CH_2)_3OC_6H_4NO_2$-*p*	$-C_6H_5$	3.5	...	...
XIV	*C*	NH_2	$-(CH_2)_3OC_6H_4NH_2$-*p*	$-C_6H_5$	8.4		
VI	*C*	NH_2	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	$-C_6H_5$	8.1	...	...
XXIV	*C*	NH_2	NH_2	$-C_6H_5$	1100[h]	...	...
XXV	*C*	NH_2	$-NH(CH_2)_3C_6H_4NH_2$-*p*	$-C_6H_5$	0.42[h]	...	...
V	*C*	NH_2	$-NH(CH_2)_3C_6H_4NHCOCH_2Br$-*p*	$-C_6H_5$	0.33[h]	...	...
IV	*D*	OH	$-(CH_2)_3OC_6H_4NHCOCH_2Br$	$-C_6H_5$	800[i]	...	...
XXVI	*D*	OH	$-(CH_2)_4C_6H_5$	$-C_6H_5$	0.54	2400	200
XXVII	*D*	OH	$-(CH_2)_3NHC_6H_5$	$-C_6H_5$	1.8[i]	3000	1200
XXVIII	*E*	OH	$-(CH_2)_4C_6H_5$	$-CH_3$	30[k]	...	...
XXIX	*E*	OH	$-(CH_2)_4C_6H_5$	$-C_3H_7$-*n*	900[g]	...	...
XXX	*E*	OH	$-(CH_2)_3NHC_6H_5$	$-CH_3$	800[l]	...	...
XXXI	*E*	OH	$-(CH_2)_3OC_6H_5$	$-CH_3$	840[k]	...	...
XXXII	*E*	OH	$-(CH_2)_4C_6H_5$	$-(CH_2)_4C_6H_4NHCOCH_2Br$-*p*	240[m]	...	...

[a] See *Footnote a*, Table I. [b] Data from *Reference 26*; K_D = dissociation constant determined by quenching of fluorescence spectra of pure enzyme. Binary binding is determined in the absence of TPNH and ternary binding in the presence of TPNH. [c] Data from *Reference 7*. [d] Data from *Reference 15*. [e] Data from *Reference 24*. [f] Data from *Reference 6*. [g] Data from *Reference 10*. [h] Data from *Reference 16*. [i] Data from *Reference 2*. [j] $K_i \times 10^6\ M$ measured at pH 6 with folic acid as substrate; data from *Reference 17*. [k] Data from *Reference 27*. [l] Data from *Reference 22*. [m] Data from *Reference 1*.

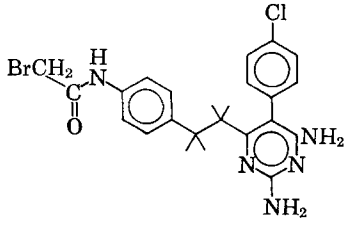
TABLE III—SOME POSSIBLE CONFORMATIONS FOR BINDING TO DIHYDROFOLIC REDUCTASE



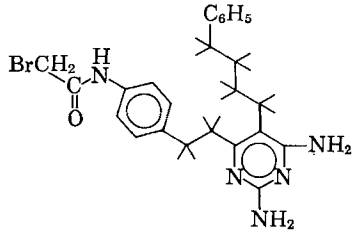
IV
Conformation *A*



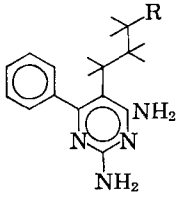
II, $n=0$
XXXII, $n=1$
Conformation *B*



XVI
Conformation *C*

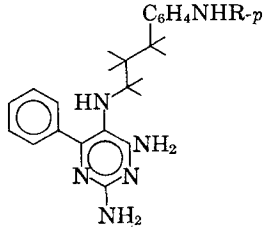


III
Conformation *C*



Conformation *D*

VI, R = —$OC_6H_4NHCOCH_2Br$-*p*
XIII*b*, R = —OC_6H_5
XXII, R = —$CH_2C_6H_5$
XXIII, R = —NHC_6H_5



Conformation *D*

V, R = —$COCH_2Br$
XXV, R = —H

phenyl of XX was replaced by methylene as in XVIII.

Therefore, the conformations outlined in Table III will be used as a working hypothesis. The synthesis of compounds V and XXV (16) is quite adaptable to variation of side chain in order to determine the ideal bridge distance for irreversible inhibition (3, 4); such studies are currently being pursued. Such active-site-directed irreversible inhibitors, if successful, would give another parameter to the assignment of binding conformations, as noted in the comparison of IV and VI.

REFERENCES

(1) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **55**, 1417(1966).
(2) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422 (1966).
(3) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, New York, N. Y., 1967.
(4) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(5) *Reference 3*, chap. X.
(6) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(7) Baker, B. R., and Jordaan, J. H., *ibid.*, **54**, 1740 (1965).
(8) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(9) Baker, B. R., Schwan, T. J., Novotny, J., and Ho B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308 (1966).
(11) *Reference 3*, chap. VIII.
(12) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(13) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).
(14) Bertino, J. R., Booth, B. A., Bieber, B. L., Cashmore, A., and Sartorelli, A. C., *J. Biol. Chem.*, **239**, 479(1964).
(15) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **4**, 31(1967).
(16) Baker, B. R., Jackson, G. D. H., and Meyer, R. B., Jr., *J. Pharm. Sci.*, **56**, 566(1967).
(17) Baker, B. R., Shapiro, H. S., and Werkheiser, W. C., *J. Med. Chem.*, **8**, 283(1965).
(18) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(19) Baker, B. R., and Shapiro, H. S., *J. Med. Chem.*, **6**, 664(1963).
(20) DeGraw, J., Ross, L. O., Goodman, L., and Baker, B. R., *J. Org. Chem.*, **26**, 1933(1961).
(21) Baker, B. R., Santi, D. V., and Shapiro, H. S., *J. Pharm. Sci.*, **53**, 1317(1964).
(22) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(23) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714 (1964).
(24) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(25) Perkins, J. P., and Bertino, J. R., *Biochemistry*, **5**, 1005(1966).
(26) Hillcoat, B., and Bertino, J. R., to be published.
(27) Baker, B. R., Ho, B.-T., and Chheda, G. B., *J. Heterocyclic Chem.*, **1**, 88(1964).

Application of Regression Analysis to Hypoglycemic Activities of 12 Piperidinesulfamylsemicarbazides and Activity Predictions for 12 Analogs

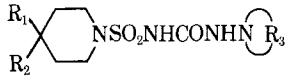
By W. RONALD SMITHFIELD and WILLIAM P. PURCELL

A regression analysis is applied to the hypoglycemic activity data for a series of 12 piperidinesulfamylsemicarbazides. The results are used to estimate the hypoglycemic activity for 12 untested analogs. The following seven untested compounds are predicted to have greater hypoglycemic activity than chlorpropamide: 1,1-pentamethylene-4-(4-ethyl-4-methyl-1-piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4-methoxy-4-methyl-1-piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4,4-diethyl-1-piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4-ethyl-4-methoxy-1-piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4,4-dimethoxy-1-piperidinesulfamyl)semicarbazide; 1,1-hexamethylene-4-(4-methoxy-4-methyl-1-piperidinesulfamyl)semicarbazide; 1,1-hexamethylene-4-(4-ethyl-4-methoxy-1-piperidinesulfamyl)semicarbazide.

THE POSSIBILITY of designing molecules for specific therapeutic uses by the application of regression analyses to structure-activity data (1–3) holds considerable interest for the authors. Recently, Free and Wilson's method (3) has been applied to an analogous series of cholinesterase inhibitors (4) synthesized in these laboratories.

Several prerequisites are necessary for the satisfactory application of regression analysis to structure-activity data: (*a*) the series to be studied should be composed of closely related analogs (thereby increasing the probability of having a similar mechanism of action), (*b*) accurate biological data measured under uniform conditions must be available, and (*c*) the activity parameter chosen must be intrinsically additive. Naturally, it is also desirable to have a maximum number of degrees of freedom and, therefore, to have a high ratio of the number of observations to the number of unknown terms in the linear equations.

McManus and Gerber (5) have recently reported activity data which appear suitable for the application of the regression analysis technique. They evaluated a series of 12 substituted piperidinesulfamylsemicarbazides (I) as possible hypoglycemic agents, and the activity data (Table I), including standard deviations, are presented as the maximum per cent fall in blood glucose at a dose level of 100 mg./Kg. in groups of 8–10 rats of the Sprague-Dawley strain. The authors analyzed these results using a method similar to that reported by Free and Wilson (3) with the purpose of (*a*) calculating and ranking group contributions to activity for the series of piperidinesulfamylsemicarbazides and (*b*) estimating the hypoglycemic activity of analogous molecules which have not been screened.



I

REGRESSION ANALYSIS

If one assumes that the substituents on the molecules evaluated by McManus and Gerber (5) make a constant and additive contribution to the over-all activity, a simultaneous equation may be written for each of the 12 observations (Eqs. 1–12).

$$a[\mathrm{H}] + b[\mathrm{H}] + c[\mathrm{C_5}] + \mu = 14.8 \quad \text{(Eq. 1)}$$

$$a[\mathrm{H}] + b[\mathrm{H}] + c[\mathrm{C_6}] + \mu = 11.1 \quad \text{(Eq. 2)}$$

$$a[\mathrm{H}] + b[\mathrm{Me}] + c[\mathrm{C_5}] + \mu = 26.1 \quad \text{(Eq. 3)}$$

$$a[\mathrm{H}] + b[\mathrm{Me}] + c[\mathrm{C_6}] + \mu = 33.9 \quad \text{(Eq. 4)}$$

$$a[\mathrm{Me}] + b[\mathrm{Me}] + c[\mathrm{C_5}] + \mu = 39.1 \quad \text{(Eq. 5)}$$

$$a[\mathrm{Me}] + b[\mathrm{Me}] + c[\mathrm{C_6}] + \mu = 34.9 \quad \text{(Eq. 6)}$$

$$a[\mathrm{Me}] + b[\mathrm{Et}] + c[\mathrm{C_6}] + \mu = 42.0 \quad \text{(Eq. 7)}$$

$$a[\mathrm{Et}] + b[\mathrm{Et}] + c[\mathrm{C_6}] + \mu = 34.4 \quad \text{(Eq. 8)}$$

$$a[\mathrm{C_2}] + b[\mathrm{C_2}] + c[\mathrm{C_5}] + \mu = 35.6 \quad \text{(Eq. 9)}$$

$$a[\mathrm{C_2}] + b[\mathrm{C_2}] + c[\mathrm{C_6}] + \mu = 30.8 \quad \text{(Eq. 10)}$$

$$a[\mathrm{C_{2.5}}] + b[\mathrm{C_{2.5}}] + c[C_6] + \mu = 25.0 \quad \text{(Eq. 11)}$$

$$a[\mathrm{H}] + b[\mathrm{OCH_3}] + c[\mathrm{C_6}] + \mu = 24.8 \quad \text{(Eq. 12)}$$

In Eqs. 1–12 the brackets indicate activity contributions, while the letters *a*, *b*, and *c* refer to positions R_1, R_2, and R_3, respectively. Therefore, $a[\mathrm{H}]$ is the activity contribution of a hydrogen atom at position R_1; $a[\mathrm{C_2}]$ is the activity contribution of half of a tetramethylene group at position

Received December 21, 1966, from the Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee, Memphis, TN 38103

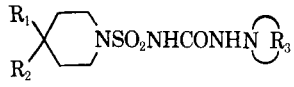
Accepted for publication February 13, 1967.

The authors acknowledge support by grant GB-4453 from the National Science Foundation and research contract DA-49-193-MD-2779 from the U. S. Army Medical Research and Development Command. Computer facilities were provided by grant HE-09495 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors thank Dr. C. W. Sheppard and Mrs. A. B. McEachran, University of Tennessee Medical Units Computer Center, for help in processing the data.

Contribution No. 143 from the Army Research Program on malaria.

TABLE I—OBSERVED HYPOGLYCEMIC ACTIVITY FOR 12 SUBSTITUTED PIPERIDINESULFAMYLSEMICARBAZIDES AND PREDICTED ACTIVITIES FOR 12 SIMILARLY SUBSTITUTED DERIVATIVES



No.	Substitution at R_1[a]						Substitution at R_2[a]						Substitution at R_3		Activity[b]	
	H	CH_3	C_2H_5	OCH_3	$(CH_2)_2$	$(CH_2)_{2.5}$	H	CH_3	C_2H_5	OCH_3	$(CH_2)_2$	$(CH_2)_{2.5}$	$(CH_2)_5$	$(CH_2)_6$	Obs.[c]	Calcd.
1	+	...	...	...	...	...	+	...	...	...	...	...	+	...	14.8 ± 7.0	14.8
2	+	...	...	...	...	...	+	...	...	...	...	...	...	+	11.1 ± 1.9	14.1
3	+	...	...	...	...	...	...	+	...	...	...	...	+	...	26.1 ± 2.6	27.4
4	+	...	...	...	...	...	...	+	...	...	...	...	...	+	33.9 ± 1.5	26.7
5	+	...	...	...	...	...	...	...	+	...	...	...	+	...	...	26.0
6	+	...	...	...	...	...	...	...	+	...	...	...	...	+	...	25.3
7	+	...	...	...	...	...	...	...	...	+	...	...	+	...	...	25.0
8	+	...	...	...	...	...	...	...	...	+	...	...	...	+	24.8 ± 3.1	24.3
9	...	+	...	...	...	...	...	+	...	...	...	...	+	...	39.1 ± 2.8	39.9
10	...	+	...	...	...	...	...	+	...	...	...	...	...	+	34.9 ± 3.3	39.2
11	...	+	...	...	...	...	...	...	+	...	...	...	+	...	...	38.5
12	...	+	...	...	...	...	...	...	+	...	...	...	...	+	42.0 ± 3.3	37.8
13	...	+	...	...	...	...	...	...	...	+	...	...	+	...	...	37.5
14	...	+	...	...	...	...	...	...	...	+	...	...	...	+	...	36.8
15	...	...	+	...	...	...	...	...	+	...	...	...	+	...	...	37.2
16	...	...	+	...	...	...	...	...	+	...	...	...	...	+	34.4 ± 2.8	36.5
17	...	...	+	...	...	...	...	...	...	+	...	...	+	...	...	36.2
18	...	...	+	...	...	...	...	...	...	+	...	...	...	+	...	35.4
19	...	...	...	+	...	...	...	...	...	+	...	...	+	...	...	35.1
20	...	...	...	+	...	...	...	...	...	+	...	...	...	+	...	34.4
21	...	...	...	...	+	...	...	...	...	...	+	...	+	...	35.6 ± 2.4	33.5
22	...	...	...	...	+	...	...	...	...	...	+	...	...	+	30.8 ± 3.7	32.8
23	...	...	...	...	...	+	...	...	...	...	...	+	+	...	...	25.7
24	...	...	...	...	...	+	...	...	...	...	...	+	...	+	25.0 ± 3.7	25.0

[a] The 4,4-tetramethylene group is denoted by two units of $(CH_2)_2$, and the 4,4-pentamethylene group is denoted by two units of $(CH_2)_{2.5}$. [b] Activities are reported (5) as maximum per cent blood glucose lowering at dose of 100 mg./Kg. McManus and Gerber (5) found chlorpropamide to have an activity of 35 ± 3.3%. [c] Observed values are reported with standard deviations.

R_1 and is necessarily paired with $b[C_2]$; $a[C_{2.5}]$ is the activity contribution of half of a pentamethylene group at position R_1 and is paired with $b[C_{2.5}]$; $c[C_5]$ is the activity contribution of a pentamethylene group at position R_3; $c[C_6]$ is the activity contribution of a hexamethylene group at position R_3, *etc.*; μ represents the activity contribution of the parent structure. In each equation the sum of the appropriate group contributions and μ are set equal to the maximum per cent fall in blood glucose at a dose level of 100 mg./Kg. as reported by McManus and Gerber (5).

If one assumes that positions R_1 and R_2 are equivalent in the substituted piperidinesulfamylsemicarbazides, the following six simplifying equations may be written:

$$a[H] = b[H] \quad \text{(Eq. 13)}$$

$$a[Me] = b[Me] \quad \text{(Eq. 14)}$$

$$a[Et] = b[Et] \quad \text{(Eq. 15)}$$

$$a[C_2] = b[C_2] \quad \text{(Eq. 16)}$$

$$a[C_{2.5}] = b[C_{2.5}] \quad \text{(Eq. 17)}$$

$$a[OCH_3] = b[OCH_3] \quad \text{(Eq. 18)}$$

Substitution of these relationships into Eqs. 1–12 results in 12 equations with nine unknowns. In addition, two restrictions (Eqs. 20, 21) result from the summation to zero (3) of the group contributions at positions R_1 and R_2 and position R_3:

$$5a[H] + 2b[H] + 3a[Me] + 4b[Me] + a[Et] + 2b[Et] + 2a[C_2] + 2b[C_2] + a[C_{2.5}] + b[C_{2.5}] + b[OCH_3] = 0 \quad \text{(Eq. 19)}$$

or, from Eqs. 13–18,

$$7a[H] + 7a[Me] + 3a[Et] + 4a[C_2] + 2a[C_{2.5}] + a[OCH_3] = 0 \quad \text{(Eq. 20)}$$

and

$$4c[C_5] + 8c[C_6] = 0 \quad \text{(Eq. 21)}$$

The 14 simultaneous equations with nine unknowns were solved by the method of least squares by employing the program Scrap and the IBM 1620 computer.

RESULTS AND DISCUSSION

Simultaneous solution of the 12 linear equations gives the calculated activity contribution for each group (Table II) and the value for the constant, μ (35.25%).

The appropriate group contributions and μ may be used in Eq. 22 to calculate hypoglycemic activities (Table I) and

$$\text{hypoglycemic activity} = \mu + \Sigma \text{ group contributions} \quad \text{(Eq. 22)}$$

the agreement between calculated and observed values may be used as a test for the analysis.

From Table II one can rank[1] the groups at positions R_1 and R_2 in order of decreasing contribution to hypoglycemic activity as follows: $CH_3 > C_2H_5 > OCH_3 > (CH_2)_2 > (CH_2)_{2.5} > H$. The hydrogen substituent has a deleterious effect on activity (−8.99%), while the methyl group contributes most to activity (3.56%). It is interesting to note that the addition of one methylene group produces a substantial reduction in contribution between the tetramethylene group, $(CH_2)_4$ (2 ×

[1] Free and Wilson have emphasized that "The rank order of related substituents within a position would be expected to have meaning" (3).

TABLE II—SUBSTITUTENT GROUP CONTRIBUTION TO HYPOGLYCEMIC ACTIVITY

Position(s)	Group[a]	Contribution[b]
R_1 and R_2	H	−8.99
R_1 and R_2	CH_3	3.56
R_1 and R_2	C_2H_5	2.20
R_1 and R_2	OCH_3	1.17
R_1 and R_2	$(CH_2)_2$	0.38
R_1 and R_2	$(CH_2)_{2.5}$	−3.54
R_3	$(CH_2)_5$	−2.47
R_3	$(CH_2)_6$	−3.17

[a] See *Footnote a*, Table I. [b] See *Footnote b*, Table I.

0.38% = 0.76%), and the pentamethylene group, $(CH_2)_5$ (2 × −3.54% = −7.08%). At the R_3 position, both the pentamethylene and hexamethylene groups have a negative contribution (−2.47% and −3.17%, respectively).

It is apparent from Table I that reasonable agreement exists between the calculated and observed hypoglycemic activities. The *F*-ratio between the variance of the estimated activity and the variance of the observed activity is significant at the 99% level. The coefficient of the multiple correlation is 0.977. In four of the 12 compounds (No. *2*, *4*, *10*, and *12*), however, the differences between the calculated and observed values are greater than the standard deviations reported by McManus and Gerber (5).

The primary interest in this type of study is the selection of untested molecules which have a high probability of possessing useful therapeutic activity. The seven untested molecules which are predicted to be more active than chlorpropamide [activity as determined by McManus and Gerber (5), 35 ± 3.3%], are 1,1-pentamethylene-4-(4-ethyl-4-methyl-1-piperidinesulfamyl)semicarbazide; 1,1 - pentamethylene - 4 - (4 - methoxy - 4 - methyl-1 - piperidinesulfamyl)semicarbazide; 1,1 - hexamethylene - 4 - (4 - methoxy - 4 - methyl - 1 - piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4,4-diethyl - 1 - piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4-ethyl- 4 - methoxy-1-piperidinesulfamyl)semicarbazide; 1,1-hexamethylene-4-(4-ethyl-4-methoxy-1-piperidinesulfamyl)semicarbazide; 1,1-pentamethylene-4-(4,4-dimethoxy-1-piperidinesulfamyl)semicarbazide (No. *11*, *13*, *14*, *15*, *17*, *18*, and *19*, respectively). These compounds appear to be worthy of synthesis and testing as possible hypoglycemic agents.

REFERENCES

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Kinetics of Reaction of Dehydroacetic Acid I

Reaction with Primary Amines I

By SHIGERU GOTO, SADAO IGUCHI, AKIRA KONO, and HIDEKO UTSUNOMIYA

Dehydroacetic acid (DHA), one of the officially recognized food preservatives in Japan, has been found in some instances to readily react with amino compounds under mild and physiological conditions to produce Schiff's base type compounds. The kinetic study of the reaction between DHA and β-phenethylamine (PE-NH), with the formation of 3-(1-phenethylamino)ethylidene-6-methyl-3H-pyran-2,4-dione, was investigated in various water–ethanol mixtures, and a linear relationship was observed between the reciprocal of the dielectric constant of the solvent and log *k*. The *k*–pH profiles for the reaction in 80 per cent ethanol solution at 40 and 60° were completed in the acetate buffer regions. No general acid-base catalysis was observed but salt effects were not negligible. The rate-determining step in 80 per cent ethanol solution was probably the attack of free PE-NH on the protonated carbonyl carbon atom in free DHA.

DEHYDROACETIC ACID (DHA) (I) has been accepted as one of the officially recognized food preservatives in Japan and is at present being used widely not only in some foodstuffs and refreshing drinks but also in pharmaceutical preparations as an antiseptic. It has been pointed out that one of the most remarkable chemical behaviors of DHA is its affinity toward compounds possessing an amino radical. When they coexist in solution, Schiff's base type compounds (II) are initially readily produced by the reaction between DHA and amino compounds, and then finally transformed, in some cases, to lutidone derivatives (V). This has been reported in the preceding papers (1, 2) and the mechanism described in Scheme I was proposed.

In some instances the conversion proceeds under mild and physiological conditions.

Other similar qualitative and synthetic investigations (3–5) have been published, but no

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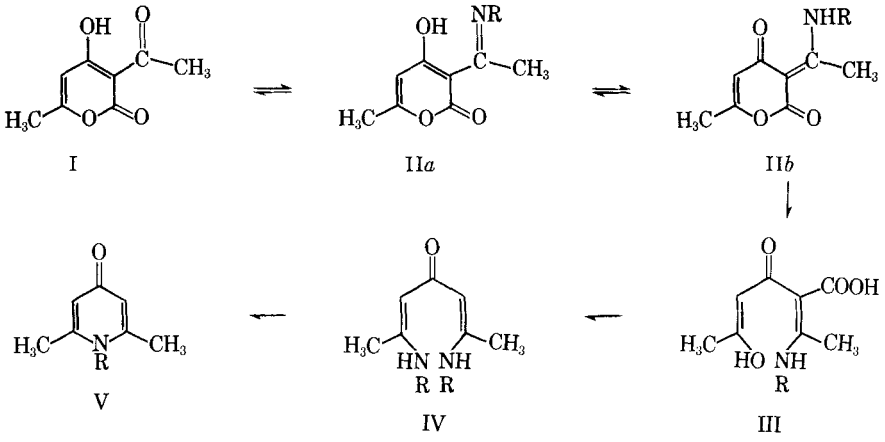


R = CH_3, C_2H_5, $CH_2C_6H_5$, and $(CH_2)_2C_6H_5$

Proposed Route for Reaction of Dehydroacetic Acid with β-Phenethylamine

Scheme I

kinetic data on this transformation have been reported. It seems natural, based on the unique chemical behavior of DHA, especially from a kinetic viewpoint, to extend studies to cover the re-estimation of its practical usages in Japan.

In this experiment the authors selected β-phenethylamine (PE-NH) as a representative primary amine because of its easiness of treatment. Reactions of DHA were kinetically studied in water–ethanol mixtures.

This paper considers the kinetics in the first step of the transformation reaction, the formation of 3-(1-phenethylamino)-ethylidene-6-methyl-3H-pyran-2,4-dione (NR-DHA).

EXPERIMENTAL

Materials—Ethanol of superior special grade for precision analysis was used. PE-NH (Wako pure reagent) was redistilled twice (b.p. 178–179°). DHA (Taito Pfizer Co.) was recrystallized from ethanol–water, m.p. 109–110°. Commercial acetic acid was redistilled, and sodium acetate and sodium chloride of pure reagent grade were used for the kinetic studies.

Determination of DHA—A 1-ml. sample was diluted with 9 ml. of cold distilled water. Three drops of a 10% hydrochloric acid solution and 0.1 ml. of a 17% titanium chloride solution were added to the sample solution. The observed pH of the final sample solution was in the range of 1.5 to 2.0. The absorbance of the sample was measured against a blank solution at 550 mμ using 1-cm. cells in a Hitachi photoelectric spectrophotometer of the EPU 2A type. A plot of absorbance *versus* concentration gave a straight line graph over the range 1×10^{-4} to 2×10^{-3} *M*. The percentage standard deviation for repeated determinations was ±1.0%. The coexistences of NR-DHA (II), lutidones (V), 2,6-bis(phenethylamino)-2,5-heptadien-4-one (IV), and PE-NH did not interfere with DHA determinations.

Kinetic Studies—The rates were measured by following the decrease of DHA. Twenty-five milliliters of a 2×10^{-2} *M* DHA solution in an appropriate acetate buffer was mixed with 25 ml. of a 2×10^{-2} *M* PE-NH solution in a 50-ml. glass volumetric flask, and thermostated at the temperature of the prospective kinetic study. The pH of the reaction mixture was measured over the entire period of a kinetic run, and the average of these pH values was used for further kinetic calculations. The ionic strengths of the reactions were generally constant at 0.1. The effect of change in the ionic strength on the reaction was studied by changing the ionic strength of the solutions with sodium chloride, and the effect of the general acid-base catalysis was evaluated in eightfold ranges of acetate buffer concentration at the constant ionic strength ($\mu = 0.1$). Aliquots (1 ml.) were pipeted out at regular time intervals, and the above for the DHA assay was used. Verification of the fact that the spectrophotometric absorption band at 550 mμ characterized the intact DHA was obtained by thin-layer chromatographic determination of an aliquot of the residual DHA and the reaction product, NR-DHA, in 99.7% ethanol at 40°. Calibration curves of known concentrations (1×10^{-5} to 2×10^{-4} *M*) for the two components in ethanol were prepared by spectrophotometric absorbances at 305 mμ for DHA and 314 mμ for NR-DHA. The thin-layer plates (20 × 20 cm.) were coated with a 0.4-mm. layer of silica gel B-5 (Wako pure reagent), and a 100-μl. sample solution was spotted on the plate. The plates were developed with benzene–acetic acid–water (1:1:2) twice. The solvent front was allowed to advance 13 cm. Detection for DHA and NR-DHA was performed under ultraviolet light at 253.6 mμ. Each spot was cut off and extracted with warm ethanol in a small glass apparatus for about 5 min. The extracts were filtered through filter paper (Toyo Roshi Co.). The residues on the paper were washed twice by warm ethanol. The washings were added to the filtrates, and the total solutions were adequately diluted by ethanol and measured by the spectrophotometric method. The results of the analysis from colorimetric and thin-

layer chromatographic methods coincided well. Since the sum of the mole concentrations of DHA and NR-DHA at each time interval was equal to the initial mole concentration of DHA, the reaction was terminated at the formation of the Schiff's base type compounds.

RESULTS AND DISCUSSION

Reaction in 99.73 Per Cent v/v[1] Ethanol Solution—A mixture of DHA and an equivalent mole of PE-NH ($1 \times 10^{-2}M$) was placed in the constant-temperature bath, and the proceeding reaction was studied by estimating the concentration of DHA by means of the color assay developed with titanium chloride. The reaction rate was expressed as:

$$v = k_1 (\text{DHA})(\text{PE-NH}) \qquad \text{(Eq. 1)}$$

and satisfied the rate equation,

$$dx/dt = k_1 (a - x)^2 \qquad \text{(Eq. 2)}$$

and the integrated form is

$$1/(a - x) = k_1 t + 1/a \qquad \text{(Eq. 3)}$$

where a is the initial concentration for DHA and PE-NH, x represents the consumed concentration at t for DHA and PE-NH, and k_1 is the apparent second-order rate constant. The second-order plots for the reaction are given in Fig. 1.

Effect of Addition of Water—The addition of water retarded the apparent rate constant, k_1, and accelerated the hydrolysis of NR-DHA (k_2).

$$\text{PE-NH} + \text{DHA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{NR-DHA} + \text{H}_2\text{O}$$

The observed apparent equilibrium constant, K, in the presence of an excess amount of water is

$$K = \frac{(\text{NR-DHA})}{(\text{DHA})(\text{PE-NH})} \qquad \text{(Eq. 4)}$$

and the apparent rate constants for the forward and the reverse reactions, k_1 and k_2, are calculated by means of the following equations:

$$k_1 = \frac{1}{t} \frac{2.303\, x_e}{a^2 - x_e^2} \log \frac{x_e (a^2 - x x_e)}{a^2 (x_e - x)} \qquad \text{(Eq. 5)}$$

$$k_2 = \frac{1}{K} k_1 = \frac{(a - x_e)^2}{x_e} k_1 \qquad \text{(Eq. 6)}$$

where x_e is the concentration of consumed DHA and PE-NH at equilibrium. The values of k_1 are calculated from the slope of $2.303\, x_e/(a^2 - x_e^2) \log x_e(a^2 - x x_e)/a^2(x_e - x)$ *versus* t.

It was observed that the rate constant for forward reaction, k_1, decreased with a decrease of concentration of ethanol in solvent. This fact may be due to an increase in ionizing power of solvent by the addition of water to solvent. The plot of log k_1 *versus* the reciprocal of dielectric constants,[2] 1/D, is an approximate straight line (Fig. 2). This phenomenon may be ascribed to the degree of ionization of both DHA and PE-NH at initial stage, namely, the relative concentration of free DHA and free PE-NH.

[1] Measured by titrimetric (Karl Fischer) method.
[2] Dielectric constants of ethanol–water mixtures were obtained from Akerlöf, G., *J. Am. Chem. Soc.*, **54**, 4125(1932).

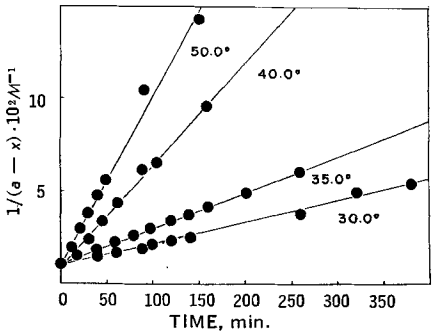


Fig. 1—Second-order plots for the reaction of dehydroacetic acid with β-phenethylamine in 99.73% ethanol.

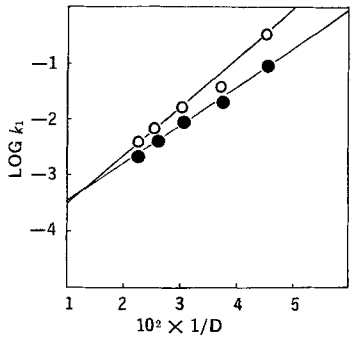


Fig. 2—Influence of the dielectric constant on the reaction rate constants for the reaction of dehydroacetic acid with the primary amines. Key: ●, β-phenethylamine; ○, benzylamine.

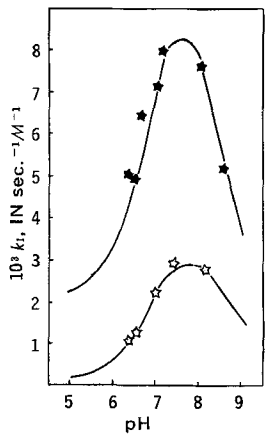


Fig. 3—The k_1-pH profiles for the reaction of dehydroacetic acid with β-phenethylamine. Key: ☆, experimental values at 40°; ★, experimental values at 60°; ———, theoretical lines.

Effect of pH—In Fig. 3, the apparent second-order rate constants in 80% ethanol at 40 and 60° are plotted against the observed pH values. All points on the pH-rate profile have been obtained in the acetate buffer solution or no buffer solution. When

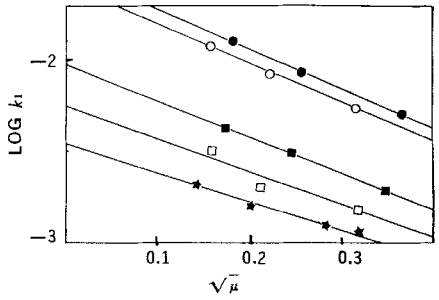


Fig. 4—Effect of the ionic strength on the reaction rate constants for the reaction of dehydroacetic acid with β-phenethylamine. Key: ●, pH 6.84 at 60°; ○, pH 6.58 at 60°; ■, pH 6.98 at 40°; □, pH 6.57 at 40°; ★, pH 6.37 at 40°.

TABLE I—OBSERVED RATE CONSTANT ($10^3 k_1$ IN M^{-1} sec.$^{-1}$) FOR THE REACTION OF DHA WITH PE-NH IN 80% ETHANOL[a]

Observed pH	Observed Rate Constant
40° C.	
6.37[b]	0.97
6.57[b,c]	1.44
6.98[b,c]	2.25
7.50	2.90
8.13	2.80
60° C.	
6.58[b,c]	5.01
6.84[b]	6.45
7.07	7.17
7.19[c]	7.80
8.10[c]	7.67
8.63	5.00

[a] Initial concentration: DHA, 1×10^{-2} *M*; PE-NH, 1×10^{-2} *M* ($\mu = 0.1$). [b] When the ionic strength was changed, a significant difference in the apparent rate constant was observed (see Fig. 4). [c] When the concentration of acetate buffer solution was at eightfold ranges, no significant differences in the apparent rate constants were observed.

the concentration of acetate buffer solution (μ = 0.1) was changed at eightfold ranges, no significant difference in the apparent rate constants was observed. But a significant ionic strength effect was observed for the reaction at the same pH and buffer (Fig. 4); therefore, the ionic strength was adjusted to 0.1 by the addition of sodium chloride. As the concentration of the protonated PE-NH increases with a decrease in pH, the ability of PE-NH to act as a nucleophilic agent is lost, and thus a decrease in the rate constant is observed. On the other hand, in alkaline media where the concentration of the protonated PE-NH is at a minimum, a reduction in the rate is again observed. This is attributed to a decrease in the concentration of free DHA. Since a maximum of the pH-profile occurred at about a pH of 7.5 (Fig. 3 and Table I), these results suggest that a probable mechanism would be the attack of a nucleophilic reagent such as free undissociated PE-NH onto the protonated carbonyl carbon atom in free undissociated DHA. Assuming that the relative species are free PE-NH and DHA, the forward rate v_f is expressed as:

TABLE II—APPARENT pKa VALUES[a] IN ETHANOL–WATER MIXTURES AT 40°C.

Vol. % of Ethanol[b]	pKa
β-Phenethylamine	
97.5	8.00
88.2	8.37
78.4	8.61
68.6	8.71
49.0	8.80
Dehydroacetic Acid	
98.5	7.55
89.3	7.25
79.2	6.80
69.5	6.40
59.2	6.25

[a] The titrations were conducted on 40-ml. sample solutions with 0.1 *N* HCl and 0.1 *N* NaOH aqueous solutions and pKa values were calculated by Shima's method (see Shoji, R., and Yoshimura, H., "Theory and Measurement of pH," Maruzen, Tokyo, Japan, p. 130.). Estimated pKa values for β-phenethylamine and dehydroacetic acid in 80% ethanol were 8.58 and 6.83, respectively. [b] The per cent ethanol at each pKa was calculated from the known volume in the original solution and the known volume of aqueous titer added to achieve the apparent pH corresponding to that pKa value.

$$v_f = k_f (\text{DHA})_f (\text{PE-NH})_f \quad \text{(Eq. 7)}$$

$$= k_f \frac{K_1}{K_1 + (\text{H}^+)} \frac{(\text{H}^+)}{K_2 + (\text{H}^+)} (a - x)^2 \quad \text{(Eq. 8)}$$

where k_f is the true rate constant for the forward reaction. The K_1 and K_2 are the dissociation constants of the protonated PE-NH and DHA, respectively. The apparent rate constant, k_1, for the forward reaction can be written:

$$k_1 = k_f \frac{K_1}{K_1 + (\text{H}^+)} \frac{(\text{H}^+)}{K_2 + (\text{H}^+)} \quad \text{(Eq. 9)}$$

Moreover, k_f is expressed as:

$$k_f = k_0 + k_{\text{H}^+} (\text{H}^+) \quad \text{(Eq. 10)}$$

where k_0 may be the rate constant for the solvent catalyzed, and k_{H^+} is the bimolecular rate constant for hydrogen ion. A plot of k_f against the hydrogen-ion concentration estimates k_{H^+} from the slope and k_0 from the intercept. The values of k_0 and k_{H^+} were calculated to be 2.2×10^{-1} mole^{-1} sec.$^{-1}$ and 5.5×10^4 mole^{-1} sec.$^{-1}$ at 40°, 6.0×10^{-1} mole^{-1} sec.$^{-1}$ and 8.0×10^5 mole^{-1} sec.$^{-1}$ at 60°, respectively. The values of K_1 and K_2 in 80% ethanol were calculated by titration in various ethanol concentrations at 40°. The results are shown in Table II. The values of K_1 and K_2 in 80% ethanol at 40° applied to Eqs. 8 and 9.

In conclusion, it has been suggested that the rate-determining step for the reaction between DHA and PE-NH in 80% ethanol is the attack of free PE-NH on the protonated carbonyl carbon atom in free DHA.

REFERENCES

(1) Iguchi, S., Inoue, A., and Kurahashi, T., *Chem. Pharm. Bull. (Tokyo)*, **11**, 385(1963).
(2) Iguchi, S., and Inoue, A., *ibid.*, **11**, 390(1963).
(3) Garrat, S., *J. Org. Chem.*, **28**, 1886(1963).
(4) Schut, R. N., Strycker, W. G., and Thomas, M. H. L., *ibid.*, **28**, 3046(1963).
(5) Cook, D., *J. Chem. Soc.*, **41**, 1453(1963).

Behavior of Erythrocytes in Various Solvent Systems IV

Water–Dimethylsulfoxide

By DONALD E. CADWALLADER and JOHN P. DRINKARD*

Hemolytic behavior of human erythrocytes in water–DMSO solutions was investigated. Complete hemolysis of erythrocytes took place in all DMSO solutions, with discoloration and precipitation occurring at DMSO concentrations greater than 35 per cent. The addition of 0.9 per cent NaCl or isotonic concentrations of other compounds ($CaCl_2$, dextrose, lactose, KBr, Na citrate, NaBr, NaI, and Na salicylate) prevented hemolysis in solutions containing 0.0 to 40 per cent DMSO. The addition of these compounds to solutions containing more than 40 per cent DMSO did not prevent hemolysis, discoloration, or precipitation of human red blood cells. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in various aqueous DMSO solutions. The DMSO concentration which afforded the greatest protection to erythrocytes against osmotic hemolysis was 26–27 per cent. In aqueous ternary solvent systems, DMSO did not affect the concentrations at which propylene glycol and polyethylene glycol 300 damage erythrocytes.

To PREPARE a safe, stable, and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous cosolvent. With this in mind, the hemolytic behavior of rabbit and human erythrocytes in aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols has been investigated (1–3).

The solvent system selected for this investigation was water–dimethylsulfoxide (DMSO). In recent years, DMSO has been the subject of many research articles concerning its effect on biological systems and its unusual and numerous medicinal properties. In addition, a great deal of interest has been expressed in the excellent solvent properties of DMSO. Of the aprotic solvents, DMSO has one of the highest dielectric constants, 48.9 at 20°. Thus, DMSO is completely miscible in all proportions with water and most common organic solvents, including glycerin, acetone, and ethanol (4). DMSO has been found to be an unusually effective solvent for a variety of compounds, including proteins (5), glycogen (6), cellulose and related substances (7, 8), steroids (9), sugar and sugar esters (10), and alphaglucochloralose (11).

Results of preliminary pharmacological tests indicate that drugs dissolved in DMSO and administered systemically do not differ significantly in their lethality or cellular penetration (12). Willson *et al.* (13) carried out toxicity studies to determine if DMSO would be useful as a vehicle for the intravenous administration of water-insoluble antitumor agents. His and other reports (14, 16) indicate that DMSO has a very low toxicity; however, it appears that blood cells are hemolyzed in the presence of high concentrations of this vehicle.

The purpose of this investigation was to study the behavior of red blood cells in aqueous DMSO solutions. The hemolytic method was employed, and the experiments were designed so that standard hemolysis curves obtained for human blood in experiments using aqueous saline solutions could be compared to hemolysis curves obtained from experiments using sodium chloride–water–DMSO solutions. From these data it was possible to calculate *hemolytic* isotonic coefficients for sodium chloride in various water–DMSO solutions. Experiments were also carried out to observe what effect the addition of isotonic quantities of various compounds had in preventing hemolysis of erythrocytes in water–DMSO systems.

EXPERIMENTAL

Materials—Reagent grade dimethylsulfoxide supplied by the J. T. Baker Chemical Co. was used. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Blood Samples—Human blood was used in all experiments and was obtained from several 20–25 year old male Caucasian donors just prior to each experiment. Approximately 10 ml. of blood was obtained from the forearm vein and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. After gently rotating the flask for approximately 5 min., the defibrinated blood was decanted into a 50-ml. conical flask and aerated by gently swirling the flask for about 5 min.

Received October 10, 1966, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication December 15, 1966.

Previous paper: Smith, L. B., and Cadwallader, D. E., *J. Pharm. Sci.*, **56**, 351(1967).

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

* Student participant in a 1965–1966 Mead Johnson undergraduate research award.

Preparation of Solutions—Dimethylsulfoxide solutions were calculated and prepared as volume–volume percentage solutions. Solutions containing glycerin, polyethylene glycol, and propylene glycol were prepared on a weight-volume basis. Concentrations of electrolytes and solid nonelectrolytes in solution were based on weight-in-volume calculations. Solutions containing isotonic concentrations of various compounds were prepared using data from an earlier paper [*viz.*, Table I (2)].

Quantitative Determination of Per Cent Hemolysis—The method used to determine the amount of hemolysis of erythrocytes in various solutions was described in earlier papers in this series (1, 2). Hemolysis experiments were carried out at 25 ± 1 and 37 ± 1°.

To determine the effect of time on hemolysis, several rate studies were carried out in which the blood-test solution mixtures were removed at 10-min. intervals, during a period of 60 min., centrifuged, and the amount of hemolysis determined colorimetrically. These rate experiments were carried out at 37 ± 1, 25 ± 1, and 20 ± 1° so that the effect of temperature on rate could be ascertained.

Calculation of i Values—An objective of this paper was to calculate *apparent i* values for sodium chloride when the salt was present in a water–DMSO system, *e.g.*, sodium chloride in 20% DMSO. When the concentrations of sodium chloride in water and water–DMSO solutions causing the same degree of hemolysis are known, the value of i (isotonic coefficient) for sodium chloride in aqueous DMSO solutions can be calculated according to a previously described equation (1) modified as follows:

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml. water} \end{pmatrix} = \begin{pmatrix} i \text{ value for} \\ \text{NaCl in DMSO soln.} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl in} \\ \text{100 ml. DMSO soln.} \end{pmatrix} \quad \text{(Eq. 1)}$$

The value of i for sodium chloride in water was taken as 1.86.

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–DMSO solutions were plotted on rectangular coordinate paper. From these curves the concentrations of sodium chloride in Gm./100 ml. of water and the other solvent, causing 25, 50, and 75% hemolysis were determined. These values were inserted into Eq. 1, and the values of i for sodium chloride in a particular water–DMSO solution, at concentrations giving 25, 50, and 75% hemolysis were determined. The various i values for sodium chloride in aqueous DMSO solutions are shown in Table I.

Preparation of Hemolysis Curves—Twenty experiments employing human blood were carried out. The average readings of these experiments were used to construct a standard hemolysis curve (left-hand side of Fig. 1.) Hemolysis curves of the various DMSO solutions shown in Fig. 1 were prepared in the same manner as described in previous papers (1, 3).

To determine DMSO concentrations that offered greatest protection to red blood cells, hemolysis experiments were carried out in various water–DMSO–sodium chloride solutions and the data portrayed on rectangular coordinate paper as described above. From these hemolysis curves the amounts of sodium chloride preventing hemolysis of 50% of the erythrocytes (or where 50% hemolysis occurred) in various DMSO solutions were determined and the results are shown in Fig. 2.

TABLE I—VALUES OF i FOR SODIUM CHLORIDE IN VARIOUS WATER–DMSO SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

DMSO, % v/v	Hemolysis 25%	50%	75%	Av.
	Expt. at 25°			
5	1.87	1.88	1.87	1.87
10	1.94	1.97	1.97	1.97
15	1.99	1.98	1.99	1.99
20	2.16	2.15	2.13	2.15
30	2.13	2.11	2.11	2.12
35	2.06	2.06	2.05	2.06
40	1.96	2.08	2.07	2.04
	Expt. at 37°			
5	1.86	1.87	1.86	1.86
10	1.89	1.88	1.89	1.89
15	1.91	2.00	1.96	1.95
20	2.09	2.11	2.09	2.10
30	2.08	2.07	2.06	2.07
35	2.01	1.99	2.02	2.01
40	1.90	2.00	2.00	1.97

[a] Each i value represents an average of at least two blood samples.

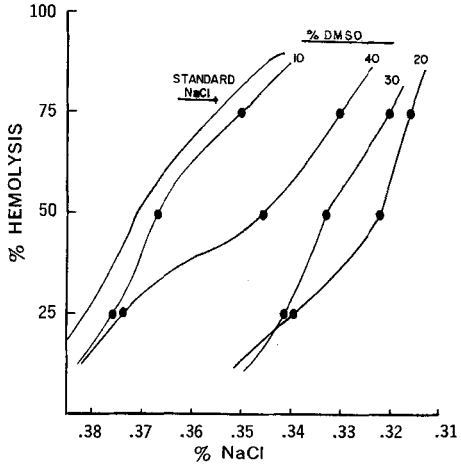


Fig. 1—Hemolysis of human erythrocytes after 45 min. at 37° in various DMSO–saline solutions.

RESULTS AND DISCUSSION

Complete hemolysis of human erythrocytes occurred in 0.0 to 100% DMSO solutions after 45 min. at 25 or 37°. Hemolysis in aqueous solutions containing 0.0 to 30% DMSO gave normal, clear red solutions; however, in solutions containing 35 to 45% DMSO, red-brown solutions were observed. Red-brown solutions containing a brown precipitate resulted when blood was placed in solutions containing 50% or more of DMSO. Precipitation became very heavy when the DMSO concentration reached 75 to 100%. These observations were

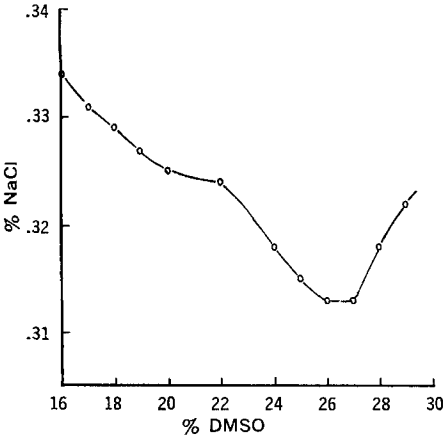


Fig. 2—Amount of sodium chloride preventing hemolysis of 50% of erythrocytes in various DMSO solutions at 37°.

similar to those reported by Ansel and Leake for rabbit blood (17).

Time studies showed that hemolysis and/or damage of red blood cells occurred immediately upon the addition of blood to aqueous DMSO solutions. The rate of hemolysis and cell damage was independent of experimental temperatures (20, 25, and 37°).

The addition of 0.9% sodium chloride prevented hemolysis of human erythrocytes in solutions containing 0.0 to 40% DMSO, however, in experiments where the concentration of DMSO was greater than 25%, trace hemolysis (less than 10%) occurred with several blood samples. Increased hemolysis was observed in solutions containing 40–45% DMSO, and brown-green solutions containing a dark brown precipitate resulted when blood was added to saline solutions containing between 46 and 49% DMSO. Discoloration and precipitation occurred in solutions containing higher concentrations of DMSO. This phenomenon was not prevented by the addition of 2 or 3% sodium chloride.

Aqueous DMSO solutions containing isotonic concentrations of various substances (1.15% calcium chloride, 9.39% dextrose, 8.16% lactose, 1.91% potassium bromide, 1.96% sodium citrate, 1.51% sodium bromide, 2.20% sodium iodide, and 2.45% sodium salicylate) gave results similar to those described above for DMSO–saline solutions. These compounds prevented hemolysis in 0.0 to 40% DMSO solutions but not in the more concentrated solutions.

The fragility of human erythrocytes in various water–DMSO solutions was modified by the addition of hypotonic quantities of sodium chloride. Typical sigmoid hemolysis curves (*viz.*, Fig. 1) resulted when blood was added to saline solutions containing 0.0 to 40% DMSO. These curves were constructed in the manner described under *Experimental* utilizing the data presented in Table I.

Hemolysis in 0.0 to 40% DMSO solutions is an osmotic phenomenon since addition of 0.9% sodium chloride to these solutions prevents hemolysis, and addition of hypotonic quantities of saline allows partial hemolysis to occur.

It was possible to calculate i values for sodium chloride in various water–DMSO solutions. The average i values for sodium chloride in these systems (Table I) were slightly greater than 1.86, the accepted value for 0.9% sodium chloride in water. This indicated that DMSO contributed very little to the tonicity of the extracellular aqueous solutions; however, it did not have a negative effect until, of course, the DMSO concentration became greater than 40%. This slight increase in i values can probably be attributed either to some effect of DMSO on the red cell membrane or to increased activity of sodium chloride in DMSO solutions. Sears *et al.* (18) determined the equivalent conductances in DMSO of a number of sodium and potassium salts including halides, but not sodium chloride, and found them to be completely dissociated in DMSO.

The i values at 37° were consistently slightly greater (approximately 1–3%) than the corresponding values at 25°. In previous studies (1, 2) with glycerin and propylene glycol systems, slightly greater i values were found at 37° than at 25°.

Although the range of hemolytic i values of sodium chloride in DMSO solutions was narrow, there was an optimum DMSO concentration which gave the most protection to erythrocytes against osmotic hemolysis. As can be seen in Fig. 2 the amount of sodium chloride needed to protect 50% of the erythrocytes against hemolysis decreased as the concentration of DMSO increased from 16 to 26–27%; the protective concentrations then increased with further increases in DMSO concentrations.

Kinetic studies were carried out in DMSO solutions (10, 20, and 30%) containing between 0.32 and 0.37% sodium chloride. Hypotonic concentrations of sodium chloride were used so that partial hemolysis of erythrocytes would occur in DMSO test solutions. Hemolysis did not appreciably increase from the first 10-min. reading to the 60-min. reading; final hemolysis readings were about 5% greater than initial readings. Essentially the same results were obtained for all experimental temperatures (20, 25, and 37°). These rate studies showed that complete or partial hemolysis in DMSO solutions occurs rapidly and is independent of time and temperature, indicating simple diffusion. The slight increases in hemolysis which did occur with time were probably due to some effect of DMSO on the red cell membrane.

Throughout this investigation it was observed that high concentrations of DMSO caused damage to red blood cells and that diluting the DMSO solutions with saline lowered the *in vitro* toxicity. This damage was not inhibited or prevented by the addition of sodium chloride or isotonic concentrations of various electrolytes and nonelectrolytes. DiStefano and Klahn (15) have shown that DMSO is a potent hemolytic agent when administered intravenously to cats. However, when DMSO was injected in a diluted form, it was far less hemolytic, and hemolysis was almost negligible when DMSO was diluted 8 times in isotonic saline. Willson *et al.* (13) reported that perivascular inflammation and local thrombosis resulted from repeated intravenous administration of undiluted DMSO into dogs, but that these reactions did not occur when the material was diluted adequately before use. In this current study the authors have found that the addition of 0.9% sodium chloride to solu-

tions containing more than 40% DMSO does not prevent damage to erythrocytes and/or the precipitation of blood components. It appears that aqueous solutions containing more than 40% DMSO would be unsafe as a vehicle for intravenous preparations.

To determine the effect of a ternary solvent system on erythrocytes, hemolysis experiments were run at 37° in solutions containing 20% DMSO, 0.9% sodium chloride, and various amounts of either propylene glycol or polyethylene glycol 300 (PEG 300). Solutions containing 0.0 to 30% propylene glycol did not hemolyze red blood cells, however, complete hemolysis with slight discoloration took place in those solutions containing more than 30% propylene glycol. Hemolysis did not take place in solutions containing 0.0 to 20% PEG 300; however, when blood was placed in solutions containing 25% or more PEG 300, the solutions became green-brown, and a brown precipitate formed. In these ternary solvent systems, the addition of DMSO did not alter the critical concentrations (in 0.9% saline) (2, 3) at which propylene glycol and PEG 300 have been reported to hemolyze red blood cells. The damaging effect of the glycols appeared to be solely dependent on their concentration in solution, and there was no additive effect contributed by the DMSO present.

REFERENCES

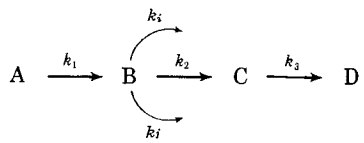
(1) Cadwallader, D. E., *J. Pharm. Sci.*, **52**, 1175(1963).
(2) Cadwallader, D. E., Wickliffe, B. W., and Smith, B. L., *ibid.*, **53**, 927(1964).
(3) Smith, B. L., and Cadwallader, D. E., *ibid.*, **56**, 351 (1967).
(4) "Dimethyl Sulfoxide (DMSO) Technical Bulletin," Crown Zellerbach Corp., Camas, Wash.
(5) Rees, E. C., and Singer, S. J., *Arch. Biochem. Biophys.*, **63**, 144(1956).
(6) Whistler, R. L., and BeMiller, J. N., *ibid.*, **98**, 120 (1962).
(7) Hagglund, E., Lindberg, B., and McPherson, J., *Acta Chem. Scand.*, **10**, 1160(1956).
(8) Draus, A., *Farbe Lack*, **64**, 487(1958).
(9) Rosenkrantz, H., Hadidian, Z., Seay, H., and Mason, M. M., *Cancer Chemotherapy Rept.*, **31**, 7(1963).
(10) Everett, W. W., and Foster, J. F., *J. Am. Chem. Soc.*, **81**, 3459(1959).
(11) Bradue, M. C., and Monroe, R. R., *Current Therap. Res.*, **7**, 502(1965).
(12) Dixon, R. L., Adamson, R. H., Ben, M., and Rall, D. P., *Proc. Soc. Exptl. Biol. Med.*, **118**, 756(1965).
(13) Willson, J. E., Brown, D. E., and Timmens, E. K., *Toxicol. Appl. Pharmacol.*, **7**, 104(1965).
(14) Herschler, R. J., and Jacob, S. W., *Tappi*, **48**, 43A (1965).
(15) DiStefano, V., and Klahn, J. L., *Toxicol. Appl. Pharmacol.*, **7**, 660(1965).
(16) Gerhards, E., Gibian, H., and Raspé, G., *Arzneimittel-Forsch.*, **15**, 3(1965).
(17) Ansel, H. C., and Leake, W. F., *J. Pharm. Sci.*, **55**, 685(1966).
(18) Sears, P. G., Lester, G. R., and Dawson, L. R., *J. Phys. Chem.*, **60**, 1433(1956).

Fallacy in Concluding There are Zero-Order Kinetics from Blood Level and Urinary Excretion Data

By JOHN G. WAGNER

During the past several years there have been a number of reports in which the authors concluded their data proved zero-order formation of a metabolite or zero-order absorption of a drug in the animal or human body. For two of these reviewed in this paper—namely, the conjugation of benzoate with glycine in the rabbit following doses of 500 mg./Kg. of benzoic acid and the conversion of salicylate to salicyluarate in rats at doses above 20 mg./Kg.—the evidence is very convincing. However, the principal evidence in the remainder of the reports is the apparent linearity of a segment of a cumulative urinary excretion curve and/or the curvature of semilogarithmic plots of drug blood levels or of amounts of drug not excreted against time. Model studies and simulations presented here show that neither of the latter is sufficient evidence for concluding there is zero-order rate of formation of a metabolite or zero-order absorption of a drug. The simulations of pharmacokinetic data have far-reaching implications which go much beyond the zero-order–first-order problem. They show one cannot disregard first-order rate constants of relatively large magnitude in many cases; that graphical fitting of pharmacokinetic, and even chemical kinetic, data may often lead to serious misinterpretations; and that "Lineweaver-Burk" plots, which are artifacts, are produced by plotting the reciprocal of excretion rate of a metabolite against the reciprocal of the amount of metabolite remaining in the body.

To illustrate how one may erroneously conclude that there are parallel zero-order and first order kinetics when, in fact, all kinetics are first order, a model simulating the real situation is presented. Assume there is a catenary chain with branching of parallel paths at one point in the chain and that all rate constants are first-order rate constants. Then we may write the model as shown in Scheme I.



Scheme I

Received November 16, 1966, from the Medical Research Division, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication January 10, 1967.

tions containing more than 40% DMSO does not prevent damage to erythrocytes and/or the precipitation of blood components. It appears that aqueous solutions containing more than 40% DMSO would be unsafe as a vehicle for intravenous preparations.

To determine the effect of a ternary solvent system on erythrocytes, hemolysis experiments were run at 37° in solutions containing 20% DMSO, 0.9% sodium chloride, and various amounts of either propylene glycol or polyethylene glycol 300 (PEG 300). Solutions containing 0.0 to 30% propylene glycol did not hemolyze red blood cells, however, complete hemolysis with slight discoloration took place in those solutions containing more than 30% propylene glycol. Hemolysis did not take place in solutions containing 0.0 to 20% PEG 300; however, when blood was placed in solutions containing 25% or more PEG 300, the solutions became green-brown, and a brown precipitate formed. In these ternary solvent systems, the addition of DMSO did not alter the critical concentrations (in 0.9% saline) (2, 3) at which propylene glycol and PEG 300 have been reported to hemolyze red blood cells. The damaging effect of the glycols appeared to be solely dependent on their concentration in solution, and there was no additive effect contributed by the DMSO present.

REFERENCES

(1) Cadwallader, D. E., *J. Pharm. Sci.*, **52**, 1175(1963).
(2) Cadwallader, D. E., Wickliffe, B. W., and Smith, B. L., *ibid.*, **53**, 927(1964).
(3) Smith, B. L., and Cadwallader, D. E., *ibid.*, **56**, 351 (1967).
(4) "Dimethyl Sulfoxide (DMSO) Technical Bulletin," Crown Zellerbach Corp., Camas, Wash.
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(6) Whistler, R. L., and BeMiller, J. N., *ibid.*, **98**, 120 (1962).
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(9) Rosenkrantz, H., Hadidian, Z., Seay, H., and Mason, M. M., *Cancer Chemotherapy Rept.*, **31**, 7(1963).
(10) Everett, W. W., and Foster, J. F., *J. Am. Chem. Soc.*, **81**, 3459(1959).
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(13) Willson, J. E., Brown, D. E., and Timmens, E. K., *Toxicol. Appl. Pharmacol.*, **7**, 104(1965).
(14) Herschler, R. J., and Jacob, S. W., *Tappi*, **48**, 43A (1965).
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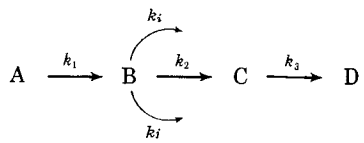
To illustrate how one may erroneously conclude that there are parallel zero-order and first order kinetics when, in fact, all kinetics are first order, a model simulating the real situation is presented. Assume there is a catenary chain with branching of parallel paths at one point in the chain and that all rate constants are first-order rate constants. Then we may write the model as shown in Scheme I.



Scheme I

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Let k be the first-order rate constant for over-all loss from the B compartment. Hence, as written, we have $k = k_2 + k_i + k_j$. If there are no parallel paths, then $k = k_2$. If there is an additional parallel path initiating at B, with rate constant k_p, then $k = k_2 + k_i + k_j + k_p$. The kinetics of the catenary chain depend only on the values of k, k_1, k_2, and k_3; hence, it is necessary only to know k and not its components k_i, k_j, k_p, *etc.* The solutions below are applicable in all cases where no two or more of the constants k, k_1, k_2, and k_3 are exactly equal.

In the model, A represents a drug at the absorption site and B represents the drug in blood and other fluids of distribution. In the real world, the A → B process may be quite complicated and the process may only be approximated by first-order kinetics. However, it is well documented (1–7) that the over-all effects of dissolution of solid drug, stomach emptying, and absorption of the drug through the stomach and/or small intestinal barriers may be represented, usually by a first-order rate constant with or without a small lag time. In the case of acetylsalicylic acid (ASA) in tablet form, A would represent ASA in tablets, and B would represent salicylate in blood and other fluids of distribution. The over-all effects of tablet disintegration, dissolution of ASA from the resulting granules, stomach emptying, absorption of ASA from the stomach and small intestine, and hydrolysis of the ASA to salicylate have been shown (2, 5) to be represented by a combination of a first-order rate constant and a small lag time.

In the model, C represents a metabolite which is formed from B, and D represents the cumulated metabolite excreted in the urine. Interpretation of blood level and urinary excretion data has revealed that *usually* the apparent rate constant for absorption (k_1 in the model) and the rate constant for urinary excretion of metabolites (k_3 in the model) are greater in magnitude than the rate constant for over-all loss of drug from the body (k in the model). To simulate the usual kinetic situation the numerical values of k_1 and k_3 have been made larger than the numerical value of k in the examples. The rate constant for formation of metabolite (k_2 in the model) will always be less than k if there are parallel paths, and the ratio k_2/k gives the fraction of the total drug reaching the circulation which is converted to the metabolite. Hence, the ultimate amount in D will be this fraction multiplied by total amount of drug reaching the circulation. It is assumed that the amounts in compartments A, B, C, and D are all in the same mass units and in terms of one of the substances, since the substance in C and D is different than the substance in A and B.

The principal purpose of the model and the examples is to show that even when k_1 and k_3 are relatively large they become important in the kinetics in those situations when $1 < k_1/k < 100$ and/or $1 < k_3/k < 100$. Also, neither k_1 nor k_3 can be ignored, without appreciable error, when $1 < k_1/k < 10$ and/or $1 < k_3/k < 10$! These statements are valid when k_1 and k_3 are both greater than k. Since k_1 is usually in the range 0.5–2 hr.$^{-1}$, as estimated from actual data (1–7), and k_3 is usually in the range 0.07–2 hr.$^{-1}$ as estimated from actual data (8–11), then k_1 and k_3 cannot be ignored, without appreciable error, with drugs (simulated by B) whose k values range from 0.007 to 2 hr.$^{-1}$ or whose $t_{1/2}$ values ($0.693/k$) range from 0.3 to 99 hr.! This includes the vast majority of drugs which the author has been privileged to investigate and read about during the past 15 years. Another purpose of the examples is to show, as stated above, how one may erroneously conclude there are parallel zero-order and first-order processes when, in fact, all processes are first order. This error can be made readily when $1 < k_1/k < 10$ and $1 < k_3/k < 10$ since, in such cases, plots of both the cumulative amount of B converted to C *versus* time and the cumulative excretion plot are only slightly curved for a considerable period of time and may be erroneously interpreted as being linear. But this interpretation is incorrect. Following the assumption of linearity the conclusion can be made that the formation step, B → C, is obeying zero-order kinetics and, hence, this leads to the conclusion that there are parallel zero-order and first-order kinetics for loss of the drug from the body. Proof of zero-order rate of formation of a metabolite or zero-order rate of absorption of a drug requires, for any given drug, more evidence than just the appearance of the cumulative urinary excretion and semilogarithmic blood level plots. The experiments needed to provide the necessary additional data are usually feasible and have been performed with specific drugs by some investigators.

EXPERIMENTAL

Refer to Scheme I. Let X_A, X_B, X_C, and X_D be the amounts in compartments A, B, C, and D, respectively, at time t. Let X_A^0 be the initial amount in compartment A at time zero. Assume that $X_B = X_C = X_D = 0$ at time zero.

The differential equations and their solutions for the model are as follows:

$$\frac{-dX_A}{dt} = k_1 X_A \qquad \text{(Eq. 1)}$$

and

$$X_A = X_A^0 e^{-k_1 t} \quad \text{(Eq. 2)}$$

$$\frac{dX_B}{dt} = k_1 X_A - k X_B \quad \text{(Eq. 3)}$$

and

$$X_B = X_A^0 \left(\frac{k_1}{k_1 - k}\right)[e^{-kt} - e^{-k_1 t}] \quad \text{(Eq. 4)}$$

$$\frac{dX_C}{dt} = k_2 X_B - k_3 X_C \quad \text{(Eq. 5)}$$

and

$$X_C = X_A^0 \left(\frac{k_2}{k}\right)\left[\frac{-k_1 k e^{-k_1 t}}{(k_1 - k)(k_3 - k_1)} + \frac{k_1 k e^{-kt}}{(k_1 - k)(k_3 - k)} + \frac{k_1 k e^{-k_3 t}}{(k_3 - k_1)(k_3 - k)}\right] \quad \text{(Eq. 6)}$$

$$\frac{dX_D}{dt} = k_3 X_C \quad \text{(Eq. 7)}$$

and

$$X_D = X_A^0 \left(\frac{k_2}{k}\right)\left[\frac{-k k_3 (1 - e^{-k_1 t})}{(k_1 - k)(k_3 - k_1)} + \frac{k_1 k_3 (1 - e^{-kt})}{(k_1 - k)(k_3 - k)} + \frac{k_1 k (1 - e^{-k_3 t})}{(k_3 - k_1)(k_3 - k)}\right] \quad \text{(Eq. 8)}$$

For computational and instructional purposes, it is best to let $t = n/k$. Hence, n is the number of time constants where the time constant is $1/k$. Also, if we let $t_{1/2} = 0.693/k$, then $1/k = 1.44 t_{1/2}$. Since, in both examples below, $k = 0.25$ hr.$^{-1}$, then $t = 4n$ and $6n$ represents 24 hr. All graphs have been drawn with two abscissa scales; one scale is in terms of n, the other in terms of t. With k the smallest of the rate constants appearing in the exponential terms of Eqs. 4, 6, and 8, the n scale indicates it requires about 6 time constants or 8.6 half-lives of the drug to clear essentially all of the drug and metabolite from compartments B and C, respectively, and to reach essentially the asymptotic amount in compartment D.

Levy (11) has implied that the critical ratio is k_3/k_2; that is, the ratio of the excretion rate constant to the formation rate constant of the metabolite. This ratio is *not* critical unless $k_2/k \to 1$, *i.e.*, unless $k_2 \to k$. This can be seen by substituting n/k for t in the exponential e^{-kt} which yields e^{-n}; similar substitutions change $e^{-k_1 t}$ to $e^{-(k_1/k)n}$ and change $e^{-k_3 t}$ to $e^{-(k_3/k)n}$. Hence, the critical ratios are k_1/k and k_3/k, not k_3/k_2. The ratio k_2/k is equivalent to the fraction of B converted to C and which ultimately appears in D. In the case of the salicylurate–salicylate system $k_2/k \simeq 0.6$ and the ratios k_1/k and k_3/k become quite critical due to the order of magnitudes of the rate constants.

Example 1—Assignments made were $k_1 = 0.5$ hr.$^{-1}$, $k = 0.25$ hr.$^{-1}$, $k_2 = 0.15$ hr.$^{-1}$, and $k_3 = 0.75$ hr.$^{-1}$. Hence, $k_1/k = 2$, $k_2/k = 0.6$, and $k_3/k = 3$. For these numerical values, Eqs. 2, 4, 6, and 8 are reduced to Eqs. 9, 10, 11, and 12, respectively, if we let $X_A^0 = 100$ units.

$$X_A = 100e^{-2n} \quad \text{(Eq. 9)}$$

$$X_B = 200[e^{-n} - e^{-2n}] \quad \text{(Eq. 10)}$$

$$X_C = 60[e^{-n} - 2e^{-2n} + e^{-3n}] \quad \text{(Eq. 11)}$$

$$X_D = 60[1 - 3e^{-n} + 3e^{-2n} - e^{-3n}] \quad \text{(Eq. 12)}$$

Also, the rate of formation of C is given by

$$k_2 X_B = 30[e^{-n} - e^{-2n}] \quad \text{(Eq. 13)}$$

And the rate of appearance of C in D is given by

$$\frac{dX_D}{dt} = k_3 X_C = 45[e^{-n} - 2e^{-2n} + e^{-3n}] \quad \text{(Eq. 14)}$$

The cumulative amount of B converted to C in time T is given by

$$k_2 \int_0^T X_B \cdot dt = \left[\left(\frac{1}{k_3} \cdot \frac{dX_D}{dn}\right) + X_D\right] \quad \text{(Eq. 15)}$$

It should be clear that in Eqs. 9 through 14 terms with exponent $[-n]$ are associated with k, terms with exponent $[-2n]$ are associated with k_1, and terms with exponent $[-3n]$ are associated with k_3 in this example. The constants 2 and 3 in these terms are just the ratios k_1/k and k_3/k, respectively. As n increases, the order of magnitudes will always be $e^{-n} > e^{-2n} > e^{-3n}$. Also, as t increases, the order of magnitudes will be $e^{-kt} > e^{-(k_1/k)t} > e^{-(k_3/k)t}$ if $k_3 > k_1 > k$, and the order will be $e^{-kt} > e^{-(k_3/k)t} > e^{-(k_1/k)t}$ if $k_1 > k_3 > k$. Hence, the ratios k_1/k and k_3/k are critical as indicated above. If the numerical values of these ratios are very close, there will be very little curvature in a large segment of the X_D, t plot.

Equations 9 through 15 were solved for $n = 0.05$, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.55, 0.6, 0.65, 0.7, 0.8, 0.9, 0.95, 1, 1.125, 1.25, 1.375, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, and 6. In addition, values of $\Delta X_D/\Delta t$ were calculated corresponding to midpoint times of 0.2, 0.6, 1, 1.6, 2.2, 2.6, 3.2, 3.8, 4.5, 5.5, 7, 10, 14, 18, and 22 hr. The latter were intended to simulate the estimation of urinary excretion rates from analysis of fractional urine collections made at $t = 0.4$, 0.8, 1.2, 2, 2.4, 2.8, 3.6, 4, 5, 6, 8, 12, 16, 20, and 24 hr.

Example 2—Assignments made were $k_1 = 1.25$ hr.$^{-1}$, $k = 0.25$ hr.$^{-1}$, $k_2 = 0.15$ hr.$^{-1}$, and $k_3 = 1.0$ hr.$^{-1}$. Hence $k_1/k = 5$, $k_2/k = 0.6$, and $k_3/k = 4$. For these numerical values of the constants, Eqs. 2, 4, 6, and 8 become Eqs. 16, 17, 18, and 19, respectively, if $X_A^0 = 100$ units.

$$X_A = 100e^{-5n} \quad \text{(Eq. 16)}$$

$$X_B = 125[e^{-n} - e^{-5n}] \quad \text{(Eq. 17)}$$

$$X_C = 60[0.416e^{-n} - 1.66e^{-4n} + 1.25e^{-5n}] \quad \text{(Eq. 18)}$$

$$X_D = 60[1 - 1.66e^{-n} + 1.66e^{-4n} - e^{-5n}] \quad \text{(Eq. 19)}$$

RESULTS

Example 1—The time course of the amounts in compartments A, B, C, and D is shown in Fig. 1. The amount, X_A, drops rapidly with increase in time so that after 4 hr. only 13.5% of the initial amount remains in A. The amount, X_B, reaches a peak in the 2.5–3-hr. region then falls off without a great deal of curvature until 12 hr. The X_C curve rises slowly, reaches a peak of about 8.9 units at 4.5 hr., then falls off with little curvature until about 12 hr. The low peak of the X_C curve illustrates why it is often very difficult to measure blood levels of a metabolite. The X_D curve rises very slowly during the first 2 hr., but from 2 to 7 hr. has only *slight* curvature; during the latter period

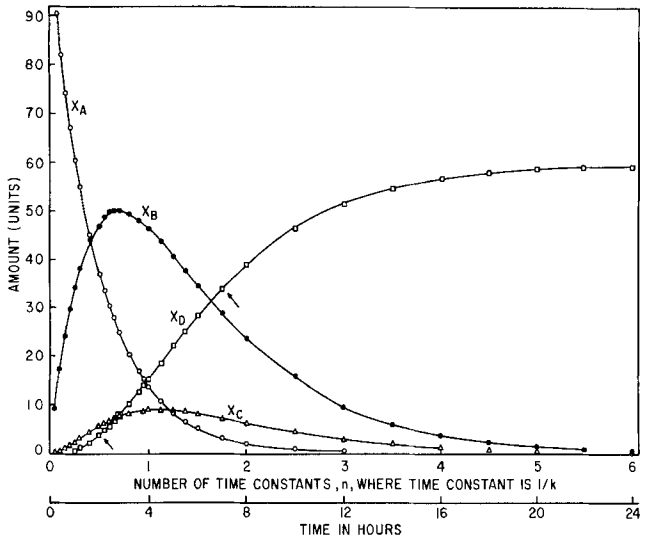


Fig. 1—The time course of the amounts X_A, X_B, X_C, and X_D in compartments A, B, C, and D, respectively, for the model shown as Scheme I and the constants of Example 1.

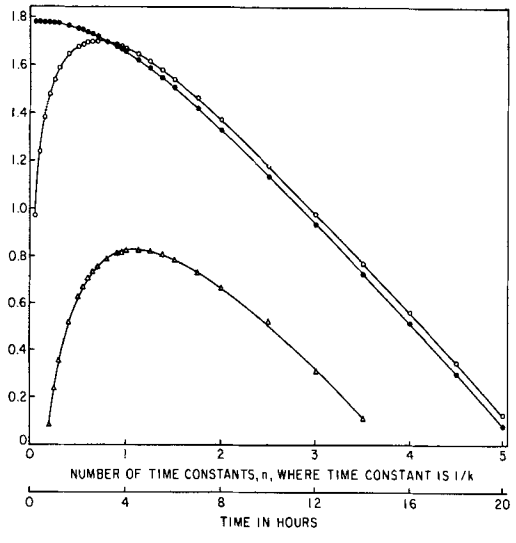


Fig. 2—Plots of $\log_{10}(60-X_D)$, $\log_{10}(X_B)$, and $\log_{10}(k_3 \cdot X_C)$ against time. These correspond to plots of the logarithm of the amount of metabolite not excreted, the logarithm of the plasma concentration of drug, and the logarithm of the excretion rate of metabolite against time. The constants of Example 1 were used. Key: ●, logarithm of amount not excreted = $\log_{10}(60 - X_D)$; ○, logarithm of amount in "B" = $\log_{10}(X_B)$; Δ, logarithm of excretion rate = $\log_{10}(K_3 \cdot X_C)$.

about 30 units or 50% of the C destined for the D compartment is contributed. It is not difficult to see how such a segment of a cumulative urinary excretion curve could be interpreted as being linear, particularly when analytical error, incomplete urine collections due to bladder retention, and other factors are contributing scatter to the points in the case of actual biological data.

Figure 2 is a plot of $\log_{10}(60 - X_D)$, $\log_{10}(X_B)$, and $\log_{10}(k_3X_C)$ *versus* time. These are comparable to plots of the logarithm of the amount of metabolite not excreted, the logarithm of the plasma concentration of drug, and the logarithm of the excretion rate of a metabolite. The curvature of the plots and the "plateau" in the excretion rate plot from about 3 to 6 hr. are noteworthy since proponents of zero-order formation have used these observations to support their claims.

Plots of the cumulative amount of B converted to C and the cumulative amount in D *versus* time are compared in Fig. 3. The first curve mentioned (the formation curve) could be interpreted, erroneously, as being linear in the region from 1 to 6 hr., while the excretion curve could be interpreted, also erroneously, as being linear in the region from 2 to 7 hr. The proper curve to resolve is the formation curve, not the excretion curve. The excretion curve was used by Levy (11), although Eq. 15 was published (12) for a different purpose; namely, to estimate absorption rate from measurement of unchanged drug in the urine and Levy (2) has made use of the equation.

Figure 4 is a plot of k_3X_C (the rate of excretion of metabolite) against k_2X_B (the rate of formation of metabolite). If formation rate were equal to excretion rate, all points would lie on the 45° line drawn through the origin; but, of course this is not true. Cummings and Martin (13) pointed out that in respect to drugs which are excreted predominantly as metabolites, the rate of excretion of a metabolite in urine will only parallel the rate of formation some time after the attainment of the maximum plasma concentration of the metabolite which is about 4 hr. in this example. This time corresponds to the change-over from diamonds to squares on the graph. One can estimate the relationship between rate of formation and rate of excretion if $k < k_3$ since at later times

$$k_2X_B \simeq \left(1 - \frac{k}{k_3}\right) k_2X_C \qquad \text{(Eq. 20)}$$

Substituting the values of k and k_3 for Example 1 into Eq. 20 yields

$$k_2X_B \simeq 0.67k_2X_C \qquad \text{(Eq. 21)}$$

The average ratio, k_2X_B/k_2X_C is 0.686 based on eight pairs of values in the 10–24-hr. interval.

The "mathematical error" involved in fractional urine collections may be gauged by the ratios $(\Delta X_D/\Delta t)/(dX_D/dt)$ where the derivatives are those at the midpoints of the Δt intervals. For the 15 "fractional collections" simulated the average value of this ratio was 0.99 with a range of 0.62 to 1.30. The extremes of the range were those corresponding to the midpoints of 0.4 and 0.2 hr., respectively. For the 13 "fractional collections" made in the 1.2–24-hr. range, the average ratio was 1.0 with a range of 0.95 to 1.17. It is heartening to know that, except at the very early times, $\Delta X_D/\Delta t$ estimates dX_D/dt within a reasonably small error.

Figure 5 is a "Lineweaver-Burk" (14) type plot of the reciprocal of $\Delta X_D/\Delta t$ against the reciprocal of X_B taken at the midpoints of the $\Delta X_D/\Delta t$ intervals. The midpoints used were those at 7, 10, 14, 18, and 22 hr. Levy (15) and Nelson *et al.* (16) interpreted such a plot as indicating a metabolite was being formed according to Michaelis-Menton kinetics (17). But the apparent "$V_{max.}$" and apparent "K_m" one calculates from the intercept and slope in the conventional manner (14) are purely artifacts. From the intercept one estimates an apparent "$V_{max.}$" of 19 units/hr. which is higher than the maximum excretion rate of about 6.67 units/hr. Hence, if one assumes that the maximum excretion rate is "$V_{max.}$," as Levy did, then this type of plot overestimates "$V_{max.}$" exactly as Levy found (15). The reciprocal of the slope of the line in Fig. 5, also equivalent to apparent "$V_{max.}$/apparent 'K_m'," since apparent "K_m" = slope x apparent "$V_{max.}$," is a meaningless number in the context of the model. As a result of the method of plotting and Eq. 21, the value of the reciprocal, 0.238, multiplied by 0.67,

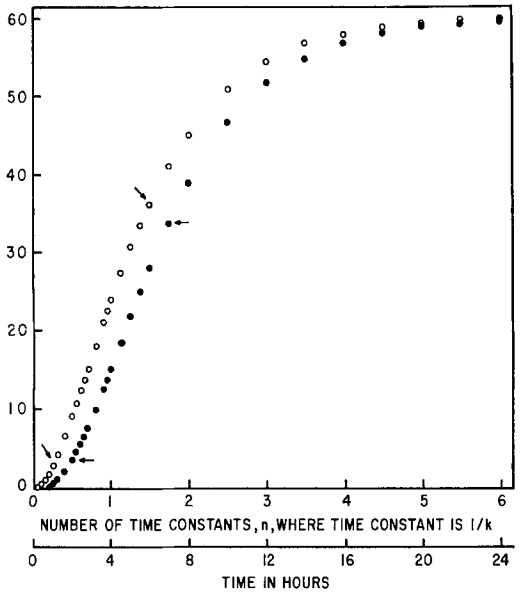


Fig. 3—Plots of the cumulative amount of B converted to C and the cumulative amount in D against time. These correspond to plots of the cumulative amount of drug converted to metabolite and the cumulative urinary excretion of the metabolite against time. The constants of Example 1 were used. Key: ○, cumulative amount of B converted to C; ●, cumulative amount in D.

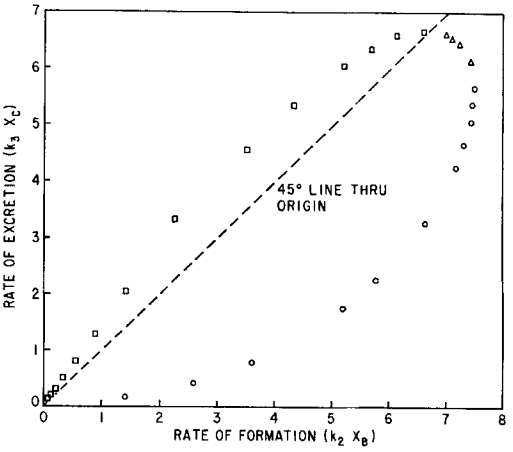


Fig. 4—A plot of the rate of excretion of metabolite (k_3X_C) against the rate of formation of metabolite (k_2X_B). Key: ○, values in the range $0 < n < 0.7$ and $0 < t < 2.8$; Δ, values in the range $0.8 < n < 1$ and $3.2 < t < 4$; □, values in the range $1.125 < n < 6$ and $4.5 < t < 24$. The plot shows that initially excretion rate underestimates formation rate then, beyond the time of occurrence of the peak metabolite concentration in blood, excretion rate overestimates formation rate. If excretion rate equalled formation rate, all points would lie on the 45° line drawn through the origin; but it is obvious this is not the case. The constants of Example 1 were used.

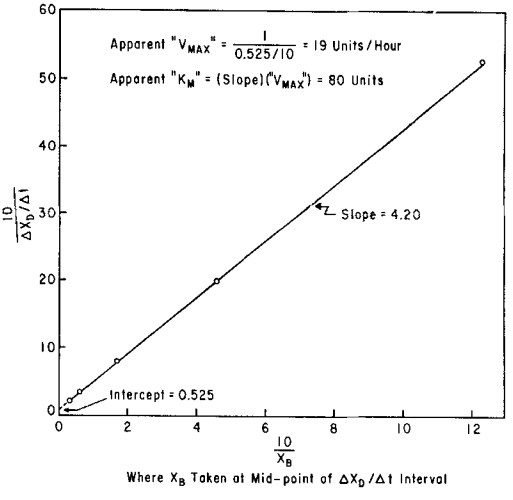


Fig. 5—Showing how a "Lineweaver-Burk" plot, which is an artifact, results from plotting the reciprocal of the excretion rate, $1\ \frac{\Delta X_D}{\Delta t}$, against the reciprocal of the amount of B, $1/X_B$, where X_B is taken at the midpoints of the excretion intervals. The constants of Example 1 were used.

is 0.16; the resultant number is essentially the same as the value of 0.15 obtained for k_2 from the correct plot shown in Fig. 6.

If Michaelis-Menton kinetics apply, one must use the reciprocal of the formation rate, *i.e.*, $1/k_2X_B$, rather than the reciprocal of the excretion rate for making a Lineweaver-Burk plot. From Eq. 20 can be calculated the approximate error in the ordinate value of such a plot if $1/k_2X_C$ is used in place of $1/k_2X_B$. If the kinetics are truly those of Michaelis and Menton (17), use of the reciprocal of the excretion rate causes rotation of the true line, so that the slope of the line drawn from excretion rate data has a slope which is less, and an intercept which is greater, than those of the true line. In such a case $V_{max.}$ is not estimated accurately, but K_m is estimated accurately since K_m = (slope) ($V_{max.}$). However, K_m will not have the same significance as it has usually (18). This is discussed in more detail under *Discussion*.

The proper plot employing $1/k_2X_B$ and $1/X_B$ is shown in Fig. 6. As expected, the reciprocal of the slope is k_2.

Example 2—The time course of the amounts in compartments A, B, C, and D is shown in Fig. 7. Since $k_1 = 1.25$ hr.$^{-1}$ in this example, compared with 0.5 hr.$^{-1}$ in the first example, X_A drops more rapidly in Example 2 than in Example 1, so that after only 1.6 hr. 13.5% of the initial amount remains in A. Hollister and Levy (19) reported an average k of 0.258 hr.$^{-1}$, an average k_2 of 0.153 hr.$^{-1}$, and Levy (11) reported an average k_3 of 1.1 hr.$^{-1}$ for the salicylate–salicylurate system in man. Hence, except for k_1, which Levy ignored, and the initial value $X_A{}^0$, the values of the constants in this example are very similar to those actually calculated for a specific drug–metabolite system as a result of the analysis of biological samples obtained from man.

Plots of the cumulative amount of B converted to C and the cumulative amount in D *versus* time are compared in Fig. 8. It should be noticed that the effect of increasing k_1 and k_3, but keeping k constant, is to cause the curves to rise more steeply and to become almost linear at an earlier time. One could interpret the formation curve as being linear in the 0.4 to 4.0-hr. period and the excretion curve similarly in the 1.2 to 5-hr. period. As in the previous example, during the latter period about 50% of the total metabolite is excreted.

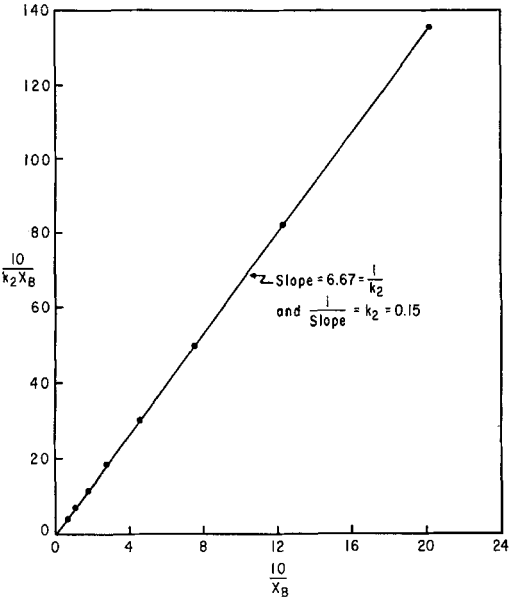


Fig. 6—A plot of the reciprocal of the formation rate, $1/k_2X_B$, against the reciprocal of the amount of B, $1/X_B$. The constants of Example 1 were used.

DISCUSSION

The implications of the simulations and equations presented above are as follows.

(*a*) The conclusion of Cummings *et al.* (20), Levy (11, 15), Nelson *et al.* (16), and Elliott (8) that the formation of salicylurate from salicylate becomes a zero-order process for some period of time following administration of either single oral doses of 1.5 or 2 Gm., or multiple oral doses of about 1 Gm., of acetylsalicylic acid to *adult humans* is most

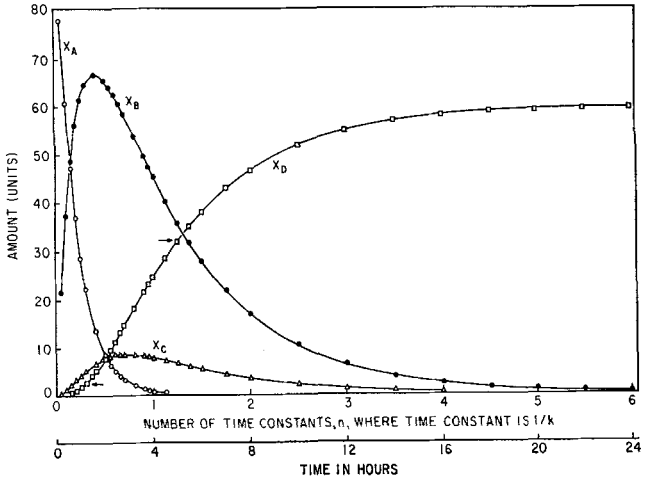


Fig. 7—The time course of the amounts X_A, X_B, X_C, and X_D in compartments A, B, C, and D, respectively, for the model shown as Scheme I and the constants of Example 2.

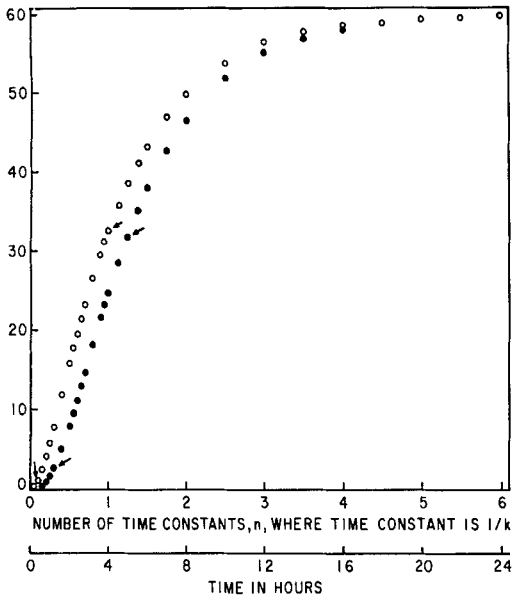


Fig. 8—Plots of the cumulative amount of B converted to C and the cumulative amount in D against time. These correspond to plots of the cumulative amount of drug converted to metabolite and the cumulative urinary excretion of the metabolite against time. The constants of Example 2 were used. Key: O, cumulative amount of B converted to C; ●, cumulative amount in D.

probably in error. Most probably only first-order rate constants are involved at all times. The data of Levy (11) are fit quite well by Eq. 8 based on Scheme I.

(*b*) The conclusion of Nelson (21) that the absorption of tetracycline becomes a zero-order process for some period of time following oral administration of tetracycline mucate in a special dosage form is most probably in error.

(*c*) Large errors can be made in estimating the half-life of a drug (*i.e.*, $0.693/k$) if the estimation is made in a time period when more than one exponential term is making a significant contribution. The wide variability of the reported half-life of salicylate (11) is explained better on this basis than the explanations presented by Levy (11) and Cummings *et al.* (20).

(*d*) When analyzing such pharmacokinetic data by graphical methods using semilogarithmic graph paper one tends to underestimate the over-all rate constant for loss from the body (k) if it is the smallest rate constant appearing in Eqs. 7, 9, and 11 or, in the case of other models, in equations similar to these. This in turn means one tends to overestimate the half-life of the drug. This is due to the fact that the semilogarithmic plots are actually gently curving when one assumes they are linear. Hence, when residuals are taken by extrapolating the terminal line drawn, one also obtains biased residuals. This ultimately results in estimates of the other rate constants, like k_1 and k_3, which are different from their real or "best" values. The best approach is to treat the graphical estimates of the rate parameters as "purely estimates" which are used as input data to a digital computer; with the proper iterative procedure programmed into the computer, the data may be fit by the method of least squares so that "least squares" estimates of all the rate constants may be obtained simultaneously. Such sets of rate constants are readily shown to be better estimates than the graphical ones since, in almost all cases, there is a significant reduction in the variance associated with the fit.

(*e*) The method of subtracting amounts of unchanged drug and metabolite excreted to 4 hr. postadministration from the total amounts excreted and elaborating a pharmacokinetic model from the residuals, as practiced by Nelson and O'Reilly (9), may introduce significant errors into the estimates of the rate constants from some, but not necessarily all, subjects' data. The magnitude of the errors depends upon the effective first-order rate constant for absorption (k_1), and this will vary widely from subject to subject (2, 3, 6).

(*f*) The conclusion of Levy (15) and Nelson *et al.* (16) that a plot of the reciprocal of the excretion rate against the reciprocal of the amount of total drug left in the body is a "Lineweaver-Burk" plot, in the accepted sense, is spurious. It has been shown above that when formation rate of the metabolite is first order, such a plot yields an intercept and a slope, but the plot is an artifact in the Michaelis-Menton sense. If the disappearance of B is truly describable by the extended Michaelis-Menton equation (18), namely

$$\frac{-dC_B}{dt} = \frac{V_{max.} \cdot C_B}{K_m + C_B} \qquad \text{(Eq. 22)}$$

where C_B is the concentration in compartment B at time t, and the system is as in Scheme II, where

$$E + B \underset{K_2}{\overset{K_1}{\rightleftarrows}} EB \xrightarrow{K_3} E + C \xrightarrow{K_4} D$$

Scheme II

E denotes the enzyme, EB the enzyme–drug complex, and K_i ($i = 1, 2, 3, 4$) are first order-rate constants, then the K_m obtained would have a different significance than in the classical case. In the classical case, the system is as shown in Scheme III,

$$E + B \underset{K_2}{\overset{K_1}{\rightleftarrows}} EB \xrightarrow{K_3} E + C$$

Scheme III

and the "extended Michaelis constant," $K_m = (K_2 + K_3)/K_1$ (18). The application of the Michaelis-Menton equation to metabolite data derived from the intact animal or human will always be complicated by the C → D step unless $K_4 > 100K_3$ in Scheme II; in addition, there may be additional errors if the application is made in a time period when B is being supplied from a reservoir such as the absorption site or from a precursor.

A Critique of History—Bray *et al.* (22) showed that in the conjugation of benzoic acid with glycine to form hippuric acid the availability of glycine for conjugation controlled the rate of hippuric acid formation and that formation of hippuric acid apparently proceeded at a constant rate when rabbits were given relatively high doses of 500 mg./Kg. This was a significant contribution. However, these authors made the following statement: "Hippuric acid formation takes place at a constant rate and *the acid is excreted immediately*

up to a limiting rate which appears to vary considerably between different rabbits." In their mathematical treatment they completely ignored the rate constant for urinary excretion of hippuric acid. This reference, which has been cited extensively, may be the source of the problem. Some scientists seem to have reached the conclusion that if a rate constant is relatively large, say 1.0 hr.$^{-1}$, it can be ignored. The presentation in this paper should dispel this idea.

Nelson *et al.* (16) showed that following 20, 40, and 60 mg./Kg. doses of salicylic acid *to rats*, the rate of excretion of the metabolite, salicyluric acid, was the same, within error, at all three doses. In addition, they showed that salicyluric acid, administered intraperitoneally at doses of 200 mg./Kg. to rats, was rapidly excreted; the excretion obeyed first-order kinetics with an excretion rate constant of 1.15 hr.$^{-1}$ which is essentially the same as Levy (11) reported for one human given 2 doses of salicyluric acid, but considerably higher than the average value of 0.654 hr.$^{-1}$ reported by Elliott (8) for five humans. The additional rat study showed that saturation of the active secretory pathway in the kidney could not have occurred for salicylurate in the rats dosed with sodium salicylate. Hence, their evidence is very convincing that saturation of the enzyme system which converts salicylate to salicylurate occurred *in rats* at the dose of 20 mg./Kg. and above. One should not conclude, however, that a comparable dose, *i.e.*, about 1.4 Gm. of salicylic acid in a 70-Kg. man, will produce the same effect!

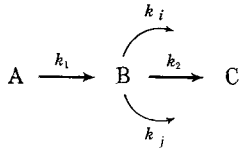
In the same paper (16) the conclusion was reached that the cumulative salicylurate excretion plots were linear following doses 1.5 and 2.0 Gm. of acetylsalicylic acid administered to two humans. Careful analysis of these plots reveals that the data points form a *curve;* the points start below the straight line drawn through them, then the points go above the line, then they go below the line again as time increases. The same result would be obtained if one were to draw a straight line through the points of the apparently linear segments of the X_D, t curves of Figs. 1 and 7! Levy (11) had previously drawn the conclusion that salicylurate was being formed at a constant rate from the same sets of experimental data. Later he (15) constructed "Lineweaver-Burk" plots from the same sets of data. He utilized the reciprocals of the salicylurate *excretion* rates, not the formation rates, in the time period 15 to 27 hr. postadministration. In light of the above presentation it appears that there is convincing proof of zero-order formation of salicylurate from salicylate in the rat, but the same type of proof has not been presented for man. It is obvious that there must be some critical dose level of acetylsalicylic acid or salicylate for man above which there will be zero-order formation of salicylurate for some time postadministration; such levels, for example, could be reached in poisoning cases when children ingest large quantities of acetylsalicylic acid. However, the administration of doses of up to 4 Gm. of sodium salicylate to adult subjects provided no data which supported zero-order formation of salicylurate from salicylate (23). Unfortunately, Levy (11) completely misinterpreted the data of Schachter and Manis (23).

Cummings and Martin (24) made some erroneous statements about metabolite formation which should be corrected. They stated that one could estimate the maximum rate constant for renal excretion of a metabolite from the volume of distribution (V) and the maximum renal clearance (R) employing the equation of Butler (25). But this is not so. The Butler equation, transferred into the terminology of this paper, is

$$R = Vkf = Vk_2 \qquad \text{(Eq. 23)}$$

where f is the fraction of B converted to C (*i.e.*, $f = k_2/k$). Hence, from renal clearance one can only estimate k or k_2 and not k_3. They also stated that the slope of the terminal segment of a plot of the logarithm of the excretion rate of metabolite would yield an estimate of k_2 when $k_2 < k_3$. But this is not true. The constant determined by this method of plotting will be k, providing k is less than k_1 and k_3. This is so because k, and not k_2, appears in the exponents of exponential terms of the solutions of the differential equations. Later, these authors (26) stated that if there was zero-order formation of one metabolite and first-order formation of another metabolite, then a plot of the logarithm of formation rate of total metabolites *versus* time would be a straight line having the same slope as if the latter metabolite were being formed alone. Their equations on which this conclusion is based are in error, and their own plots show this is not true!

The same type of error with respect to linearity of cumulative excretion curves, as discussed above for metabolities, can lead to the conclusion that constant rate (zero-order) absorption is operative, when, in fact, all rate constants are first-order rate constants. If a drug is partly metabolized and partly excreted unchanged in the urine and one measures unchanged drug in the urine, then one pertinent model may be written as in Scheme IV



Scheme IV

where A represents the solid drug administered, B represents the drug in blood and other fluids of distribution, and C represents the drug in urine. If k has the same meaning as for Scheme I, and k_2 is the rate constant for urinary excretion of unchanged drug, then a plot of the cumulative amount of unchanged drug in the urine (*i.e.*, X_C *versus* t) will be apparently linear, but actually slightly curved, during some time period if $1 < k_1/k < 10$. An example is shown in Fig. 9 for the case when $k_1 = 0.5$ hr.$^{-1}$, $k = 0.25$ hr.$^{-1}$, and $k_2 = 0.15$ hr.$^{-1}$. It may be seen that if a smooth line was drawn through the points, the line would be only slightly curved in the period 1.2 to 6 hr. postadministration. This region is marked off by the arrows on the graph. If one assumed linearity of the plot in this time period, one would conclude the A $\rightarrow$ B step was zero order—*i.e.*, that absorption was proceeding at a constant rate. This probably explains the results of Nelson (21) obtained with tetracycline mucate. The writer once made a similar interpretation from data derived following administration of a different drug and lived to regret it. As in Scheme I, the critical ratio for Scheme IV is k_1/k and not k_1/k_2.

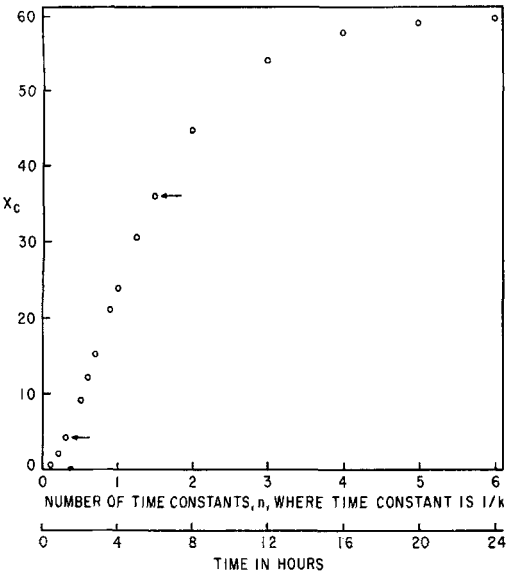


Fig. 9—A plot of the cumulative amount of unchanged drug in the urine, X_C versus time for the model shown as Scheme IV and the constants given in the text.

REFERENCES

(1) Wagner, J. G., and Nelson, E., *J. Pharm. Sci.*, **53**, 1392(1964).
(2) Levy, G., and Hollister, L. E., *ibid.*, **53**, 1447(1964).
(3) Krüger-Thiemer, E., and Bünger, P., *Chemotherapia*, **10**, 61(1965–1966).
(4) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).
(5) Levy, G., Leonards, J. R., and Procknall, J. A., *ibid.*, **54**, 1719(1965).
(6) Wagner, J. G., "Design and Data Analysis of Biopharmaceutical Studies in Man," presented to the Industrial Pharmacy Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
(7) Carpenter, O. S., Northam, J. I., and Wagner, J. G., "Antibiotics—Advances in Research, Production and Clinical Use," Herold, M., and Gabriel, Z., eds., Butterworth & Co., Ltd., London, England, 1966, pp. 165–172.
(8) Elliott, H. C., *Proc. Soc. Exptl. Biol. Med.*, **121**, 861(1966).
(9) Nelson, E., and O'Reilly, I., *J. Pharmacol. Exptl. Therap.*, **129**, 368(1960).
(10) Nelson, E., *Antibiot. Chemotherapia*, **12**, 29(1964).
(11) Levy, G., *J. Pharm. Sci.*, **54**, 959(1965).
(12) Wagner, J. G., and Nelson, E., *ibid.*, **52**, 610(1963).
(13) Cummings, A. J., and Martin, B. K., *Biochem. Pharmacol.*, **13**, 767(1964).
(14) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658(1934).
(15) Levy, G., *J. Pharm. Sci.*, **54**, 496(1965).
(16) Nelson, E., Hanano, M., and Levy, G., *J. Pharmacol. Exptl. Therap.*, **153**, 159(1966).
(17) Michaelis, L., and Menton, M. L., *Biochem. Z.*, **49**, 333(1913).
(18) Bartholomay, A. F., "Stochastic Models in Medicine and Biology," Proceedings of a Symposium, Mathematics Research Center, U. S. Army, University of Wisconsin, Madison, Wis., June 1963.
(19) Hollister, L., and Levy, G., *J. Pharm. Sci.*, **54**, 1126 (1965).
(20) Cummings, A. J., Martin, B. K., and Renton, R., *Brit. J. Pharmacol.*, **26**, 461(1966).
(21) Nelson, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 54 (1960).
(22) Bray, H. G., Thorpe, W. V., and White, K., *Biochem. J.*, **48**, 88(1951).
(23) Schachter, D., and Manis, J. G., *J. Clin. Invest.*, **37**, 800(1958).
(24) Cummings, A. J., and Martin, B. K., *Nature*, **200**, 1296(1963).
(25) Butler, T. C., *Federation Proc.*, **17**, 1158(1958).
(26) Cummings, A. J., Martin, B. K., and Park, G. S., *Nature*, **202**, 779(1964).

Pharmacokinetic Model for Nalidixic Acid in Man III

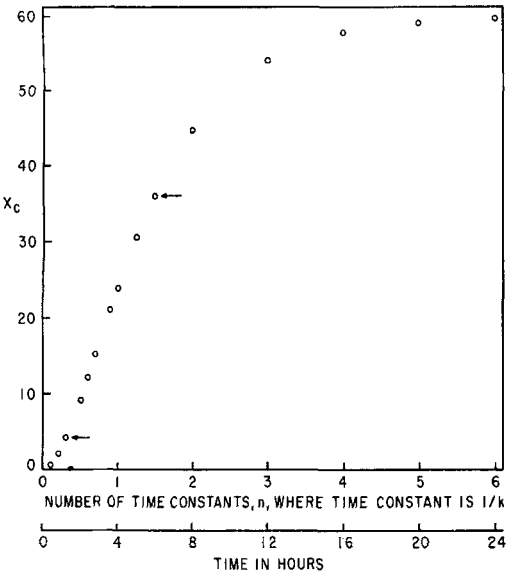
Effect of Repeated Oral Dosage

By E. W. MCCHESNEY, G. A. PORTMANN, and R. F. KOSS

In studies which involved the administration of 1-Gm. doses of nalidixic acid to human volunteers four times daily for a period of 10 days, it has been shown that the resulting plasma levels of nalidixic and of hydroxynalidixic acid could be predicted reasonably well from a model derived from single-dose studies. More satisfactory blood levels are obtained if the doses are taken at least 1 hr. before meals. Repeated dosage results in no important change in the absorption-excretion-metabolism patterns of the drug.

IN PREVIOUS communications (1, 2) a model describing the absorption and elimination of nalidixic acid[1] (NA) in man was presented. This model was derived from observations in seven subjects following the ingestion of single 1-Gm. doses of NA in several physical forms. There remained, however, the question of how well such a model would describe the situation in which the drug is given as a standard therapeutic course, *i.e.*, 1 Gm. four times daily. In particular there was the problem of whether such a dosage regimen would result in a significant carry-over of the drug and its metabolites from one day to another [it was already known (3) that following single 1-Gm. doses the excretion is usually not quite complete within 24 hr.], and whether the repeated administration of 4 Gm. per day would result in significant changes in the way the body handles

Received October 21, 1966, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144
Accepted for publication January 24, 1967.
The authors thank Dr. D. A. Berberian and Ruth Graham, R.N., for supervision of the medications and collection of the biological materials.
Previous paper: Portmann, G. A., McChesney, E. W., Stander, H., and Moore, W. E., *J. Pharm. Sci.*, **55**, 72(1966).
[1] Nalidixic acid is 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid. Marketed as NegGram by Winthrop Laboratories, New York, N.Y.



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REFERENCES

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(2) Levy, G., and Hollister, L. E., *ibid.*, **53**, 1447(1964).
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(4) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).
(5) Levy, G., Leonards, J. R., and Procknall, J. A., *ibid.*, **54**, 1719(1965).
(6) Wagner, J. G., "Design and Data Analysis of Biopharmaceutical Studies in Man," presented to the Industrial Pharmacy Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
(7) Carpenter, O. S., Northam, J. I., and Wagner, J. G., "Antibiotics—Advances in Research, Production and Clinical Use," Herold, M., and Gabriel, Z., eds., Butterworth & Co., Ltd., London, England, 1966, pp. 165–172.
(8) Elliott, H. C., *Proc. Soc. Exptl. Biol. Med.*, **121**, 861(1966).
(9) Nelson, E., and O'Reilly, I., *J. Pharmacol. Exptl. Therap.*, **129**, 368(1960).
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By E. W. MCCHESNEY, G. A. PORTMANN, and R. F. KOSS

In studies which involved the administration of 1-Gm. doses of nalidixic acid to human volunteers four times daily for a period of 10 days, it has been shown that the resulting plasma levels of nalidixic and of hydroxynalidixic acid could be predicted reasonably well from a model derived from single-dose studies. More satisfactory blood levels are obtained if the doses are taken at least 1 hr. before meals. Repeated dosage results in no important change in the absorption-excretion-metabolism patterns of the drug.

IN PREVIOUS communications (1, 2) a model describing the absorption and elimination of nalidixic acid[1] (NA) in man was presented. This model was derived from observations in seven subjects following the ingestion of single 1-Gm. doses of NA in several physical forms. There remained, however, the question of how well such a model would describe the situation in which the drug is given as a standard therapeutic course, *i.e.*, 1 Gm. four times daily. In particular there was the problem of whether such a dosage regimen would result in a significant carry-over of the drug and its metabolites from one day to another [it was already known (3) that following single 1-Gm. doses the excretion is usually not quite complete within 24 hr.], and whether the repeated administration of 4 Gm. per day would result in significant changes in the way the body handles

Received October 21, 1966, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144
Accepted for publication January 24, 1967.
The authors thank Dr. D. A. Berberian and Ruth Graham, R.N., for supervision of the medications and collection of the biological materials.
Previous paper: Portmann, G. A., McChesney, E. W., Stander, H., and Moore, W. E., *J. Pharm. Sci.*, **55**, 72(1966).
[1] Nalidixic acid is 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid. Marketed as NegGram by Winthrop Laboratories, New York, N.Y.

the drug. To answer these questions three separate studies have been conducted as outlined in studies *A*, *B*, and *C*.

Study A—The results of giving 1-Gm. doses four times daily for 3 consecutive days have been determined.

Study B—A comparison of the results obtained when the drug (single 1-Gm. dose) is taken on a full *versus* an empty stomach has been made.

Study C—The results of giving 1-Gm. doses four times daily for 10 consecutive days have been determined, at intervals during the course of this dosage regimen. (*Note:* the daily time schedules for studies *A* and *C* were not identical; see under *Experimental*.)

METHODS OF ANALYSIS

The methods used in the present work have been described (2, 3) and no further details are given here except as noted below.[2]

THEORETICAL

The equation used for calculating the theoretical NA plasma levels following the first 3 doses (in studies *A* and *C*) was that derived by Dost (4) for the case where equal doses are taken at equal time intervals. The average rate constants and apparent volumes of distribution which had been determined previously for another set of seven volunteers (2) were used in making the present calculations. For hydroxynalidixic acid (HNA) the general equation given below was derived by methods similar to those of Dost and of Wiegand *et al.* (5). The model, the basic equations, and the applicable constants have been presented (2). The urinary outputs of NA and of its several metabolic products were calculated in the same manner as the plasma levels.

The elimination rate constants for NA and HNA are designated as k_d and k_{d_2}, respectively. The availability rate constant for NA is designated as k_A, and the rate constant for the oxidation of NA to HNA as k_{M_1}. A_0 represents the total amount absorbed (in this case A_0 = dose administered) and n the number of doses given. The time interval between doses is designated as T, and the time elapsed after the administration of each dose as t. Division of the term HNA-B (that is, HNA in the body) by the apparent volume of distribution gives the plasma concentration:

$$\text{HNA-B} = \frac{k_{M_1}k_A A_0}{k_A - k_d} \times \left[\frac{(k_A - k_d)\, e^{-k_{d_2}t}}{(k_d - k_{d_2})(k_A - k_{d_2})}\left(\frac{1 - e^{-nk_{d_2}T}}{1 - e^{-k_{d_2}T}}\right) + \frac{e^{-k_A t}}{(k_A - k_{d_2})}\left(\frac{1 - e^{-nk_A T}}{1 - e^{-k_A T}}\right) - \frac{e^{-k_d t}}{(k_d - k_{d_2})}\left(\frac{1 - e^{-nk_d T}}{1 - e^{-k_d T}}\right)\right]$$

The average values for the various constants are as follows: k_d = 0.0112^{-1} min.; k_{M_1} = 0.00453 min.$^{-1}$; k_{d_2} = 0.00746 min.$^{-1}$; k_A = 0.0180 min.$^{-1}$; V_d for NA = 34% of body weight; V_d for HNA = 49% of body weight; mean body weight for study *A* = 74 Kg., for study *C* = 65.5 Kg.; lag time = 18 min. There is an almost perfect correlation between the values for V_d and per cent protein-bound: per cent protein-bound NA/HNA = 93/63 = 1.48 (2) V_d(HNA)/V_d(NA) = 49/34 = 1.45.

EXPERIMENTAL

Study A—Four male human volunteers took 1-Gm. doses of NA four times daily for 3 consecutive days, without control of the timing of the meals, or of the amounts eaten. The doses were taken, as 0.5-Gm. tablets, at 8:15 a.m., 1:15 p.m., 6:15 p.m., and at bedtime (about 11:15 p.m.). Oxalated plasma samples were obtained at 8:15 a.m. on each of the 3 days, at 2-hr. intervals thereafter until 4:15 p.m., and at 8:15 a.m. on the fourth day. Urine samples were collected for the interval 8:15 a.m.–4:15 p.m. for the first 4 days, and for the 4:15 p.m.–8:15 a.m. interval of the first 3 nights, (*i.e.*, for 80 hr. following the first dose). The compounds determined in these samples were NA and HNA in both free and ester glucuronide forms.

Study B—Three human volunteers took 1-Gm. doses of NA on an empty stomach, and were provided breakfast 75 min. later, while three other volunteers took 1-Gm. doses immediately after a full breakfast. Three days later the groups of subjects were reversed, to complete the experimental cross-over design. Oxalated plasma samples were obtained from each subject just prior to medication and at intervals of 0.5, 1, 2, and 3 hr. thereafter. These samples were analyzed for free NA and HNA.

Study C—Six human volunteers (four males, two females) took 1-Gm. doses of NA four times daily for 10 consecutive days. On days 1, 7, and 10 a strict schedule of dosage and meals was followed, with the drug being taken at 7:30 and 11:30 a.m., 3:30 and 9:00 p.m., and the meals being eaten at 8:30 a.m., 12:30 p.m., and 6–7 p.m. On the other 7 days the subjects were permitted a wider latitude of choice in the timing of their meals and of the medications, but record cards which they were required to fill out showed that the greatest deviation from the dosage schedule in any case was 1 hr. On days 1, 7, and 10 of this regimen oxalated plasma samples were obtained at 7:30, 9:00, and 11:30 a.m., and at 1:00, 3:30, and 5:00 p.m. Quantitative urine collections were made under toluene for the intervals 7:30 a.m.–4:30 p.m. and 4:30 p.m.–7:30 a.m. of each of these days. The plasma samples were analyzed for free, (*i.e.*, extractable) NA and HNA; the urine samples were analyzed for both free and conjugated NA and HNA, and for the dicarboxylic acid metabolite (3). (Analysis for the last-named is somewhat nonspecific, and is subject to some interference from other drugs which the volunteers may take; *e.g.*, salicylates.)

RESULTS AND DISCUSSION

The data obtained in study *A* are presented in Tables I and II. The plasma levels observed for NA and HNA and their glucuronides are presented in Table I, as are the values for NA and HNA which were calculated from the model (2). Table II pre-

[2] In *Reference 2*, p. 73, the volume ratio of buffer solution (pH 5.63) to toluene was inadvertently not stated. The ratio used is 45/30.

TABLE I—PLASMA LEVELS OF NA AND HNA, FREE AND CONJUGATED, IN FOUR HUMANS TAKING 1 GM. OF NA 4 TIMES DAILY FOR 3 CONSECUTIVE DAYS[a]

Substance Determined[b]	Day of Expt.	hr. After Morning Dose[c] 0	2	4	6	8
NA	1	...	15.6 ± 7.2 (17.3)	13.7 ± 3.4 (6.6)	8.1 ± 5.1 (17.2)	9.6 ± 2.7 (12.0)
HNA	1	...	3.8 ± 1.5 (3.6)	5.0 ± 0.6 (4.7)	3.6 ± 0.7 (3.6)	4.4 ± 2.1 (5.9)
NA-G	1	...	26.5 ± 10.5	9.0 ± 1.3	6.5 ± 2.6	9.6 ± 3.3
HNA-G	1	...	5.9 ± 2.6	2.3 ± 0.8	1.0 ± 0.5	1.2 ± 0.6
NA	2	1.2 ± 0.7[d] (0)	14.5 ± 6.3 (17.3)	11.8 ± 3.4 (6.6)	9.6 ± 4.5 (17.2)	13.2 ± 7.7 (12.0)
HNA	2	1.5 ± 0.5[d] (1.0)	4.9 ± 1.0 (3.9)	6.5 ± 2.3 (4.7)	7.1 ± 4.4 (3.6)	6.4 ± 2.5 (5.9)
NA-G	2	2.1 ± 0.8[d]	10.0 ± 3.2	9.4 ± 0.6	12.4 ± 4.1	9.2 ± 2.4
HNA-G	2	2.1 ± 0.8[d]	2.3 ± 1.7	3.1 ± 1.6	4.7 ± 2.5	1.9 ± 0.8
NA	3	1.5 ± 0.5 (0)	17.8 ± 6.5 (17.3)	9.5 ± 1.8 (6.6)	11.5 ± 6.6 (17.2)	6.2 ± 1.9 (12.0)
HNA	3	2.8 ± 0.8 (1.0)	6.4 ± 1.7 (3.9)	5.8 ± 0.9 (4.7)	8.1 ± 4.3 (3.6)	5.8 ± 2.8 (5.9)
NA-G	3	2.0 ± 0.9	15.8 ± 5.2	9.4 ± 1.3	9.1 ± 3.4	9.4 ± 2.7
HNA-G	3	1.3 ± 0.5	2.3 ± 0.5	1.5 ± 0.6	1.2 ± 0.8	1.2 ± 0.7
NA	4	1.9 ± 1.0 (0)	...	...	...	...
HNA	4	3.4 ± 0.8 (1.0)	...	...	...	...
NA-G	4	4.2 ± 1.8	...	...	...	...
HNA-G	4	1.1 ± 0.2	...	...	...	...

[a] Doses given at 0, 5, 10, and 15 hr. of each day; initial dose given at 8:15 a.m. [b] NA, nalidixic acid; HNA, hydroxynalidixic acid; NA-G and HNA-G, corresponding glucuronides. [c] Values given as mcg./ml. ± S.E.; the calculated values are given in parentheses and were derived from observations on a panel of seven subjects which had one subject common to the present panel (2). [d] Subject who omitted fourth dose on day 1 not included in the means.

TABLE II—URINARY EXCRETION OF NA AND THREE METABOLIC PRODUCTS IN FOUR HUMANS TAKING 1-GM. DOSES OF NA 4 TIMES DAILY FOR 3 CONSECUTIVE DAYS[a]

Substance Determined[b]	Day of Expt.	Interval After Morning Dose[c] 0–8 hr.	8–24 hr.	0–24 hr.
NA	1	−2 ± 7 (13)	−5 ± 8 (22)	−7 ± 14 (35)
HNA		82 ± 51 (130)	255 ± 29 (302)	337 ± 65 (432)
NA-G		485 ± 130	1500 ± 230	1985 ± 310 (2142)
HNA-G		189 ± 80	918 ± 100	1107 ± 35 (911)
Total		754 ± 230	2668 ± 173	3422 ± 130 (3520)
NA	2	1 ± 3 (13)	−5 ± 3 (22)	−4 ± 6 (35)
HNA		66 ± 20 (139)	210 ± 63 (302)	276 ± 49 (441)
NA-G		595 ± 79	1140 ± 229	1735 ± 288 (2142)
HNA-G		267 ± 63	735 ± 194	1002 ± 257 (931)
Total		929 ± 110	2080 ± 215	3009 ± 315 (3549)
NA	3	5 ± 2 (13)	5 ± 3 (22)	10 ± 2 (35)
HNA		70 ± 48 (139)	146 ± 28 (302)	216 ± 42 (441)
NA-G		571 ± 103	1474 ± 217	2045 ± 240 (2142)
HNA-G		330 ± 105	680 ± 97	1010 ± 171 (931)
Total		976 ± 152	2305 ± 139	3281 ± 310 (3549)
NA	4	7 ± 7	—	8 ± 8[d] (105)[d]
HNA		22 ± 5	—	839 ± 135[d] (1314)[d]
NA-G		105 ± 28	—	5941 ± 208[d] (6426)[d]
HNA-G		109 ± 38	—	3191 ± 136[d] (2773)[d]
Total		243 ± 37	—	9979 ± 125[d] (10618)[d]

[a] Doses given at 0, 5, 10, and 15 hr. of each day; initial dose at 8:15 a.m. [b] For definition of terms see Table I. [c] Values given as mg. ± S.E., and in terms of NA equivalents. Figures in parentheses are the calculated values derived from the model. [d] Total for 80 hr.

sents the corresponding urinary excretion data for NA, HNA, and their glucuronides, along with the values for each which were calculated from the model. The results obtained in study *B* are presented in Table III, while the results obtained in study *C* are presented in Tables IV and V. The latter tables are quite comparable to Tables I and II, the principal difference being that Table V includes data on the actual and calculated excretion of the dicarboxylic acid metabolite. The following comments may be made on the data.

There are numerous time schedules which might be followed in taking 1-Gm. doses of any drug four times daily. In the case of a drug which has a half-life of about 60 min., it is especially desirable to use a regimen which will maintain the maximum possible plasma levels over the longest period of time, within the limits of safety. [In this connection it should be noted that the antibacterial spectrum of HNA both *in vitro* and *in vivo* is very similar to that of NA (3, 6, 7); therefore, the biologically effective plasma level is a function of the sum of the concentrations of free NA plus HNA.] As a practical matter, however, there are essentially only two schedules of dosage which are likely to be prescribed: (*a*) 1 Gm. after meals and at bedtime, and (*b*) 1 Gm. between meals and at bedtime. Study *A* was based on the former schedule and the data obtained suggested that, as a result of the absence of any control of the amount of food in the stomach when the doses were taken, some variation in the rate (and possibly completeness) of absorption had occurred. Nevertheless, certain general conclusions could be drawn from the study, as follows.

(*a*) The plasma levels of free NA were generally about equal to those of conjugated NA (Table I). For the 15 separate intervals studied the ratio of free to conjugated NA averaged 1.0 ± 0.3 (S.D.),

TABLE III—PLASMA LEVELS OF NA AND HNA[a] IN MAN AT 4 INTERVALS AFTER INGESTION OF 1-GM. DOSES OF NA

Subject	State of Stomach[b]	Time After Medication, hr. 0.5 NA	0.5 HNA	1.0 NA	1.0 HNA	2.0 NA	2.0 HNA	3.0 NA	3.0 HNA
1	Full	1.5	0.8	4.0	2.3	4.4	3.9	4.4	5.7
2	Full	2.2	0.1	32.5	3.2	16.2	7.8	7.1	6.6
3	Full	0.6	0.2	5.7	0.7	10.1	4.4	17.2	5.4
4	Full	1.1	0.3	7.8	2.1	12.1	3.4	26.7	10.4
5	Full	33.9	1.9	17.6	4.4	11.9	4.4	4.8	4.6
6	Full	0.1	0.1	0	1.1	5.5	0.5	10.4	2.3
Mean	Full	6.6	0.6	11.3	2.3	10.0	4.1	11.8	5.8
S.E.		5.5	0.3	4.9	0.6	1.8	1.0	3.6	1.1
NA/HNA		11.0		4.9		2.4		2.0	
1	Empty	19.5	11.4	13.4	16.4	7.8	15.4	1.7	10.2
2	Empty	9.4	1.6	14.3	3.6	20.8	9.4	9.8	7.0
3	Empty	18.8	4.2	17.8	3.5	15.4	8.8	8.8	7.4
4	Empty	18.6	4.2	24.8	7.6	11.0	8.2	7.0	6.3
5	Empty	18.8	3.4	20.4	5.3	14.8	5.8	5.4	4.1
6	Empty	0.6	0.3	7.6	1.7	11.4	3.5	5.0	3.8
Mean	Empty	14.3	4.2	16.3	6.4	13.5	8.5	6.3	6.5
S.E.		3.2	1.6	2.5	2.1	1.8	1.6	1.2	1.0
NA/HNA		3.4		2.5		1.6		0.97	

[a] Values given as mcg./ml.; zero-hr. blank values (0.1–0.2 mcg./ml.) subtracted. [b] When dose was ingested. The term "full stomach" means that the subjects were allowed as big a breakfast as they wished. The dose was taken immediately after the meal was finished.

TABLE IV—PLASMA LEVELS OF NA AND HNA IN SIX HUMANS TAKING 1 GM. OF NA 4 TIMES DAILY FOR 10 CONSECUTIVE DAYS[a]

Substance Determined[b]	Day of Expt.	hr. After Morning Dose[c] 0	1.5	4	5.5	8	9.5
NA	1	...	12.1 ± 2.6 (21.8)	4.3 ± 0.8 (7.7)	13.5 ± 3.1 (24.5)	10.9 ± 2.3 (8.1)	16.4 ± 5.8 (25.1)
HNA	1	...	5.0 ± 1.5 (2.9)	2.8 ± 0.4 (4.7)	3.7 ± 0.5 (6.8)	7.3 ± 1.2 (6.2)	9.5 ± 1.7 (7.6)
NA	7	1.1 ± 0.7 (0)	21.7 ± 2.8 (21.8)	4.3 ± 0.8 (7.7)	21.0 ± 7.1 (24.5)	7.9 ± 1.2 (8.1)	32.2 ± 6.1 (25.1)
HNA	7	4.5 ± 0.6 (0.9)	12.2 ± 1.6 (3.2)	8.2 ± 1.3 (4.7)	8.4 ± 1.8 (6.8)	8.5 ± 1.3 (6.2)	17.0 ± 1.9 (7.6)
NA	10	1.1 ± 0.2 (0)	17.5 ± 2.9 (21.8)	3.6 ± 1.0 (7.7)	32.2 ± 3.2 (24.5)	6.6 ± 2.0 (8.1)	24.2 ± 4.1 (25.1)
HNA	10	2.9 ± 1.0 (0.9)	11.9 ± 4.1 (3.2)	5.8 ± 1.1 (4.7)	12.6 ± 3.6 (6.8)	10.8 ± 1.9 (6.2)	14.6 ± 2.4 (7.6)

[a] Doses taken at zero (7:30 a.m.), 4, 8, and 13.5 hr. each day. The subjects had breakfast at 1 hr., lunch at 5 hr., and dinner at approximately 11 hr. of each day, based on this time schedule. [b] NA, nalidixic acid; HNA, hydroxynalidixic acid. [c] Values given as mcg./ml. ± S.E.; those values given in parentheses were calculated on the basis of rate constants derived from a panel which included one of the same subjects (2).

and no individual subject showed any consistent change in these ratios as related to time of day or the number of days on medication.

(*b*) There was a small but definite carry-over of naphthyridine in the plasma from one day to another, although the equations derived from the earlier single-dose studies (see under *Theoretical*) suggested that the only carry-over should be a small amount of HNA. The mean carry-over at 24 hr. in all forms determined, for example, was 6.9 mcg./ml.; at 48 hr. it was 7.6 mcg./ml. and at 72 hr. it was 10.6 mcg./ml. Free naphthyridine represented about half of the carry-over and in this category HNA predominated over NA in a ratio of about 3/2 (Table I).

(*c*) The urinary output of free naphthyridine (NA + HNA) per hour was greatest in the 8–24-hr. period of the first day, but otherwise fluctuated according to no obvious pattern. The per hour outputs of this item for the seven successive periods of observation were: 10.0, 15.6, 8.5, 12.8, 9.5, 9.4, and 3.7 mg. The 8–24-hr. period of the first day was also characterized by the largest output of total naphthyridine, for any of the periods studied. The ratio of free naphthyridine to total naphthyridine also varied from one period to another, from a low of 0.065 to a high of 0.119, but according to no consistent pattern.

(*d*) With allowance for the excretion of 4% of the dose in the feces (3) and 8% as the dicarboxylic acid[3] (2), the daily urinary output of total naphthyridine should have averaged 3540 mg. This rate of excretion was not achieved for any one of the 3 days studied, reflecting the fact that in two of the subjects the over-all recoveries were only 84 and 93% of the expected amounts. The subject who gave the lowest total recovery of naphthyridine in the urine also generally gave the lowest plasma levels. (NA + HNA = 29.4 mcg./ml. at 4 hr. and 27.2 mcg./ml. at 26 hr.; otherwise there was no level above 18. The expected peak level for a subject of his body weight, following a single 1-Gm. dose of NA would be about 32.) This subject, considered reliable, was very positive that he had taken all of the doses.

The reasons for the somewhat erratic results obtained in study *A* were revealed in part in study *B*,

[3] In terms of NA equivalents an output of 8 mg. as dicarboxylic acid would be reduced to 7 mg.

TABLE V—URINARY EXCRETION OF NALIDIXIC ACID AND FOUR METABOLIC PRODUCTS IN SIX HUMANS TAKING 1-Gm. DOSES OF NA 4 TIMES DAILY FOR 10 CONSECUTIVE DAYS[a]

Substance Determined[b]	Day of Expt.	Interval After Morning Dose[c] 0–9 hr.	9–24 hr.	0–24 hr.
NA	1	9 ± 7 (17)	18 ± 4 (20)	27 ± 6 (35)
HNA		180 ± 58 (156)	249 ± 48 (280)	429 ± 53 (432)
NA-G		716 ± 65	1145 ± 68	1861 ± 112 (2142)
HNA-G		393 ± 108	862 ± 89	1255 ± 176 (911)
DCA		78 ± 15	165 ± 43	244 ± 51 (320)
Total		1376 ± 117	2439 ± 160	3816 ± 148 (3840)
NA	7	16 ± 5 (17)	15 ± 5 (20)	31 ± 8 (35)
HNA		229 ± 42 (166)	253 ± 46 (280)	482 ± 94 (442)
NA-G		1152 ± 191	1176 ± 91	2328 ± 251 (2142)
HNA-G		528 ± 122	708 ± 158	1236 ± 151 (933)
DCA		84 ± 25	148 ± 39	232 ± 36 (328)
Total		2009 ± 200	2300 ± 91	4309 ± 249 (3880)
NA	10	0 ± 4 (17)	9 ± 3 (20)	9 ± 7 (35)
HNA		189 ± 79 (166)	248 ± 74 (280)	437 ± 71 (442)
NA-G		789 ± 85	1122 ± 156	1911 ± 132 (2142)
HNA-G		471 ± 83	769 ± 98	1240 ± 107 (933)
DCA		104 ± 25	166 ± 70	270 ± 63 (328)
Total		1553 ± 178	2314 ± 218	3867 ± 90 (3880)

[a] Doses taken at 0, 4, 8, and 13.5 hr. of each day (zero hr. = 7:30 a.m.). The subjects had breakfast at 8:30 a.m., lunch at 12:30 p.m., and dinner at approximately 6:30 p.m. each day on which samples were to be taken. One subject failed to take the fourth dose on the first day and is not included in the means for that day. [b] For definition of terms see Table I; DCA, dicarboxylic acid. [c] Values given as mg., in terms of NA equivalents.

which showed definitely that the presence of food in the stomach delays the absorption of NA. In the "full stomach" part of the experiment, one subject gave a peak plasma level at 0.5 hr., and another subject at 1 hr., but in half of the subjects the 3-hr. level was the highest observed (Table III). In contrast, when in the postprandial state the mean plasma level for the same six subjects at 0.5 hr. postmedication exceeded any which was attained when they ingested the dose immediately after a full breakfast, and in this state only one of the subjects reached his peak level as late as 2 hr. postmedication.

In study *C* the expected urinary excretion rate of about 3860 mg./day (including the dicarboxylic acid) was fully realized, as is evident in Table V. The mean outputs of the items determined, as per cent of the total amount excreted, were approximately as follows, for the 3 days studied: conjugated NA, 51; conjugated HNA, 31; free HNA, 11; dicarboxylic acid, 6.5; free NA, 0.5 (all values given in terms of equivalents of NA). On the basis of the single-dose studies (2) the expected partition of these excretory products, listed in the same order, was: 56, 24, 11, 8, 1. Thus, the only important difference between the present and the earlier set of values would be the shift of about 6% of the output from the conjugated NA to the conjugated HNA fraction. This shift was also reflected in the smaller than expected output of free NA. No consistent change in the fraction of the total output represented by any of these compounds becomes evident in a comparison of the results for days 1, 7, and 10. For example, free HNA remained constant throughout at 11.2% of the total, while conjugated NA increased from 48.7 to 54% and then decreased to 49.4% on day 10, and conjugated HNA decreased from 32.9 to 28.6% on day 7 and then increased again to 32.1% on day 10. Meanwhile the per cent of the output represented by DCA varied within the narrow limits of 5.2 and 7%.

With regard to the plasma level curves for the 3 days which were studied, the following generalizations may be made.

(*a*) On the first day the levels for NA deviated considerably from the expected course. Generally the observed values were lower than the calculated, one possible reason being that as a result of the very rapid absorption-elimination kinetics of the drug, the true peak level for each individual could only have been established by multiple blood sampling at times around the expected peak. Practical considerations rendered it impossible to do this. Other contributing factors might be the small number of subjects used relative to the complexity of the pharmacokinetic model, and the fact that only one of the experimental subjects was included in the panel from which the model was derived. The last-named option was deliberate, in the hope that the results obtained on the panel would be sufficiently representative to permit a prediction of what would be observed in the general population. This hope was only partially realized in the present experiments, but in kinetic studies such as those of Engberg-Pedersen (8) and of Krüger-Thiemer and Bunger (9) the possibility of predicting plasma levels of a chemotherapeutic agent from a kinetic model has been well established.

(*b*) Also on the first day, the observed values for HNA never coincided exactly with the calculated values, although the means of the two sets of figures were about equal. In general, therefore, the expected peaks and valleys developed, but these peaks and valleys did not invariably occur at the predicted times, for reasons set forth above.

(*c*) On the seventh and tenth days of the experiment the observed values for NA followed the calculated path quite closely.

(*d*) On the seventh and tenth days of the experiment the observed values for HNA exceeded the calculated values by rather large amounts (exception: day 10, 4 hr.). This discrepancy was due in part to the fact that the daily lows for HNA were considerably higher than the calculated 0.9 mcg./ml. However, in each of the six subjects the carryover of HNA from day 9 was actually less than that from day 6, indicating that there was not a progres-

sive accumulation of the drug in the plasma. For days 7 and 10 the overnight lows for NA in the six subjects ranged from 0.1 to 1.7 mcg./ml., and those for HNA ranged from 1.6 to 12.1 mcg./ml.[4]

(*e*) HNA represented a more constant constituent of the plasma than NA. Evidence for this is the fact that at practically every low point in the NA curves, the level of HNA was higher than that of NA, and *vice versa*. Thus, following each dose of NA the ratio NA/HNA increased to a peak of about 3/1, and then gradually decreased, so that by the time the next dose was taken the ratio was less than 1. (*Note:* at the equilibrium high the calculated plasma levels for subjects of the average weight used in this study would be: NA = 25 mcg./ml.; HNA = 7.5 mcg./ml.)

SUMMARY

The absorption and elimination of nalidixic acid have been studied in subjects receiving the drug four times daily on two different dosage schedules: "after meals" and "between meals." More satisfactory and predictable results were obtained when the doses were given at least 1 hr. before meals, rather than at variable times after meals. Plasma levels and excretion patterns of nalidixic and hydroxynalidixic acids resulting from the administration of 1 Gm. of the former four times daily for 10 days followed quite closely a model derived from single-dose studies. This dosage regimen did not result in any important change in the characteristics of the absorption, excretion, or metabolism of the drug, as based on a comparison of the data for the first, seventh, and tenth days of medication. The multiple dosage regimen resulted in some carry-over of naphthyridine in the plasma from one day to another, but the carry-over did not appear invariably to increase or decrease with the number of days on medication. Sensitivity of the plasma levels to small changes in the rate constants has been shown. The inverse relationship of plasma levels and body weights suggests that dosage should be adjusted to individual body weights, with the initial adult dosage regimen being approximately 15–16 mg./Kg. four times daily.

[4] Since the number of metabolic and excretion processes involved in the over-all picture is considerable, relatively small changes in the rates of several of these could grossly alter the overnight values. For example, a 30% decrease in the mean elimination rate constant for HNA (k_{H2}) would increase the overnight low from 0.8 to 1.9 mcg./ml., and would increase the 9.5-hr. value from 7.6 to 10 mcg./ml. A 1.5-fold increase in the average rate constant for NA → HNA (k_{M1}) without a corresponding change in k_d would increase HNA 1.5 times. Such differences in rate constants have been observed (2).

REFERENCES

(1) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).
(2) Portmann, G. A., McChesney, E. W., Stander, H., and Moore, W. E., *ibid.*, **55**, 72(1966).
(3) McChesney, E. W., Froelich, E. J., Lesher, G. Y., Crain, A. V. R., and Rosi, D., *Toxicol. Appl. Pharmacol.*, **6**, 292(1964).
(4) Dost, F. H., "Der Blutspiegle," Arbeitsgemeinschaft Medizinische Verlag, G. M. B. A., Leipzig, Germany, 1953, pp. 41, 254.
(5) Wiegand, R. G., Buddenhagen, J. D., and Endicott, C. J., *J. Pharm. Sci.*, **52**, 268(1963).
(6) Lesher, G. Y., Proceedings of the 3rd International Congress of Chemotherapy, Stuttgart (July 23–27, 1963), G. Thieme Verlag, Stuttgart, Germany, 1964.
(7) Goss, W. A., and Deitz, W. H., *Bacteriol. Proc.*, **1963**, 93.
(8) Engberg-Pedersen, H., Morch, P., and Tybring, L., *Brit. J. Pharmacol.*, **23**, 1(1964).
(9) Krüger-Thiemer, E., and Bunger, P., *Chemotherapia*, **10**, 129(1965).

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The increase of calcium/potassium ratios in nutrient solutions reduces growth of belladonna plants. This is evident in the reduced elongation of all stems, particularly the sympodial flowering branches, and in the fresh and dry weights of all plant parts. There appears to be a concurrent increase in the proportion of leaf to total plant at the expense of stem and root. The increase in calcium/potassium ratio results in higher yields of total nitrogen and alkaloids.

THE IMPORTANCE of calcium and potassium in the nutrition of plants is well established (1–4). The authors' interest was directed to the effect of varying ratios of calcium/potassium concentration on the metabolic reactions in the alkaloid-producing plant *Atropa belladonna* L. The influence of the concentration of one of these cations on the other has been expressed in connection with: (*a*) their being bound on soil particles (5–7), (*b*) their absorption from the soil solution by roots (7), (*c*) their translocation through the plant (8), and (*d*) the rate of a number of metabolic reactions within the plant (9, 10).

Brown (11) reported that interactions involving nutrient elements may directly or indirectly affect all of the major metabolic pathways in plants and animals. The ratio of iron/phosphorus may drastically alter the ability of cells to grow by controlling oxidative mechanisms.

Received August 15, 1966, from the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois, Chicago, IL 60680
Accepted for publication February 16, 1967.

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REFERENCES

(1) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).
(2) Portmann, G. A., McChesney, E. W., Stander, H., and Moore, W. E., *ibid.*, **55**, 72(1966).
(3) McChesney, E. W., Froelich, E. J., Lesher, G. Y., Crain, A. V. R., and Rosi, D., *Toxicol. Appl. Pharmacol.*, **6**, 292(1964).
(4) Dost, F. H., "Der Blutspiegle," Arbeitsgemeinschaft Medizinische Verlag, G. M. B. A., Leipzig, Germany, 1953, pp. 41, 254.
(5) Wiegand, R. G., Buddenhagen, J. D., and Endicott, C. J., *J. Pharm. Sci.*, **52**, 268(1963).
(6) Lesher, G. Y., Proceedings of the 3rd International Congress of Chemotherapy, Stuttgart (July 23–27, 1963), G. Thieme Verlag, Stuttgart, Germany, 1964.
(7) Goss, W. A., and Deitz, W. H., *Bacteriol. Proc.*, **1963**, 93.
(8) Engberg-Pedersen, H., Morch, P., and Tybring, L., *Brit. J. Pharmacol.*, **23**, 1(1964).
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Likewise, the calcium/boron ratio can markedly influence development of meristems and metabolism of sugars in all living cells.

James (12), in 1945, detailed a factorial experiment, involving additions of nitrogen, calcium, potassium, and phosphate to potted plants of belladonna. He reported that the application of nitrogen increased the total plant yield to its greatest magnitude. The addition of potassium and calcium increased the yield of alkaloids. Calcium was present at 40 Gm. of lime per plant, and potassium at 8 Gm. of potassium sulfate. This implies that a relationship of the two (Ca–K) exerted an effect on the plant in addition to that stimulated by the other nutrients.

Olsen (13) studied the effects of calcium and potassium on the growth and production of dry matter on *Sinapis* and *Hordeum* in nutrient solutions. He found the accumulation of dry weight greatest when the plants received Ca–K in a ratio of approximately 10:1.

It was the purpose of this study to determine what effects the ratios of concentrations of calcium to potassium have on the growth of the belladonna plant and its alkaloid production. In order to eliminate effects of soil binding of ions, plants were grown in nutrient solutions. Thus, concentrations of each of the ions available to the plant could be controlled.

EXPERIMENTAL

Plants and Containers—Seeds of a selected strain of *A. belladonna* were scarified by exposure to concentrated sulfuric acid for 1 min., followed by vigorous rinsing with tap water. They were then germinated in a sand–vermiculite mixture (1:1) maintained at 70°F. Germination occurred within 3 weeks. Following germination, the young seedlings were transferred to a full nutrient solution (14) for 3 weeks. They had developed 5 leaves, 2–3 cm. tall, averaging 2 Gm. fresh weight. The plants were then transferred to the experimental treatments.

Plants were grown in the greenhouse in glazed crocks which contained 12 L. of nutrient solution. The plants, which were supported on specially prepared Masonite covers, were lightly fastened by plant ties to a heavy wire frame. Four plants were grown in each of the four crocks used, making a total of 16 plants for each treatment.

Nutrient Solutions[1]—Solutions were prepared from reagent grade chemicals and distilled water on a milligram-equivalent per liter basis. All solutions contained the following ions in milligram-equivalents per liter: magnesium, 4 ml.; phosphate, 1 ml.; nitrate, 20 ml.; sulfate, 4 ml.; iron as EDTA, 1 ml.; and trace elements,[2] 1 ml. Experimental solutions contained, in addition to these, quantities of calcium and potassium in mg.-equivalents/liter, as adapted for each of the treatments.

Distilled water was added periodically to maintain the crock volumes at the same level as that at the beginning of the experiments. The pH of the solutions was adjusted weekly to 5.8 by the addition of dilute hydrochloric acid.

Nutritional Treatments—Three experiments were performed. In the first, the ratio of calcium to potassium was varied by maintaining the potassium concentration at the same level as that of the full nutrient solution. The following seven treatments were selected: Ca–K: 30:4, 20:4, 10:4, 5:4, 4:4, 2:4, and 1:4.

In the second experiment, the ratio was varied by maintaining the calcium level but by introducing a wide range of potassium concentration. The following eight treatments were chosen: Ca–K: 10:0, 10:1, 10:2, 10:4, 10:5, 10:10, 10:20, and 10:30

In the third experiment, five ratios were selected. Based on the observations of the preceding two experiments, it was found that they would support growth satisfactorily. They were, as follows: Ca–K: 1:10, 1:4, 4:10, 4:4, and 10:4.

Measurement and Sampling—Measurements of the length of the primary shoot and of the first sympodial flowering shoot were made weekly during the growth period. Plants were harvested 35 to 42 days after having been transferred to the nutrient solutions. Fresh weights of separated parts were made at harvest; dry weights were recorded after the parts were air-dried and then oven-heated at 100°C. for 1 hr.

All of the dried leaves of a single treatment were combined and made into a homogeneous powder (20 mesh). Three-gram samples of these were taken and prepared for total nitrogen (Kjeldahl) determinations. Two separate determinations were conducted, and agreed within 3%.

In order to determine the total alkaloids, the authors followed the method of Brown, Kirch, and Webster (15), which involved aliquot portions from an extract of a 10-Gm. sample. However, they modified it by utilizing only 2-Gm. sample extractions for each determination, and chromatographing the total extract.

RESULTS

Growth Responses

Shoot Elongation—(Table I)—With an increase of the calcium/potassium ratio, there was a progressive reduction of primary shoot length. In experiment *A*, the growth of plants was greatest at the lowest ratios of Ca–K: 1:4 and 2:4. In experiment *B*, ratios of 10:20 and 10:4 produced optimum growth. In experiment *C*, selected ratios of 1:10 and 1:4 resulted in the best growth. Otherwise, fairly uniform growth was observed.

Sympodial Flowering Branches—(Table I)—Differences in elongation of the sympodial flowering branches were of greater magnitude than those of the primary shoot, with the greatest elongation occurring at the intermediate ratio of 5:4 in experiment *A*.

[1] These nutrient solutions were prepared from gram-equivalent stock solutions of the following salts: calcium nitrate, potassium nitrate, potassium acid phosphate (Sorensen's), magnesium sulfate, and iron ethylenediaminetetraacetate. The final solutions were made by adding 1.0 ml. of the stock solution per liter to a large volume of distilled water, and then filling each crock to the desired level with distilled water.

[2] The trace elements were prepared according to the Hoagland and Arnon (14) formula and added weekly.

In experiment *B*, due to the low potassium concentration (1 or 2 mg. equivalent/liter), the growth of the sympodial flowering branches was not so great as that of the primary shoot.

TABLE I—EFFECT OF CALCIUM/POTASSIUM RATIOS ON SHOOT LENGTH IN THE NUTRIENT MEDIUM

Expt.	Ratio Ca:K	Length of Main Erect Primary Stem, cm.	Length of First Sympodial Flowering Branch, cm.
A[a]	1:4	61.5[b] ± 1.7[c]	63.4[b] ± 9.1[c]
	2:4	60.9 ± 2.2	86.4 ± 16.7
	4:4	57.0 ± 4.3	85.3 ± 9.3
	5:4	57.6 ± 4.3	94.6 ± 8.4
	10:4	54.6 ± 1.6	93.3 ± 8.8
	20:4	53.7 ± 1.7	69.3 ± 11.7
	30:4	49.5 ± 2.4	65.6 ± 9.6
B[d]	10:30	55.4 ± 6.0	39.3 ± 6.2
	10:20	61.5 ± 4.8	39.6 ± 9.7
	10:10	60.8 ± 3.0	43.3 ± 10.3
	10:5	61.5 ± 6.3	54.4 ± 7.3
	10:4	62.0 ± 2.3	53.7 ± 12.5
	10:2	57.5 ± 2.6	59.5 ± 15.2
	10:1	54.5 ± 1.7	34.0 ± 9.1
	10:0	22.3 ± 3.4	...
C[e]	1:10	54.2 ± 1.6	48.2 ± 5.9
	1:4	53.6 ± 1.3	43.1 ± 7.0
	4:10	51.7 ± 2.2	52.3 ± 11.0
	4:4	50.2 ± 1.6	44.4 ± 7.6
	10:4	51.4 ± 1.6	32.0 ± 5.8

[a] Experiment *A*, 6 weeks of growth. [b] Shoot lengths are mean of 16 plants. [c] Standard error of the mean. [d] Experiment *B*, 5 weeks of growth. [e] Experiment *C*, selected ratios.

In experiment *C*, the length of the sympodial flowering branches at the intermediate ratio of Ca–K 4:10 was significantly greater than the others ($p = 0.001$).

Fresh Weight—(Table II)—Increase of the Ca–K ratio resulted in decreased growth as measured by fresh weight of leaves, stems, and total plant at harvest. This was especially noted in experiment *C*, when selected ratios were used.

Fresh weights of roots appeared to be less responsive to the effect of this environmental ratio. The heaviest root systems were obtained at the intermediate ratios.

Dry Weight—(Table III)—In experiment *C*, increased Ca–K ratios produced less reduction in dry weights than was observed in fresh weights. On a percentage basis, the decrease in dry weights and in fresh weights was essentially the same.

Ratios of Plant Parts to Each Other—Increase in the Ca–K ratio resulted in a greater proportion of leaf to total plant with a corresponding decrease in stem. The ratio of shoot to root increased slightly, but none of the results were statistically significant.

Total Nitrogen (Kjeldahl) of Leaves—(Table IV)—Increase of the ratio of calcium/potassium resulted in a distinct rise in the total nitrogen content in the leaves of the plant, except in the case of the lowest ratio, 1:10. This increased amount of nitrogen per gram of dry tissue was in direct contrast

TABLE II—EFFECT OF CALCIUM/POTASSIUM RATIOS ON FRESH WEIGHT OF PLANT PARTS IN THE NUTRIENT MEDIUM

Expt.	Ratio Ca:K	Fresh Wt., Gm. Leaves	Stems	Roots	Total Plant
A[a]	1:4	71.9[b] ± 4.3[c]	56.1[b] ± 9.2[c]	44.3[b] ± 2.6[c]	189.3[b] ± 16.1[c]
	2:4	64.1 ± 4.5	51.0 ± 4.5	56.1 ± 5.1	171.3 ± 14.1
	4:4	55.7 ± 2.8	53.6 ± 4.8	55.6 ± 7.7	161.4 ± 15.3
	5:4	63.0 ± 3.4	60.8 ± 3.4	59.1 ± 3.2	183.0 ± 10.0
	10:4	69.3 ± 4.3	57.5 ± 4.0	78.4 ± 3.2	186.0 ± 11.5
	20:4	52.0 ± 3.7	46.8 ± 4.1	48.1 ± 3.7	147.0 ± 11.5
	30:4	44.0 ± 4.3	41.4 ± 5.5	35.4 ± 3.7	120.9 ± 13.5
B[d]	10:30	73.8 ± 7.1	47.4 ± 7.2	36.2 ± 4.1	157.2 ± 19.0
	10:20	75.8 ± 7.0	54.7 ± 5.9	36.5 ± 3.6	166.8 ± 15.6
	10:10	80.8 ± 6.5	63.6 ± 6.2	40.9 ± 3.4	185.4 ± 16.1
	10:5	58.8 ± 4.0	52.2 ± 3.5	37.3 ± 2.5	148.5 ± 9.4
	10:4	59.9 ± 4.6	49.7 ± 4.6	44.6 ± 4.6	153.9 ± 13.8
	10:2	53.3 ± 4.5	40.6 ± 4.7	37.8 ± 4.3	134.7 ± 13.5
	10:1	43.9 ± 3.1	37.2 ± 2.7	39.0 ± 3.6	120.3 ± 9.4
	10:0	8.6 ± 0.57	6.8 ± 0.48	7.2 ± 1.4	21.5 ± 3.4
C[e]	1:10	78.3 ± 3.5	60.8 ± 3.2	42.5 ± 2.5	181.6 ± 9.0
	1:4	69.5 ± 8.9	51.0 ± 3.6	34.8 ± 2.4	155.3 ± 9.5
	4:10	65.6 ± 6.3	51.1 ± 6.1	35.4 ± 4.1	152.0 ± 16.6
	4:4	65.2 ± 4.5	43.5 ± 4.0	37.4 ± 3.1	146.4 ± 11.3
	10:4	54.9 ± 3.8	38.0 ± 3.0	27.4 ± 2.7	120.3 ± 9.2

[a] Experiment *A*, 6 weeks of growth. [b] Weights are the means of the plant parts of 16 plants. [c] Standard error of the mean. [d] Experiment *B*, 5 weeks of growth. [e] Experiment *C*, selected ratios.

TABLE III—EFFECT OF SELECTED CALCIUM/POTASSIUM RATIOS ON DRY WEIGHT OF PLANT PARTS IN THE NUTRIENT MEDIUM (EXPERIMENT *C*)

Ratio Ca:K	Dry Wt., Gm. Leaves	Stems	Roots	Total
1:10	7.0[a] ± 0.07[b]	5.4[a] ± 0.35[b]	2.4[a] ± 0.04[b]	14.8[a] ± 0.59[b]
1:4	6.5 ± 0.32	4.8 ± 0.43	2.0 ± 0.08	13.3 ± 1.21
4:10	6.8 ± 0.49	4.7 ± 0.59	1.8 ± 0.06	13.3 ± 1.26
4:4	6.4 ± 0.39	4.4 ± 0.52	2.3 ± 0.06	13.1 ± 1.05
10:4	5.3 ± 0.32	3.3 ± 0.08	1.4 ± 0.003	10.1 ± 0.59

[a] Weights are the means of plant parts of 16 plants. [b] Standard error of the mean.

TABLE IV—EFFECT OF SELECTED CALCIUM/POTASSIUM RATIOS ON TOTAL NITROGEN (KJELDAHL) AND TOTAL NITROGEN IN THE LEAF IN THE NUTRIENT MEDIUM (EXPERIMENT *C*)

Ratio Ca:K	Total Nitrogen, mg./Gm. Dry Wt.	Alkaloids, mg./Gm. Dry Wt.
1:10	44.8[a] ± 1.4[b]	1.03[a] ± .0017[b]
1:4	36.4 ± 1.3	0.91 ± .0014
4:10	44.1 ± 0.25	0.94 ± .0009
4:4	46.45 ± 0.16	1.35 ± .0010
10:4	48.2 ± 0.03	1.05 ± .0015

[a] Mean of 4 determinations. [b] Standard error of the mean.

to the amount of growth obtained with the same ratios.

Total Alkaloids—(Table IV)—The pattern of alkaloid yield in general followed that of the total nitrogen synthesized in leaves. The 1:10 ratio revealed an alkaloid yield similar to that of the 10:4. It should be noted that on a mg./Gm. dry weight basis, the amount of alkaloids at the lowest ratio was similar to that produced at the highest ratio.

DISCUSSION

The use of nutrient solutions for growing plants for this type of study is advantageous. It eliminates the problem of binding of solute ions on soil particles which affects their availability to the plant. Soluble salts were used in this work because absorption of calcium by roots in the soil is slower than that of potassium. Except where noted, ratios were unreasonably low in calcium or potassium ions. No morphological deficiency symptoms were observed in any of the plants.

The upper sympodial flowering shoots were more responsive to the different nutrient treatments than were the main primary shoots. This might be partially explained on the basis that the primary shoot had already begun its elongation pattern at the beginning of the experimental treatment, whereas the sympodial branches had not yet been initiated. However, many experimental treatments were so drastic that the few centimeters of shoot length at the beginning of an experiment were not significant, considering the results. Lack of responsiveness of the main primary shoot would then indicate a certain inherent genetic quality which to a large extent operated independently of environmental nutritional stimuli. By the time sympodial branches were in the process of developing, growth had been more profoundly influenced by the nutritional supply.

Elongation of both primary and secondary stems appeared to be somewhat retarded by increased ratios of calcium/potassium. Percentage increases which occurred in the second, third, and fourth weekly measurements were greater at the higher ratios. This is in contrast to the general tendency of the shoot which would normally approach its maximum length at progressively slower rates.

Effects attributed to the ratio of concentrations might be considered in terms of one of the following: (*a*) the result of the calcium concentration irrespective of the potassium concentration, or *vice versa;* (*b*) the difference between hydration of the tissue at the lower ratios, or less hydration and more woodiness at higher ratios; or (*c*) the result of total concentration of solutes in the solution.

In experiment *A* when plants were grown at varying calcium concentrations but with potassium constant, the differences in succulence *versus* woodiness were not apparent even at high concentrations of calcium. Likewise in experiment *B*, where the potassium concentration was varied, differences in succulence were not apparent. This indicated that the effect was due to the ratio of these ions to each other, rather than to either ion by itself.

When considering the water content in the tissue, the total range from the lowest to the highest ratio indicated a variation of less than 0.5%. Likewise, the effect of total ion concentration on hydration of the tissue resulted in a difference of 0.6% between the lowest and the highest ratios of calcium/potassium.

Except for the samples determined at the lowest ratio (Ca–K 1:10), the total nitrogen and the alkaloids present in the leaves of the plants resulted in a higher yield, as the ratio of calcium/potassium increased. Because the results of determinations were surprisingly high at the low ratio, they were redetermined and verified. High percentages of nitrogen and total alkaloids were also found by Schermeister in work done in this laboratory (16).

Ratios of leaf to stem, and shoot to root, showed that higher calcium/potassium ratios favored leaf development and decreasing stem. They also tended to favor shoot over root. The smaller plant at the higher calcium/potassium ratio produced more leaf or photosynthetic material, and less stem and root structure.

CONCLUSIONS AND SUMMARY

Growth of belladonna is affected by the ratio of calcium to potassium in the nutrient medium. Production of all plant parts is reduced as the ratio of calcium/potassium is increased. Reduced elongation occurred in the sympodial flowering shoots to a more marked extent than in the primary erect stems. Fresh and dry weights of all plant parts were lower as the ratio increased.

As the calcium/potassium ratio increased, the yield of total nitrogen and alkaloids was higher. There was one exception: the 1:10 ratio.

REFERENCES

(1) Hoagland, D. R., "Lecture on the Inorganic Nutrition of Plants," Chronica Botanica Co., Waltham, Mass., 1944, p. 173.

(2) Wallace, T., "The Diagnosis of Mineral Deficiencies in Plants," H. M. Stationery Office, London, England, 1951, pp. 19–23.

(3) "Mineral Nutrition of Plants," Troug, E., ed., University of Wisconsin Press, Madison, Wis., 1951, p. 65.

(4) "Plant Analysis and Fertilizer Problems," Reuther, W., ed., American Institute of Biological Sciences, Washington, D. C., 1960, p. 215.

(5) York, E. T., Bradfield, R., and Reed, M., *Soil Sci.*, **76**, 379(1953).

(6) *Ibid.*, **76**, 481(1953).

(7) Smith, R. L., and Wallace, A., *ibid.*, **81**, 97(1956).

(8) Jacobson, L., Hannapel, R. J., Moore, D. P., and Schaedle, M., *Plant Physiol.*, **36**, 58(1961).

(9) Rasmussen, G. K., and Smith, P. F., *ibid.*, **36**, 99 (1961).

(10) Crane, F. A., and Steward, F. C., *Cornell Univ., Agr. Expt. Sta. Mem., 379*, 1962.

(11) Brown, J. C., *Ann. Rev. Plant Physiol.*, **14**, 98(1963).

(12) James, W. O., *et al.*, "Oxford Medicinal Plants Scheme," Annual Report, 1945.

(13) Olsen, C., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **24**, 69(1942).

(14) Hoagland, D. R., and Arnon, D. I., *Calif. Univ. Agr. Expt. Sta. Circ. 347*, 1950.

(15) Brown, N. G., Kirch, E. R., and Webster, G. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **37**, 24(1948).

(16) Schermeister, L. J., Crane, F. A., and Voigt, R. F. *ibid.*, **49**, 698(1960).

Tumor Inhibitors XXI

Active Principles of *Acer negundo* and *Cyclamen persicum*

By S. MORRIS KUPCHAN, RICHARD J. HEMINGWAY, JOHN R. KNOX, STANLEY J. BARBOUTIS, DIETER WERNER, and MAUREEN A. BARBOUTIS

An alcoholic extract of leaves and stems of *Acer negundo* L. was found to show significant inhibitory activity against sarcoma 180 in mice. Systematic fractionation of the extract led to concentration of the activity and separation of two chromatographically homogeneous active acidic saponin fractions. One of the latter saponin fractions also showed significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 tumor in rats. An alcoholic extract of the tubers of *Cyclamen persicum* Mill. was found to show significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB). Systematic fractionation led to separation of a chromatographically homogeneous saponin fraction with significant inhibitory activity against the Walker 256 tumor in rats. Preliminary characterization of the active saponin fractions is described.

In the course of the continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Acer negundo* L.[1] (*Aceraceae*) was found to show significant inhibitory activity against sarcoma 180 in mice,[2] and an alcoholic extract of *Cyclamen persicum* Mill.[3] (*Primulaceae*) was found to show significant cytotoxic activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB).[2] The present report describes the systematic fractionation and the isolation and preliminary chemical characterization of growth-inhibitory saponin fractions from each extract. The observations confirm and extend earlier reports of the tumor-inhibitory (1, 2) and cytotoxic (3) activity of saponins.

The fractionation of the alcoholic extract of *Acer negundo* is summarized in Schemes I and II. Fraction H material, dissolved in 10% methanol in chloroform, was subjected to chromatography on adsorbent magnesium silicate,[4] and the active fractions from this column were further purified on silicic acid columns, to yield two one-spot but noncrystalline acidic saponin materials. Attempts to further purify these materials by chromatography and countercurrent distribution were unsuccessful.

The *in vivo* assay data for fractions obtained in a typical experiment are reported in Table I. The evaluation of assay results by the CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less.[2] The inhibitory activity of fraction P against the Walker carcinosarcoma 256 tumor in rats over a wide dosage range is noteworthy.

The preliminary fractionation of the alcoholic extract of *Cyclamen persicum* is summarized in Scheme III. Fraction X material dissolved in 10% methanol in chloroform was subjected to chromatography on silicic acid columns, to yield a one-spot but noncrystalline saponin material. Attempts to further purify this material by chromatography and countercurrent distribution were unsuccessful.

The cytotoxicity data are reported in Table II. Evaluation of the tissue culture assay results by the CCNSC is such that a material is considered active if the ED_{50} (dose inhibiting growth to 50% of control growth) equals or is less than 4 mcg./ml.[2] The Walker 256 assay data for fraction E′ are reported in Table III. It is noteworthy that the homogeneous saponin fraction E′ showed significant inhibitory activity against the Walker 256 tumor system *in vivo*, even though the cytotoxicity against KB cells was not marked; no explanation is readily apparent.

EXPERIMENTAL

Melting points were determined on a Hoover Uni-Melt melting point apparatus and were corrected. Infrared spectra were determined in chloroform solution on a Beckman IR5A infrared spectrophotometer. Specific rotations were measured on a Zeiss-

Received December 14, 1966, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison, WI 53706

Accepted for publication February 28, 1967.

This investigation was supported by grant CA-04500 from the National Cancer Institute, grant T-275 from the American Cancer Society, and contract PH 43-64-551 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

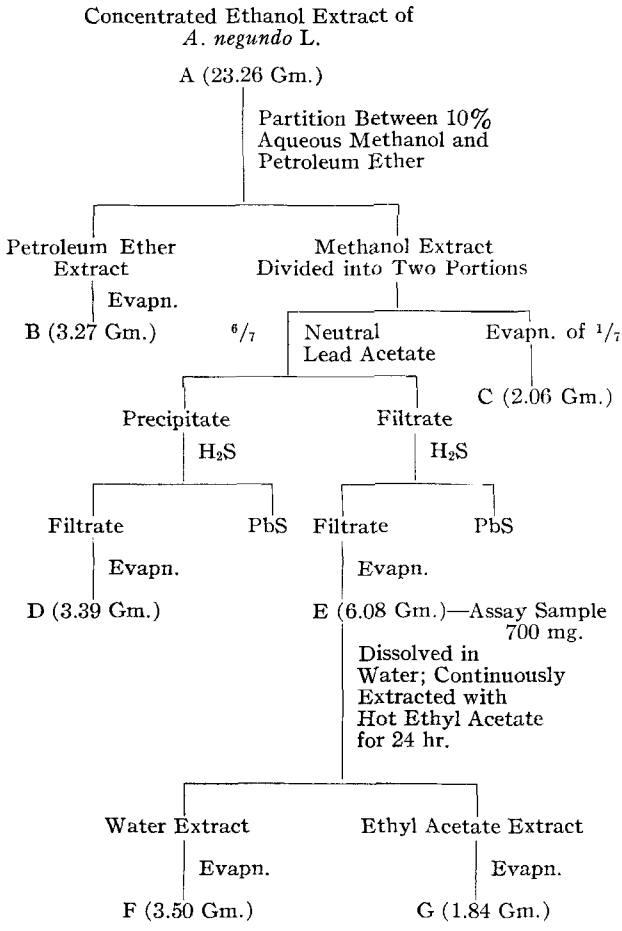
Previous paper: Kupchan, S. M., Hemingway, J. C., Cassady, J. M., Knox, J. R., McPhail, A. T., and Sim, G. A., *J. Am. Chem. Soc.*, **89**, 465(1967).

[1] Leaves and stems, gathered in the vicinity of Madison, Wis., July 1961. The authors thank Professor H. H. Iltis, University of Wisconsin, for confirming the identity of the plant. A voucher specimen is deposited in the University of Wisconsin Herbarium.

[2] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

[3] Tubers, gathered in Israel in April 1962. The authors acknowledge with thanks receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center.

[4] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.



Flow Sheet for Preliminary Fractionation of Tumor-Inhibitory Extract from A. negundo *L.*

Scheme I

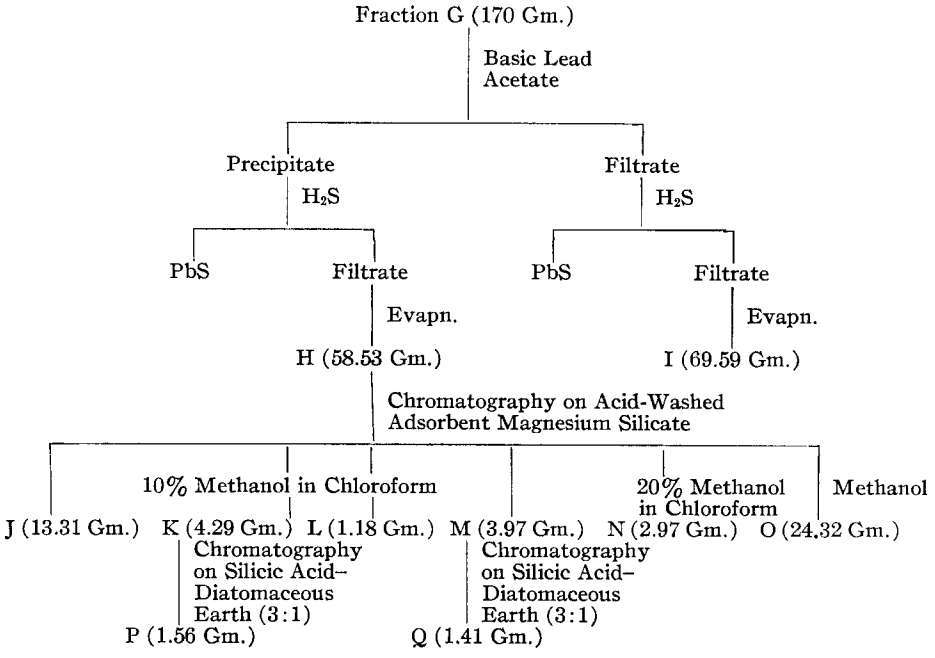
Winkel polarimeter. The petroleum ether used was Skellysolve B (b.p. 60–68°). Diatomaceous earth[5] was used for chromatography.

Extraction and Preliminary Fractionation of A. negundo—Coarsely ground leaves and twigs of *A. negundo* (2.8 Kg.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump pressure to a thick dark syrup (A, 370 Gm.). A portion of the extract (23.26 Gm.) was partitioned between petroleum ether (600 ml.) and 10% aqueous methanol (400 ml.); the petroleum ether solution and one seventh of the 10% aqueous methanol layer were evaporated under reduced pressure (B, 3.27 Gm., and C, 2.06 Gm., respectively). The remaining 10% aqueous methanol layer was treated with a methanol solution of neutral lead acetate (25% w/v) to complete precipitation. The precipitate was removed by centrifuging and washed with a little methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered after precipitation had been completed, and the filtrate was concentrated to dryness under reduced pressure (D, 3.39 Gm.). The supernatant liquid from lead acetate precipitation was also treated with hydrogen sulfide, filtered, and evaporated (E, 6.08 Gm.). The material not precipitated by neutral lead acetate (after removal of an assay sample) was partitioned between water (30 ml.) and ethyl acetate (150 ml.) and the aqueous layer continuously extracted for 20 hr. with ethyl acetate. The two layers were concentrated under reduced pressure (F, 3.50 Gm., and G, 1.84 Gm., respectively).

A larger batch of fraction G (245 Gm.) was prepared from the crude extract (1450 Gm.) and a portion (170 Gm.) was dissolved in methanol (1500 ml.) and treated with a saturated methanolic solution of basic lead acetate to complete precipitation. The precipitate was removed by centrifugation and washed with a little methanol before suspending in methanol (1400 ml.) and bubbling in hydrogen sulfide. The lead sulfide was filtered after precipitation had been completed, and the filtrate was evaporated (H, 58.53 Gm.). The supernatant liquid from basic lead acetate precipitation was also treated with hydrogen sulfide, filtered, and evaporated (I, 69.59 Gm.).

Chromatography of Active Fraction H—Trial chromatographies on adsorbent magnesium silicate and silicic acid indicated that two materials giving

[5] Marketed as Celite 545 by Johns-Manville, New York, N. Y.



Flow Sheet for Final Fractionation and Chromatography of Active Principles of A. negundo *L.*

Scheme II

a brown color with 3% ceric sulfate in 3 *N* sulfuric acid were responsible for the activity of fraction H.

Fraction H (58.53 Gm.) was dissolved in methanol (100 ml.) and diluted with chloroform (900 ml.). The solubles (51.43 Gm.) were added to a column of acid-washed adsorbent magnesium silicate (3 Kg.) in chloroform. The material was eluted with 10% methanol in chloroform, and fractions were collected and examined by thin-layer chromatography on Silica Gel G using ethyl acetate–acetic acid–water (3:1:3) upper phase as developing solvent and ceric sulfate spray reagent. Fraction J (13.31 Gm.) was eluted before the first active material. Fraction K (4.29 Gm.) contained the first active material. Fraction L (1.18 Gm.) contained a mixture of the two active materials. Fraction M (3.97 Gm.) contained the second active material. The solvent was changed to 20% methanol in chloroform, fraction N (2.97 Gm.) was collected, and the remaining material was finally removed with methanol (O, 24.32 Gm.).

Fraction K (4.28 Gm.), dissolved in 25 ml. of 25% methanol in chloroform, was added to a column of silicic acid–diatomaceous earth (3:1, 800 Gm.) and the column was eluted with 7% methanol in chloroform (2.5 L.). The solvent was changed to 10% methanol in chloroform (12 L.), and fractions were collected and examined by TLC, using ethyl acetate–acetic acid–water (3:1:3) upper phase as solvent, and ceric sulfate as spray reagent. The fractions richest in the first active material were combined (P, 1.56 Gm.). Elution was continued with 20% methanol in chloroform (2 L.) and the remaining material was finally removed with methanol. Fraction P accounted for the activity of fraction K, and was essentially one-spot material on TLC.

Fraction M (3.9 Gm.), dissolved in 25 ml. of 25% methanol in chloroform, was added to a column of silicic acid–diatomaceous earth (3:1, 750 Gm.) and the column was eluted with 8.5% methanol in chloroform (2 L.). The solvent was changed to 10% methanol in chloroform (9 L.), fractions were collected and examined by TLC using ethyl acetate–acetic acid–water (3:1:3) upper phase as solvent, and ceric sulfate as spray reagent. The fractions richest in the second active material were combined (Q, 1.41 Gm.). Elution was continued with 20% methanol in chloroform (2 L.) and the remaining material was removed with methanol. Fraction Q accounted for the activity of the fraction M, and was one-spot material on TLC.

Fractions P and Q were also tested against Walker carcinosarcoma 256 (see Table II); only fraction P showed activity.

Fraction P was a yellow powder, m.p. 198–205° dec., $[\alpha]_D^{28} = +28.5°$ (c 1.79, MeOH).

Fraction Q was a beige powder, m.p. 230–237° dec., $[\alpha]_D^{27} = +8.6°$ (c 1.45, MeOH).

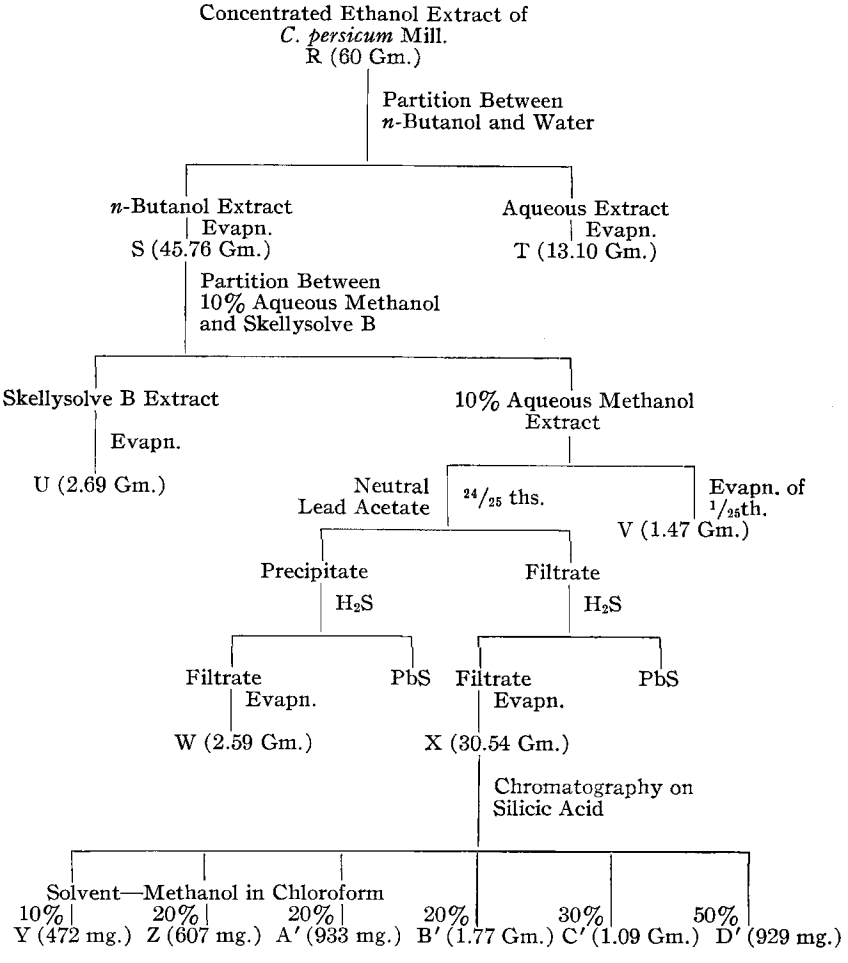
The acidic nature of fractions P and Q was indicated by their solubility properties. Both were readily soluble in dilute aqueous sodium bicarbonate, from which they were precipitated upon acidification with dilute hydrochloric acid.

Extraction and Preliminary Fractionation of C. persicum—Coarsely ground tubers of *C. persicum* (500 Gm.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump pressure to a brown resinous solid (R, 175 Gm.). A portion of the extract (60 Gm.) was partitioned between *n*-butanol (1 L.) and water (500 ml.), and the two solutions were evaporated separately under reduced pressure (S, 45.76 Gm., and T, 13.10 Gm., respectively). The *n*-butanol extract was further partitioned between

petroleum ether (1 L.) and 10% aqueous methanol (500 ml.), and the petroleum ether solution and one twenty-fifth part of the 10% aqueous methanol were evaporated under reduced pressure (U, 2.69 Gm., and V, 1.47 Gm., respectively). The remaining 10% aqueous methanol layer was treated with a saturated

TABLE I—ACTIVITY OF FRACTIONS FROM *A. negundo* L.

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change, Gm. Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C × 100
			Against Sarcoma 180		
A	500	0/4			Toxic
	250	1/4	−0.3	265/1563	Toxic
	125	4/4	−3.0	603/1563	38
B	160	4/4	+0.8	521/879	59
	80	4/4	+1.7	474/879	53
	40	4/4	+1.3	738/879	83
C	175	4/4	−2.7	290/1062	27
	87.5	4/4	+1.3	396/1062	37
	43.7	4/4	+0.3	771/1062	72
D	200	4/4	+1.5	253/879	28
	100	4/4	+1.5	655/879	74
	50	3/4	−1.2	1030/879	117
E	350	0/4			Toxic
	175	4/4	−3.1	46/879	5
	87.5	4/4	−2.1	715/879	81
F	350	4/4	+1.1	1323/1361	97
	175	4/4	+3.4	1244/1361	91
	87.5	4/4	+0.3	1045/1361	76
G	190	0/4			Toxic
	95	1/4	−1.7	205/1361	Toxic
	47.5	3/4	−1.8	330/1361	24
H	100	0/4			Toxic
	50	0/4			Toxic
	25	4/4	+1.2	561/1438	39
I	100	4/4	+0.9	1409/1438	97
	50	4/4	−1.1	1473/1438	102
	25	4/4	+1.4	1615/1438	112
J	80	4/4	+1.7	2124/2101	101
	40	4/4	+2.2	1598/2101	76
	20	4/4	+1.0	2653/2101	126
K	16	0/4			Toxic
	8	0/4			Toxic
	4	4/4	−3.4	1311/1867	70
L	12	0/4			Toxic
	6	3/4	−4.9	950/1867	50
	3	4/4	−3.0	874/1867	46
M	16	0/4			Toxic
	8	0/4			Toxic
	4	2/4	−7.2	243/1867	Toxic
N	14	3/4	−4.2	820/1867	43
	7	3/4	−2.0	932/1867	49
	3.5	3/4	+0.1	1828/1867	97
O	40	4/4	−0.7	1139/2101	54
	20	4/4	+0.2	2441/2101	116
	10	4/4	+0.7	2295/2101	109
P	10	0/4			Toxic
	5	1/4	−4.5	485/1192	Toxic
	2.5	3/4	−4.8	317/1192	26
Q	6	2/4	−3.3	568/1192	Toxic
	5	1/4	−3.8	565/1192	Toxic
	4	4/4	−5.4	270/1192	22
	3	3/4	−4.4	478/1192	40
	2	4/4	−3.9	346/1192	29
	1	4/4	−3.5	484/1192	40
			Against Walker Carcinosarcoma 256		
P	6	4/4	−24	1500/4000	37
	3	4/4	−20	1100/4000	27
	1.5	4/4	−12	2500/4000	62
	7.5	5/6	−5	2600/9100	28
	5.0	6/6	−6	3000/9100	32
	3.3	6/6	−10	2000/9100	21
	2.2	6/6	−6	3500/9100	38
Q	8	2/4	−20	1700/5800	Toxic
	4	4/4	−13	5000/5800	86
	2	4/4	0	5200/5800	89



Flow Sheet for Fractionation of Cytotoxic Extracts of C. persicum *Mill.*

Scheme III

methanol solution of neutral lead acetate to complete precipitation. The precipitate was removed by centrifuging and washed with a little methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered off after precipitation had been completed, and the filtrate was evaporated (W, 2.59 Gm.). The supernatant liquid from lead acetate precipitation was also treated with hydrogen sulfide, filtered, and concentrated to dryness under reduced pressure (X, 30.54 Gm.).

TABLE II—CYTOTOXICITY OF FRACTIONS FROM *C. persicum* MILL.

Fraction	ED_{50}, mcg./ml.
R	25
S	17
T	>100
U	>100
V	2.1
W	10
X	2.7
Y	22
Z	13
A′	7.4
B′	18
C′	13
D′	18
E′	24

Chromatography of Active Fraction X—Fraction X (6.0 Gm.) dissolved in methanol (20 ml.) was diluted with chloroform (180 ml.) and the solution was introduced onto a column of silicic acid (600 Gm.) in chloroform. The column was eluted with 10% methanol in chloroform until the yellow bands were eluted (fraction Y, 472 mg.). The solvent was changed to 20% methanol in chloroform, and fractions were collected and examined by thin-layer chromatography on Silica Gel G using 50% methanol in chloroform as solvent and ceric sulfate as spray reagent. Fraction Z (607 mg.) was eluted before a one-spot active material. Fraction A′ (933 mg.) contained the one-spot active material and fraction B′ (1.77 Gm.) was eluted after the active material. The solvent was changed to 30% methanol in chloroform, when fraction C′ (1.09 Gm.) was eluted. The remaining material was removed with 50% methanol in chloroform, to yield fraction D′ (929 mg.).

TABLE III—ACTIVITY OF THE ACTIVE FRACTION FROM *C. persicum* MILL. AGAINST THE WALKER INTRAMUSCULAR CARCINOSARCOMA 256

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change, Gm. Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C × 100
E′	50	2/4	−14	1400/7400	Toxic
	40	4/4	−11	1900/7100	26%
	30	4/4	−7	4400/7100	61%

A larger batch of fraction A′ (9.67 Gm.), prepared from crude extract (84 Gm.), was dissolved in 5% methanol in chloroform (400 ml.) and introduced onto a column of silicic acid (1 Kg.) in chloroform. The column was eluted with 5% methanol in chloroform (4 L.), 7% methanol in chloroform (6 L.), and with 10% methanol in chloroform (10 L.), to yield fractions which were examined by TLC as before. The fractions richest in the one-spot active material were combined (E′, 4.84 Gm.) and tested in the Walker carcinosarcoma 256 assay (see Table III).

Fraction E′ was a white amorphous powder, (m.p. 229–233°); $[\alpha]_D^{28}$ = +0.8° (c 1.20 MeOH), I.R. (Nujol) 3.04 μ, 5.81 μ, 6.10 μ.

Determination of the Hemolytic Index of Fractions P, Q, and E′—The determination was performed by the method outlined in the Swiss Pharmacopoea (4) using a 1:20 dilution of blood with normal saline and Fisher standard saponin as reference. Using these conditions, fraction P had a hemolytic index of 0.8; fraction Q, 0.5; and fraction E′, 316; with reference to standard saponin at 100. Hence the *Acer negundo* active principles have low hemolytic activity.

Test for the Glycosidic Nature of Fractions P, Q, and E′—Fractions P, Q, and E′ (5 mg. of each, separately), when heated for 2 min. with a 0.5% aqueous solution of triphenyltetrazolium chloride (2 drops) and 0.5 *N* sodium hydroxide (1 drop), gave no pink color or precipitate.

Further 5 mg. portions of fractions P, Q, and E′ were hydrolyzed by refluxing for 30 min. with 0.5 *N* hydrochloric acid in 50% aqueous alcohol (0.2 ml). Each hydrolyzate was neutralized and the triphenyltetrazolium chloride test performed. The deep red solutions and red precipitates indicated the presence of reducing sugars in each of the hydrolyzates (5).

REFERENCES

(1) Nigrelli, R. F., *Zoologica, Part 2*, **37**, 89(1952).
(2) Chanley, J. D., Mezzetti, T., and Sobotka, H., *Tetrahedron*, **22**, 1857(1966).
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(5) Feigl, F., "Spot Tests in Organic Analysis," 6th ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 1960, p. 424.

Molecular Orbital Calculations on Sulfonamide Molecules

By E. C. FOERNZLER* and A. N. MARTIN†

The LCAO-MO method has been used to compute the electronic characteristics for a series of 50 sulfonamide derivatives. The results of the calculations have been compared with experimental parameters through correlation with pKa. For those compounds where the substituent R group was an alternate ring, the correlation of pKa and the electronic charge at the ionizing (N^1) nitrogen atom was considered significant. However, it was necessary to classify the compounds by type of R group, plotting each class separately. The simple Hückel MO treatment was inadequate for those molecules where the substituent R group attached to the N^1 nitrogen atom was a nonalternate ring or a straight chain.

IN RECENT YEARS it has become possible to study the electronic structure of large organic molecules by using the principles of quantum mechanics. This field is known as quantum chemistry, and when applied to molecules of biological interest, has been called quantum biology or quantum biochemistry (1–4). The molecular orbital (MO) method, involving a linear combination of atomic orbitals (LCAO), is ordinarily used for calculating the energies of the π or delocalized electrons in a molecule, and the abbreviation LCAO-MO is used to designate this type of calculation. In this study, a modification of the LCAO-MO method, the simple Hückel molecular orbital (HMO) approximation (3–6), has been employed.

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Received April 25, 1966, from the Department of Physical Pharmacy, Purdue University, Lafayette, IN 47907
Accepted for publication January 12, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
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behavior in going into a transition state along with a specific receptor or reagent (7). However, where such an approach is either impossible or too complex, useful information may be obtained by considering various static properties of the molecules, such as the electronic charge densities relative to the total π or mobile electron systems of the molecules in their ground states. Calculation of the distribution of electronic charge indicates the average density of mobile electrons present on different atoms in the molecule. The higher the calculated charge, the greater the π electron density associated with a particular atom. It is also possible to compute bond orders, free valence, and the relative values of the highest occupied (homo) and lowest empty (lemo) molecular orbitals. This information, together with other chemical knowledge may be useful for predicting various physical-chemical properties and even biological activities of drug molecules.

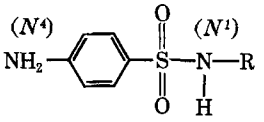
During the past two and one-half decades, a number of theories have been advanced on the mechanism of sulfonamide action. Bell and Roblin (8), Kumler and associates (9–11), Klotz and Bordwell (12–13), and Krüger-Thiemer *et al.* (14) have proposed possible modes of action. Although these theories present different explanations of the mechanism of sulfonamide action, they are all based on the original suggestion of Woods (15) and Fildes (16) that the sulfonamides interfere with the utilization of *p*-aminobenzoic acid in enzyme systems through competitive enzyme inhibition.

In this study the authors have computed the electronic charge densities for 50 sulfonamide compounds. The results of the computations have allowed an examination of the utility of HMO calculations applied to complex drug molecules, and have resulted in additional knowledge which may be useful for further elucidating the mechanism of sulfonamide action.

TREATMENT OF SULFONAMIDE MOLECULES

Compounds Studied—The 50 sulfonamide molecules selected for the calculations were those studied by Bell and Roblin (8). These molecules are all N^1-substituted sulfanilamide type compounds of the general structure I, and are listed in Table I according to nomenclature and numbering used by Bell and Roblin. The chemical structures of these compounds are given in a text by Northey (17). The experimental data, including the acid dissociation constants and the *in vitro* activities, C_R, were taken from the published results of Bell and Roblin.

The activity index, C_R, in Table I represents the minimum molar concentration of drug necessary to cause bacteriostasis of *E. coli* in a buffered (pH 7) synthetic medium under standardized conditions. The smaller the number, the greater the activity. As pointed out by Bell and Roblin, it is difficult to obtain a high degree of precision in such tests and small differences in bacteriostatic activity (a factor of 2) are within experimental error.



I

Sulfanilamide type compounds are weak acids because the amide (N^1) nitrogen atom of these molecules is sufficiently negative in solution to liberate a proton. Due to their high electronegativity, the oxygen atoms of the sulfonyl (SO_2) group greatly attract electrons. The resulting electron deficiency of the sulfur atom results in the electrons of the N:H bond being held more closely to the nitrogen atom. The hydrogen, therefore, is bound less firmly and readily ionizes to liberate a proton in solution. In addition to the acid dissociation constants, Bell and Roblin also measured the basic dissociation constants which are due to the ionization of the *p*-amino group or amino (N^4) nitrogen atom. These values were found to be approximately constant [$(0.5–2.3) \times 10^{-12}$]. A striking difference was thus observed between the acid and basic dissociation constants.

The R group is the only variable involved in the N^1-substituted sulfonamide derivatives, and it must be the factor controlling the acid dissociation constants of these compounds (8). In other words, the greater the electronegativity of the R group, the more R attracts electrons from the adjacent amide (N^1) nitrogen so that the hydrogen escapes more easily as a proton in solution leaving the anion form of the molecule. However, according to Bell and Roblin, as the electron-attracting power of different R groups increases, the SO_2 group should become less negative (thereby decreasing activity), because under these conditions a greater part of the ionic charge of the amide (N^1) nitrogen will be taken by the R group which will be competing more strongly with the SO_2 group for the ionic charge. As the SO_2 group becomes less negative, its ion should be less active than an ion whose R group is a weaker electron-attracting group. Similar conclusions are obtained by applying the same reasoning to the molecular form.

By calculating the electronic charge densities of the sulfanilamide type compounds, we would expect to find a relationship between the pKa and the electronic charge at the ionizing (N^1) nitrogen atom. The relationship of pKa to electronic charge has been discussed by Daudel *et al.* (3, 18–20), Longuet-Higgins (21), the Pullmans (22–24), and others (25).

Calculations—The linear combination of atomic orbitals-molecular orbital (LCAO-MO) method was employed for the calculations, using the Hückel molecular orbital (HMO) approximation with neglect of overlap (3–6). For the heteroatom (or substituent group) X, the Coulomb integral (α_X) and resonance or bond integral (β_{CX}) were given the usual forms shown in Eqs. 1 and 2:

$$\alpha_X = \alpha_C + \delta_X\beta_{CC} \qquad \text{(Eq. 1)}$$

TABLE I—SULFONAMIDE DERIVATIVES USED FOR LCAO-MO CALCULATIONS (8)

No.	Compd.[a]	pKa	K_a	$C_R \times 10^6$
1	*p*-Aminobenzoic acid	4.68	2.1×10^{-5}	...
2	Sulfanilamide	10.43	3.7×10^{-11}	20.0
3	N^1-Methylsulfanilamide	10.77	1.7×10^{-11}	30.0
4	N^1,N^1-Dimethylsulfanilamide	...	...	30.0
5	N^1-Hydroxyethylsulfanilamide	10.92	1.2×10^{-11}	50.0
6	Sulfanilylglycine	3.52	3.0×10^{-4}	[>90.0
7	N^1-Phenylsulfanilamide	9.60	2.5×10^{-10}	3.0
8	N^1-*o*-Tolylsulfanilamide	9.96	1.1×10^{-10}	10.0
9	N^1-*m*-Tolylsulfanilamide	9.74	1.8×10^{-10}	5.0
10	N^1-*p*-Tolylsulfanilamide	9.82	1.5×10^{-10}	5.0
11	N^3-Sulfanilylmetanilamide	8.23	5.9×10^{-9}	2.0
12	N^4-Sulfanilylmetanilamide	7.85	1.4×10^{-8}	0.5
13	N^1-*p*-Aminophenylsulfanilamide	10.22	0.6×10^{-10}	5.0
14	N^1-Furfurylsulfanilamide	10.88	1.3×10^{-11}	20.0
15	Sulfapyridine	8.43	3.7×10^{-9}	0.6
16	3-Sulfanilamidopyridine	7.89	1.3×10^{-8}	0.2
17	2-S-5-Bromopyridine	7.15	7.1×10^{-8}	0.5
18	5-S-2-Bromopyridine	7.12	7.6×10^{-8}	0.2
19	2-S-5-Aminopyridine	8.47	0.34×10^{-8}	0.6
20	5-S-2-Aminopyridine	8.82	0.15×10^{-8}	2.0
21	2-Sulfanilamidoimidazole	9.72	1.9×10^{-10}	40.0
22	3-Sulfanilamidopyridazine	7.06	0.87×10^{-7}	0.08
23	Sulfadiazine	6.48	3.3×10^{-7}	0.08
24	2-S-4-Methylpyrimidine	7.06	0.87×10^{-7}	0.2
25	2-S-4,6-Dimethylpyrimidine	7.37	0.43×10^{-7}	0.3
26	2-S-4-Aminopyrimidine	9.44	3.6×10^{-10}	20.0
27	4-S-Pyrimidine	6.17	6.7×10^{-7}	0.1
28	5-S-Pyrimidine	6.62	2.4×10^{-7}	0.2
29	5-S-2-Chloropyrimidine	5.80	1.6×10^{-6}	0.1
30	2-Sulfanilamidopyrazine	6.04	0.91×10^{-6}	0.08
31	4-S-1,2,4-Triazole	4.66	2.2×10^{-5}	>80.0
32	2-Sulfanilamidooxazole	6.50	3.2×10^{-7}	0.08
33	5-S-3-Methylisoxazole	4.20	6.3×10^{-5}	0.6
34	Sulfathiazole	7.12	7.6×10^{-8}	0.08
35	2-S-4-Methylthiazole	7.79	1.6×10^{-8}	0.2
36	3-S-4-Methylfurazan	4.10	7.9×10^{-5}	1.0
37	3-S-5-Methyloxadiazole	4.40	4.0×10^{-5}	2.0
38	2-S-1,3,4-Thiadiazole	4.77	1.7×10^{-5}	0.6
39	2-S-5-Methylthiadiazole	5.45	3.5×10^{-6}	0.2
40	Sulfanilylcyanamide	2.92	1.2×10^{-3}	100
41	Sulfanilylurea	5.42	3.8×10^{-6}	10.0
42	Sulfanilylguanidine	...	...	10.0
43	Sulfanilylaminoguanidine	...	...	0.9
44	N^1-Acetylsulfanilamide	5.38	4.2×10^{-6}	0.7
45	N^1-Chloroacetylsulfanilamide	3.79	1.6×10^{-4}	10.0
46	N^1-Benzoylsulfanilamide	4.57	2.7×10^{-5}	0.3
47	N^1-*p*-Aminobenzoylsulfanilamide	5.20	6.3×10^{-6}	0.5
48	N^1-Ethylsulfonylsulfanilamide	3.10	7.9×10^{-4}	1000
49	N^1-Sulfanilylsulfanilamide	2.89	1.3×10^{-3}	60.0
50	4,4′-Diaminodiphenylsulfone	...	...	2.0

[a] S = Sulfanilamido.

$$\beta_{CX} = \eta_{CX}\beta_{CC} \qquad \text{(Eq. 2)}$$

where δ_X (heteroatom Coulomb integral coefficient) and η_{CX} (heteroatom bond integral coefficient) are the adjustable, semiempirical parameters of the HMO treatment. The δ and η values used in this study were similar to those which have been suggested by the Pullmans (24). A complete listing of these parameters is given in Table II.

For each molecule, the appropriate δ_X and η_{CX} values must be selected. The HMO matrix (A) must then be set up and solved for the eigenvalues and eigenvectors. The electronic charges may then be computed from the coefficients contained in the eigenvectors. Although a number of different procedures may be employed to manipulate such matrices, the most reasonable approach for large molecules as those studied here is through the use of a digital computer. To solve the matrices on a computer, a program is required which will determine the eigenvalues (x) and eigenvectors (v) of a real symmetric matrix (A) for an eigenvalue problem which has the form $Av = xv$ (26). Initial calculations were made using a Jacobi routine (27) and an IBM 1620 computer. However, this method was too slow, and the procedure was changed to a modified Givens routine (28) written for an IBM 7090. In this case a subroutine for the Gram-Schmidt orthogonalization procedure (29) was added to the main program in order to insure an orthonormal basis for the eigenvectors computed by the Givens method. A second subroutine was also added to the main program which computed the electronic charges directly according to Eq. 3, thereby eliminating most of the hand computations. In Eq. 3, q_J is the electronic charge density of atom J,

$$q_J = \sum_{i=1}^{n} e_i c_{iJ}^2 \qquad \text{(Eq. 3)}$$

and c_{iJ} is the LCAO coefficient of atom J in the i^{th} molecular orbital which is occupied by e_i electrons. The sum is taken over all molecular orbitals, $i = 1, 2, \ldots, n$, however, only the occupied MO's contribute to the charge, since for the unoccupied orbitals, $e_i = 0$. For the HMO matrix, A, the roots (x) of the characteristic equation of A, *i.e.*, $|A - xI| = 0$, were obtained in the form shown in Eq. 4, where E_i is the energy level of the i^{th} molecular orbital, α is the Coulomb integral, β is the resonance integral, and x_i is the molecular orbital energy coefficient of the i^{th} MO.

$$E_i = \alpha + x_i\beta \text{ or } x_i = \frac{\alpha - E_i}{\beta} \qquad \text{(Eq. 4)}$$

TABLE II—δ AND η VALUES

Atom[a]	δX	Bond	ηCX
=N—	0.4	C=N	1.0
—N—	1.0	C—N	0.9
≡N	1.5[b]	C≡N	2.0[b]
=N$^+$—	2.0[c]	C=N$^+$	1.0[c]
NO_2 (—N$^+$, ···O$^-$)	0.6	C—N$^+$	1.0
	1.6	N$^+$ ··· O$^-$	1.0
=O	1.2	C=O	2.0
—O—	2.0	C—O	0.9
=S	0	C=S	1.2
—S—	0[d]	C—S	0.9[d]
—F	3.0[e]	C—F	0.7[e]
—Cl	1.4–2.0	C—Cl	0.4
—Br	1.5	C—Br	0.3
$\rangle C_{arom.} - C_{aliph.} = H_2$			
$C_{arom.}$	−0.1		
$C_{aliph.}$	0	$C_{arom.}$—$C_{aliph.}$	0.7
=H_2	−0.2[f]	C=H_2	2.0[f]
$\rangle C_{arom.} - C_{aliph.} = H_2$			
$C_{arom.}$	−0.1		
$C_{aliph.}$	0	$C_{arom.}$—$C_{aliph.}$	0.7
=H_2	−0.2[f]	C=H_2	2.0[f]
$\rangle C_{arom.}=H_2$			
$C_{arom.}$	0		
=H_2	−0.2[f]	C=H_2	2.0
$\rangle C_{arom.}=CH-C_{arom.}$			
$C_{arom.}$	0	$C_{arom.}$=CH	1.0
=CH	0	=CH—$C_{arom.}$	1.0
—N$^+$—C—C≡H_2			
—N$^+$—	2.0	N$^+$——C	1.0
—C—	0.2	C—CH_3	1.0
CH_3 C	0	C≡H_3	2.0[f]
H_3	−0.2[f]		
$\rangle C_{arom.}$—CH=CH_2			
$C_{arom.}$	0		
—CH=	0	$C_{arom.}$—CH=	1.0
=CH_2	0	—CH=CH_2	1.0
		$C_{aliph.}$—$C_{aliph.}$	0.7
		$C_{aliph.}$—N$^+$—	0.7
		$C_{aliph.}$—N—	0.7
		=N—N—	0.9
		$C_{arom.}$—COOH	0.7
		$C_{arom.}$—$CONH_2$	0.7

[a] The dots above the atoms refer to the number of π electrons initially assigned to the atom. [b] For $C_{arom.}$—$C_{aliph.}$ ≡N; $C_{arom.}$ has $\delta C = 0.15$; $C_{aliph.}$ has $\delta C = 0.30$; $C_{arom.}$—$C_{aliph.}$ has $\eta CC = 0.7$. [c] For C atoms adjacent to =N$^+$—, $\delta C = 0.3$. [d] For the most part, $\eta CS = 0.9$ has been used for a ring sulfur, *e.g.*, in thiazole or phenothiazine, but $\eta CS = 0.6$ has also been used in a few cases. [e] For $C_{arom.}$ — F; $C_{arom.}$ has $\delta C = 0.2$. [f] Hyperconjugation.

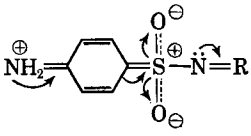
Table II—δ and η Values

Atom[a] | δX | Bond | ηCX

=N— | 0.4 | C=N | 1.0
—N— | 1.0 | C—N | 0.9
≡N | 1.5[b] | C≡N | 2.0[b]
=N$^+$— | 2.0[c] | C=N$^+$ | 1.0[c]
NO_2 (—N$^+$, ···O$^-$) | 0.6 | C—N$^+$ | 1.0
 | 1.6 | N$^+$ ··· O$^-$ | 1.0
=O | 1.2 | C=O | 2.0
—O— | 2.0 | C—O | 0.9
=S | 0 | C=S | 1.2
—S— | 0[d] | C—S | 0.9[d]
—F | 3.0[e] | C—F | 0.7[e]
—Cl | 1.4–2.0 | C—Cl | 0.4
—Br | 1.5 | C—Br | 0.3
$\rangle C_{arom.} - C_{aliph.} = H_2$ | | |
$C_{arom.}$ | −0.1 | |
$C_{aliph.}$ | 0 | $C_{arom.}$—$C_{aliph.}$ | 0.7
=H_2 | −0.2[f] | C=H_2 | 2.0[f]
$\rangle C_{arom.} - C_{aliph.} = H_2$ | | |
$C_{arom.}$ | −0.1 | |
$C_{aliph.}$ | 0 | $C_{arom.}$—$C_{aliph.}$ | 0.7
=H_2 | −0.2[f] | C=H_2 | 2.0[f]
$\rangle C_{arom.}=H_2$ | | |
$C_{arom.}$ | 0 | |
=H_2 | −0.2[f] | C=H_2 | 2.0
$\rangle C_{arom.}=CH-C_{arom.}$ | | |
$C_{arom.}$ | 0 | $C_{arom.}$=CH | 1.0
=CH | 0 | =CH—$C_{arom.}$ | 1.0
—N$^+$—C—C≡H_2 | | |
—N$^+$— | 2.0 | N$^+$——C | 1.0
—C— | 0.2 | C—CH_3 | 1.0
CH_3 C | 0 | C≡H_3 | 2.0[f]
H_3 | −0.2[f] | |
$\rangle C_{arom.}$—CH=CH_2 | | |
$C_{arom.}$ | 0 | |
—CH= | 0 | $C_{arom.}$—CH= | 1.0
=CH_2 | 0 | —CH=CH_2 | 1.0
 | | $C_{aliph.}$—$C_{aliph.}$ | 0.7
 | | $C_{aliph.}$—N$^+$— | 0.7
 | | $C_{aliph.}$—N— | 0.7
 | | =N—N— | 0.9
 | | $C_{arom.}$—COOH | 0.7
 | | $C_{arom.}$—$CONH_2$ | 0.7

[a] The dots above the atoms refer to the number of π electrons initially assigned to the atom. [b] For $C_{arom.}$—$C_{aliph.}$ ≡N; $C_{arom.}$ has $\delta C = 0.15$; $C_{aliph.}$ has $\delta C = 0.30$; $C_{arom.}$—$C_{aliph.}$ has $\eta CC = 0.7$. [c] For C atoms adjacent to =N$^+$—, $\delta C = 0.3$. [d] For the most part, $\eta CS = 0.9$ has been used for a ring sulfur, *e.g.*, in thiazole or phenothiazine, but $\eta CS = 0.6$ has also been used in a few cases. [e] For $C_{arom.}$ — F; $C_{arom.}$ has $\delta C = 0.2$. [f] Hyperconjugation.

The molecular form of the sulfonamide molecules was used as a model for the LCAO-MO calculations.[1] In order to consider the lone pair of electrons at the N^1 nitrogen atom as part of the mobile electron system, the resonance structure (II) was assumed.



II

The number of molecular orbitals and the number of π electrons considered for each molecule are given in Table III. The HMO matrix A for any particular compound listed in Table I may be set up by referring to Northey (17) for the structure of the R group, by allocating to the molecule the number of π electrons shown in Table III, and by using the δ and η values given in Table II. An example of the HMO matrix is given in Table IV for sulfapyridine (sulfa No. *15*). The numbering of the rows and columns in the matrix corresponds to the numbering of the atoms shown in structure III.[2] All other molecules were treated in the same manner as the sulfapyridine example, with exception of the δ and η values for the R groups. The δ and η values for the sulfanilamido part of the molecule in each case were the same as those given for the sulfapyridine example, that is, up to and including δ_{11} and η_{11-12}.

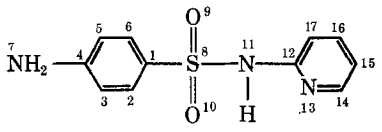
Results—The results of the LCAO-MO calculations on 30 of the sulfonamides studied are given in Table V. The tabulation includes the electronic charges at the amide nitrogen (q_{N1}), the *p*-amino nitrogen (q_{N4}), the SO_2 group oxygen atoms (q_{oxy}), and the sulfur atom (q_S). The formal π charges at these atoms may be computed by subtracting the tabulated electronic charges (the q's) from 2, 2, 1, and 2, respectively, according to Eq. 5, where f_J denotes the net or formal charge on atom J, atom J contributing e_J π electrons to the π electron pool.

[1] Calculations on the ionic forms did not differ appreciably from the molecular forms, and the authors were not able to improve the correlations by accounting for the ionic species in the calculations. Also, the omega technique (5, 30) was used for another series of compounds; however, it did not change the results significantly. The authors have found more success in accounting for ionic forms by moderately adjusting the δx parameter rather than by using the iterative process of the omega method.

[2] The *d*-orbitals of sulfur have been neglected. There is some controversy in the literature about the contribution of these orbitals (34). However, an article by Cammarata [*J. Pharm. Sci.*, **55**, 1469(1966)] suggests that if the effects of the *d*-orbital overlap are taken into account (using $\delta x = -2.60$ and $\beta CX = 0.99$ for the entire SO_2 group), small variations in the electronic charge at the *p*-amino position of the sulfonamides could be noted.

$$f_J = (e_J - q_J) \qquad \text{(Eq. 5)}$$

The results of the MO calculations on the sulfanilamide type compounds generally agree with chemical facts. In terms of the formal charge for sulfanilamide, for example (sulfa No. *2*), the electronic configuration shown in structure IV is found. The results indicate: (*a*) a deficiency (+) of π electrons at the *p*-amino group due to the fact that this group has donated its electrons to the adjacent benzene ring; (*b*) that due to their high electronegativity, the sulfonyl oxygen atoms have withdrawn electrons from the sulfur atom and also from the N^1 nitrogen atom, resulting in an excess (−) of electrons at these atoms; and (*c*) that the N^1 nitrogen atom is deficient (+) in π charge with



III

Table III—Data Used for LCAO-MO Calculations on Sulfonamide Derivatives

Sulfa	No. of MO's	No. of π Electrons
1 (PABA)	10	12
2	11	14
3	13	16
4	15	18
5	16	20
6	16	20
7	17	20
8	19	22
9	19	22
10	19	22
11	21	26
12	21	26
13	18	22
14	18	22
15	17	20
16	17	20
17	18	22
18	18	22
19	18	22
20	18	22
21	16	20
22	17	20
23	17	20
24	19	22
25	21	24
26	18	22
27	17	20
28	17	20
29	18	22
30	17	20

Table V—Electronic Charges for Sulfonamide Derivatives

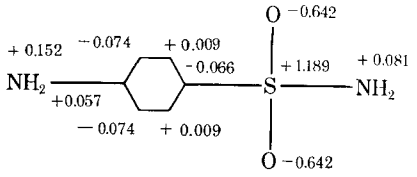
Sulfa	Electronic Charges			
	q_{N^1}	q_{N^4}	q_{oxy}	q_S
1 (PABA)	...	...	...	...
2	1.919	1.848	1.642	0.811
3	1.853	1.848	1.640	0.811
4	1.795	1.848	1.638	0.812
5	1.851	1.848	1.642	0.811
6	1.855	1.848	1.640	0.811
7	1.794	1.848	1.637	0.810
8	1.791	1.848	1.637	0.809
9	1.792	1.848	1.637	0.811
10	1.793	1.848	1.638	0.810
11	1.792	1.848	1.637	0.811
12	1.790	1.848	1.637	0.811
13	1.805	1.848	1.638	0.811
14	1.856	1.848	1.640	0.811
15	1.781	1.848	1.636	0.810
16	1.793	1.848	1.637	0.810
17	1.781	1.848	1.637	0.810
18	1.794	1.848	1.638	0.811
19	1.793	1.848	1.637	0.811
20	1.805	1.848	1.638	0.811
21	1.790	1.848	1.637	0.811
22	1.780	1.848	1.637	0.810
23	1.770	1.848	1.636	0.810
24	1.770	1.848	1.635	0.808
25	1.769	1.848	1.638	0.810
26	1.769	1.848	1.637	0.810
27	1.768	1.848	1.636	0.809
28	1.793	1.848	1.637	0.811
29	1.797	1.848	1.636	0.810
30	1.779	1.848	1.636	0.810

Table IV—HMO Matrix for Sulfapyridine[a]

$A =$

Atom ←No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	↓
	0	1.0	0	0	0	1.0	0	0.6	0	0	0	0	0	0	0	0	0	1
	1.0	0	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	0	1.0	0	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
	0	0	1.0	0	1.0	0	0.9	0	0	0	0	0	0	0	0	0	0	4
	0	0	0	1.0	0	1.0	0	0	0	0	0	0	0	0	0	0	0	5
	1.0	0	0	0	1.0	0	0	0	0	0	0	0	0	0	0	0	0	6
	0	0	0	0.9	0	0	1.0	0	0	0	0	0	0	0	0	0	0	7
	0.6	0	0	0	0	0	0	0	2.0	2.0	0.9	0	0	0	0	0	0	8
	0	0	0	0	0	0	0	2.0	1.2	0	0	0	0	0	0	0	0	9
	0	0	0	0	0	0	0	2.0	0	1.2	0	0	0	0	0	0	0	10
	0	0	0	0	0	0	0	0.9	0	0	1.0	0.9	0	0	0	0	0	11
	0	0	0	0	0	0	0	0	0	0	0.9	0	1.0	0	0	0	1.0	12
	0	0	0	0	0	0	0	0	0	0	0	1.0	0.4	1.0	0	0	0	13
	0	0	0	0	0	0	0	0	0	0	0	0	1.0	0	1.0	0	0	14
	0	0	0	0	0	0	0	0	0	0	0	0	0	1.0	0	1.0	0	15
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.0	0	1.0	16
	0	0	0	0	0	0	0	0	0	0	0	1.0	0	0	0	1.0	0	17

[a] Numbering of rows and columns corresponds to the numbering of structure III.



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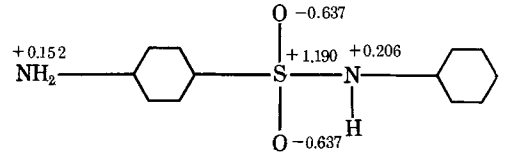

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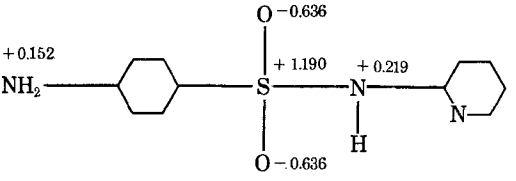

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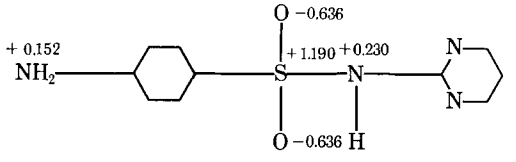
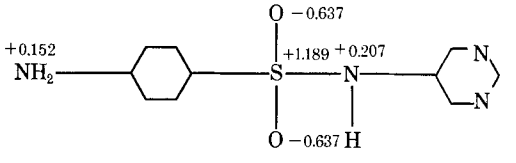


VII



VIII

respect to its original contribution of 2π electrons to the π electron pool.

Now looking at the derivative whose R group is a phenyl radical, N^1-phenylsulfanilamide (sulfa No. 7) (V), the results change accordingly. In this case, the electronegative phenyl group as well as the SO_2 group oxygen atoms withdraw electrons from the sulfanilamide system. A much greater deficiency of π charge results at the N^1 nitrogen atom. The phenyl group has also reduced the charge on the oxygen atoms, further indicating its effect as an electron acceptor.

An even greater deficiency of π charge results at the N^1 nitrogen atom where the R group is pyridine with the pyridine nitrogen at the *ortho* position in sulfapyridine (sulfa No. *15*) (VI). The greater electronegativity of this R group is thereby accounted for. A still greater change occurs for sulfadiazine (sulfa No. *23*) (VII). The excess π charge at the oxygen atoms reacts accordingly when these atoms as well as the electronegative R group compete for the available delocalized electrons. For the compound 5-sulfanilamidopyrimidine (sulfa No. *28*) (VIII), the electron attracting effect of the R group is less than that for sulfadiazine, apparently because the electronegative nitrogen atoms in this substituent group are further removed from the point of attachment at the N^1 nitrogen.

Table V also shows that for all of the sulfonamide derivatives studied, the electronic charge at the N^4 nitrogen atom is constant, $q_{N^4} = 1.848$ (formal π charge $= +0.152$). Although this group seems to be necessary for biological activity, it appears that no correlation exists between the degree of activity and the electronic charge density calculated by the HMO method for this position of the compounds studied. This seems to agree with Bell and Roblin's finding of constant basic dissociation; however, it is possible that differences in the charges at the N^4 nitrogen atom of various sulfonamide derivatives would become evident if a more refined method of calculation were established.[2]

CORRELATION AND DISCUSSION OF RESULTS

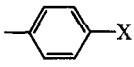
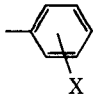
A plot of pKa *versus* the electronic charge at the ionizing N^1 nitrogen atom (q_{N^1}) for the 50 sulfanilamide type compounds indicated considerable scatter. The correlation expected in this case would be a linear relationship between pKa and q_{N^1}. The scatter was due, at least in part, to the effect of solvation energy which was not included in the calculations. More important, however, is the fact that the R groups of the 50 sulfonamide derivatives are of widely varying types, such as alternate rings, nonalternate rings, rings with and without substituent groups, and straight chains with and without heteroatoms. The correlation of pKa and electronic charge depends upon whether certain partition functions cancel (3, 19, 20). If this is not the case, the relationship may be much less direct, especially in the case of changing molecular skeletons. Also, the Hückel approximation does not provide equally good results in all of these cases. It is known, for instance, that the simple Hückel treatment gives a better account of alternate than nonalternate hydrocarbons because in the latter case the presence of a nonself-consistent field really requires that the Coulomb integral of each atom be corrected for the charges on neighboring atoms (3, 4). Also, the treatment of straight chains requiring the use of hyperconjugation is often less reliable. The R groups of those compounds which were poorly accounted for by the simple Hückel method were all of the nonalternate or straight chain types (types *D*, *E*, and *F* as defined below), and hence, less reliable results were to be expected in these cases.

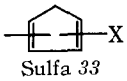
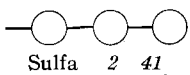
In order to examine the relationship between pKa and electronic charge in more detail, the 50 sulfonamide derivatives were classified into groups dependent on the type of substituent R group attached to the N^1 nitrogen atom. The classes are given in Table VI. Following this classification, individual plots were constructed for each type of R group. The results are shown in Figs. 1–3, for the *A*, *B*, and *C* type substituents. Accordingly, the categorization by the type of R group seems to be an important factor in explaining the correlation of

the pKa–electronic charge relationship. The results illustrate that if one anticipates using the electronic charge at the ionizing atom of sulfanilamide type compounds (or similarly complex drug molecules) for theoretically predicting pKa, it will probably be necessary to employ a more exact treatment in the cases of nonalternate R groups and hyperconjugation than the simple Hückel approximation. Procedures for the treatment of hyperconjugation and nonalternate molecules are available (5, 30, 31); however, the problems involved become increasingly difficult with the number and types of heteroatoms.

A knowledge of the electronic characteristics of a molecule may also allow prediction of drug activity. This would be possible even for compounds which have never been synthesized, once a basic relationship has been established by pharmacological testing of a representative sample of compounds. Table VII shows the relationship between sulfonamide activity and the charge $q_N{}^1$. The trend exhibited by the data indicates that, in general, a decrease in bacteriostatic activity may be associated with a decrease in the formal π charge at

TABLE VI—CLASSIFICATION OF SULFONAMIDE DERIVATIVES ACCORDING TO TYPE OF R GROUP

Type A[a]	Type B	Type C
		
Sulfa *7*	Sulfa *10*	Sulfa *8*
15	*12*	*9*
16	*13*	*11*
22	*17*	*24*
23	*18*	*25*
27	*19*	*26*
28	*20*	
30	*29*	

Type D	Type E	Type F
		
Sulfa *14*	Sulfa *33*	Sulfa *2* *41*
21	*35*	*3* *42*[b]
31	*36*	*4*[b] *43*[b]
32	*37*	*5* *44*
34	*39*	*6* *45*
38		*40* *48*

Compd. Not Classified
Sulfa *1* (PABA)
46[c]
47[c]
49
50[b]

[a] The ring may contain heteroatoms. This note applies to B, C, D, and E also. [b] No pKa available. [c] These compounds may not be permitted to form the theorized resonance structure, and therefore, may require a slightly different MO treatment.

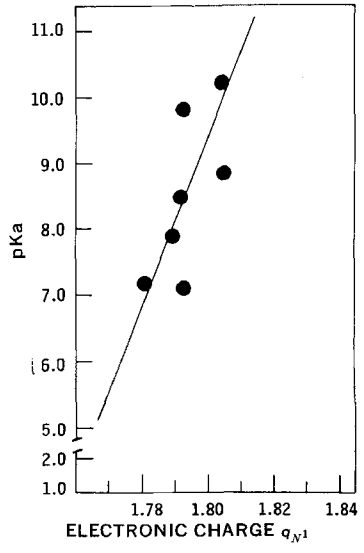


Fig. 2—pKa-electronic charge relationship for the type B sulfonamide derivatives.

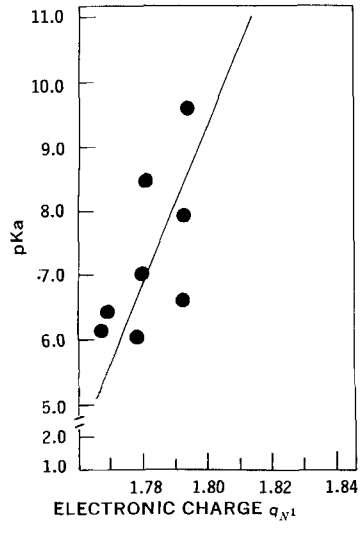


Fig. 1—pKa-electronic charge relationship for the type A sulfonamide derivatives.

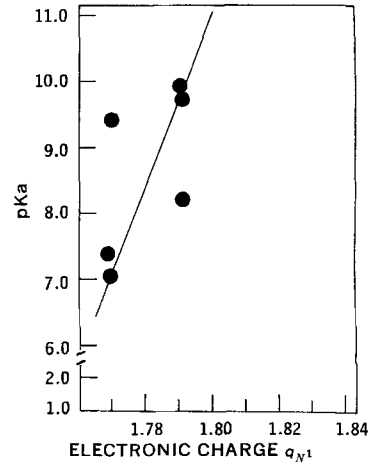


Fig. 3—pKa-electronic charge relationship for the type C sulfonamide derivatives.

TABLE VII—RELATIONSHIP BETWEEN BIOLOGICAL ACTIVITY AND ELECTRONIC CHARGE

Sulfa	Activity Index ($C_R \times 10^5)^{-1}$	Decreasing Activity	Formal π Charge $f_N{}^1$	Decreasing Charge
23	12.5		(+) 0.230	
30	12.5		0.221	
22	12.5		0.220	
27	20.0		0.232	
29	10.0		0.203	
16	5.0		0.207	
28	5.0		0.207	
18	5.0		0.206	
17	2.0		0.219	
12	2.0		0.210	
15	1.67		0.219	
19	1.67		0.207	
11	0.50		0.208	
20	0.50		0.195	
7	0.33		0.206	
9	0.20		0.208	
10	0.20		0.207	
13	0.20		0.195	

N^1 (or an increase in $q_N{}^1$). A better correlation of these parameters is probably dependent upon a more complete calculation procedure, including the use of partition coefficients as substituent constants (32, 33) in order to account for the polar characteristics of the molecules. Further work is being performed to correlate the electronic characteristics of the sulfonamide molecules with biological activity.

REFERENCES

(1) Pullman, A., and Pullman, B., "Les Theories Electroniques de la Chimie Organique," Masson et Cie., Paris, France, 1952.
(2) Szent-Györgyi, A., "Introduction to Submolecular Biology," Academic Press Inc., New York, N. Y., 1960.
(3) Daudel, R., Lefebvre, R., and Moser, C., "Quantum Chemistry," Interscience Publishers, Inc., New York, N. Y., 1959.
(4) Pullman, A., and Pullman, B., "Quantum Biochemistry," John Wiley & Sons, Inc., New York, N. Y., 1963.
(5) Streitwieser, A., Jr., "Molecular Orbital Theory for Organic Chemists," John Wiley & Sons, Inc., New York, N. Y., 1961.
(6) Roberts, J. D., "Notes on Molecular Orbital Calculations," W. A. Benjamin Co., New York, N. Y., 1961.
(7) DeHeer, J., *J. Am. Chem. Soc.*, **76,** 4802(1954).
(8) Bell, P. H., and Roblin, R. O., *ibid.*, **64,** 2905(1942).
(9) Kumler, W. D., and Daniels, T. C., *ibid.*, **65,** 2190 (1943).
(10) Kumler, W. D., and Eiler, J. J., *ibid.*, **65,** 2355 (1943).
(11) Kumler, W. D., and Halverstadt, I. F., *ibid.*, **63,** 624(1941).
(12) Klotz, I. M., *ibid.*, **66,** 459(1944).
(13) Bordwell, F. G., and Klotz, I. M., *ibid.*, **66,** 660 (1944).
(14) Seydel, J., Krüger-Thiemer, E., and Wempe, E., *Z. Naturforsch.*, **15b,** 628(1960).
(15) Woods, D. D., *Brit. J. Exptl. Pathol.*, **21,** 74(1940).
(16) Fildes, P., *Lancet*, **238,** 955(1940).
(17) Northey, E. H., "The Sulfonamides and Allied Compounds," Reinhold Publishing Corp., New York, N. Y., 1948, pp. 512–514.
(18) Daudel, R., *Compt. Rend.*, **227,** 1241(1948).
(19) Daudel, R., and Chalvet, O., *J. Chim. Phys.*, **46,** 332 (1949).
(20) Chalvet, O., Daudel, R., Pages, M., Roux, M., Buu-Hoi, N. P., and Royer, R., *ibid.*, **51,** 548(1954).
(21) Longuet-Higgins, H. C., *J. Chem. Phys.*, **18,** 275 (1950).
(22) Nakajima, T., and Pullman, B., *Bull. Soc. Chim. France*, **1958,** 1502.
(23) Pullman, A., and Pullman, B., *Biochim. Biophys. Acta*, **36,** 343(1959).
(24) Pullman, A., and Pullman, B., *Rev. Mod. Phys.*, **32,** 428(1960).
(25) Dasgupta, M. M., and Basu, S., *J. Chem. Phys.*, **58,** 186(1961).
(26) White, P. A., *J. Soc. Ind. Appl. Math.*, **6,** 393(1958).
(27) Lewis, N. J., "Eigenvalues of Real Symmetric Matrices on the 1620 Data Processing System," File No. 5.0.003, IBM SPS-1620, IBM 1620 Program Library, New York, N. Y., 1961.
(28) Garbow, B. S., "Eigenvalues and Eigenvectors of a Real Symmetric Matrix," F2 AN F202, IBM SHARE No. 664, IBM 704 SHARE Program Library, New York, N. Y., 1959.
(29) Bellman, R., "Introduction to Matrix Analysis," McGraw-Hill Book Co., New York, N. Y., 1960.
(30) Streitwieser, A., Jr., and Nair, P. M., *Tetrahedron*, **5,** 149(1959).
(31) Wheland, G. W., and Mann, D. E., *J. Chem. Phys.*, **17,** 264(1949).
(32) Iwasa, J., Fugita, T., and Hansch, C., *J. Med. Chem.*, **8,** 150(1965).
(33) Fugita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86,** 5175(1964).
(34) Kaiser, E. T., Urberg, M. M., and Eargle, D. H., Jr., *ibid.*, **88,** 1037(1966).

Hemolysis of Erythrocytes by Antibacterial Preservatives IV

Hemolytic Activity of Chlorhexidine Diacetate

By HOWARD C. ANSEL

Chlorhexidine diacetate has been shown, *in vitro*, to be destructive to rabbit erythrocytes in a range of concentrations similar to that required for its antimicrobial effect, with total hemolysis and bacteriolysis occurring at similar dosage levels. It appears that the mode of action of chlorhexidine diacetate against microorganisms and erythrocytes is similar; that is, through the disruption of the structure and function of the cellular membrane. The hemolytic activity of chlorhexidine diacetate was reduced by the presence of polyethylene glycols of various molecular weights and by dimethylsulfoxide. This interference in the activity of chlorhexidine diacetate was considered from two possible modes: (*a*) chemical interaction between the chlorhexidine diacetate and the added substances, and (*b*) cellular effects of the added substances and chlorhexidine diacetate, making the hemolytic activity of the latter less pronounced. The latter mode of interference by the added substances is more strongly suspected.

CHLORHEXIDINE is a potent antibacterial compound, the properties of which were first described in 1954 (1). Since that time, investigations conducted mainly in Great Britain have been directed at determining the mode of action of chlorhexidine and its efficacy as a preservative agent particularly for ophthalmic preparations (2–10).

The present investigation represents a continuation of studies (11–14) to determine the hemolytic activity of antibacterial preservatives alone and in the presence of pharmaceutical adjuncts. Thus far, the work in the series has demonstrated a similarity between the drug concentrations required of certain preservatives to act effectively as antibacterial and hemolytic agents. Also, certain pharmaceutical adjuncts as the polyethylene glycols and dimethylsulfoxide (DMSO) have been shown to interfere with the hemolytic activity of certain preservatives. The information gathered in this series is expected to contribute both to the understanding of the cellular effects of preservative agents and to the effective preservation of pharmaceutical products, including those intended for intravenous administration. Chlorhexidine was selected for inclusion in the over-all study principally because its chemical structure, 1,6-di(4-chlorophenyldiguanido)hexane, is sufficiently diverse from those antibacterial agents already examined for hemolytic activity. Also, the bacteriological data for chlorhexidine, as reported in the current literature, provided a ready means for the comparison and possible correlation between the effects of the compound on microorganisms and on red blood cells.

Received December 19, 1966, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication February 9, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by the office of General Research, University of Georgia, Athens.

Previous paper: Ansel, H. C., and Leake, W. F., *J. Pharm. Sci.*, **55**, 685(1966).

EXPERIMENTAL

Materials—The chlorhexidine diacetate employed in this investigation was pharmaceutical grade obtained through the courtesy of Ayerst Laboratories, Inc., New York, N. Y., and the dimethylsulfoxide was reagent grade obtained commercially.

Blood Samples—Rabbit blood, obtained by heart puncture and defibrinated in the manner previously described (15), was used throughout the study. Washed erythrocytes, prepared as reported in an earlier communication (14), were employed in certain experiments to reveal the influence of serum proteins on the hemolytic activity of chlorhexidine diacetate.

Each blood sample was collected just prior to its use and was verified for osmotic normalcy (11) during the course of each experiment.

Quantitative Determination of Per Cent Hemolysis—The colorimetric method employed for the determination of the degree of hemolysis occurring in each test solution has recently been described (11). In brief, it involved the addition of 0.05 ml. of defibrinated blood to duplicate pairs of colorimeter tubes, each containing 5 ml. of test solution. The test mixtures were incubated in a water bath for 45 min. at 37° after which the unhemolyzed cells were settled by centrifugation and the absorbance readings of the hemolysate determined with a Klett-Summerson photoelectric colorimeter. Each absorbance reading was compared with a total hemolysis reading obtained by laking red cells in distilled water. The degree of hemolysis occurring in each test solution was calculated as a per cent of total hemolysis. The data reported represent the average of a minimum of two like experiments. During and after the 45-min. incubation period each test mixture was macroscopically observed for color changes, precipitation, and other signs of denaturation.

Experimental Solutions—Aqueous solutions were employed throughout the investigation. Chlorhexi-

dine diacetate test solutions of varying concentration were prepared in 0.6% sodium chloride. The sodium chloride afforded the erythrocytes protection against osmotic hemolysis, and therefore any hemolysis occurring in the test solutions could be attributed to the cytotoxic effects of the preservative agent (11). The data thus obtained established the minimum concentrations of chlorhexidine diacetate required to induce trace and total hemolysis of rabbit erythrocytes. The latter concentration, producing the optimum effect, was subsequently employed in experiments designed to determine the influence of polyethylene glycols of various molecular weights and of DMSO on the hemolytic activity of chlorhexidine diacetate.

Test solutions were prepared to contain the optimum hemolytic concentration of chlorhexidine diacetate in 0.6% sodium chloride and varying concentrations of polyethylene glycols 400, 1540, and 4000, or dimethylsulfoxide. The ability of the polyethylene glycols or of DMSO to interfere with the hemolytic activity of chlorhexidine diacetate would be reflected by decreased hemolytic response. The test solutions were equilibrated overnight at room temperature prior to conductance of hemolysis experiments at 37°. Appropriate control solutions were employed concurrently to affirm the effects attributed to the presence of the polyethylene glycols and DMSO.

The concentration of chlorhexidine diacetate in the various test solutions is expressed in terms of mcg./ml., in keeping with the precedent established by other investigators of the properties and activities of this preservative agent. In doing so, a direct comparison was facilitated between the data obtained in this study employing erythrocytes and that of previous studies employing microorganisms.

Kinetic Studies—The general experimental design of the research series is such that the hemolytic effects of the preservative agents and the influences of the pharmaceutical adjuncts are determined after a constant 45-min. incubation period. In the current project the hemolytic activities of certain representative test solutions were also determined at incubation intervals of from 5 to 20 min., during periods up to 120 min.

RESULTS AND DISCUSSION

Hemolytic Activity of Chlorhexidine Diacetate—Chlorhexidine diacetate at concentrations as low as 20 mcg./ml. induced trace hemolysis of both washed and unwashed rabbit erythrocytes (Fig. 1). As the concentrations of the test solutions were increased, hemolysis increased and at a concentration of 110 mcg./ml., total hemolysis first occurred. As can be seen in Fig. 1, the washed cells appeared slightly more sensitive to the lytic effects of chlorhexidine diacetate than did the unwashed cells. The absence of serum proteins and the mechanical effects of the repeated washings during the preparation of the washed cell samples could account for the increased cellular fragility and sensitivity to chlorhexidine diacetate.

It is interesting to note that in various studies (4, 5, 10) involving the application of chlorhexidine to the preservation of ophthalmic solutions, the successful concentrations (depending on such conditions of the experiment as: cell count, micro-

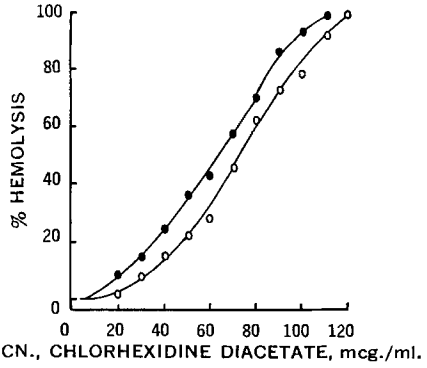


Fig. 1—Hemolytic activity of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: ○, using unwashed erythrocytes; ●, using washed erythrocytes.

organism tested, temperature, and duration of incubation) ranged between 20 and 100 mcg./ml., a range which is nearly identical to that determined in the present investigation for trace to total destruction of erythrocytes. In practice, the most popular concentration of chlorhexidine employed as a preservative in ophthalmic solutions is reported to be 50 mcg./ml. (10).

Hugo and Longworth (2, 4) have investigated the mode of action of chlorhexidine diacetate against *Escherichia coli* and *Staphylococcus aureus*. They found chlorhexidine to be rapidly adsorbed by the cells. This adsorption was accompanied by cytological changes involving the permeability of the cells and their optical properties. The two microorganisms responded similarly to the effects of various dosage levels of chlorhexidine diacetate. At concentrations of 20 mcg./ml., the cells showed little damage to biochemical properties as well as an unchanged appearance as revealed by electron microscopy. At concentrations of 90 mcg./ml., chlorhexidine caused cytological damage resulting in widespread cellular leakage and in the formation of cellular ghosts devoid of cytoplasmic constituents. At concentrations of 200 and 500 mcg./ml., which greatly exceeded that required for cellular damage, the cells failed to exude their cytoplasmic constituents; however, compared to the controls, the cytoplasm appeared granular to electron microscopy and the cellular membranes appeared altered. They concluded from their studies that chlorhexidine exerts its bactericidal action by combining with the cell surface in a manner which lethally affects its structure and function thereby causing a disruption in its permeability barriers and over-all integrity. The release of cellular constituents by the bacterial cells was related to the level of drug adsorption. However, cellular release was not necessarily the sign of cellular death. This was demonstrated with highly bactericidal concentrations of chlorhexidine diacetate which as a consequence of their high concentration failed to induce bacteriolysis.

In the present study in which erythrocytes rather than microorganisms were exposed to various concentrations of chlorhexidine diacetate a similar pattern of cell response was noted (Fig. 1) with one exception. At high (500 mcg./ml.) chlorhexidine diacetate concentrations, which affected the bacterial cell membrane without inducing bacteriolysis, the

erythrocytes hemolyzed completely as they had at drug levels as low as 110 mcg./ml. In general, however, the red blood cells behaved similarly to the microorganisms, an observation which had been made before in this laboratory for other preservative agents (11–14). It is not unlikely that the mode of action suggested for chlorhexidine diacetate against microorganisms is similar against erythrocytes, that is, the disruption and disorganization of the structure and function of the cell membrane.

Kinetic Study of Chlorhexidine Diacetate—As depicted in Fig. 2, the hemolytic effects of chlorhexidine diacetate are manifest after a lag time apparently required for drug adsorption and consequent disruption of the permeability barriers of the cell. Gross cytolytic damage supported by cellular leakage proceeds rapidly, providing that the appropriate level of drug is present. In their observations on the effects of chlorhexidine diacetate on *E. coli*, Hugo and Longworth (4) concluded that of the two parmeters, time and concentration, the latter was the more important with respect to induced cytological damage.

Influence of Polyethylene Glycols and DMSO on the Hemolytic Activity of Chlorhexidine Diacetate—As noted in previous communications (12, 14), the presence of pharmaceutical adjuncts including the polyethylene glycols and dimethylsulfoxide interfere with the hemolytic activities of certain preservative agents suggesting like interference with preservative function.

The hemolytic activities of solutions containing 110 mcg./ml. of chlorhexidine diacetate, an amount sufficient to induce total hemolysis of erythrocytes (Fig. 1), in 0.6% sodium chloride and varying amounts of polyethylene glycols 400, 1540, and 4000 were determined along with the appropriate control solutions. As can be seen in Fig. 3, the presence of polyethylene glycols in increasing amounts and in increasing molecular weights was increasingly effective in preventing chlorhexidine-induced hemolysis. A similar influence of the polyethylene glycols in preventing hemolysis induced by antimicrobial preservatives was noted for phenolic compounds and was reported in an earlier communication (12). In that study a chemical interaction between the phenolic compound and the various polyethylene glycols was demonstrated titrimetrically and verified through the literature. In the current study, the possibility of a chemical interaction between the polyethylene glycols and the chlorhexidine diacetate, thereby rendering the preservative agent ineffective in its hemolytic capability, should be considered especially in light of the incompatible nature of both the polyethylene glycols (12) and chlorhexidine (5, 10) with other chemical materials. No visible signs of incompatibility were seen during the study. A second possibility for the protective action of the polyethylene glycols is a cellular effect of this chemical which interferes in some manner with the adsorption of chlorhexidine diacetate onto the erythrocytic membrane. This interference will be explored to a greater extent in a subsequent presentation.

The controversial chemical dimethylsulfoxide has been the subject of conflicting reports concerning its ability to act as a penetrant carrier of drugs through biologic membranes (16–20). Although DMSO freely penetrates the erythrocytic membrane

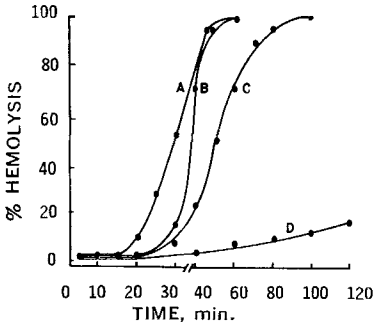


Fig. 2—Kinetic study of the hemolytic activity of various concentrations of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: A, *110 mcg./ml.;* B, *90 mcg./ml.;* C, *70 mcg./ml.;* D, *20 mcg./ml.*

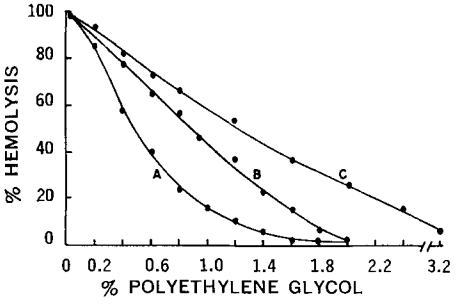


Fig. 3—Influence of PEG's on the hemolytic activity of 110 mcg./ml. of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: A, *PEG 4000;* B, *PEG 1540;* C, *PEG 400.*

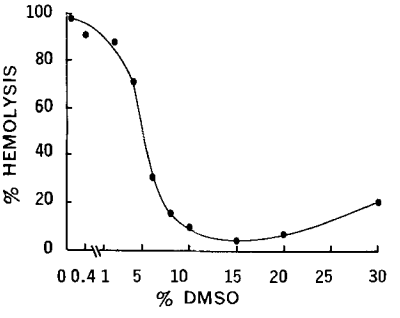


Fig. 4—Influence of DMSO on the hemolytic activity of 110 mcg./ml. of chlorhexidine diacetate in the presence of 0.6% NaCl.

and, in high concentrations, is destructive to red blood cells, it was shown (14) to greatly reduce, not enhance, the phenol-induced hemolysis of both washed and unwashed erythrocytes. It was suggested that this interference may be manifest through chemical interaction with the phenol or through a direct cellular effect. The present experiment involving the combination of DMSO and chlorhexidine diacetate was an outgrowth of the prior study. As can be seen in Fig. 4, the presence of DMSO reduced the hemolytic capability of an otherwise hemolytic concentration of chlorhexidine diacetate (110 mcg./ml.). All test solutions contain-

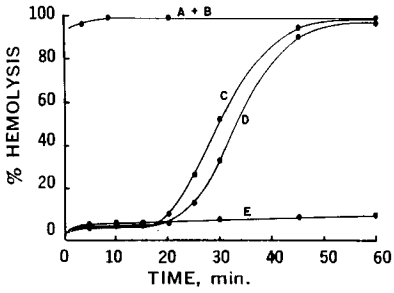


Fig. 5—Kinetic study of the hemolytic activity of various test solutions. Key: A, water; B, 15% DMSO; C, 110 mcg./ml. chlorhexidine diacetate in 0.6% NaCl; D, 110 mcg./ml. chlorhexidine diacetate, 0.6% NaCl, and 1% DMSO; E, 110 mcg./ml. chlorhexidine diacetate, 0.6% NaCl, and 15% DMSO.

ing concentrations of DMSO exceeding 35% caused the denaturation of blood, as noted in an earlier report (14).

As can be seen in Fig. 5, both distilled water and 15% DMSO induced total hemolysis within the first 5 min. of the study. Hemolysis occurring in the 15% DMSO test solution can be largely attributed to osmotic hemolysis resulting from the penetration of this hygroscopic material through the erythrocytic membrane (14). The addition of 0.6% sodium chloride to DMSO solutions of less than 35% concentration prevents osmotic hemolysis (14); hemolysis occurs in solutions containing greater amounts of DMSO due to the cytotoxic action of the more concentrated solutions. The addition of 1% DMSO to a solution containing 0.6% sodium chloride and 110 mcg./mg. of chlorhexidine diacetate slowed the rate of hemolysis induced by the latter and the addition of 15% DMSO all but prevented the chlorhexidine diacetate-induced hemolysis. The suspicion that DMSO exerts an independent cellular effect such as to prevent the preservative from exerting its own cellular activity has been bolstered by recent data obtained in this laboratory (21) showing a similar influence of DMSO on the hemolytic activities of such chemically diverse preservatives as phenylethyl alcohol, benzalkonium chloride, *p*-chlorophenol, benzyl alcohol, phenylmercuric acetate, and *m*-cresol. In each instance, the concentration of DMSO required to all but eliminate the hemolysis normally induced by the hemolytic concentrations of these preservatives was between 7 and 15%. Spectrophotometric analysis failed to reveal complexation between the DMSO and the chlorhexidine diacetate, further adding support to interference by DMSO by a cellular mechanism.

REFERENCES

(1) Davies, G. E., Francis, J., Martin, A. R., Rose, F. L., and Swain, G., *Brit. J. Pharmacol.*, **9**, 192(1954).
(2) Hugo, W. B., and Longworth, A. R., *J. Pharm. Pharmacol.*, **16**, 655(1964).
(3) *Ibid.*, **16**, 751(1964).
(4) *Ibid.*, **17**, 28(1965).
(5) Hugo, W. B., and Foster, J. H. S., *ibid.*, **16**, Suppl. 124T(1964).
(6) Brown, M. R. W., and Richards, R. M. E., *ibid.*, **16**, Suppl. 41T(1964).
(7) *Ibid.*, **16**, Suppl. 51T(1964).
(8) Wiseman, D., *ibid.*, **16**, Suppl. 56T(1964).
(9) Rye, R. M., and Wiseman, D., *ibid.*, **17**, 295(1965).
(10) Brown, M. R. W., and Norton, D. A., *J. Soc. Cosmetic Chemists*, **16**, 369(1965).
(11) Ansel, H. C., and Cadwallader, D. E., *J. Pharm. Sci.*, **53**, 169(1964).
(12) Ansel, H. C., *ibid.*, **54**, 1159(1965).
(13) Cadwallader, D. E., and Ansel, H. C., *ibid.*, **54**, 1010 (1965).
(14) Ansel, H. C., and Leake, W. F., *ibid.*, **55**, 685(1966).
(15) Grosicki, T. S., and Husa, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 632(1954).
(16) Herschler, R. J., unpublished data; through Jacob, S. W., Bischel, M., and Herschler, R. J., *Current Therap. Res.*, **6**, 193(1964).
(17) Jacob, S. W., Bischel, M., and Herschler, R. J., *Current Therap. Res.*, **6**, 193(1964).
(18) *Ibid.*, **6**, 134(1964).
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(20) Ben, M., *et al.*, *Pharmacologist*, **6**, 189(1964).
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Drug Standards

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By JOSEPH LEVINE and THOMAS D. DOYLE

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THE ISOLATION and determination of phenylephrine by the usual methods for the analysis of alkoids[1] is not feasible because of the highly unfavorable extraction characteristics of the compound. Most of the published procedures for its analysis are not applicable in the presence of the drugs with which it is compounded in many formulations. Pratt (2), applying the acetylation procedure of Welsh (3), converted phenylephrine in aqueous solution to the diacetyl derivative, which is readily extracted with im-

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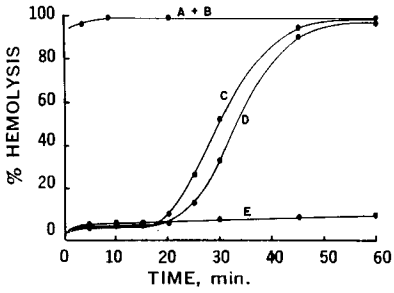

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REFERENCES

(1) Davies, G. E., Francis, J., Martin, A. R., Rose, F. L., and Swain, G., *Brit. J. Pharmacol.*, **9**, 192(1954).
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THE ISOLATION and determination of phenylephrine by the usual methods for the analysis of alkoids[1] is not feasible because of the highly unfavorable extraction characteristics of the compound. Most of the published procedures for its analysis are not applicable in the presence of the drugs with which it is compounded in many formulations. Pratt (2), applying the acetylation procedure of Welsh (3), converted phenylephrine in aqueous solution to the diacetyl derivative, which is readily extracted with im-

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miscible organic solvents. Clark and Rosenberg (4) and Hyatt (5) modified the acetylation procedure to permit its application to combinations containing various antihistamines. In these procedures, an alkaline solution of the sample is used as the immobile phase in a chromatographic column. A preliminary chloroform elution removes the antihistamines only. A chloroform solution of acetic anhydride passed over the column acetylates the phenylephrine *in situ*, and the resultant diacetyl compound is readily eluted. This procedure is inapplicable to samples containing acetaminophen, with which phenylephrine is frequently combined. Smith (6) separated phenylephrine from a number of other drugs by eluting them with chloroform from a partition chromatographic column in which the ratio of diatomaceous earth[2] to immobile phase is much higher than that usually employed; he then washed off the phenylephrine with ethanol. He reported the method to be unsuccessful for mixtures containing several accompanying drugs, including sodium benzoate and potassium guaiacolsulfonate. Hiskey and Levin (7) described a colorimetric procedure applicable directly to solutions without prior isolation of the phenylephrine. Reaction with 4-aminoantipyrine and potassium ferricyanide in sodium bicarbonate solution produces a red color, which can be measured directly. This method must be applied with great care, since the intensity of the color developed varies significantly with the concentration of each of the reagents, and both the intensity of the color and the wavelength of maximum absorbance change rapidly with time. Acetaminophen also produces a color with the reagent, while phenolphthalein is converted to its red salt; therefore, this method is inapplicable in the presence of these drug materials.

Phenylephrine may be extracted readily by converting it to an ion-pair with di-(2-ethylhexyl) phosphoric acid (DEHP).[3] Use of this reagent is based upon a report by Temple and Gillespie (8) that chloroform solutions of this "liquid ion-exchanger" will extract physiologically active amines from aqueous solutions at pH 4–8; in turn, the amines will be re-extracted by aqueous solutions at low pH levels. However, the procedure as described by Temple and Gillespie is not directly applicable to the determination of phenylephrine in pharmaceutical combinations, since other alkoids will be extracted together with that substance. Also, the unfavorable partition coefficient of ion-pairs formed with phenolic amines would require multiple extractions for complete recovery of phenylephrine.

Quantitative extraction of the phenylephrine–DEHP complex is readily achieved through the application of partition chromatography. A solution of the sample itself adjusted to a pH of 5–6 constitutes the immobile phase of the system. An ether solution of DEHP passed over the column elutes the phenylephrine; this is quantitatively extracted from the eluate with dilute sulfuric acid and determined spectrophotometrically. Because alkaline solutions of phenylephrine absorb more strongly and with a maximum at a longer wavelength than acid solutions, the extract is made alkaline prior to measurement.

This partition system is effective in the analysis of phenylephrine in combination with other drugs. A chloroform or ether wash of the column prior to the elution of phenylephrine with the DEHP solution will remove a variety of substances, including acetaminophen, sulfonamides, aspirin, phenolphthalein, glyceryl guaiacolate, dextromethorphan, dihydrocodeinone, and the antihistamines, as well as flavoring matter present in syrups.

Phenylpropanolamine is not completely removed by this treatment. This compound is separated from phenylephrine by passing the DEHP complex of both compounds through a sodium hydroxide column. This will trap only the phenylephrine, which will subsequently be released when the column is neutralized.

EXPERIMENTAL

Chromatographic Columns—Attach a 50-mm. length of 6 or 8-mm. tubing to a 25 × 250-mm. test tube. Use a tamping rod consisting of a disk of stainless steel, aluminum, or glass, of a diameter 1 mm. less than that of the column, attached to a rod 12 to 18 in. long. Pack fine glass wool[4] in the base of the column as support.

Buffers—*pH 5.8 Buffer*—Mix 1 vol. of 1 *M* dibasic potassium phosphate with 9 vol. of 1 *M* monobasic potassium phosphate. Adjust to pH 5.80 ± 0.05, using a pH meter.

pH 5.1 Buffer—Mix 2 vol. of 1 *M* dibasic potassium phosphate with 1 vol. of 1 *M* citric acid. Adjust to pH 5.10 ± 0.05.

Preparation of Samples—*Syrups*—For samples containing the usual concentration of 5 mg. of phenylephrine HCl per 5 ml., measure 10.0 ml. of pH 5.8 buffer into a 25-ml. volumetric flask. Add syrup to volume, being careful to avoid wetting the flask above the graduation mark.

Tablets—Weigh a ground sample containing about 2 mg. of phenylephrine into a 50-ml. beaker. If components of tablet are water-soluble, add 2 ml. of water, warm slightly to dissolve, and add 1 ml. of pH 5.8 buffer. If some components are not water-soluble (*e.g.*, acetaminophen), add 1 ml. of dimethyl-

[2] Marketed as Celite 545 by the Johns-Manville Corp., New York, N. Y.

[3] Listed in some catalogs as bis-2(ethylhexyl) phosphate.

[4] Pyrex Filtering Fibre, Corning Glass catalog No. 3950.

sulfoxide, warm to dissolve, and then add 2 ml. of pH 5.8 buffer. For tablets containing antacids such as magnesium and aluminum hydroxides, heat the powdered sample with 5 ml. of ethanol and 1 ml. of concentrated HCl to dissolve the alkaline material; add 10 ml. of *n*-butanol and evaporate to dryness.[5] Dissolve residue in 1 ml. of dimethylsulfoxide and add 2 ml. of pH 5.8 buffer.

Procedure

Use water-saturated solvents throughout.

A—*Samples Without Phenylpropanolamine*—Transfer mixture of 1 Gm. of acid-washed diatomaceous earth with 0.8 ml. of pH 5.1 buffer to column containing a pledget of glass wool, and tamp to a uniform mass. Mix 4 Gm. of diatomaceous earth with 3 ml. of syrup sample or with tablet preparation and transfer to column directly above pH 5.1 layer. Dry-wash beaker with 1 Gm. of diatomaceous earth, add to column, and tamp. Cover with wad of glass wool.

Pass 75 ml. of $CHCl_3$ over column, followed by 100 ml. of ether. Evaporate final 10 ml. of eluate separately; if residue remains, pass additional ether over column.

With a rubber bulb, draw DEHP[6] into a graduated 1-ml. pipet to a level estimated to provide a total of about 1.2 ml.[7] Transfer DEHP into 50 ml. of ether; then flush the pipet several times with the ether. Avoid contact with the reagent.

Place a separator containing about 20 ml. of approximately 0.1 *N* H_2SO_4, as a receiver under the column. Elute the column with the DEHP–ether solution, then follow with 25 ml. of ether. Shake the separator, and transfer aqueous layer to 50-ml. volumetric flask containing 4 ml. of 1 *N* NaOH. Re-extract ether with 15 ml. of 0.1 *N* H_2SO_4, transfer extract to the flask, and adjust to volume with water. (If sample contains magnesium hydroxide, gel will separate from this solution; remove by filtration or centrifugation.) Determine absorbance with a spectrophotometer at maximum at about 291 mμ, and compare with that of a standard solution containing about 2 mg. of phenylephrine HCl in 50 ml. of approximately 0.01 *N* NaOH.

B—*Samples Containing Phenylpropanolamine*—Prepare sample column, and wash with $CHCl_3$ and ether as under *A*. Mount a second column, prepared with 3 Gm. of diatomaceous earth and 2 ml. of 0.5 *N* NaOH, directly below sample column. Elute phenylpropanolamine with a solution of 0.1 ml. of DEHP in 50 ml. of ether, followed by 25 ml. of ether. Finally, elute the phenylephrine from the column train with 1.2 ml. of DEHP in ether exactly as described above.

RESULTS AND DISCUSSION

Standard quantities of phenylephrine, both alone and combined with other drugs, were analyzed by the procedure as described. Recoveries are presented in Table I. The analysis of phenylephrine in commercial samples of various combinations is presented in Table II. In several cases, analyses of selected accompanying materials were made as outlined below. The ultraviolet spectra of these were identical with those of the pure material, and the recoveries were in accord with the label claims. Since no standard recovery data were collected for these compounds, the results of the analyses are not included.

In accord with their distribution coefficients and dissociation constants, the majority of pharmaceutically important bases can be completely eluted from chromatographic columns at pH levels ranging down to about pH 3, using moderate volumes of ether or chloroform; the rate of elution increases rapidly with increase of pH up to the pKa value of the base. At pH 5.8 alkoids such as most of the antihistamines are readily removed; phenylpropanolamine is eluted slowly with chloroform and phenylephrine very slowly with ether at this pH level. The latter two compounds are retained on the pH 5.1 trap while the others pass through.

Phenylpropanolamine is separated from phenylephrine by passing the ether solution of their DEHP complexes over a diatomaceous earth–sodium hydroxide column. Phenylephrine is retained as its phenol salt while the phenylpropanolamine complex is eluted. For this separation, the quantity of DEHP used initially must be sufficient to remove the phenylpropanolamine completely while being insufficient to neutralize the entire NaOH column. This column must, in turn, be completely neutralized by the DEHP used to elute the phenylephrine. The proper balance is achieved with the concentrations specified under *Experimental*. It can be noted that phenylephrine can be quantitatively determined with procedure *A* even though the phenylephrine fraction is not free of phenylpropanolamine, since solutions of the latter

Table I—Standard Recovery of Phenylephrine Hydrochloride

Other Drugs Present	Phenylephrine HCl Taken, mg.	Recovery Found, mg.	Recovery %
...	4.38	4.43	101.0
...	2.37	2.39	100.6
Phenylpropanolamine HCl, 24.8 mg.	5.31	5.34	100.4
Phenylpropanolamine HCl, 25.8 mg.	5.83	5.74	98.4
Phenylpropanolamine HCl, 19.3 mg.	5.22	5.14	98.3
Phenylpropanolamine HCl, 9.8 mg.	2.05	2.07	101.0
Dextromethorphan HBr, 9.9 mg. Glyceryl guaiacolate, 37.3 mg. Acetaminophen, 65.8 mg.	2.39	2.40	100.4
Chlorpheniramine maleate, 12.5 mg. Phenyltoloxamine citrate, 150 mg.	2.14	2.15	100.4
Aspirin, 132.6 mg.	2.06	2.08	101.0
Aspirin, 82.8 mg. Phenolphthalein, 24.5 mg. Magnesium hydroxide, 220.5 mg.	2.39	2.38	99.6

[5] Butanol is used to effect azeotropic evaporation of water, thereby insuring complete removal of HCl.

[6] No differences were observed from using DEHP from two sources. One, with no grade indicated, was colorless; the other, labeled "Practical Grade," was quite yellow.

[7] One milliliter of DEHP was found to be a marginal amount in several instances.

TABLE II—ANALYSIS OF COMMERCIAL SAMPLES

Sample	Drugs Present		Phenylephrine HCl Found, mg.	Phenylephrine HCl % of Labeled Claim
Syrup	Phenylephrine HCl, 25 mg.		24.12	96.4
	Glyceryl guaiacolate, 250 mg.		24.05	96.2
	Pyrilamine maleate, 30 mg.			
	Methapyrilene fumarate, 30 mg./fl. oz.			
Syrup	Phenylephrine HCl, 5 mg.	Sample A	4.96	99.2
	Dextromethorphan HBr, 15 mg.			
	Chlorpheniramine maleate, 1 mg./5 ml.	Sample B	4.72	94.6
	Alcohol, 10%		4.78	95.6
Syrup	Phenylephrine HCl, 5 mg.		5.08	101.6
	Phenylpropanolamine HCl, 20 mg.		5.12	102.4
	Phenyltoloxamine citrate, 7.5 mg.			
	Chlorpheniramine maleate, 2.5 mg./5 ml.			
	Sodium benzoate, 0.1%			
Nasal soln.	Phenylephrine HCl, 2.5 mg.		2.60	104.0
	Sulfisoxazole, 40 mg./ml. (as diethanolamine salt)		2.58	103.2
Expectorant	Phenylephrine HCl, 5.0 mg.		4.86	97.2
	Brompheniramine maleate, 2.0 mg.		4.94	98.8
	Phenylpropanolamine HCl, 5.0 mg.			
	Glyceryl guaiacolate, 100 mg./5 ml.			
Tablets	Phenylephrine HCl, 10 mg.		10.45	104.5
	Dihydrocodeinone bitartrate, 5 mg.		10.62	106.2
	Chlorpheniramine maleate, 2 mg.			
	Acetaminophen, 250 mg.			
	Caffeine, 30 mg.			
	Homatropine methylbromide, 1.5 mg./tab.			
Tablets	Phenylephrine HCl, 5 mg./tab.		4.24	84.8
	Aspirin	Sample A	4.21	84.2
	Yellow phenolphthalein		4.24	84.8
	Magnesium hydroxide	Sample B	4.80	96.0
			4.86	97.2
Tablets	Phenylephrine HCl, 5 mg.		4.84	96.8
	Phenindamine tartrate, 10 mg./tab.		4.82	96.4
	Aspirin		4.88	97.6
	Caffeine			
	Aluminum–magnesium hydroxide gel			

do not absorb at 291 mμ, the wavelength at which phenylephrine is measured.

With minor modification, the procedure as described can be adapted to the concurrent determination of many of the drugs which accompany phenylephrine in commercial preparations. Phenylpropanolamine, for example, can be extracted from the initial DEHP eluate with sulfuric acid in the same manner as phenylephrine. To compensate for the low absorbance of phenylpropanolamine, smaller volumes of acid may be used for the extraction. To determine acetaminophen, the initial chloroform and ether eluates from the sample column are passed over a diatomaceous earth–1 *N* NaOH column, which will retain the acetaminophen while alkoids, together with flavoring material, pass through. The acetaminophen is eluted after acidification of the column *in situ* with acetic acid, as previously described (9). For syrups containing dextromethorphan, a diatomaceous earth–2 *N* HCl column is placed in series with the sample column, and 100 ml. of ether is passed over the columns. This will elute the greater part of the dextromethorphan, together with flavoring matter, from the sample column; the dextromethorphan alone will be retained on the HCl column. Then 75 ml. of chloroform is passed over both columns to elute the total quantity of dextromethorphan. The eluate can be received directly in a volumetric flask containing a small amount of acidified methanol (1) and its absorbance measured directly. Chlorpheniramine, if present in this same combination, will be retained on the HCl column, and it can be eluted with ammoniacal chloroform by the procedure described for other alkoids (10). Finally, the phenylephrine is eluted with DEHP as above.

REFERENCES

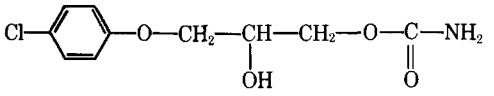
(1) Levine, J., *J. Pharm. Sci.*, **54**, 485(1965).
(2) Pratt, E. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 505 (1957).
(3) Welsh, L. H., *ibid.*, **44**, 507(1955).
(4) Clark, W. R., and Rosenberg, L. A., *J. Assoc. Offic. Agr. Chemists*, **48**, 579(1965).
(5) Hyatt, R., *ibid.*, **48**, 594(1965).
(6) Smith, D. J., *J. Assoc. Offic. Anal. Chemists*, **49**, 536 (1966).
(7) Hiskey, C. F., and Levin, N., *J. Pharm. Sci.*, **50**, 393(1961).
(8) Temple, D. M., and Gillespie, R., *Nature*, **209**, 714 (1966).
(9) Levine, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 687(1957).
(10) Levine, J., and Roe, J. E., *J. Assoc. Offic. Agr. Chemists*, **42**, 693(1959).

Qualitative and Quantitative Tests for Chlorphenesin Carbamate

By EDWARD F. SALIM* and ROGER E. BOOTH†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

3-(P-CHLOROPHENOXY)-2-HYDROXYPROPYL CARBAMATE; $C_{10}H_{12}ClNO_4$; mol. wt. 245.67. The structural formula of chlorophenesin carbamate may be represented as:



Physical Properties—Chlorphenesin carbamate occurs as a white to off-white, odorless powder, m.p. 86–92° (U.S.P. class Ia). It is very slightly soluble in water, freely soluble in alcohol, and sparingly soluble in chloroform.

Identity Tests—Mix 500 mg. of chlorphenesin carbamate with 1 ml. of acetic anhydride, add 1 drop of sulfuric acid, stir until solution is effected, and allow the solution to stand at room temperature for 30 min., stirring occasionally. Pour the solution into 50 ml. of water while stirring the mixture vigorously, and allow to crystallize. Filter off the crystals, wash with water until the odor of acetic acid is no longer perceptible, and dry at about 60°: the acetyl chlorphenesin carbamate so obtained melts at about 133°.

A 1 in 100,000 solution of chlorphenesin carbamate in alcohol exhibits ultraviolet absorbance maxima at about 227 mμ [absorptivity (*a*) about 53], 280 mμ, and 288 mμ and absorbance minima at about 245 mμ and 284 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a chlorphenesin carbamate dispersion in liquid petrolatum is shown in Fig. 2.

Purity Tests—Dry about 2 Gm. of chlorphenesin carbamate, accurately weighed, over silica gel and at a pressure not exceeding 5 mm. of mercury for 6 hr.: it loses not more than 0.5% of its weight.

Char about 1 Gm. of chlorphenesin carbamate, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.5%.

Received October 28, 1966, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION, Washington, DC 20037

Accepted for publication December 19, 1966.

† The Upjohn Co., Kalamazoo, MI 49002. The Upjohn Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The Drug Standards Laboratory gratefully acknowledges the assistance of Miss Carolyn Damon and Miss Hannah Klein.

Weigh accurately about 600 mg. of chlorphenesin carbamate and transfer to a Kjeldahl flask with 20 ml. of sulfuric acid, 200 mg. of selenium, and 15 Gm. of anhydrous sodium sulfate. After the mixture has become colorless, digest for 1 hr., cool, and dilute to about 300 ml. with water. Cool, make alkaline with sodium hydroxide solution (1 in 2), and distil the liberated ammonia into 40.0 ml. of 0.1 *N* sulfuric acid. Add methyl red T.S. and titrate the excess acid with 0.1 *N* sodium hydroxide. Each milliliter of 0.1 *N* sulfuric acid is equivalent to 1.401 mg. of nitrogen (N). The amount of nitrogen found is not less than 5.50% and not more than 5.90% calculated on the dried basis.

Using about 20 mg. of chlorphenesin carbamate, accurately weighed, proceed as directed under "Oxygen Flask Combustion," U.S.P. XVII, p. 884, using a mixture of 1 ml. of sodium hydroxide T.S., 5 ml. of hydrogen peroxide T.S., and 15 ml. of water as the absorbing liquid. After the combustion is complete, rinse the stopper and sample holder with water and boil the solution for about 5 min. Cool, acidify with diluted nitric acid adding 2 ml. in excess, add 10.0 ml. of 0.05 *N* silver nitrate, 2 ml. of nitrobenzene, and 2 ml. of ferric ammonium sulfate T.S. Shake well, and titrate the excess silver nitrate with 0.05 *N* ammonium thiocyanate. Each milliliter of 0.05 *N* silver nitrate is equivalent to 1.773 mg. of chlorine (Cl). The chlorine content is not less than 13.9% and not more than 14.9%, calculated on the dried basis.

Assay—Dissolve about 125 mg. of chlorphenesin carbamate, accurately weighed, in 25 ml. of chloroform in a 50-ml. volumetric flask, add the solvent to volume, and mix. Similarly, dissolve an accurately weighed quantity of chlorphenesin carbamate reference standard in chloroform to obtain a standard solution having a concentration of about 2.5 mg./ml. Concomitantly determine the absorbances of both solutions in 1.0-mm. cells at the wavelength of maximum absorbance at about 5.80 μ, with a suitable infrared spectrophotometer, using chloroform as the blank. Calculate the quantity, in milligrams, of $C_{10}H_{12}ClNO_4$ in the portion of chlorphenesin carbamate taken by the formula $50C \times (A_u/A_s)$, in which C is the concentration, in milligrams per milliliter, of chlorphenesin carbamate reference standard in the standard solution, and A_u and A_s are the absorbances of the solution of chlorphenesin carbamate and the standard solution, respectively. The amount of chlorphenesin carbamate found is

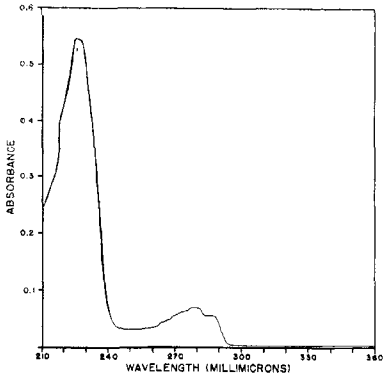


Fig. 1—Ultraviolet absorption spectrum of chlorphenesin carbamate in alcohol (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

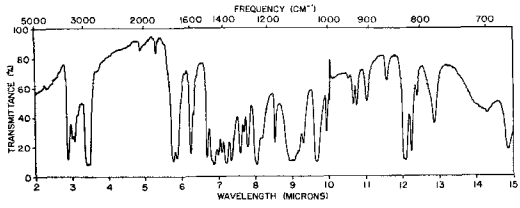


Fig. 2—Infrared spectrum of a chlorphenesin carbamate dispersion in liquid petrolatum; Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

not less than 97.0% and not more than 103.0%, calculated on the dried basis.

DOSAGE FORMS OF CHLORPHENESIN CARBAMATE

Chlorphenesin Carbamate Tablets

Identity Test—Triturate a quantity of finely powdered chlorphenesin carbamate tablets, equivalent to about 400 mg. of chlorphenesin carbamate, with 50 ml. of chloroform, and filter. Evaporate the clear filtrate on a steam bath to dryness: a liquid petrolatum dispersion of the residue exhibits infrared absorption maxima only at the same wavelengths as that of a similar preparation of chlorphenesin carbamate shown in Fig. 2.

Assay—*Standard Preparation*—Transfer 50.0 mg. of chlorphenesin carbamate reference standard to a glass-stoppered, 50-ml. conical flask and dissolve in 20.0 ml. of chloroform.

Assay Preparation—Weigh and finely powder not less than 10 chlorphenesin carbamate tablets. Weigh accurately a portion of the powder, equivalent to about 50 mg. of chlorphenesin carbamate, and transfer to a glass-stoppered, 50-ml. conical flask. Add 20.0 ml. of chloroform, stopper, and shake mechanically for 15 min. Filter a portion through paper into a glass-stoppered flask.

Procedure—Determine the absorbance of the *Assay Preparation* and that of the *Standard Preparation* in 1.0-mm. cells at the wavelength of maximum absorbance at about 5.80 μ, with a suitable infrared spectrophotometer, using chloroform as the blank. Calculate the quantity, in milligrams, of $C_{10}H_{12}ClNO_4$ in the portion of the tablets taken by the formula $50 \times (A_u/A_s)$, in which A_u is the absorbance of the *Assay Preparation* and A_s is the absorbance of the *Standard Preparation*. The amount of chlorphenesin carbamate found is not less than 92.5% and not more than 107.5% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Chlorphenesin carbamate[1] is an orally active skeletal muscle relaxant useful in the relief of discomfort associated with skeletal muscle trauma and inflammation.

Purity Tests—The synthesis of chlorphenesin carbamate involves the opening of a cyclic carbonate intermediate (1). An isomeric secondary carbamate is also formed in the reaction and constitutes a possible impurity in the final product. The limit of 3-(*p*-chlorophenoxy)-1-hydroxypropyl carbamate as a contaminant in chlorphenesin carbamate is controlled by quantitative nuclear magnetic resonance (NMR) spectroscopy (2) and should not exceed 1.5% of the weight of the bulk material.

Quantitative Methods—The nitrogen content of chlorphenesin carbamate as determined by the Kjeldahl procedure and the chlorine content by oxygen flask combustion gave average values of 5.61 ± 0.05%[2] and 14.2 ± 0.1%,[2] respectively.

Analyses for bulk chlorphenesin carbamate and the tablet formulation are based on infrared absorption properties of the carbonyl group in the molecule. Commercial tablets (400 mg.) gave an average recovery value of 99.7 ± 0.4%[2] of the labeled amount. Infrared analysis of the bulk drug yielded assay results of 100.2 ± 0.2%[2] which compares favorably to the recoveries (100.3 ± 0.3%[2]) obtained by the assay procedure reported for tybamate (3). A 500-mg. sample of chlorphenesin carbamate is subjected to sodium methoxide cleavage in a nonaqueous medium and the excess alkali determined by back titration with standard acid.

REFERENCES

(1) Collins, R. J., and Matthews, R. J., U.S. pat. 3,161,567 (December 15, 1964); through *Chem. Abstr.*, **62**, 5149(1965).
(2) Slomp, G., Baker, R. H., Jr., and MacKellar, F. A., *Anal. Chem.*, **36**, 375(1964).
(3) Salim, E. F., Bodin, J. I., Zimmerman, H. B., and Reisberg, P., *J. Pharm. Sci.*, **55**, 1439(1966).

[1] Marketed as Maolate by The Upjohn Co., Kalamazoo, Mich.
[2] Maximum deviation from the mean value.

Selective Colorimetric Determination of Microgram Amounts of Quinestrol and Ethinyl Estradiol in Dosage Forms

By DIMITRI C. TSILIFONIS and LESTER CHAFETZ

Ethinyl estradiol and its 3-cyclopentyl ether, quinestrol, react with a methanol–sulfuric acid reagent of critical composition to form a stable color with $\lambda_{max.}$ 540 mμ and ε 36,000. The chromogenic reaction appears to be specific for these estrogens and the related steroid, mestranol. Separations required utilize only simple solvent extraction, and the use of the nonpolar solvent, petroleum ether, affords additional selectivity in the assay of dosage forms. Application of the method for the assay of quinestrol and ethinyl estradiol in representative dosage forms is described. The assay has sensitivity, precision, and accuracy suitable for its use in content uniformity testing of dosage forms declaring 10 mcg. or more without resort to micro techniques.

A NEED FOR a facile and accurate means for assaying microgram amounts of quinestrol in the numerous formulations produced in the course of process evaluation, stability testing, and clinical studies led to the examination of procedures for isolating and quantitating the estrogen from individual dosage units. Work on the assay of quinestrol, the 3-cyclopentyl ether of ethinyl estradiol, led to a superior assay for ethinyl estradiol itself. The procedure appears equally applicable to assay of mestranol, the 3-methyl ether of ethinyl estradiol.

The Iron-Kober assay for ethinyl estradiol used in U.S.P. XVII (1) is equally applicable to quinestrol, but the procedure requires meticulous technique for satisfactory results, and it is insufficiently rapid and precise for the analysis of single tablets. The azo dye method for phenolic estrogens (2) is a general chromogenic reaction for phenols, inapplicable to quinestrol determination and lacking in sensitivity adequate for process control studies. Gas-liquid chromatographic assays for ethinyl estradiol and mestranol (3–7) provide both sensitivity and specificity, but the procedures suffer from excipient interference, and the precision reported (7) was unsatisfactory for our purposes. Thin-layer chromatography (3, 8) and paper chromatography (9) were less suitable from the standpoints of time, convenience, and accuracy than the colorimetric method reported here.

Treatment of many estrogens with concentrated sulfuric acid results in a red color with green fluorescence (10), a procedure used in U.S.P. as an identification reaction for ethinyl estradiol. Reaction of ethinyl estradiol or quinestrol with the ethanol–sulfuric acid reagent described by Heusghem and Jehotte (11) affords an intense red color with green fluorescence, but the color fades rapidly with detriment to the precision and accuracy of the method. Study of this reaction led to the development of a methanol–sulfuric acid chromogenic reagent of definite and critical proportions which provided a stable and intense color suitable for measurement of microgram amounts of the estrogens.[1] The reaction was found surprisingly specific for ethinyl estradiol and its 3-ethers among a number of representative estrogens, although noninterfering chromophores were produced with three other estrogenic steroids.

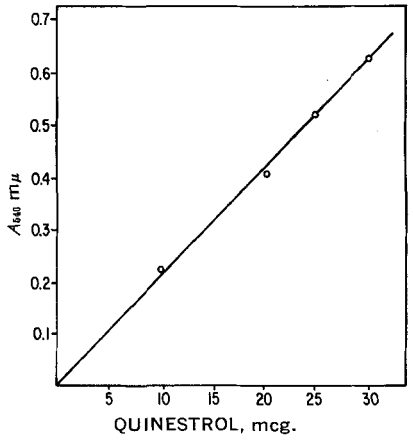


Fig. 1—Beer's law plot for the quinestrol chromophore.

EXPERIMENTAL

Equipment and Supplies—Absolute methanol and sulfuric acid were analytical reagent grade. The petroleum ether should give no color with concentrated sulfuric acid (Mallinckrodt's Nanograde was

Received October 28, 1966, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950
Accepted for publication February 16, 1967.
Presented at the Sixth Annual Conference on Pharmaceutical Analysis, Land O'Lakes, Wis., September 1966.

[1] Subsequent to presentation of this manuscript we learned that a similar method has been used for mestranol at Eli Lilly and Co. (12).

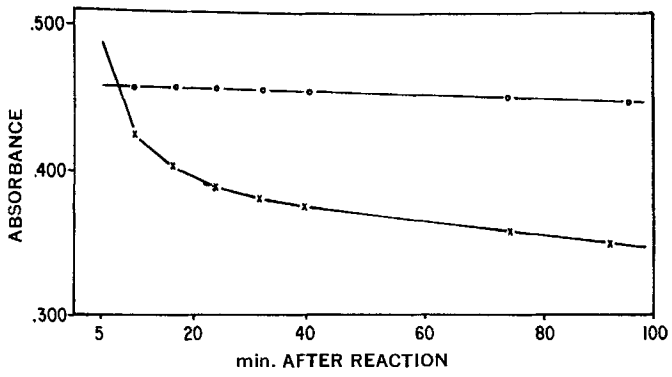


Fig. 2—Stability of color produced in the Heusghem and Jehotte and in this procedure for 20 mcg. of ethinyl estradiol. Key: ×, Heusghem and Jehotte; O, this procedure.

used). The separators used were fitted with Teflon stopcocks. A Beckman DU spectrophotometer and a Beckman DK-2 recording spectrophotometer were employed with 1-cm. silica cells for absorbance measurements.

The steroids used were official grades or comparable purity. These included reference standard quinestrol, ethinyl estradiol U.S.P., mestranol (Syntex), estrone N.F., estrone 3-cyclopentyl ether (Vismara Terapeutici, Casatenovo, Como, Italy), and β-estradiol N.F. α-Estradiol, equilin, 17-α-dihydroequilin, and equilinin were obtained from Ayerst Laboratories.

Chromogenic Reagent—Cautiously add concentrated sulfuric acid to 30.0 ml. of chilled absolute methanol in a 100-ml. volumetric flask. Adjust to room temperature and take the solution to the mark with acid. The reagent appears stable for at least 7 days.

Standard Preparation—Dissolve about 50 mg. of quinestrol or ethinyl estradiol, accurately weighed, in methanol and dilute to 100.0 ml. Further dilute 5.0 ml. to 50.0 ml. with absolute methanol to obtain a solution containing about 25 mcg. in 0.50 ml.

Assay of Tablets and Capsules—Determine the average unit weight of not less than 10 units for composite sample assay, and reduce the sample to a fine powder in a glass mortar. Transfer a weighed sample equivalent to about 25 mcg. of quinestrol or ethinyl estradiol to a 125-ml. separator containing 10 ml. of water, add 4–5 drops of concentrated hydrochloric acid, and extract the mixture with two 75-ml. vol. of petroleum ether, shaking vigorously for 2 min. during each extraction. Combine the solvent extracts in a second separator, and wash with one 10-ml. vol. of 1% sodium carbonate to remove colorants, if present. Discard the aqueous phase, and quantitatively transfer the combined extracts to a third, *scrupulously dry* separator through a cotton pledget filter with the aid of an additional 10 ml. of petroleum ether. Add exactly 0.5 ml. of anhydrous methanol, then add exactly 5.0 ml. of chromogenic reagent. Shake the mixture vigorously for 4 min., and allow the phases to separate. Discard the first few drops of the colored lower phase through the bore of the stopcock, and deliver about 4.5 ml. of the lower phase into a dry test tube. Withdraw exactly 4.0 ml. of the solution by pipet into a dry glass-stoppered centrifuge tube, add exactly 0.3 ml. of anhydrous methanol, mix well, and clarify if necessary by centrifugation. Determine the absorbance of the solution at the maximum, about 540 mμ, designating the absorbance A_u.

Concomitantly develop a color with the standard preparation, using 0.5 ml. of the standard preparation and 150 ml. of petroleum ether in a separator and following the procedure described above, beginning with "... add exactly 5.0 ml. of chromogenic reagent." Designating the concentration of the standard preparation in mcg./0.50 ml. as C and the absorbance of the color produced from it as A_s:

$$\text{estrogen, mcg./unit} = A_u/A_s \times C \times \text{mg. av. unit wt./mg. sample wt.}$$

Individual Unit-Dose Assay—Use the procedure described above with an individual intact tablet or the contents of one capsule as sample in place of the composite, adjusting the volumes of reagents appropriately for dosage forms containing more than 25 mcg. of estrogen.

Assay of Ethinyl Estradiol in a Complex Dosage Form—The example chosen was a tablet[2] declaring 5 mcg. of ethinyl estradiol, 2.5 mg. of methyltestosterone, and 16 mg. of thyroglobulin. Transfer an accurately weighed amount of pulverized tablet composite equivalent to about 15 mcg. of ethinyl estradiol to a 250-ml. separator containing 10 ml. of water, add 1-ml. of concentrated hydrochloric acid, and mix thoroughly. Extract the mixture with two 100-ml. vol. of petroleum ether, shaking about 2 min. during each extraction. Combine the extracts in a second 250-ml. separator, and wash the extract with three 5-ml. vol. of water, discarding the washings. Extract the organic phase with two 10-ml. vol. of 10% sodium hydroxide, shaking for 5 min. each time. Transfer the aqueous phases through a small cotton pledget into a third separator containing 7 ml. of concentrated hydrochloric acid. Discard the organic phase, then continue as described above for the general procedure, starting with "... extract with two 75-ml. vol. of petroleum ether. ..."

RESULTS AND DISCUSSION

Validity of Beer's Law at 540 mμ—Concentration was found to be proportional to absorbance for the chromophore produced by reaction of quinestrol or ethinyl estradiol with the chromogenic reagent. A typical Beer's law plot is shown in Fig. 1. It may be noted that assay of individual 10-mcg. tablets affords

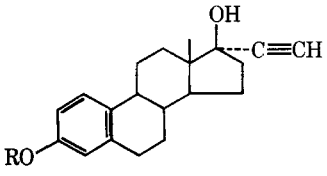
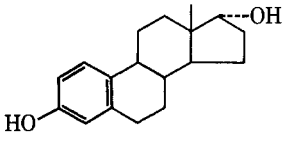
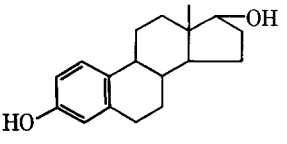
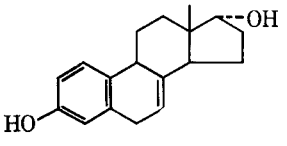
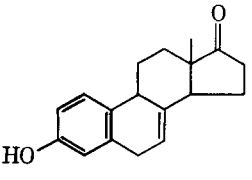
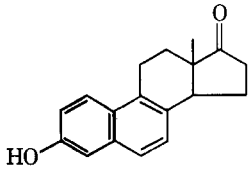
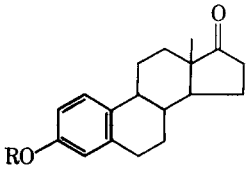
[2] Marketed as Plestran Tablets by Warner-Chilcott Laboratories.

absorbance values at the low end of the optimum range for spectrophotometric accuracy. While the use of micro cells would have afforded better absorbance values by permitting smaller volumes of chromogenic reagent, this expedient was not resorted to in this study.

Color Stability—The stability of the color produced in the Heusghem and Jehotte procedure (11) and the color produced in the procedure reported here are compared in Fig. 2. The initial rapid fading of the color in the published procedure led to erratic results in routine assays. The difference in the methods was more marked with assay samples than with standards, for the petroleum ether solvent affords more selectivity than the more polar and hygroscopic diethyl ether used in the published method. Moreover, the new procedure obviates a solvent evaporation step.

Selectivity of the Color Reaction—The remarkable selectivity of the chromogenic reaction is illustrated in Table I. The absorptivity values reported are approximations obtained by assuming no volume changes on mixing reagents. Spectra of the ethinyl estradiol chromophore and the yellow chromophore from 17-α-dihydroequilin are shown in Fig. 3. The reaction is more selective than reactions using similar

TABLE I—VISIBLE SPECTRAL DATA FOR THE METHANOL–SULFURIC ACID CHROMOPHORE OF VARIOUS ESTROGEN STEROIDS

	Estrogen	λ max., mμ	a	ε
	Ethinyl estradiol (R = H)	540	110.2	36000
	Quinestrol (R = cyclopentyl)	540	92.8	36000
	Mestranol (R = methyl)	540	110	34000
	α-Estradiol	452	91.4	24000
	β-Estradiol	Nil	...	...
	17-α-Dihydroequilin	452	155	42000
	Equilin	452	67.2	18000
	Equilinin	Nil	...	...
	Estrone (R = H)	Nil	...	...
	Estrone 3-cyclopentyl ether (R = cyclopentyl)	Nil	...	...

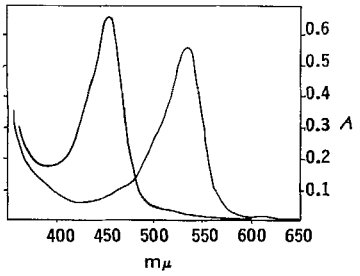


Fig. 3—Visible spectra of the chromophore from 25 mcg. of ethinyl estradiol (peak at right) and 17-α-dihydroequilin.

reagents, such as the Kober reaction to which all estrogens respond (13) and the Wintersteiner-Pfiffner green fluorescence in sulfuric acid (14). The lack of color formation in the reaction with estrone or its 3-ether suggests that the chromogenic reaction involves the groups at the 17-position of ethinyl estradiol and its ethers, but it is more difficult to adduce a structural feature common to the three other estrogens which results in the yellow chromophore. Elucidation of the apparent selectivity of the reactions of estrogens with methanol–sulfuric acid awaits characterization of the chromophores.

A possible, although not observed, degradation route for quinestrol in dosage forms is ether hydrolysis to form ethinyl estradiol. The latter might also be a by-product of quinestrol or mestranol synthesis. Extraction from alkaline solution, where any ethinyl estradiol would be retained as phenolate ion, would provide a selective assay for quinestrol or mestranol. As noted above, use of a nonpolar solvent in the isolation procedure confers a high degree of selectivity. No interference was encountered from red colorants used in tablet formulations, and spectrophotometric scans of the chromophore indicated absence of interferences in every formulation assayed.

Effect of Water on the Chromogenic Reaction—The necessity for employing dry reagents and glassware in the reaction was tested by adding varying amounts of water in the reaction of 20 mcg. of ethinyl estradiol with the chromogenic reagent. The effect is evident in Table II.

Recovery of Quinestrol Added to Placebo Tablets—Essentially quantitative recovery of added quinestrol was obtained. This was demonstrated by experiments using 20.0 mcg. of quinestrol and 150 mg. of excipients. These data are presented in Table III.

Table II—Effect of Added Water on the Color Obtained with 20 mcg. Ethinyl Estradiol

Water Added, ml.	0	0.05	0.10	0.15	0.20
A_{540} mμ	0.455	0.430	0.410	0.390	0.335

Table IV—Sextuplicate Assays of Commercial Ethinyl Estradiol Tablets

Brand	Declared, mcg.	Found, mcg.	±S.D.	Relative S.D., %
A	10.0	10.5	0.12	1.2
B	20.0	19.8	0.39	2.0

Table V—Assay of Individual Quinestrol Tablets

	Declared, mcg./Tablet	
	10.0	25.0
	Found, mcg./Tablet	
1	10.0	24.2
2	9.3	24.2
3	10.3	24.8
4	9.5	23.2
5	9.5	25.2
6	11.0	23.2
7	9.8	26.0
8	9.5	24.2
9	10.5	23.4
10	9.3	24.8
	Av., mcg./Tablet	
	9.9	24.3
	Relative S. D., %	
	5.8	3.4

Assay of Quinestrol in Tablets—Seventeen trials on composites of tablets declaring 25 mcg. gave a mean of 24.4 mcg. (97.6%) with a relative standard deviation of 0.5 mcg. (2%). Fifteen trials on tablets declaring 10 mcg. provided a mean value of 9.6 mcg. Here the relative standard deviation from the mean was 0.28 mcg. or 2.9%.

Assay of Ethinyl Estradiol Tablets—Composite samples of two commercially available tablet formulations of ethinyl estradiol marketed by different reputable domestic manufacturers were assayed by the proposed procedure in sextuplicate. Table IV shows the results obtained. The precision compares quite favorably with that obtained in other procedures. Boughton *et al.* (7) reported over-all standard deviations of ±5.5% by the U.S.P. method and ±8.6% for their gas chromatographic procedure in assays of ethinyl estradiol tablets declaring 100 mcg. Heusser (8) reported a ±5% precision for assays of 20 mcg. tablets using colorimetry following a thin-layer chromatographic separation of the drug.

Content Uniformity Assays—Since process control studies require the assay of individual dosage units to insure uniform distribution of microgram amounts of drugs in mg. amounts of excipient matrix, the proposed procedure was applied to quinestrol tablets. The results of individual tablet assay on two typical quinestrol formulations are presented in Table V. Since the precision obtained in recovery

Table III—Recovery of 20.0 mcg. of Added Quinestrol

Trial	1	2	3	4	5	6	7
Found, mcg.	19.5	19.8	20.2	20.2	20.0	19.3	20.0

Av. recovery ± S.D. = 19.9 ± 0.34 mcg. or 99.5 ± 1.7%

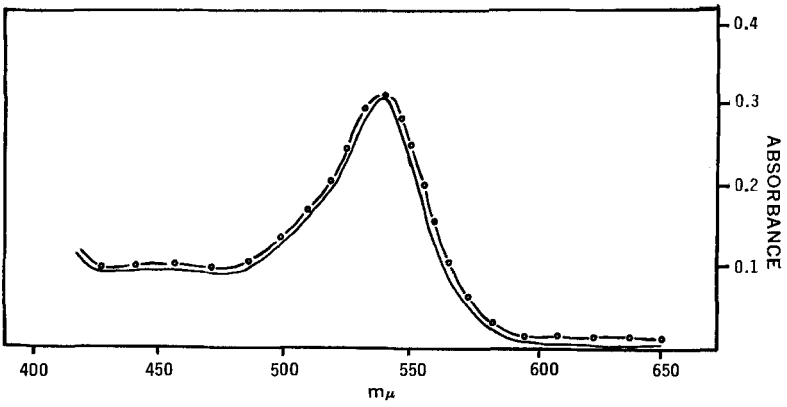


Fig. 4—Recovery of 15 mcg. of ethinyl estradiol from the tablets. (See Footnote 2). *Key: —, ethinyl estradiol standard; —·—·—·—, sample.*

experiments with quinestrol was 1.7%, the assay appears quite suitable for use in process control studies.

Ethinyl Estradiol Assay in the Estrogen–Androgen–Thyroglobulin Tablets—Recovery experiments for 10.0 mcg. of ethinyl estradiol added to the equivalent of two placebo tablets yielded values of 10.1 and 9.9 mcg.; for 15.0 mcg. added to the equivalent of three placebo tablets, a value of 15.6 mcg. was obtained. Assay of a production lot of tablets provided a value of 5.03 mcg./tablet or 102% of the declared amount. The absorption spectra of the sample and standard shown in Fig. 4 indicate the procedure provides clean separation from interferences.

SUMMARY AND CONCLUSION

Ethinyl estradiol, its 3-cyclopentyl ether, quinestrol, and its 3-methyl ether, mestranol, react with a methanol–sulfuric acid reagent of critical composition to form a stable chromophore with $\lambda_{max.}$ 540 mμ and ϵ 3.6 X 10^4. The chromogenic reaction appears to be specific for these estrogens among a number of others tested. A noninterfering yellow color with $\lambda_{max.}$ 452 mμ and varying absorptivities is produced by the reagent with α-estradiol, equilin, and 17-α-dihydroequilin, but no color is obtained with β-estradiol, estrone, estrone 3-cyclopentyl ether, and equilinin.

Examples are given of the use of the reaction for colorimetric assay of microgram amounts of quinestrol and ethinyl estradiol in dosage forms. The sensitivity, precision, accuracy, and manipulative ease of the method compare favorably with the U.S.P. colorimetric assay for ethinyl estradiol and the various chromatographic assays for it and mestranol in the literature. Solvent extraction with petroleum ether is used for isolation of the estrogens from dosage forms; this procedure itself affords considerable selectivity, since most excipients and many co-formulated drugs are excluded. An example of the selectivity of the procedure is presented in the assay of mcg. amounts of ethinyl estradiol in the presence of mg. amounts of methyltestosterone and thyroglobulin in a commercial dosage form.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 245.
(2) Urbanyi, T., and Rehm, C. R., *J. Pharm. Sci.*, **55,** 501(1966).
(3) Schulz, E. P., *ibid.*, **54,** 144(1965).
(4) Talmage, J. M., Penner, M. H., and Geller, M., *ibid.*, **54,** 1194(1965).
(5) France, J. T., and Knox, B. S., *J. Gas Chromatog.*, **1966,** 173.
(6) Umbreit, A. R., and Wisniewski, J. V., *Facts and Methods (F & M)*, **5,** 9(October 1964).
(7) Boughton, O. D., Bryant, R., Ludwig, W. J., and Timma, D. L., *J. Pharm. Sci.*, **55,** 951(1966).
(8) Heusser, D., *Deut. Apoth. Ztg.*, **106,** 2111(1966); through *Chem. Abstr.*, **65,** 3670*e*(1966).
(9) Roberts, H. R., and Siino, M. R., *J. Pharm. Sci.*, **52,** 370(1963).
(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 244.
(11) Heusghem, C., and Jehotte, J. M., *J. Pharm. Belg.*, **12,** 418(1957); through *Chem. Abstr.*, **52,** 3259*b*(1958).
(12) Comer, J. P., personal communication.
(13) Kober, S., *Biochem. Z.*, **239,** 209(1931); Kober, S., *Biochem. J.*, **32,** 357(1938); Bauld, W. S., *ibid.*, **56,** 426 (1954).
(14) Wintersteiner, O., and Pfiffner, J. J., *J. Biol. Chem.*, **116,** 291(1936).

Application of Reverse Phase Partition Chromatography to the Analysis of Testosterone Propionate in Oil Injectables

By EDWARD SMITH

In the analysis of testosterone propionate in oil injectables, reverse phase chromatography on silanized purified siliceous earth provides a convenient method for separating testosterone propionate from the bulk of the oil. The latter is retained on the column, while the steroid, together with the sterol and triterpenoid components of the oil, is eluted. The testosterone propionate is quantitatively separated from this mixture by direct phase chromatography, with nitromethane as immobile phase and heptane as eluant; the testosterone propionate is then determined spectrophotometrically.

THE ANALYSIS of steroid in oil solutions has been a problem for many years. Because of the lack of a suitable chemical analysis, the U.S.P. XIV (1) testosterone propionate injection monograph specified a biological assay for the solution in vegetable oil. The assay in the U.S.P. XV (2) and subsequent revisions has been a gravimetric measurement of the semicarbazone derivative, based on the procedure of Madigan *et al.* (3). For samples containing less than 10 mg./ml. of oil, a preliminary liquid–liquid extraction is used to separate the testosterone propionate from the bulk of the oil prior to the formation of the semicarbazone (3). The melting point of the semicarbazone is used as the criterion of identification.

Quantitative procedures have been described (4–6) based on colorimetric determination of the 2,4-dinitrophenylhydrazone after preliminary separation. Umberger (7) used an adsorbent magnesium silicate[1] to chromatographically separate testosterone propionate and progesterone from their oil solution prior to colorimetric determination of their isonicotinylhydrazones. Tappi *et al.* (8) also used the adsorbent magnesium silicate to separate steroids from olive oil.

The application of partition chromatography to the separation of drugs from oil dosage forms was investigated in these laboratories. Levine (9), Heftmann (10), Neher (11), and Bush (12) have reviewed the application of partition chromatography to the analysis of steroids. Jones and Stitch (13) used silicic acid–nitromethane partition columns to separate mixtures of steroids, with 3% $CHCl_3$ in petroleum ether as the mobile phase. Wolff (14) isolated progesterone from its oil injection with nitromethane as the stationary phase on purified siliceous earth[2] and *n*-heptane as the mobile phase. He found that the background ultraviolet absorbance due to the sterol fractions of the oil was thereby reduced to negligible proportions. This procedure was modified in our laboratory (15) and applied to the assay of chlorotrianisene capsules N.F.; the modified procedure is official in the N.F. XII (16). In these separations there is an interval of at least 40 ml. between the complete removal of the oil and the emergence of the desired substance.

Determination of testosterone propionate in oil injectables by this procedure proved to be difficult. The testosterone propionate follows the elution of the oil fraction so closely that sharp separation may not be achieved. The presence of such adjuvants as benzyl alcohol further interferes with this partitioning. No significant improvement was obtained by varying column dimensions or by substituting dimethylsulfoxide for nitromethane as the stationary phase.

Testosterone propionate can be separated from the bulk of the oil in these preparations by reverse phase partition chromatography. In this system, the hydrophobic support retains the nonpolar solvent as stationary phase and a polar solvent is used as the mobile phase. Silanized siliceous earth was first reported by Howard and Martin (17), who used it to separate C_{12} to C_{18} fatty acids. Other workers have used this type of silanized support to separate mixtures of adrenal steroids extracted from blood (18), to purify labeled steroids (19), and to separate these labeled steroids from urine (20). The application of silanized purified siliceous earth to the determination of steroids in oil dosage forms has not been previously described.

Silanized purified siliceous earth provides a convenient method for separating testosterone propionate from the glycerides, which constitute the major portion of the oil. The eluate from the

Received August 25, 1966, from the General Methods Branch, Division of Pharmaceutical Chemistry, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, DC 20204

Accepted for publication February 9, 1967.

Presented in part to the Scientific Section, A.PH.A., Detroit meeting, April 1965.

[1] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.

[2] Marketed as Celite 545, acid-washed, by the Johns-Manville Corp., New York, N. Y.

reverse phase column contains the testosterone propionate, together with the sterols and triterpenoids from the vehicle. It also contains any free testosterone which may be present. The testosterone propionate is separated from these materials by direct phase partition chromatography as previously described (14, 16).

EXPERIMENTAL

Materials—*Purified Siliceous Earth.*[2]

Redistilled n-Heptane—The absorbance *versus* ethanol in 1-cm. cells should be less than 0.500 in the range 250–360 mμ (limit of aromatic content). The residue from the evaporation of 25 ml. of distillate, dissolved in 10 ml. of ethanol, should have an absorbance not greater than 0.01 from 230 to 360 mμ (nonvolatile residue limit).

Nitromethane—Reagent grade.

Silanized Purified Siliceous Earth—Prepared essentially by the procedure of Howard and Martin (17). Place about 450 Gm. of purified siliceous earth in a large open glass crystallizing dish in a vacuum desiccator containing 30 ml. of silane.[3] Apply vacuum (water aspirator) intermittently for several hours until no liquid silane remains. Float the treated purified siliceous earth on distilled water and gently agitate to allow any uncoated particles to sink. Skim the coated purified siliceous earth off the surface, wash it on a sintered-glass funnel with warm methanol until the filtrate is no longer acidic, and dry at 110°.

Solvents—Shake 200 ml. of 90% ethanol with 100 ml. of redistilled *n*-heptane to equilibrate. Use the upper phase as the immobile phase on the reverse phase column and the lower phase as the eluant.

Assay Preparation—Dilute an accurately measured sample with *n*-heptane tc obtain a solution containing 0.5 mg. of testosterone propionate per ml.

Column I (Reverse Phase Column)—Pack a pledget of fine glass wool in the base of a chromatographic tube (25 × 250 mm. test tube to which is fused a 5-cm. length of 6 or 8-mm. tubing) with the aid of a tamping rod of stainless steel, aluminum, or glass at least 12 in. long and having a disk with a diameter about 1 mm. less than that of the chromatographic tube. To 3.0 Gm. of silanized purified siliceous earth in a 100-ml. beaker add 3 ml. of equilibrated *n*-heptane and incorporate by kneading thoroughly with a flexible spatula blade until the mixture is fluffy. Transfer to a chromatographic tube and tamp gently to compress the material to a uniform mass. In a like manner mix a 2-ml. aliquot of the diluted sample with 3.0 Gm. of silanized purified siliceous earth and pack above the lower layer. Dry-wash the beaker with about 1 Gm. of silanized purified siliceous earth and add this to the column. Place a small pad of glass wool above the column packing.

Colume II (Direct Phase Column)—Mix 8.0 Gm. of purified siliceous earth with 9.0 ml. of nitromethane and pack in a column as described above.

Procedure—Pass 35 ml. of equilibrated ethanol over column I, collecting the eluate in a 50-ml. beaker or conical flask. Evaporate the eluate just to dryness on a steam bath with the aid of a gentle stream of air. If the sample contains benzyl benzoate, wash down the side of the container with 5 ml. of methanol, add 5 ml. of distilled water, and re-evaporate. Repeat this evaporation step to insure complete removal of the benzyl benzoate.

Dissolve the residue in 5 ml. of *n*-heptane, heating on a steam bath and stirring to dissolve. Cool the solution to room temperature and transfer it quantitatively to column II. Wash the container with 5 ml., followed by 30 ml. of *n*-heptane and then continue the column elution with *n*-heptane. Discard the first 30 ml. of eluate. Collect the next 10-ml. fraction separately as a check[4] and then the next 150 ml. of eluate in a 250-ml. conical flask. Evaporate the 150-ml. fraction to dryness on a steam bath under a gentle stream of air. Dissolve the residue in small portions of 95% ethanol and transfer quantitatively to a 100-ml. volumetric flask. Add 95% ethanol to volume and mix. Concomitantly determine the absorbance of this solution and of a solution of the U.S.P. testosterone propionate reference standard in the same solvent at a concentration of about 10 mcg./ml. in 1-cm. cells at the absorbance maximum at about 240 and at 300 mμ in a suitable spectrophotometer, using 95% ethanol as the blank. Calculate the quantity of testosterone propionate, in milligrams, in the eluate by the formula $0.1\ C_s A_u / A_s$, where A_u is the absorbance of the sample at 240 mμ minus the absorbance of the sample at 300 mμ, A_s is the corresponding value for the standard, and C_s is the concentration of the standard solution in mcg./ml.

Identification Test—Evaporate the alcoholic solution of the isolated testosterone propionate to dryness in a suitable container. Transfer the residue obtained to a mullite mortar and prepare a potassium bromide disk in the usual manner. The infrared spectrum obtained should be identical with that obtained from a disk of testosterone propionate reference standard.

RESULTS AND DISCUSSION

For the application of reverse phase chromatography to the separation of the steroid from the bulk of the oil, several supporting media were investigated. Silanized purified siliceous earth was very effective in achieving the separation; no differences were shown by purified siliceous earth silanized with the combinations of several alkyl chlorosilanes. Nonsilanized purified siliceous earth cannot be used because it does not retain the *n*-heptane as the stationary phase. A fluorocarbon support did not give the desired separations.

During the reverse phase chromatographic partitioning, essentially all of the steroid is eluted in the first 10 ml. of eluate; however, 35 ml. of eluant is used to insure complete recovery of the steroid. Additional eluant up to 150 ml. does not remove any further quantities of ultraviolet-absorbing material from samples containing as low as 10 mg. of testosterone propionate per ml. of oil. This is the

[2] Various silanes used included 15 ml. each of dimethyldichlorosilane and trimethylchlorosilane or 30 ml. of Dow-Corning 1208 (a mixture of methyl trichlorosilane and dimethyldichlorosilane in the approximate weight ratio of 1:2).

[4] This fraction should be free of both testosterone propionate and oil. Evaporate it to dryness and dissolve the residue in 10.0 ml. of 95% ethanol. If the solution has any significant absorbance in the 230–240 mμ range, the column is not functioning properly.

TABLE I—ANALYSIS OF TESTOSTERONE PROPIONATE IN OIL BY COLUMN PROCEDURES

Concn., mg./ml. of Oil	Vehicle Compn.[a]	Testosterone Propionate mg. Taken	mg. Found	% Recovered	Av.
10	*A*	1.044	1.036	99.2	
			1.040	99.6	
			1.034	99.0	99.3
10	*A*	0.994	0.997	100.1	
			0.983	98.7	
			0.989	99.3	99.4
25	*A*	0.984	0.973	98.9	
			0.963	97.9	
			0.969	98.5	98.4
25	*B*	1.012	1.008	99.6	
			1.021	100.9	
			0.995	98.3	99.6
25	*B*	0.994	0.984	99.0	
			0.982	98.8	
			0.979	97.6	98.5
50	*B*	1.000	0.985	98.5	
			0.981	98.1	
			0.991	99.8	98.8
50	*C*	0.958	0.956	99.8	
			0.950	99.2	
			0.940	99.0	99.3
50	*D*	0.958	0.964	100.6	
			0.952	99.4	
			0.940	98.1	99.4
50	*E*	0.958	0.954	99.6	
			0.944	98.5	
			0.938	97.9	98.7

[a] *A*, Five per cent benzyl alcohol in sesame oil; *B*, 3% benzyl alcohol in sesame oil; *C*, sesame oil: sample heated on water bath for 5 min. to dissolve testosterone propionate; *D*, sesame oil: sealed in ampul, heated at 100° for 2 hr.; *E*, sesame oil: sealed in ampul, heated at 160° for 2 hr.

lowest concentration found in commercial samples, and hence the concentration containing the greatest amount of oil in the aliquot analyzed.

About 99% of the oil, essentially fatty acid glycerides, is retained on the reverse phase column. The sterols and triterpenoid components of the oil are eluted together with the testosterone propionate, as are free testosterone and a number of the common adjuvants. Benzyl alcohol, chlorobutanol, and phenol are readily removed azeotropically during the evaporation of the ethanol eluate from the reverse phase column. Benzyl benzoate is partially removed in this step, but the subsequent evaporation of the 50% methanol added to the residue is necessary to completely volatilize this high boiling liquid. None of the testosterone propionate is lost during this evaporation. The parabens are not removed in this step, but are retained on the direct phase column in the ensuing step of the procedure.

The final separation of the testosterone propionate from the sterols and triterpenoids of the oil and any free testosterone on the purified siliceous earth—nitromethane column gives quantitative yields of pure testosterone propionate which is quantitated by its ultraviolet absorbance and identified by its infrared spectrum. The sterols are completely removed by the first 15 ml. of *n*-heptane. A distinct interval of about 30 ml. then follows before the appearance of testosterone propionate in the eluate. The triterpenoids, such as sesamin in sesame oil, are not eluted with *n*-heptane but are retained on the purified siliceous earth–nitromethane column. Testosterone is also retained on this column.

A series of standard samples were prepared to contain 10 to 50 mg. of testosterone propionate per ml. of sesame oil. The recoveries obtained by the column procedure are shown in Table I. Twelve commercial samples with concentrations ranging from 10 to 100 mg./ml. of oil were also analyzed by the column procedure (Table II).

The same commercial samples (Table II) and a number of standard samples (Table III) were also analyzed by the U.S.P. procedure. The results of these U.S.P. analyses were almost always higher than those obtained by the proposed method. Some of these standard samples (samples 1–11, Table III) were prepared by simply adding the weighed testosterone propionate to the measured volume of vehicle in the reaction flask, with no intermediate treatment prior to adding the reagents and completing the analysis. The analyses of these standards at the 50 mg./ml. level by the U.S.P. procedure averaged 3% higher than those by the proposed procedure, while those of the commercial samples averaged 8% higher. In order to resolve this anomaly the effects of the usual procedures used in the manufacturing of commercial samples were investigated.

TABLE II—ANALYSIS OF COMMERCIAL SAMPLES OF TESTOSTERONE PROPIONATE IN OIL

Labeled mg./ml. of Oil	Vehicle Compn.[a]	Column Procedure Found mg./ml.	Column Procedure Found % Label	U.S.P. Procedure Found mg./ml.	U.S.P. Procedure Found % Label
10	*A*	9.32	93.2	10.15	101.5
		9.24	92.4	9.28	92.8
		9.18	91.8		
25	*B*	24.48	97.9	26.78	106.9
		24.68	98.7	29.13	116.5
		24.63	98.5		
25	*C*	23.62	94.5	25.10	100.4
		23.55	94.2	24.07	96.3
		24.12	96.5		
25	*D*	22.95	91.8	24.45	97.8
		23.10	92.4	23.75	95.0
		23.10	92.4		
25	*E*	24.48	97.9	27.30	109.2
		24.40	97.6	26.29	105.2
		24.15	96.6		
50	*B*	45.80	91.6	48.30	96.6
		46.40	92.8	49.15	98.3
		46.15	92.3	49.50	99.0
				51.40	102.8
50	*C*	47.65	95.3	52.40	104.8
		47.45	94.9	51.60	103.2
		48.90	97.8		
50	*D*	47.30	94.6	52.20	104.4
		47.00	94.0	51.50	103.2
		48.40	96.8		
50	*E*	45.85	91.7	48.90	97.8
		45.45	90.9	50.60	101.2
		47.20	94.4		
50	*F*	47.45	94.9	49.00	98.0
		46.65	93.3	49.75	99.5
		46.60	93.2	49.50	99.0
		46.50	93.0	48.80	97.6
100	*G*	90.4	90.4	91.1	91.1
		91.7	91.7	92.7	92.7
		92.5	92.5		
100	*H*	90.2	90.2	93.1	93.1
		90.6	90.6	91.0	91.0
		92.3	92.3		

[a] *A*, Three per cent benzyl alcohol, 0.5% chlorobutanol in sesame oil; *B*, 0.5% chlorobutanol in sesame oil; *C*, 0.08% methylparaben, 0.1% propylparaben in sesame oil; *D*, 1:4000 benzethonium octyloxyacetate in peanut oil; *E*, 0.5% phenol in sesame oil; *F*, 0.1% propylparaben, 3% benzyl alcohol in sesame oil; *G*, 10% benzyl alcohol, 0.08% methylparaben, 0.1% propylparaben in sesame oil; *H*, 25% benzyl benzoate, 0.5% chlorobutanol in sesame oil.

A standard sample (50 mg./ml.) was prepared by dissolving testosterone propionate in sesame oil by warming on a water bath. Portions were sealed in ampuls and subjected to dry heat sterilization. The U.S.P. method gave recoveries of 110 to 112.7% as compared with the average of 102% obtained with the unheated oil. The heating had no effect on recoveries obtained with the proposed procedure.

Blank samples of 1 ml. of sesame oil which were not heated and heated oil treated by the U.S.P. procedure yielded residues which corresponded to 1.4 and 4.1%, respectively, based on a 50-mg./ml. sample of the steroid. Greater amounts of material were contributed by the oil in the presence of testosterone propionate semicarbazone, due to coprecipitation. Weighed amounts of this derivative were dissolved in the reagent prior to the addition of the blank oil. After carrying through the U.S.P. procedure the increase contributed by the heated oil was 2 to 4 times that given by the unheated oil, paralleling the findings of the standard analyses.

The isolated derivatives from the U.S.P. assay were examined by thin-layer chromatography.[5] The material obtained from the blank oils gave a number of spots, several corresponding to the sterol and triterpenoid fractions of the oil. That from the heated oil gave more than that from the unheated oil. These same spots, as well as that of testosterone propionate semicarbazone, were ob-

[5] Samples dissolved in $CHCl_3$ were spotted on silica gel GF 0.25 mm. Solvent system: $CHCl_3$–methanol–H_2O, 90:7.5:0.5 (21). Spray the developed chromatogram with 50% H_2SO_4. Heat with heat lamp until spots are visible. Examine plate with long wavelength ultraviolet light.

TABLE III—ANALYSIS OF TESTOSTERONE PROPIONATE IN OIL BY U.S.P. XVII ASSAY PROCEDURE

Concn., mg./ml. of Oil	Vehicle Compn.[a]	Testosterone Propionate mg. Taken	mg. Found	% Recovered
10	*A*	51.0	67.1	131.5
25	*A*	50.2	59.6	118.7
25	*A*	50.4	54.2	107.7
25	*B*	50.1	55.4	110.6
25	*B*	51.8	54.9	106.0
50	*A*	51.0	51.0	100.0
50	*A*	51.1	52.4	102.5
50	*B*	50.0	50.1	100.2
50	*B*	50.8	51.3	101.0
50	*C*	55.2	57.1	103.4
50	*C*	51.9	53.4	103.0
50	*D*	48.2	54.1	112.2
50	*D*	48.0	54.1	112.7
50	*E*	48.2	53.0	110.0

[a] *A*, Three per cent benzyl alcohol in sesame oil; *B*, 3% benzyl alcohol, 0.5% chlorobutanol in sesame oil; *C*, sesame oil; *D*, sesame oil: sample heated on water bath for 5 min. to dissolve testosterone propionate; *E*, sesame oil: sealed in ampul, heated at 160° for 2 hr.

tained from the standards and from the commercial samples. Several of the latter gave in addition a spot corresponding to free testosterone semicarbazone.

In contrast, the testosterone propionate isolated by the proposed procedure gives a single spot by thin-layer chromatography.

SUMMARY

Spectrophotometrically and chromatographically pure testosterone propionate is recovered quantitatively from its oil dosage forms by a combination of reverse phase and direct phase partition chromatography. The testosterone propionate, together with sterols and triterpenoids as well as any free testosterone, is separated from the bulk of the oil in the reverse phase process; it is then isolated free of these substances by direct phase partition. The procedure is applicable to samples containing such adjuvants as benzyl alcohol, benzyl benzoate, chlorobutanol, phenol, and the parabens.

REFERENCES

(1) "United States Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 609.
(2) *Ibid.*, 15th rev., 1955, p. 717.
(3) Madigan, J. J., Zenno, E. E., and Pheasant, R., *Anal. Chem.*, **23**, 1691(1951).
(4) Vieira de Abreu, M. M., *Rev. Port. Farm.*, **13**, 141 (1963).
(5) Diding, E., *Svensk Farm. Tidskr.*, **56**, 3(1952).
(6) Sonderstrom, K., *J. Pharm. Belg.*, **10**, 379(1955).
(7) Umberger, E. J., *Anal. Chem.*, **27**, 768(1955).
(8) Tappi, G., Andreoli, E. M., and Frea, E., *Pharm. Weekblad*, **93**, 231(1958).
(9) Levine, J., *J. Pharm. Sci.*, **52**, 1015(1963).
(10) Heftmann, E., *Chem. Rev.*, **55**, 679(1955).
(11) Neher, R., "Steroid Chromatography," Elsevier Publishing Co., New York, N. Y., 1964.
(12) Bush, I. E., "The Chromatography of Steroids," The Macmillan Co., New York, N. Y., 1961.
(13) Jones, J. K. N., and Stitch, S. R., *Biochem. J.*, **53**, 679(1953).
(14) Wolff, J., *J. Pharm. Sci.*, **52**, 93(1963).
(15) Smith, E., unpublished data.
(16) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 92.
(17) Howard, G. A., and Martin, A. J. P., *Biochem. J.*, **46**, 532(1950).
(18) Morris, C. J. O. R., and Williams, D. C., *ibid.*, **54**, 470(1953).
(19) Pearlman, W. H., *J. Biol. Chem.*, **236**, 700(1961).
(20) Contractor, S. F., and Pearlman, W. H., *Biochem. J.*, **76**, 36(1960).
(21) Davis, J. S., personal communication.

Technical Articles

Effects of Selected Variables on the Microencapsulation of Solids

By L. A. LUZZI* and R. J. GERRAUGHTY

The hypothesis that solid particles may be protectively encapsulated by coacervation led to the investigation of the extractability of microcapsules, which contained solids, by gastrointestinal fluids. Starting pH, starting temperature, ratio of solid to encapsulating materials, quantity of denaturant, and final pH were varied. The effects of the variables studied on microcapsules were determined by submitting samples to simulated gastrointestinal fluids for periods of up to 2.5 hr. and then comparing the extracts spectrophotometrically to known absorption spectra. Results showed that all variables effected some degree of change in microcapsules.

THE TERM "coacervation" has recently been used to describe the salting out of a lyophilic solid into liquid droplets rather than solid aggregates (1). The term was introduced into colloidal chemistry by Kruyt and Bungenberg de Jong (2) to describe the flocculation or separation of liquids from solution, where at least one of the liquids contained a colloidal solute.

Coacervation has been subdivided into simple coacervation and complex coacervation. Briefly,

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TABLE III—ANALYSIS OF TESTOSTERONE PROPIONATE IN OIL BY U.S.P. XVII ASSAY PROCEDURE

Concn., mg./ml. of Oil	Vehicle Compn.[a]	Testosterone Propionate mg. Taken	mg. Found	% Recovered
10	*A*	51.0	67.1	131.5
25	*A*	50.2	59.6	118.7
25	*A*	50.4	54.2	107.7
25	*B*	50.1	55.4	110.6
25	*B*	51.8	54.9	106.0
50	*A*	51.0	51.0	100.0
50	*A*	51.1	52.4	102.5
50	*B*	50.0	50.1	100.2
50	*B*	50.8	51.3	101.0
50	*C*	55.2	57.1	103.4
50	*C*	51.9	53.4	103.0
50	*D*	48.2	54.1	112.2
50	*D*	48.0	54.1	112.7
50	*E*	48.2	53.0	110.0

[a] *A*, Three per cent benzyl alcohol in sesame oil; *B*, 3% benzyl alcohol, 0.5% chlorobutanol in sesame oil; *C*, sesame oil; *D*, sesame oil: sample heated on water bath for 5 min. to dissolve testosterone propionate; *E*, sesame oil: sealed in ampul, heated at 160° for 2 hr.

tained from the standards and from the commercial samples. Several of the latter gave in addition a spot corresponding to free testosterone semicarbazone.

In contrast, the testosterone propionate isolated by the proposed procedure gives a single spot by thin-layer chromatography.

SUMMARY

Spectrophotometrically and chromatographically pure testosterone propionate is recovered quantitatively from its oil dosage forms by a combination of reverse phase and direct phase partition chromatography. The testosterone propionate, together with sterols and triterpenoids as well as any free testosterone, is separated from the bulk of the oil in the reverse phase process; it is then isolated free of these substances by direct phase partition. The procedure is applicable to samples containing such adjuvants as benzyl alcohol, benzyl benzoate, chlorobutanol, phenol, and the parabens.

REFERENCES

(1) "United States Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 609.
(2) *Ibid.*, 15th rev., 1955, p. 717.
(3) Madigan, J. J., Zenno, E. E., and Pheasant, R., *Anal. Chem.*, **23**, 1691(1951).
(4) Vieira de Abreu, M. M., *Rev. Port. Farm.*, **13**, 141 (1963).
(5) Diding, E., *Svensk Farm. Tidskr.*, **56**, 3(1952).
(6) Sonderstrom, K., *J. Pharm. Belg.*, **10**, 379(1955).
(7) Umberger, E. J., *Anal. Chem.*, **27**, 768(1955).
(8) Tappi, G., Andreoli, E. M., and Frea, E., *Pharm. Weekblad*, **93**, 231(1958).
(9) Levine, J., *J. Pharm. Sci.*, **52**, 1015(1963).
(10) Heftmann, E., *Chem. Rev.*, **55**, 679(1955).
(11) Neher, R., "Steroid Chromatography," Elsevier Publishing Co., New York, N. Y., 1964.
(12) Bush, I. E., "The Chromatography of Steroids," The Macmillan Co., New York, N. Y., 1961.
(13) Jones, J. K. N., and Stitch, S. R., *Biochem. J.*, **53**, 679(1953).
(14) Wolff, J., *J. Pharm. Sci.*, **52**, 93(1963).
(15) Smith, E., unpublished data.
(16) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 92.
(17) Howard, G. A., and Martin, A. J. P., *Biochem. J.*, **46**, 532(1950).
(18) Morris, C. J. O. R., and Williams, D. C., *ibid.*, **54**, 470(1953).
(19) Pearlman, W. H., *J. Biol. Chem.*, **236**, 700(1961).
(20) Contractor, S. F., and Pearlman, W. H., *Biochem. J.*, **76**, 36(1960).
(21) Davis, J. S., personal communication.

Technical Articles

Effects of Selected Variables on the Microencapsulation of Solids

By L. A. LUZZI* and R. J. GERRAUGHTY

The hypothesis that solid particles may be protectively encapsulated by coacervation led to the investigation of the extractability of microcapsules, which contained solids, by gastrointestinal fluids. Starting pH, starting temperature, ratio of solid to encapsulating materials, quantity of denaturant, and final pH were varied. The effects of the variables studied on microcapsules were determined by submitting samples to simulated gastrointestinal fluids for periods of up to 2.5 hr. and then comparing the extracts spectrophotometrically to known absorption spectra. Results showed that all variables effected some degree of change in microcapsules.

THE TERM "coacervation" has recently been used to describe the salting out of a lyophilic solid into liquid droplets rather than solid aggregates (1). The term was introduced into colloidal chemistry by Kruyt and Bungenberg de Jong (2) to describe the flocculation or separation of liquids from solution, where at least one of the liquids contained a colloidal solute.

Coacervation has been subdivided into simple coacervation and complex coacervation. Briefly,

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simple coacervation usually deals with systems containing only one colloidal solute, while complex coacervation usually deals with systems containing more than one colloid (3, 4).

Complex coacervation has been found to be primarily dependent on pH. It has been reported that in a gum arabic–gelatin system, complex coacervation into microcapsules occurred at pH values below the isoelectric point of the gelatin and would not occur above this pH regardless of how other factors were changed (5). The same was true of other systems containing two dispersed colloids of opposite electrical charge.

The optimum conditions for complex coacervation were achieved by adjusting the pH to a point at which equivalents of oppositely charged molecules of the two colloids were present, since, at this pH, the greatest number of salt bonds were formed (5). In such a system, the pH must be adjusted so that the gelatin particles are positively charged (below the isoelectric point), since gum arabic particles are always negative in colloidal dispersions (6). Therefore, the principal condition necessary for successful complex coacervation is pH. This factor is easily and precisely controlled and since the author had previous experience with this system, it was decided that encapsulation by way of complex coacervation would be suited to the purposes of this study.

Green and Schleiker made practical use of the process of coacervation in various patents (7–11). Two of the patents (7, 10) indicated that oils, some of which contained dissolved dyes, had been entrapped in gelatin–acacia microcapsules. No quantitative method to evaluate the strength of the capsules or the degree of encapsulation was given.

It appears that encapsulation through coacervation has great potential application in pharmacy. Some properties that might be especially useful include (*a*) prevention of vaporization of volatile substances (*e.g.*, volatile oils), (*b*) protection of moisture-sensitive or light-sensitive substances (*e.g.*, vitamins A and K), (*c*) separation of incompatible substances within a single system, (*d*) aid in the dispersion of water-insoluble substances in aqueous media and, (*e*) controlled release of encapsulated substances to the environment (*e.g.*, long acting medication).

Before such application could be explored, it was apparent that some methods should be available to predict necessary conditions to achieve good encapsulation. Luzzi and Gerraughty (12) examined some properties of oils used in pharmacy and found that the acid value and the surfactant properties of the oil affected the extraction of oils from microcapsules, whereas saponification values had little or no effect on extractability when subjected to ethyl ether.

With these considerations in mind, the system of Luzzi and Gerraughty (12) was examined. It was the intent of this study to develop a means to encapsulate solids through complex coacervation and to determine: (*a*) the effects of varying the starting pH, the starting temperature, the quantity of denaturant, the ratio of material to be encapsulated to encapsulating agents, and the final pH on the permeability of capsule shell when in contact with gastrointestinal fluids; and (*b*) to evaluate the effect of such variations on the system.

EXPERIMENTAL

Preparation of Microcapsules—The basic procedure employed by Luzzi and Gerraughty (12) for the preparation of microcapsules was used. The primary encapsulating solutions were made by dissolving 3.0 Gm. of acacia and 3.0 Gm. of gelatin separately in 100 ml. of purified water at 55°. The acacia used was powdered acacia U.S.P.; the gelatin used was 300 bloom pigskin gelatin[1] with an isoelectric point at pH 8.0.

A 20% sodium hydroxide solution was used to adjust the starting pH of the solution to 6.5. Acacia particles in solution are negatively charged and at this pH the gelatin particles are sufficiently negative so that they will not interact with the acacia.

A suspension was formed by adding the solid to be encapsulated to the solution of gelatin and acacia with gentle agitation. The pH of the mixture was then adjusted to 4.5 by the dropwise addition of diluted hydrochloric acid U.S.P., while stirring. At the pH, the gelatin particles were positively charged and were attracted to the negatively charged acacia particles. The combined particles coalesced about the suspended solid and isolated it from the rest of the system. At this point, 10 ml. of formaldehyde solution U.S.P. was added to the mixture and it was cooled to 10° by immersion in an ice bath.

The pH was then adjusted to a final pH of 9.0 by the dropwise addition of 20% sodium hydroxide solution. In order to remove solubilized drug and excess formaldehyde, the mixture was then diluted to approximately 400 ml. with distilled water and filtered. The wet mass was firmly wrapped in blotting paper to remove most of the remaining liquid. A coarsely granular material was obtained from the resulting soft rubbery mass by passing it through a 20-mesh screen and drying it at 50° for 24 hr.

To determine the effect of each variable all other variables were held at the values given in the above procedure. Each variable was studied within the following limits: (*a*) the starting pH was varied between 5.0 and 7.0; (*b*) the ratio of materials to be encapsulated to encapsulating materials (dry weight) was varied from 2/6 through 20/6; (*c*) the quantity of formaldehyde solution was varied from

[1] American Agricultural Chemical Co., Detroit, Mich.

1 ml. to 20 ml. for each 100 ml. of 3% gelatin dispersion; (*d*) the starting temperature was varied from 34° to 42°; and (*e*) the final pH was varied from 6.5 to 8.8.

Choice of Materials—The primary objective of the research was to determine whether a solid particle could be encapsulated by this particular process. Preliminary investigation showed that: (*a*) the solid to be encapsulated should be insoluble, or nearly so, in the system; (*b*) the solid particles should be readily dispersable in either gelatin or acacia solution; (*c*) the solid should contribute no surfactant properties; (*d*) the solid should be quickly soluble when exposed to the proper solvent; and (*e*) the solid should not degrade appreciably when exposed to aqueous systems for several hours.

The materials encapsulated for possible elucidation of one or more of the previously mentioned aims were: colloidal sulfur, special flowers of sulfur, aspirin anhydride (recrystallized from ethyl acetate solutions to a m.p. of 81°–83°), acetophenetidin U.S.P., barbituric acid, lycopodium spores, DDT, pentobarbituric acid (m.p. 127°–128°), and carbon black.

With one exception, difficulties in assay and stability precluded the use of all of these materials for use in these studies. The exception was pentobarbituric acid. The advantages in this case were those of relative insolubility in acid aqueous media and solubility, as a salt, in alkaline aqueous media. In addition, quantitative spectrophotometric determinations for sodium pentobarbital are easily accomplished.

METHOD OF EVALUATION OF MICROCAPSULES

The method used to evaluate the susceptibility of the coacervate shell to the action of U.S.P. simulated gastrointestinal fluids was based principally on the ability of the fluids to expose and/or extract the encapsulated material. In order to determine the degree of destruction or permeability, or both, caused by the gastrointestinal fluids, a spectrophotometric method of assay was used.

The microcapsules were prepared in the manner previously described and 0.25-Gm. samples of the dried capsules were exposed consecutively, then separately to 100 ml. of gastric fluid and 100 ml. of intestinal fluid, employing a Burrell wrist action shaker.[2]

In the first instance, the samples remained in contact with 100 ml. of gastric fluid for 0.5 hr., were filtered, and the filter paper and remaining sample were then transferred to 100 ml. of intestinal fluid; the filter paper was broken up prior to immersion in intestinal fluid. A 1.0-ml. sample of the intestinal fluid was withdrawn through a cotton pledget after 1 hr., and after 2 hr. the intestinal fluid was removed from the microcapsules by filtration.

The removal of the sample from gastrointestinal fluids was preceded in each instance by the adjustment of pH to 9.25. If the samples were to remain in a fluid for further extraction, the pH was again adjusted to official (U.S.P.) suggested values for that fluid using diluted hydrochloric acid U.S.P. or 20% sodium hydroxide solution.

[2] Burrell Corp., Pittsburgh, Pa.

Pentobarbituric acid has a pKa of 8.11 (13) and, therefore, at pH 9.1 the ratio of sodium pentobarbital to pentobarbituric acid becomes 10 to 1. Spectrophotometric studies of solutions of sodium pentobarbital indicated that absorption increased with increased pH values and that at pH 9.0, as may be predicted from theory, a gradual decrease in the slope of the curve occurred. Since pH values of 9.25 could be achieved with relative accuracy (±0.05 pH units) and ease, and since a great majority of the solute is in the salt form at this pH, this was the pH at which all liquid filtrates were compared.

The samples of filtered gastric fluid were assayed for content of active constituents, as were the filtered samples of intestinal fluids. The solutions were scanned for wavelength of maximum absorption using a recording spectrophotometer.[3] A broad peak was found with a maximum at 241 to 242 mμ. Beer's law plots were prepared using a Beckman spectrophotometer.[4] The concentration of the liquid filtrates, obtained by the extraction methods already described, were determined by comparing their respective Beer's law plots to the absorbance observed.

The total quantity of pentobarbituric acid in each sample was found by disintegrating a known weight of capsules in a Virtis blender[5] with a known volume of distilled water at pH 9.25, the sample was filtered and the resulting liquid adjusted, if necessary, to pH 9.25. Additional blending and maceration indicated that substantially all pentobarbituric acid had been released by the capsules. Aliquots were prepared and spectrophotometric data were collected which, when compared to the Beer's law plot yielded the information from which the calculations for total pentobarbituric acid content were carried out.

Microphotography of Microcapsules—It is the intent of this section to illustrate the typical formation and general appearance of microcapsules under various conditions.

Figure 1 shows a dispersion of pentobarbituric acid particles in a 3% gelatin solution. It can be seen that the particles are quite well dispersed.

Figure 2 is typical of groups of microcapsules found when there is nearly an optimum relationship between solid particles and encapsulating agents. The ratio of material to be encapsulated to encapsulating material was 8/6.

Figure 3 depicts both filled and unfilled microcapsules. The ratio of material to be encapsulated to encapsulating material was 4/6.

RESULTS

Effect of Starting pH on Microcapsules—A series of samples ranging from pH 5.0 to pH 7.0 was prepared and 0.25 Gm. of each was assayed in the manner previously described. Table I shows that, throughout the entire series, the maximum difference in per cent of total pentobarbituric acid released after 2.5 hr. of exposure to gastrointestinal fluids was only 4.8. The difference in per cent released was somewhat greater (9.9) after 0.5 hr. exposure to gastric fluid alone.

[3] Bausch & Lomb Spectronic 505, Bausch & Lomb Optical Co., Rochester, N. Y.

[4] Beckman DU spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

[5] Virtis "45," Precise Products Corp., Racine, Wis.

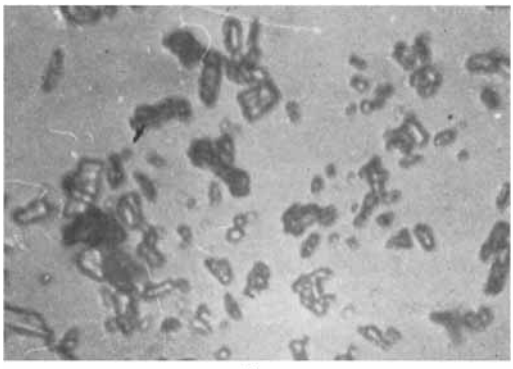

Fig. 1—Dispersion of pentobarbituric acid particles in a 3% gelatin solution.

Fig. 2—Typical group of microcapsules found when there is nearly an optimum relationship between solid particles and encapsulating agents.

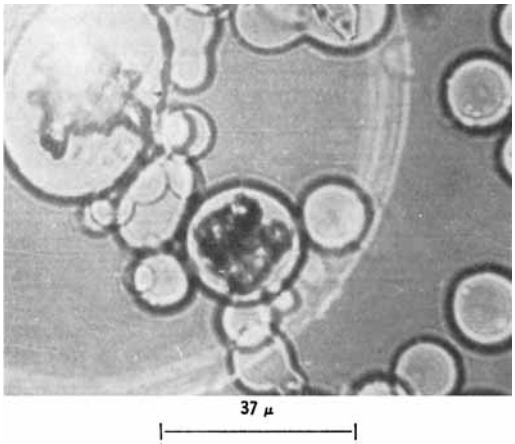

Fig. 3—Filled and unfilled microcapsules.

Effect of Starting Temperature on Microcapsules—Table II shows that the range of starting temperatures employed was between 34° and 42°. In all cases observed, except after 0.5 hr. in gastric fluid, the per cent of pentobarbituric acid that was released was found to be at a maximum at 34° starting temperature and reached a minimum at 37°.

Effect of Ratio of Solid to Encapsulating Materials—This series consisted of ratios ranging from 4.0 Gm. of pentobarbituric acid to 6 Gm. total dry weight of encapsulating materials (4/6), through 20.0 Gm. of acid to 6 Gm. total dry weight of encapsulating materials (20/6). Table III shows that the per cent of total acid which was released from the samples increased as the ratio of solid to encapsulating materials increased. Table III, it should be noted, also shows that the increase in per cent released, from 1 to 2 hr. exposures to intestinal fluid, decreased or the ratio increased.

Effect of Different Quantities of Denaturant on Microcapsules—Formaldehyde acts as a denaturant to harden and fix the wet gelatinous shell. In

TABLE I—EFFECT OF STARTING pH ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Starting pH	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
5.0	33.8	15.9	24.0	57.8
5.3	27.0	14.8	26.9	53.9
6.0	30.1	13.3	23.6	53.7
6.5	23.7	10.9	29.3	53.0
7.0	30.5	13.3	24.0	54.5

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid, the samples were transferred to intestinal fluid.

TABLE II—EFFECT OF STARTING TEMPERATURE ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Starting Temp.	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
34	44.3	24.4	27.6	71.9
36	37.8	16.4	25.3	63.1
37	36.8	13.2	22.0	58.8
40	34.8	19.2	26.3	61.1
42	38.8	18.9	26.8	65.6

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid, the samples were transferred to intestinal fluids.

TABLE III—EFFECT OF RATIO ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Ratio	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
4/6	28.7	13.3	24.0	42.7
8/6	36.8	13.2	22.0	58.8
12/6	39.1	18.4	26.6	65.7
16/6	40.2	26.1	30.7	70.9
20/6	45.2	26.2	29.1	74.3

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid, the samples were transferred to intestinal fluid.

TABLE IV—EFFECT OF FORMALDEHYDE ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Formaldehyde Soln., ml./100 ml.	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
1	Sample not collectable			
2	Sample not collectable			
4	40.2	22.6	30.0	70.2
6	38.0	16.6	26.8	64.8
8	32.9	17.3	26.4	59.3
10	37.8	16.4	25.3	63.1
20	47.8	18.2	23.8	71.6

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid, the samples were transferred to intestinal fluid.

TABLE V—EFFECT OF FINAL pH ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUID[a]

Final pH	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
6.5	27.0	14.7	23.5	50.5
7.0	23.7	28.4	29.3	53.0
7.5	29.4	13.1	22.4	51.8
8.0	28.6	17.7	27.2	55.8
8.8	36.7	33.7	36.0	72.7

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid, the samples were transferred to intestinal fluid.

order to test this effect, concentrations of from 1 to 20 ml. of 37% formaldehyde solution were added to the microencapsulation process for each 100 ml. of 3% gelatin solution. Table IV shows that 1- and 2-ml. quantities of formaldehyde solution were insufficient to allow collection of samples. When 4 ml. of formaldehyde solution was used, the samples became collectable and continued so throughout the remainder of the series. An interesting observation was that during initial exposure to either gastric or intestinal fluids, the per cent of pentobarbituric acid released was greater in those samples to which the largest quantity of formaldehyde had been added.

Effect of Final pH on Microcapsules—A series of samples was prepared with final pH values ranging between 6.5 and 8.8. Table V shows that after initial exposure to gastric fluids, the per cent of acid released from samples prepared at pH 7.0 was less than the acid released from samples prepared at pH 6.5. In intestinal fluid, the per cent of acid released after initial exposure was less for samples prepared at pH 7.5 than for those prepared at pH 6.5. Thereafter, a gradual increase was noted throughout the remainder of the series.

Table V indicates that in assays carried out after 1- and 2-hr. periods, on samples transferred from gastric fluid to intestinal fluid, the per cent of pentobarbituric acid extracted was erratic, with a slight tendency to increase as the final pH was increased. Table V also shows, however, that the over-all per cent extracted increased with increasing final pH.

DISCUSSION AND CONCLUSIONS

Encapsulation of solids occurred when oppositely charged colloidal particles agglomerated about the solid particles to form a shell which may contain one or several solid particles.

There seemed to be little difference in the quantity of solid extracted over a 2.5-hr. period when the starting pH was the variable. It was expected that maximum amounts of solid would be extracted at the extremes and lesser quantities in the median ranges. Although this was the trend, a wider range of pH values would be necessary to establish this clearly.

The minimum in amount extracted at starting temperature 37° (Table II) may have been due to a combination of adequate fluidity on the part of the colloidal particles and the relative insolubility of the solid.

The study of ratio of solid to encapsulating material (Table III) seems to indicate that the smaller the ratio the greater the retaining power of the capsule. This could be due to multiple droplet thickness of the wall or to the attraction of empty capsules to filled capsules.

Although microscopic examination of samples prepared with little or no formaldehyde indicated that capsule formation did take place, the capsules seemed to disappear when the temperature of the mixture was raised from 10° through 25° to 40°. When the capsules did remain intact the increase in the per cent of solid released at lower formaldehyde concentrations (Table IV) was probably due to two factors, both of which could result from inadequate denaturization. First, the gelatin–acacia shell might not have been sufficiently denatured, thus promoting a partial solubilization of the capsule during washing. This may have made the shell less impervious to the action of gastrointestinal fluids through lack of adequate denaturization. The increased per cent of acid that was extracted when greater amounts of formaldehyde were added was probably due to a cracking, resulting from excessive denaturization, of the capsule shell on drying.

As the final pH of the encapsulating process was increased the quantity of pentobarbituric acid extracted increased (Table V). This was probably due to a solubilization of the acid at the higher pH values and the subsequent weakening of the capsule wall by the resulting increased salt content of the mixture.

REFERENCES

(1) "The Condensed Chemical Dictionary," 4th ed., Reinhold Publishing Corp., New York, N. Y., 1950, p. 178.
(2) Kruyt, H. R., and Bungenberg de Jong, H. G., *Proc. Koninkle. Nederland Wetenschap., Amsterdam*, **32**, 849 (1929).
(3) "Colloid Science," Kruyt, H. R., ed., vol. 2, Elsevier Publishing Co., Amsterdam, The Netherlands, 1949, p. 339.
(4) *Ibid.*, p. 250.
(5) *Ibid.*, p. 341.
(6) "Textbook of Pharmaceutical Compounding and Dispensing," Lyman, R. A., and Sprowls, J. B., eds., 2nd ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 296.
(7) Green, B. K., and Schleiker, L., U. S. pat. 2,800,458 (1957).
(8) Green, B. K., and Schleiker, L., U. S. pat. 2,730,457 (1956).
(9) Green, B. K., U. S. pat. 2,712,507(1955).
(10) Green, B. K., and Schleiker, L., U. S. pat. 2,730,456 (1956).
(11) Green, B. K., and Schleiker, L., U. S. pat. 2,800,457 (1957).
(12) Luzzi, L. A., and Gerraughty, R. J., *J. Pharm. Sci.*, **53**, 429(1964).
(13) Martin, A. N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, p. 253.

Chemical Examination of *Clerodendron serratum*. Isolation and Characterization of D-Mannitol

By V. P. GARG and S. C. L. VERMA

D-Mannitol has been isolated from the root bark of *Clerodendron serratum* in a yield of 10.9 per cent and identified by mixed melting point, by analysis, chromatographic behavior, and by preparation of hexa-acetyl, hexanitro, hexabenzoyl derivatives, and benzaldehyde condensation product.

CLERODENDRON SERRATUM Spreng (N.O. *verbinaceae*) (1), popularly known in India as Bharangi, is a small flowering shrub found more or less throughout India, Ceylon, and the Malay peninsula. Various parts of the plant are credited in the Indian system of medicine with usefulness in bronchitis, asthma, eczema, catarrhal affections of lungs, febrile, and other manifestations. Aqueous extracts of the root bark of the plant have been found by Sachdev *et al.* (2) to block selectively the histamine-induced responses in isolated guinea pig ileum and on blood pressure in anesthetized dogs. The antihistaminic effects have been confirmed by Gupta *et al.* (3) in this department. Although it was considered by Sachdev *et al.* (2) that the active material could be precipitated from the aqueous extracts by addition of ethanol, a systematic chemical examination of the plant for its constituents has not been described before. During the course of fractionation of the aqueous extracts for isolating the antihistaminic principles, it has been found that the root bark contains a considerable amount (10.9%) of D-mannitol, whose isolation and identification are presently reported.

EXPERIMENTAL

Isolation of D-Mannitol

The dried root bark, freed from outer green skin and foreign matter, was reduced to about No. 20 mesh powder (500 Gm.) and was extracted with four changes of distilled water by boiling for 15 min. each. After filtering through muslin, the combined extract (3.5 L.) was allowed to stand for 3 days in an ice chest to allow complete deposition of the suspended matter. The clear supernatant was diluted 2.5 times with water and boiled briefly with active charcoal (108 Gm.). On concentrating the colorless filtrate to about 150 ml. and cooling, colorless crystalline needles of crude D-mannitol separated slowly over a number of days. The crystals were, however, collected after 3 days and further purified by passing, in aqueous solution, through a column of ion-exchange resin[1] (H) to remove traces of calcium salts that were found to be present. Yield, about 1%.

Better yield (10.9%) of D-mannitol was obtained by soxhleting the root powder with 95% ethanol for several hours, most of the extracted D-mannitol crystallizing during the extraction process. After final purification by treatment with the resin[1] and crystallization from hot 95% ethanol, the substance melted at 166° (corrected).

Anal.—Calcd. for $C_6H_{14}O_6$: C, 39.55; H, 7.74; mol. wt., 182. Found: C, 39.67; H, 7.67; mol. wt. (ebullioscopic), 188.9.

Characterization as D-Mannitol

Solubility—The isolated substance was very slightly soluble in hot 95% ethanol and insoluble in ether, chloroform, petroleum ether, benzene, and ethyl acetate. It was highly soluble in water, the solution being neutral to litmus.

Reactions—It did not reveal the presence of aromatic character or the aldehydic, ketonic, carboxyl, and phenolic groups or unsaturation. The substance, however, changed the color of ceric ammonium nitrate reagent to orange red and discharged the pink color of 1% borax solution due to phenolphthalein, the color reappearing on warming. Like D-mannitol, it prevented the precipitation of $Fe(OH)_3$ on addition of NaOH solution to a solution of $FeCl_3$ and gave a flocculent precipitate with an ammoniacal solution of copper sulfate. Its melting point on admixture with a pure authentic sample of D-mannitol was undepressed.

Optical Rotation—An aqueous solution (10%) of the substance gave no evidence of optical activity while 10% solution in 0.2 *N* borax gave $[\alpha]_D^{25}$ +30° after 3 hr.

Chromatographic Identification—The substance was found to be chromatographically homogeneous with the authentic sample on Whatman No. 1 filter paper using butanol–ethanol–water solvent (4:1.1:1.7). Its mobility was impeded on borated chromatographic strips, which is characteristic of D-mannitol.

Derivatives—The substance gave hexa-acetyl, hexanitro, hexabenzoyl derivatives, and a benzaldehyde condensation product. The properties of these derivatives agree with those given in literature for such derivatives of D-mannitol.

Hexa-acetate—Obtained readily by treatment with acetic anhydride and fused sodium acetate, m.p. 122° (corrected).

Anal.—Acetyl value: Calcd. for $C_6H_8O_6$ $(Ac)_6$: 59.47. Found: 61.5.

Hexanitrate—Prepared by treating this substance (1 part) dissolved in concentrated HNO_3 (5 parts) with concentrated H_2SO_4 (10 parts) in cold, m.p. 112° (corrected).

Received November 28, 1966, from the Pharmacology Department, Gandhi Medical College, Bhopal, India.

Accepted for publication December 30, 1966.

The authors thank Dr. S. S. Gupta of this Department, Dr. Sukh Dev, National Chemical Laboratory, Poona, India, Dr. P. L. Narasimha Rao, Indian Institute of Science, Bangalore, India, Mr. S. B. Garg and Dr. S. S. Kanhare, M. V. M. College, Bhopal, India, Dr. Sayeeda Shinde, G. R. Medical College, Gwalior, India, and the Indian Council of Medical Research for generous grants.

[1] Marketed as Amberlite IRC-50 by Rohm & Haas, Philadelphia, Pa.

Anal.—Calcd. for $C_6H_8N_6O_{18}$: N, 18.59. Found: N, 18.50.

Hexabenzoate—Prepared by treating the substance with benzoyl chloride in presence of pyridine, m.p. 148° (corrected).

Benzaldehyde Condensation Product—The condensation (4) of D-mannitol (100 mg.) with benzaldehyde (200 mg.) was more satisfactorily achieved using phosphorus pentoxide (100 mg.) or concentrated sulfuric acid (2 drops), than with concentrated HCl. After shaking the mixture for 15 min., the product was washed with dilute sodium carbonate solution, water, and ether and crystallized from ethanol. The condensation product melted at 220° (corrected) (*cf.* m.p. 223–224°) (4).

REFERENCES

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Observations Concerning the Correlation of *In Vitro* Sulfonamide Activity with pKa and the Hammett Values

By ARTHUR CAMMARATA and RICHARD C. ALLEN*

Linear correlations of the *in vitro* bacteriostatic activity of sulfonamides with pKa provide little evidence in support of there being a definite pKa which a sulfonamide must possess in order to exhibit maximum activity.

Bell and Roblin (1) first reported a correlation between the *in vitro* bacteriostatic activity of a series of sulfonamides and their pKa values. They summarized their proposition in the statement: "the more negative the SO_2 group of a sulfonamide type compound, the greater the bacteriostatic activity of the compound . . . . .; the correlation between acid dissociation (pKa) and the activity is shown to be directly associated with the negative character of the SO_2 group." Seydel, Krüger-Thiemer, and Wempe (2, 3) obtained the polarizability of the SO_2 group by infrared spectrophotometric measurement of the S-O force constant. They reported that the SO_2 polarizability had no relation to the *in vitro* bacteriostatic activity of the sulfonamides, and the hypothesis of Bell and Roblin is thus cast into doubt. However, the experimental data which led to the Bell and Roblin hypothesis—namely, the correlation of activity with pKa is widely accepted (4, 5). Implications which are derived from the data of Bell and Roblin, and which are considered to be valid are: (*a*) a sulfonamide must possess a pKa which lies within a definite pKa region (6.0–7.5) in order to exhibit maximum activity; (*b*) sulfonamides having a pKa which lies to either side of this region exhibit decreasing activities.

Seydel (6) has recently shown that the *in vitro* bacteriostatic activities for an homologous series of N^1-phenylsulfanilamides are linearly correlated with their respective Hammett σ values. The correlation obtained provided a line of negative slope. In the present report, the authors present other correlations, obtained from the data of Bell and Roblin (1) and supplemented by the data of Seydel, Krüger-Thiemer, and Wempe (2, 3, 6, 7), whose activities correlate linearly with both pKa and with σ values[1] and for which lines of both positive and negative slopes are obtained. In light of these data, it is pointed out that there is little evidence to support the implications derived from the Bell and Roblin correlation; *i.e.*, that a sulfonamide must have a *definite* pKa (in the range of 6.0–7.5) in order to exhibit maximum activity.

If one classifies the activity-pKa data of Bell and Roblin in terms of homologous series it is noted that: (*a*) in the region where pKa = 6–11, the Bell and Roblin "curve" actually consists of a number of incomplete homologous series, each of which describes a straight line of negative slope; (*b*) in the region where pKa = 2–6, a limited number of homologous compounds can be found (we were able to find no more than three) to which a line of positive slope can be ascribed. In these terms, for the maximum in the Bell and Roblin curve to constitute a true maximum, it would be expected that one set of homologous series would afford lines of positive slope (pKa 2–6), another set of homologous series would afford lines of negative slope (pKa 6–11), and each set would intercept at the accepted maximum.

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TABLE I—ACTIVITY-pKa CORRELATIONS FOR FAMILIES OF SULFONAMIDES

	pKa	$C_R \times 10^5$ (M)[a,b]	Ref.
N¹-(Substituted) Phenylsulfanilamides			
Substituents			
4-NO_2	6.8	0.8	(2)
4-CH_3NHSO_2	7.3	0.2	(2)
4-H_2N-SO_2	7.85	0.5	(1)
3-H_2N-SO_2	8.23	2.0	(1)
4-Cl	8.6	3.2	(2)
H	9.60	3.0	(1)
3-CH_3	9.74	5.0	(1)
4-CH_3	9.82	5.0	(1)
2-CH_3	9.96	10.0	(1)
4-NH_2	10.22	5.0	(1)
Substituted N¹-Benzoylsulfanilamides			
meta and/or *para* Substituents			
H	4.57	0.3	(1)
4-$(CH_3)_2CH$	4.70	0.4	(3)
4-CH_3	4.7	0.4	(3)
4-CH_3CH_2	4.7	0.24	(3)
3-CH_3	4.75	0.4	(3)
4-$CH_3CH_2CH_2$	4.76	0.67	(3)
3,4-CH_3	4.95(4.86)	0.4(0.36)	(2, 3)
4-$(CH_3)_2CH$—O	4.9	0.4	(2)
3-CH_3, 4-CH_3O	4.9	0.56	(3)
4-NH_2	5.20	0.5	(1)
ortho Substituents			
2,4,5-CH_3	5.1	0.8	(3)
2,4-CH_3	5.1	2.25	(3)
2-CH_3	4.90	2.68	(3)
2,5-CH_3	5.05	2.68	(3)
2,4,6-CH_3	5.1	about 6.4	(3)
2,3,4,5,6-CH_3	5.25	about 51.2	(3)
2-Sulfanilamido (Substituted) Thiadiazoles			
Substituents			
H	4.77	0.6	(1)
5-CH_3CH_2	5.1	0.2	(2)
5-CH_3	5.45	0.2	(1)
N¹-(Substituted) Methylsulfanilamides			
$CON(CH_2CH_3)_2$	10.1	409.6	(2)
H	10.77	30.0	(1)
	10.88	20.0	(1)
CH_2OH	10.92	50.0	(1)
$SO_3^-NH(CH_2CH_2OH)_3$[c]	7.6	51.2	(2)
2-Sulfanilamido (Substituted) Pyridines			
5-Br	7.15	0.5	(1)
H	8.43	0.6	(1)
5-NH_2	8.47	0.6	(1)
2-Sulfanilamido (Substituted) Pyrimidines			
H	6.48	0.08	(1)
4-CH_3	7.06	0.2	(1)
4,6-CH_3	7.37	0.3	(1)
4-NH_2	7.44	20.0	(1)

[a] Minimum inhibitory concentration for bacteriostasis of *E. coli*. [b] The actual concentration was employed in evaluating $\log 1/C_R$. [c] This is the only ionic substituent in this series, which may, in part, account for its departure from the line.

very end of the Bell and Roblin curve (pKa 10–11); and, the *ortho*-substituted members of the N^1-benzoylsulfanilamide series, which yields an almost vertical line bisecting the Bell and Roblin curve at about pKa 5. These last two series have not been considered as reliable test series in our study, for there is reason to believe that the mechanism of action for these compounds may differ from that of the other series considered.

Figure 2 shows the correlation of activity with σ for the N^1-benzoylsulfanilamides.[2] It demonstrates a definite break in the line when the transition is made from *meta* and *para* substituted derivatives to the *ortho* substituted ones. If the analogy can be made between the Hammett equation and the equation which correlates activity with σ:

$$\log 1/C_R = \alpha\sigma + \log 1/C_R^\circ$$

[2] Hammett values were obtained from Jaffé's compilation (8); *ortho* values are those given by Bray and Barnes (9).

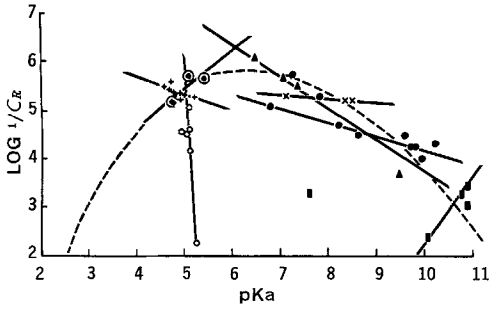
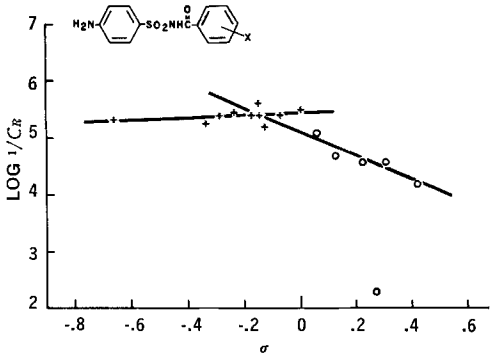


Fig. 1—Key: ⊙, *2-sulfanilamido (substituted) thiadiazoles;* +, meta *and/or* para-*substituted* N^1-*benzoylsulfanilamides;* O, ortho-*substituted* N^1-*benzoylsulfanilamides;* ▲, *2-sulfanilamido (substituted) pyrimidines;* ×, *2-sulfanilamido (substituted) pyridines;* ●, N^1-*(substituted) phenylsulfanilamides;* ■, N^1-*(substituted) methylsulfanilamides.*



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where α defines the "intrinsic" activity which may be associated with a given homologous series, and where log $1/C_R^\circ$ is an arbitrary reference activity, then the break in the line could be interpreted as a possible change in the mechanism of action. This would follow from similar considerations of the Hammett equation in relation to chemical reaction mechanisms (10).

It has been shown (11) that sulfonamides bearing an N^1-aromatic substituent are capable of binding at an enzyme site through each of their aromatic rings. Since all the series considered in this study possess an N^1-aromatic ring except the N^1-(substituted) methylsulfanilamides, the latter is not considered a reliable test series. It is quite conceivable that binding differences, coupled with diminished pKa values, may account for the divergent pattern in activities exhibited by *ortho*-substituted N^1-benzoyl-sulfanilamides.

The linear correlation of *in vitro* bacteriostatic activity with pKa, which is observed in this study for homologous series of sulfonamides, provides little evidence in support of there being a definite pKa which a sulfonamide must possess in order to exhibit maximum activity. Rather, from the data at hand, it appears that many sulfonamides lying outside of the "prescribed" Bell and Roblin maximum, notably those containing electron withdrawing substituents on an N^1-aromatic ring, are potentially capable of high *in vitro* activities, provided they can penetrate the bacterial cell wall.

REFERENCES

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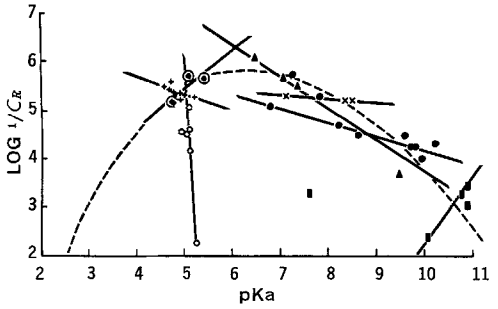
Gas Chromatography of Alkyl Ether Derivatives of *p*-Hydroxybenzoate Esters

By MERRILL WILCOX

A procedure for the separation and estimation of mixtures of the four lower normal alkyl *p*-hydroxybenzoates as derivatives is described. The esters were converted to phenyl alkyl ethers by means of diazoalkanes. Diazoethane, diazo-*n*-propane, diazoisobutane, and diazo-*n*-butane, when catalyzed with 0.007 per cent boron trifluoride, alkylated essentially quantitatively in 30 min. at room temperature. Variable yields were noted in the absence of the catalyst for reaction times as long as 18 hr. Diazomethane did not alkylate quantitatively when catalyzed in the same manner. Derivatives prepared from mixtures of the four esters were resolved on the gas chromatograph regardless of the diazoalkane used.

THE ALKYL *p*-hydroxybenzoate esters are important food and pharmaceutical preservatives. Lach and Sawardeker (1) list several reviews of the role of these esters as preservatives. They also review the general methods of analysis which have been published. An additional general method of analysis has recently appeared (2). Higuchi *et al.* (3) developed an assay using column chromatography and U.V. spectrophotometry which could be used to resolve mixtures of the esters. Lach

Received November 17, 1966, from the Agronomy Department, Agricultural Experiment Station, University of Florida, Gainesville, FL 32601
Accepted for publication January 19, 1967.
This investigation was supported in part by grant E294B from the American Cancer Society and grant GB4420 from the National Science Foundation.
Florida Agricultural Experiment Station Journal Series No. 2582.



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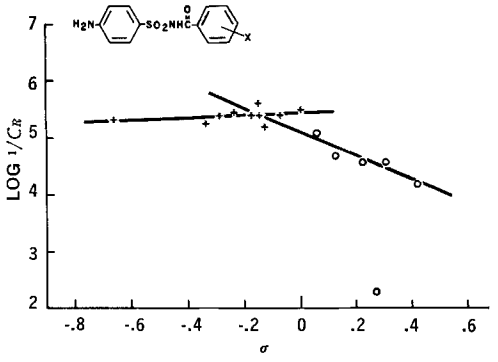

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(11) Jardetzky, O., and Wade-Jardetzky, N. G., *Mol. Pharmacol.*, **1**, 214(1965).

Gas Chromatography of Alkyl Ether Derivatives of *p*-Hydroxybenzoate Esters

By MERRILL WILCOX

A procedure for the separation and estimation of mixtures of the four lower normal alkyl *p*-hydroxybenzoates as derivatives is described. The esters were converted to phenyl alkyl ethers by means of diazoalkanes. Diazoethane, diazo-*n*-propane, diazoisobutane, and diazo-*n*-butane, when catalyzed with 0.007 per cent boron trifluoride, alkylated essentially quantitatively in 30 min. at room temperature. Variable yields were noted in the absence of the catalyst for reaction times as long as 18 hr. Diazomethane did not alkylate quantitatively when catalyzed in the same manner. Derivatives prepared from mixtures of the four esters were resolved on the gas chromatograph regardless of the diazoalkane used.

THE ALKYL *p*-hydroxybenzoate esters are important food and pharmaceutical preservatives. Lach and Sawardeker (1) list several reviews of the role of these esters as preservatives. They also review the general methods of analysis which have been published. An additional general method of analysis has recently appeared (2). Higuchi *et al.* (3) developed an assay using column chromatography and U.V. spectrophotometry which could be used to resolve mixtures of the esters. Lach

Received November 17, 1966, from the Agronomy Department, Agricultural Experiment Station, University of Florida, Gainesville, FL 32601
Accepted for publication January 19, 1967.
This investigation was supported in part by grant E294B from the American Cancer Society and grant GB4420 from the National Science Foundation.
Florida Agricultural Experiment Station Journal Series No. 2582.

and Sawardeker (1) and Donato (4) have separated and assayed the esters as their trimethylsilyl ethers by means of gas chromatography. Higher diazoalkanes have been found useful in gas chromatography of phenolic acids (5) because of their superior alkylating activity relative to diazomethane. The alkylating activity of diazomethane has been greatly increased by means of boron trifluoride catalysis (6). Alkyl ethers of phenolic acids have been demonstrated to be somewhat more stable than the corresponding trimethylsilyl ethers (7) in gas chromatography. This report describes the suitability of some higher diazoalkanes under boron trifluoride catalysis for preparation of derivatives of *p*-hydroxybenzoate esters for gas chromatography.

EXPERIMENTAL

Apparatus and Materials—A Perkin-Elmer 202 recording spectrophotometer having linear wavelength presentation was used. The gas chromatograph was a Barber-Colman 10c, equipped with a hydrogen flame ionization detector. The smallest detectable quantity of derivative was approximately 50 nanograms. Sample concentrations were chosen so that injection volumes ranged between 1 and 5 μl. Naphthalene was used as an internal standard. The injection heater and detector were maintained at a temperature 50° above that of the column.

Two columns were used. A stainless steel column 50 M. by 0.25 mm. i.d. coated internally with high vacuum paraffin grease[1] was operated isothermally at 200°, with column flow of 18 ml. argon per minute and inlet pressure of 60 p.s.i. A pre-column splitter allowed 23% of the sample to flow into the column and exhausted the remainder. In later experiments a glass column 2 M. by 4 mm. i.d. without the splitter was substituted. It was packed with 1.5% SE-30 polyester on 90–100 mesh Anakrom ABS and was operated isothermally at 135° with a column flow of 240 ml. argon per minute and an inlet pressure of 28 p.s.i.

Diazomethane was prepared without distillation from *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (5) (Aldrich Chemical Co.). Higher diazoalkanes were prepared without distillation from *N*-alkyl-*N*-nitrosoureas (5). All were used within 30 hr. of their preparation. Alkyl *p*-hydroxybenzoates were provided by Tenneco Chemicals, Inc. Methanolic boron trifluoride (14%, Applied Science Laboratories) was diluted to 0.7% with anhydrous reagent methanol.

Methods—The *p*-hydroxybenzoate esters (usually 10 mg.) were dissolved in 0.5 ml. methanol and treated at room temperature with 3 ml. ethereal diazoalkane, or enough to retain a yellow color. One drop (.03 ml.) of 0.7% methanolic boron trifluoride was added to those samples where catalysis was desired. At the end of the reaction period, the excess diazoalkane was destroyed by bubbling carbon dioxide through the solution. These samples were either injected into the gas chromatograph without further treatment or were evaporated under nitrogen and made to volume in dichloromethane which gave a very narrow solvent peak. Samples used for alkylation assays were treated similarly except that they were made to volume in water rather than dichloromethane.

A phenolate difference spectrum assay was used to measure the remaining phenolic groups after alkylation. A 5-ml. sample aliquot was added to 5 ml. of 0.2 *M* phosphate buffer (pH 10) and read against a reference solution containing an identical aliquot of sample diluted with 5 ml. of 0.2 *M* phosphate buffer (pH 7). The pH values of the solutions were checked by means of a Photovolt model 110 meter after addition of buffer. A graphic method was used to measure the absorbance (*A*). A base line was drawn between the points at 270 and 340 mμ on the spectrum. The height of the curve was then read at 320 mμ relative to the base line, giving the absorbance directly. Since the instrument had linear wavelength presentation, this was equivalent to:

$$A_{\text{corrected}} = A_{320\ m\mu} - \frac{2}{7} A_{270\ m\mu} - \frac{5}{7} A_{340\ m\mu}$$

Calibration curves were prepared in this manner for concentrations which were equivalent to a range of from 0.1 to 10% unalkylated phenol. The data followed Beer's law throughout this range, which included all values of practical interest in this work.

RESULTS AND DISCUSSION

Alkylation—The alkylation activity among the diazoalkanes generally increased with chain length but was not consistently quantitative after uncatalyzed reaction periods as long as 18 hr. Catalysis with 0.007% boron trifluoride greatly increased yields. Alkylation yields following catalyzed reaction periods of 30 min. appear in Table I. The data are averages of single determinations from each of two alkylations in a typical experiment. Reaction times of 2.5 hr. were necessary for consistent alkylation of 95% or better by catalyzed diazomethane.

Chromatography—The relative retention times of the derivatives when chromatographed on the capillary column are listed in Table II. All gave single symmetrical peaks except the *n*-butyl ethers

TABLE I—PER CENT ALKYLATION OF *p*-HYDROXYBENZOATE ESTERS BY DIAZOALKANES CATALYZED WITH .007% BORON TRIFLUORIDE FOR 30 min. AT ROOM TEMPERATURE

	% Alkylation by Diazoalkane				
Ester	Methyl	Ethyl	*n*-Propyl	Iso-butyl	*n*-Butyl
Methyl	<90	97.0	99.8	99.5	99.9
Ethyl	<90	99.2	99.9	99.8	99.6
Propyl	<90	99.6	99.9	99.9	99.9
Butyl	<90	99.5	99.9	99.9	99.9

TABLE II—RELATIVE RETENTION TIMES OF ALKYL ETHERS OF *p*-HYDROXYBENZOATE ESTERS ON PARAFFIN GREASE CAPILLARY COLUMN

	Retention Time of Alkyl Ether Relative to Naphthalene[a] Internal Std.				
Ester	Methyl	Ethyl	*n*-Propyl	Iso-butyl	*n*-Butyl
Methyl	1.3	1.7	2.5	2.9	4.0
Ethyl	1.8	2.2	3.2	3.7	4.9, 5.4[b]
Propyl	2.6	3.4	5.0	5.5	7.2, 7.8[b]
Butyl	4.0	5.2	7.7	8.6	13.3

[1] Marketed as Apiezon L by the James G. Biddle Co., Philadelphia, Pa.

[a] The absolute retention time of naphthalene was 0.90 min. at 200°, 18 ml. argon carrier per min. [b] The second peak appeared to be due to decomposition on the column.

TABLE III—RELATIVE RETENTION TIMES OF ALKYL ETHERS OF *p*-HYDROXYBENZOATE ESTERS ON COLUMN PACKED WITH 1.5% SE-30 POLYESTER ON ANAKROM ABS

Ester	Retention Time of Alkyl Ether Relative to Naphthalene[a] Internal Std.		
	Ethyl	*n*-Propyl	*n*-Butyl
Methyl	2.0	2.5	3.8
Ethyl	2.4	3.2	4.8
Propyl	3.3	4.6	7.3
Butyl	5.0	7.0	11.2

[a] The absolute retention time of naphthalene was 1.0 min. at 135°, 240 ml. argon per min.

of ethyl and propyl *p*-hydroxybenzoate. These two derivatives each gave two overlapping peaks, the first being symmetrical, the second being lower with considerable tailing. The author suspects this effect was due to decomposition on the column. Single peaks without tailing were observed for the *n*-butyl ethers when the column temperature was lowered to 170°. For this reason selected derivatives were chromatographed on a column packed with 1.5% SE-30 polyester on 90-100 mesh Anakrom ABS at a temperature designed to give similar retention times. The data which appear in Table III indicate that this packed column under these conditions is more suitable than the capillary column. The four esters thus may be resolved within each set of ether derivatives under appropriate conditions. Quantitative work was restricted to diazoethane and diazopropane since they produced stable derivatives in good yields. Plots of peak height *versus* concentration were linear for the lower three esters, as were plots of peak area *versus* concentration for the butyl ester when using ethyl derivatives on the capillary column. Plots of peak height *versus* concentration were linear when using propyl derivatives of the four esters on the packed column. This was true whether the esters were alone or mixed on either column.

The results indicate that higher diazoalkanes, when catalyzed with boron trifluoride, are useful for preparation of derivatives for gas chromatography of *p*-hydroxybenzoate esters. Now that *N*-alkyl-*N*-nitroso-*N'*-nitroguanidine precursors of higher diazoalkanes are commercially available,[2] their use should be considered for gas chromatography of these or other phenolic acids.

SUMMARY

Methyl through butyl esters of *p*-hydroxybenzoic acid have been converted to alkyl ethers by means of diazoalkanes catalyzed with .007% boron trifluoride. These ethers were easily resolved on the gas chromatograph. Ethers prepared from diazoethane and diazopropane were found suitable for quantitative work. The method seems generally applicable among phenolic acids where alkylating activity greater than that of diazomethane is needed.

REFERENCES

(1) Lach, J. L., and Sawardeker, J. S., *J. Pharm. Sci.*, **54**, 424(1965).

(2) Inamine, S., Obatake, A., and Matsuda, T., *Hakko Kogaku Zasshi*, 43, 97(1965).

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[2] Cyclo Chemical Corp., Aldrich Chemical Co., and K & K Chemical Co.

Syntheses of Some Hydroindolizines by Reductive Cyclization

By JOSEPH SAM and K. APARAJITHAN

The syntheses of dodecahydrobenzo[*b*] indolizine, octahydrocyclopenta[*b*] indolizine, decahydronaphth[2,1-*b*] indolizine, and 7-methoxydecahydronaphth[2,1-*b*]-indolizine are reported. The preparations of pyridylmethylene and pyridylmethyl derivatives of cyclopentanone, cyclohexanone, 1-tetralone, and 7-methoxy-1-tetralone also are described. Some preliminary pharmacological observations in mice are included.

THE SYNTHESIS of dodecahydrobenzo[*b*] indolizine (11-perhydroazafluorene) (II*b*) involving several steps was reported by Prelog and associates (1). Subsequently the authors reported (2) a two-step synthesis of II*b* by reductive cyclization of I at 200° and 2000 p.s.i. (Scheme I) and also the low pressure (50–60 p.s.i.) catalytic reductive cyclization of 2-pyridylmethylene-1-indanones (III) to the corresponding hydroindolizine (IV) (3, 4). (Scheme II.) The authors have now utilized the latter method for the synthesis of other hydroindolizines (II and XII) from 2-(2-pyridylmethylene)-cycloalkanones (IX and XI). The formation of IX*b* by the reaction of cyclohexanone (V*b*) with 2-pyridinealdehyde (VI) was investigated; however, only 2,6-bis-(2-pyridylmethylene) cyclohexanone (VII) was obtained. The synthesis of IX was accomplished by the reaction of 2-pyridinealdehyde with the morpholine enamine (VIII) (5, 6) of the cycloalkanone (V). The 3-, and 4-pyridylmethylenecycloalkanones also were prepared by this method using the appropriate pyridinealdehydes. The reaction of 2-pyridinealdehyde with 1-tetralones (X) occurred satisfactorily to yield the corresponding pyridylmethylene derivatives (XI). Some of the properties of the products from the reaction of pyridinealdehydes with various cycloalkanones are summarized in Table I.

A number of the unsaturated compounds (IX,

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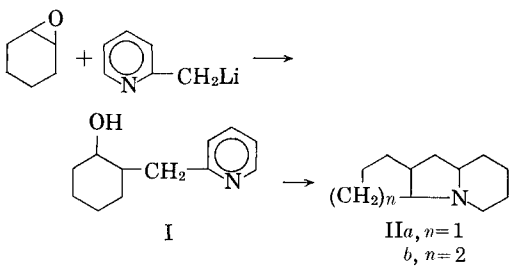
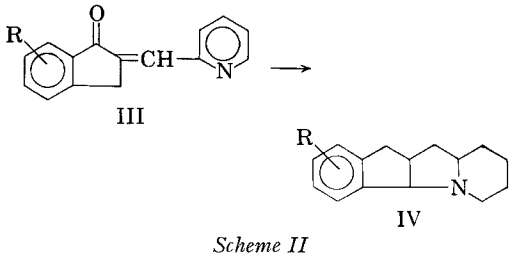


Scheme I



Scheme II

XI, and the corresponding 3- and 4-pyridyl derivatives) were catalytically (Pd-C) hydrogenated at 50–60 p.s.i. to the corresponding dihydro derivatives. The properties of the latter compounds are summarized in Table II.

The cyclization of IX and XI to the hydroindolizines (II and XII) was accomplished by low pressure catalytic (PtO_2) hydrogenation as described in earlier reports (3, 4). (Scheme III.) Elemental analysis and infrared spectrophotometry were used for the identification of the products. Gas–liquid chromatography of the hydroindolizines (Table III) indicated the presence of trace quantities of impurities. These, however, were readily removed during the recrystallization of the corresponding salts.

PHARMACOLOGY[1]

Acute effects after intraperitoneal administration to albino mice were observed for five of the pyridylmethylenecycloalkanones (Table I, compounds *13, 16, 17, 18, 20*), six of the pyridylmethylcycloalkanones (Table II, compounds *21, 22, 24, 27, 28, 33*) and three of the indolizines (Table III, compounds *38, 42, 46*). Gross symptomatology at toxic and subtoxic dosages was noted in a fashion similar to that described by Irwin (7) and approximate LD_{50}'s (48 hr.) were determined by the method of Horn (8) utilizing five mice per dosage level.

Most of the compounds showed LD_{50}'s in a moderate dosage range (147 to 562 mg./Kg.) with the exceptions of compound *20* which produced deaths only at greater than 1.0 Gm./Kg., and of the three indolizines which all had LD_{50}'s below 100 mg./Kg. (26 to 93 mg./Kg.). Excitation was prominent after compounds *13, 16, 17, 18, 20, 24, 27,* and *28*. Compound *20* differed in that it evoked convulsions which were delayed 2–3 hr. after administration. Muscular incoordination and/or tremors were prominent after compounds *21, 28, 33, 38, 42,* and *46*. Predominant symptoms of CNS depression were seen at all dosages of compounds *21* and *22*. Sedation seemed to occur at lower dosages of compounds *13* and *24*.

EXPERIMENTAL[2]

2-(Pyridylmethylene) Cycloalkanones (Table I)—*Method A*—A mixture of 0.4 mole of freshly distilled cycloalkanone, 40 Gm. (0.45 mole) of freshly distilled morpholine, 0.5 Gm. of *p*-toluenesulfonic acid, and 100 ml. of dry benzene was heated under reflux for 18 hr. with a Dean-Stark water separator. The benzene solution was distilled under reduced pressure (water-aspirator) to give the desired enamine (5) of cyclopentanone and cyclohexanone.

A solution of 0.3 mole of *N*-(1-cycloalkenyl)morpholine and 28 Gm. (0.26 mole) of pyridinealdehyde in 100 ml. of dry toluene or benzene was refluxed for 1 to 7 days with a Dean-Stark apparatus. The reaction mixture was cooled, treated with 120 ml. of 6 *N* hydrochloric acid, and thereafter stirred for 2 hr. The lower acid layer was separated, washed with ether, and then neutralized with sodium bicarbonate. The oil (or solid) that separated was extracted with benzene, washed with water, and dried (anhydrous magnesium sulfate). Distillation of the benzene solution under reduced pressure gave the desired product (Table I). The infrared spectrum showed strong absorption at about 5.75–5.9 μ.

2-(Pyridylmethylene)-1-tetralones—*Method B*—A mixture of 0.15 mole of the 1-tetralone, 4 ml. of acetic acid, 5 ml. of piperidine, and 0.15 mole of pyridinealdehyde was heated on a steam bath for 0.5 to 3 hr. The lower boiling materials were removed by distillation (water-aspirator). The residue was dissolved in benzene and extracted with dilute hydrochloric acid. The acid layer was separated and treated with dilute sodium hydroxide. The solid was removed by filtration and recrystallized from a suitable solvent (Table I). The infrared spectrum showed strong carbonyl absorption at about 5.95 μ.

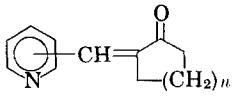
2 - Pyridylmethylcycloalkanones—*Method C*—A solution of 0.1 mole of the 2-pyridylmethylene compound in 100 ml. of ethanol was hydrogenated at 50 p.s.i. at room temperature with 1 Gm. of 5% Pd/C catalyst in a Parr low pressure hydrogenator. The product was isolated in the usual manner and distilled (Table II). The infrared spectrum showed strong carbonyl absorption at about 5.95 μ.

Indolizines (Table III)—A solution of 0.025 mole of cycloalkanone in a mixture of 50 ml. of glacial acetic acid and 15 ml. of freshly distilled acetic anhydride was hydrogenated at 47 p.s.i. with 0.1 Gm. of platinum oxide catalyst. The absorption of hydrogen was rapid in the first hr. and was complete in 24 hr. The catalyst was removed by filtration; the filtrate was treated with 15 ml. of water and then allowed to remain at room temperature for 2 hr. The solvents were distilled; the residue was treated with 75 ml. of water and filtered. The filtrate was saturated with sodium bicarbonate and extracted with chloroform. The chloroform extract was washed with water and dried over anhydrous magnesium sulfate. The chloroform solution was distilled under reduced

[1] The authors are grateful to Dr. W. Marvin Davis, Department of Pharmacology, School of Pharmacy, University of Mississippi, University, for the pharmacological data.

[2] All melting points were taken on a Fisher-Johns apparatus and are uncorrected; boiling points are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 Infracord spectrophotometer.

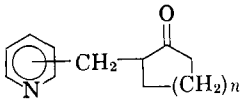
TABLE I—PYRIDYLMETHYLENE CYCLOALKANONES



Compd.	Isomer[a]	n	Method; React. Time, hr.	Recrystn. Solvent[b]	Yield, %	B.p. (mm.) and/or M.p., °C.	Molecular Formula	Anal., % Calcd. C	H	N	Found C	H	N
1	2	1	A; 24	M-W	78	132–4 (0.3) 49–51	$C_{11}H_{11}NO$	76.3	6.4	8.1	76.0	6.3	8.2
2	2	1	...	E	...	162–164	$C_{12}H_{14}INO$[c]	45.7	4.5	4.5	45.6	4.6	4.1
3	2	1	...	W	...	161–162	$C_{17}H_{14}N_4O_8$[d]						
4	3	1	A; 60	...	80	134–138 (0.8)	$C_{11}H_{11}NO$	76.3	6.4	8.1	76.3	6.6	8.2
5	3	1	...	E	...	164–166	$C_{12}H_{14}INO$[c]	45.7	4.5	4.5	45.7	4.8	4.4
6	3	1	...	W-A	...	203–205	$C_{17}H_{14}N_4O_8$[d]						
7	4	1	A; 72	...	68	158–159 (0.2)	$C_{11}H_{11}NO$	76.3	6.4	8.1	75.8	6.5	8.4
8	4	1	...	W	...	190–191	$C_{12}H_{14}INO$[c]						
9	4	1	...	W	...	161–162	$C_{17}H_{14}N_4O_8$[d]						
10	2	2	A; 108	...	29	122 (0.3)	$C_{12}H_{13}NO$	77.0	7.0	7.5	77.1	7.1	7.6
11	3	2	A; 160	...	47	166 (2.1)	$C_{12}H_{13}NO$						
12	3	2	...	W-A	...	171	$C_{18}H_{16}N_4O_8$[d]	51.9	3.9	13.5	52.6	3.9	13.2
13	3	2	...	Ac-P	...	106–107	$C_{13}H_{16}INO$[c]						
14	4	2	A; 96	...	49	141–146 (0.8)	$C_{12}H_{13}NO$						
15	4	2	...	W-A	...	167–168	$C_{18}H_{16}N_4O_8$[d]	51.9	3.9	13.5	51.9	3.9	13.5
16	4	2	...	E-Ea	...	147–148.5	$C_{13}H_{16}INO$[c]	47.4	4.9	4.3	47.4	5.2	4.1
17	2	1-Tetralone[e]	B; 0.5	M	68	119–120	$C_{16}H_{13}NO$	81.7	5.6	6.0	81.4	5.6	6.0
18	3	1-Tetralone[e]	B; 3	C	23	73.5–74	$C_{16}H_{13}NO$	81.7	5.6	6.0	81.8	5.7	5.8
19	4	1-Tetralone[e]	B; 1.5	C	51	114–115	$C_{16}H_{13}NO$	81.7	5.6	6.0	81.6	5.6	6.2
20	2	7-Methoxy-1-tetralone[e]	B; 0.5	C	68	92–93	$C_{17}H_{15}NO_2$	77.0	5.7	5.3	77.1	5.7	5.4

[a] Refers to position of the substituent in the pyridine ring. [b] A, acetic acid; Ac, acetone; C, cyclohexanone; E, ethanol; Ea, ethyl acetate; M, methanol; P, petroleum ether (30–60°); W, water. [c] Methiodide. [d] Picrate. [e] Entire cycloalkanone moiety.

TABLE II—PYRIDYLMETHYLCYCLOALKANONES

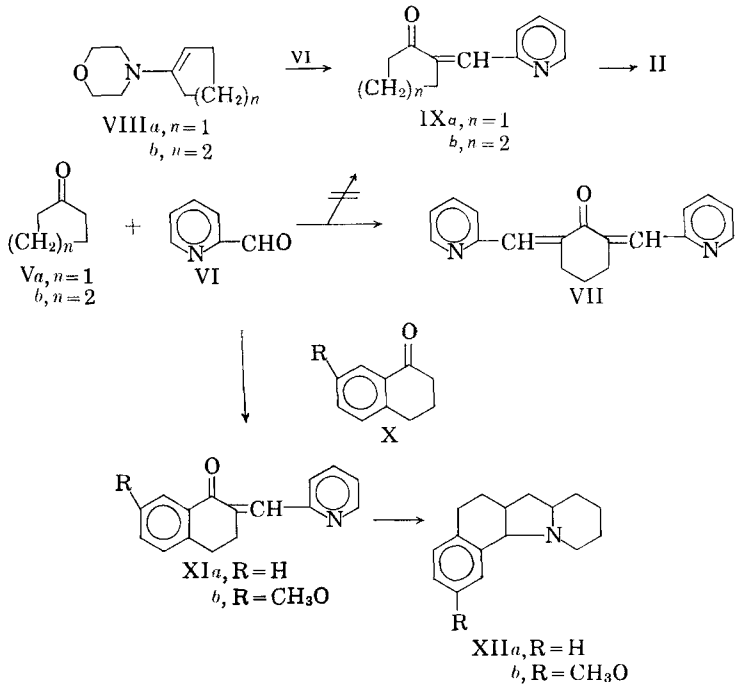


Compd.	Isomer[a]	n	React. Time, hr.[b]	Recrystn. Solvent[c]	Yield, %	B.p. (mm.) and/or M.p., °C.	Molecular Formula	Anal., % Calcd. C	H	N	Found C	H	N
21	2	1	3	...	70	132–133 (1.7)	$C_{11}H_{13}NO$						
22	2	1	...	W	...	191–193	$C_{12}H_{16}N_4O$[d]	62.0	6.9	24.1	61.3	7.1	23.8
23	2	1	...	E	...	123–124	$C_{12}H_{16}INO$[e]	45.4	5.1	4.4	45.5	5.1	4.3
24	3	1	3	...	86	122–123 (0.5)	$C_{11}H_{13}NO$						
25	3	1	...	E	...	114–115	$C_{12}H_{16}INO$[e]	45.4	5.1	4.4	45.5	5.1	4.4
26	3	1	...	W	...	118–119	$C_{17}H_{15}N_4O_8$[f]						
27	4	1	4	...	80	134–136 (1.3)	$C_{11}H_{13}NO$	75.4	7.5	8.0	75.4	7.5	8.2
28	4	1	...	Ac	...	95–96	$C_{12}H_{16}INO$[e]	45.4	5.1	4.4	45.3	5.0	4.3
29	4	1	...	W	...	136–138	$C_{17}H_{16}N_4O_8$[f]						
30	3	2	4	...	84	156–157 (3.0)	$C_{12}H_{15}NO$	76.2	8.0	7.4	75.2	7.9	7.9
31	3	2	...	W	...	142–143	$C_{18}H_{18}N_4O_8$[f]	51.7	4.3	13.4	52.2	4.2	13.3
32	4	2	4	...	80	132–133 (0.5)	$C_{12}H_{15}NO$	76.2	8.0	7.4	76.1	7.8	7.8
33	4	2	...	C	...	103–105	$C_{13}H_{18}INO$[e]	47.2	5.5	4.2	47.1	5.3	4.1
34	4	2	...	W-A	...	175–176	$C_{18}H_{18}N_4O_8$[f]	51.7	4.3	13.4	52.2	4.4	13.4
35	2	1-Tetralone[g]	0.5	P	92	181 (1.2) 70–71	$C_{16}H_{15}NO$	81.0	6.4	5.9	80.9	6.4	5.8
36	4	1-Tetralone[g]	2	P	88	174–177 (0.2) 62–64	$C_{16}H_{15}NO$	81.0	6.4	5.9	80.3	6.4	6.2

[a] Refers to position of substituent in pyridine ring. [b] Method C. [c] A, acetic acid; Ac, acetone; C, cyclohexanone; P, petroleum ether (30–60°); W, water. [d] Semicarbazone. [e] Methiodide. [f] Picrate. [g] Entire cycloalkanone moiety.

pressure to give the desired product. Carbonyl and hydroxyl group absorption was absent in the infrared spectra.

The hydrochlorides, picrates, and methiodides were prepared in the usual manner and recrystallized from a suitable solvent.



Scheme III

TABLE III—INDOLIZINES (II AND XII)

Compd.	Recrystd. from	n_D(°C.)	Yield, %	M.p., °C.	Molecular Formula	Anal., % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
37 (II*a*)	...	1.4922 (23)	65	106–108 (20 mm.)[a]	$C_{11}H_{19}N$						
38 (II*a*)	Acetonitrile	...	...	206–208	$C_{12}H_{22}IN$[b,c]	46.9	7.2	4.6	46.7	7.3	4.7
39 (II*a*)	Ethanol	...	...	211–212	$C_{17}H_{22}N_4O_7$[d]	51.8	5.6	14.2	51.9	5.8	13.9
40 (II*b*)	...	1.4967 (24)	44	126–130 (20 mm.)[a]	$C_{12}H_{22}N$[e]						
41 (II*b*)	Methanol	...	...	207–208	$C_{18}H_{28}N_4O_7$[d]						
42 (II*b*)	Acetonitrile	...	...	260–261	$C_{13}H_{24}IN$[b,f]	48.6	7.5	4.4	48.7	7.5	4.5
43 (XII*a*)	...	1.5620 (22)	68	125 (0.3 mm.)[a]	$C_{16}H_{21}N$	84.5	9.3	6.2	84.6	9.1	6.3
44 (XII*a*)	Methanol	...	...	215–217	$C_{22}H_{24}N_4O_7$[d]	57.9	5.3	12.3	58.1	5.3	12.7
45 (XII*b*)	...	1.5611 (23)	48	144–146 (0.2 mm.)[a]	$C_{17}H_{23}NO$	79.3	9.0	5.4	78.9	9.0	5.6
46 (XII*b*)	Acetonitrile	...	...	217–218	$C_{17}H_{24}ClNO$[g,h]	69.5	8.2	4.8	69.6	8.3	4.9

[a] Boiling point. [b] Methiodide. [c] Calcd. for I: 41.3. Found: 41.0. [d] Picrate. [e] *Reference 1*. [f] Calcd. for I: 39.5. Found: 39.4. [g] Calcd. for Cl: 12.1. Found: 12.1. [h] Hydrochloride.

REFERENCES

(1) Prelog, V., Frenkiel, L., and Szpilfogel, S., *Helv. Chim. Acta*, **29**, 484(1946).
(2) Sam, J., Plampin, J. N., and Alwani, D. W., *J. Org. Chem.*, **27**, 4543(1962).
(3) Sam, J., England, J. D., and Alwani, D. W., *J. Med. Chem.*, **7**, 732(1964).
(4) Sam, J., Alwani, D. W., and Aparajithan, K., *J. Heterocyclic Chem.*, **2**, 366(1965).
(5) Birkofer, L., and Barnikel, C. D., *Ber.*, **91**, 1996(1958) Hunig, S., Benzing, E., and Lucke, E., *ibid.*, **90**, 2833(1957).
(6) Szmuszkovicz, J., "Advances in Organic Chemistry: Methods and Results," vol. 4, Raphael, R. A., Taylor, E. C., and Wynberg, H., eds., Interscience Publishers, Inc., New York, N. Y., 1963, p. 1.
(7) Irwin, S., "Animal and Clinical Techniques in Drug Evaluation," Nodine, J. H., and Siegler, P. E., eds., Year Book Medical Publication, Inc., Chicago, Ill., 1964.
(8) Horn, H. J., *Biometrics*, **12**, 311(1956).

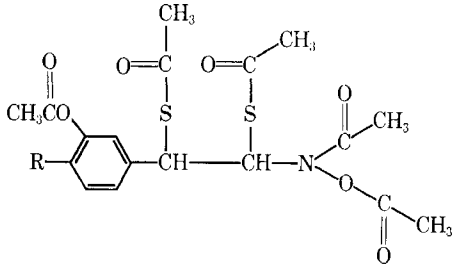
Potential Radiation Protective Agents V. Synthetic Approaches to 2-Aryl-2-mercaptoethylamines

By BENG-THONG HO and WALTER C. McCARTHY

β-Acetthiohydrocinnamoyl azide, through the Curtius degradation, gave 2-mercapto-2-phenylethylamine hydrochloride in low yield. Following the nitro-thiolacetate route to 2-aryl-2-mercaptoethylamines, two acetoxy-substituted β-nitrostyrenes were found to undergo an anomalous reaction with thiolacetic acid involving reduction of the nitro group, formation of elemental sulfur, and the isolation of products containing four more acetyl groups than the starting material.

THE CURTIUS degradation (1) route to 2-aryl-2-mercaptoethylamines was investigated. Cinnamic acid was converted through β-acetthiohydrocinnamic acid to the corresponding azide, which was rearranged and hydrolyzed to 2-mercapto-2-phenylethylamine hydrochloride. In view of the low yield, no attempt was made to extend this route to substituted members of the series.

The nitro-thiolacetate route (2, 3) to 2-aryl-2-mercaptoethylamines was studied to delineate further limitations of this synthetic sequence. Although several alkoxy-substituted β-nitrostyrenes had been shown to add thiolacetic acid to give the expected 1-aryl-1-acetthio-2-nitroethanes in good yield (3), two acetoxy-substituted β-nitrostyrenes were found to undergo a much more complex reaction under these conditions. The nitro group was reduced, elemental sulfur was formed, and the isolated products contained four more acetyl groups than the starting acetoxy-β-nitrostyrenes, as in structures I*a* and I*b*.



I

a, R = CH_3-CO_2-
b, R = H—

EXPERIMENTAL

β-Acetthiohydrocinnamoyl Chloride—β-Acetthiohydrocinnamic acid (4) was converted to its acid chloride by heating with thionyl chloride, followed by removal of the excess reagent under reduced pressure, and recrystallization from ligroin, in 99% yield, m.p. 81–82.8°.

2-Mercapto-2-phenylethylamine Hydrochloride—Activated sodium azide (5) (1.43 Gm., 0.022 mole) was added to a solution of 4.85 Gm. (0.02 mole) of β-acetthiohydrocinnamoyl chloride in 20 ml. of dry benzene, and the mixture was refluxed under nitrogen for 21 hr. After cooling, the solid matter was filtered, and the clear benzene filtrate was hydrolyzed by refluxing under nitrogen with 25 ml. of concentrated hydrochloric acid for 6 hr. Evaporation of the aqueous layer to dryness, followed by three recrystallizations from isopropyl alcohol gave 0.25 Gm. (7%) of 2-mercapto-2-phenylethylamine hydrochloride, m.p. 161–162.5°. The infrared spectrum was identical with that from the compound prepared *via* lithium aluminum hydride reduction of 1-phenyl-1-acetthio-2-nitroethane (2).

Upon cooling the separated benzene phase from the hydrolysis, a white solid crystallized, and subsequent recrystallization from a mixture of benzene and petroleum ether gave 0.56 Gm. of white crystals, m.p. 111.5–112.5°. The infrared spectrum of this product showed absorption peaks at 2565 cm.$^{-1}$ and 699 cm.$^{-1}$ characteristic of the mercapto group and at 1710 cm.$^{-1}$ characteristic of the carbonyl stretching vibration of the carboxyl group. The melting point is in agreement with that previously reported for β-mercaptohydrocinnamic acid (4, 6).

Evaporation of the benzene filtrate left 1.91 Gm. of gummy residue not further investigated.

N,O-Diacetyl-N-[β-(3,4-diacetoxyphenyl-)α,β-bis(acetthio)ethyl]hydroxylamine (Ia)—3,4-Diacetoxy-β-nitrostyrene (0.265 Gm., 0.001 mole) (7) was dissolved in 0.6 ml. (0.64 Gm., 0.008 mole) of thiolacetic acid by warming. After cooling, 5 drops of tri-*n*-butylamine was added. An exothermic reaction took place, and the mixture was allowed to stand overnight at room temperature. Filtration gave 0.295 Gm. of white solid, m.p. 166–167.5°, with a trace of yellow sulfur. (Addition of 10 ml. of 95% ethanol to the filtrate precipitated 26 mg. of yellow sulfur, m.p. 113–114.5°.) Successive recrystallization of the white solid from xylene, dioxane, and 95% ethanol raised the m.p. to 172–172.5°.

Anal.—Calcd. for $C_8H_5NO_3S_2Ac_6$: C, 49.48; H, 4.77; N, 2.88; O, 29.66; S, 13.21; acetyl, 53.2. Found: C, 49.40; H, 4.91; N, 3.01; O, 29.49; S, 13.07; acetyl, 54.0.

The infrared spectrum showed a carbonyl absorption peak at 1750 cm.$^{-1}$ and a thiolacetate peak at 1680 cm.$^{-1}$; the absorption peak of the nitro group at 1545 cm.$^{-1}$ was absent.

The NMR spectrum, in deuterochloroform at 60 Mc. with tetramethylsilane as internal reference, showed a 3-proton aromatic singlet at τ 2.84, two methine doublets centered at τ 3.67 and 4.86, J = 9.5 c.p.s., and six methyl singlets at τ 7.70, 7.74, 7.75, 7.79, 7.86, and 7.94.

3-Acetoxy-β-nitrostyrene—To a stirred solution of 10 Gm. (0.0608 mole) of *m*-acetoxybenzaldehyde, 8.2 ml. (0.152 mole) of nitromethane, and 60 ml. of 95% ethyl alcohol cooled to 5°, there was added 26 ml. of 10% (0.0604 mole) aqueous sodium hydroxide. The reaction time was limited to 30 sec. (8), after which the reaction was arrested by the addition of 200 ml. of 2% (0.067 mole) of aqueous acetic acid.

Received October 12, 1966, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication February 28, 1967.
Part of this work was done under contract DA-49-193-MD-2048 with the U. S. Army Medical Research and Development Command, Office of the Surgeon General, Washington, D. C.

A yellow oil (10.7 Gm.) separated. (Increasing the reaction time to 1 min. gave only half this yield of yellow oil.) The infrared spectrum of this material was consistent with that expected for the nitro-alcohol condensation product, but this material was sufficiently labile that it was not convenient to purify a sample for analysis. This oil was dehydrated by mixing with 10.7 Gm. of powdered fused sodium acetate and 53.4 Gm. (0.523 mole) of acetic anhydride and boiling under reflux for 10 min. After cooling, the mixture was poured into 120 ml. of water. A yellowish brown solid was obtained, 8.25 Gm., m.p. 76–79°. Recrystallization from 95% ethanol gave 6.35 Gm. (51%) of yellow crystals, m.p. 83.5–84.5°.

Anal.—Calcd. for $C_{10}H_9NO_4$: C, 57.97; H, 4.38; N, 6.76; O, 30.89. Found: C, 58.12; H, 4.50; N, 6.82; O, 30.93.

N,O-Diacetyl-N-[β-(3-acetoxyphenyl-)α,β-bis-(acetthio)ethyl]hydroxylamine (Ib)—3-Acetoxy-β-nitrostyrene (0.193 Gm.) was dissolved in 0.5 ml. of thiolacetic acid. Addition of 5 drops of tri-*n*-butylamine caused an exothermic reaction. The resulting solution was allowed to stand at room temperature for 20 hr. Upon addition of 1 ml. of 95% ethanol followed by cooling and scratching, there was precipitated 75 mg. of white solid, m.p. 110–111°. Recrystallization from ethanol did not raise the m.p.

Anal.—Calcd. for $C_8H_6NO_2S_2Ac_5$: C, 50.57; H, 4.95; N, 3.28; S, 15.00; acetyl, 50.3. Found: C, 49.76; H, 5.03; N, 3.39; S, 14.89; acetyl, 49.9.

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Oxidative Effect of Perbenzoic Acid on *N*-Methylated Pyrrole, Indole, and Carbazole

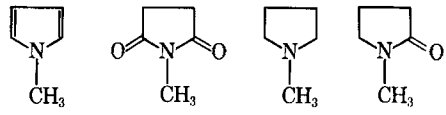
By I. NABIH and E. HELMY*

Perbenzoic has been shown to oxidize *N*-methylpyrrole to *N*-methylsuccinimide, *N*-methylpyrroline to *N*-methylpyrrolidone, and 2,3-dihydro-*N*-methylindole to *o*-dimethylaminobenzaldehyde. Under similar conditions *N*-methylcarbazole and 1,4-dihydro-*N*-methylcarbazole are not oxidized.

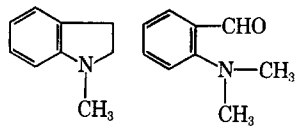
EXTENSIVE STUDIES have been carried out on the oxidation of pyrrole, indole, and carbazole using a variety of oxidizing agents (1–5).

In this work, the oxidative effect of perbenzoic acid on the *N*-methylated forms of pyrrole, indole, and carbazole is studied. Generally, peracids have been mostly used for the selective oxidation of the carbon-carbon double bond. As an electrophilic reaction, the rate is enhanced through the presence of an electron-donating group in the substrate (6). In systems where double bond is conjugated with other multiple bonds, this rate decreases since the delocalization of the π electrons reduces the electron density at all the double bonds.

Usually, the oxidation with peracids leads to the epoxide; however, the products may undergo subsequent rearrangements, as in the case of chloroolefins, where the over-all products are the chloroketones (7). Also, epoxidation of aliphatic enol-acetate leads after intermolecular rearrangements to α-acetoxy-ketones (8). By this route, the olefins *via* the epoxidation products can be converted after undergoing rearrangement to carbonyl compounds (9). In the present work, when *N*-methylpyrrole (I) was subjected to the oxidizing effect of perbenzoic acid in chloroform solution, the analytical measurements showed that two atoms of oxygen had been involved in the reaction. Preparative experiments showed that the reaction product was *N*-methyl succinimide (II) in 92% yield. It appears in this reaction that the initially formed epoxides at both double bonds, favored through the presence of the electron rich *N*-methyl group, undergo internal rearrangement to give the imide.



I II III IV



V VI

When *N*-methyl-3-pyrroline (III) was similarly treated, the product was *N*-methyl-α-pyrrolidone (IV) in 79% yield. The analytical data showed that only one oxygen atom had been consumed.

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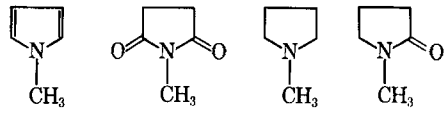
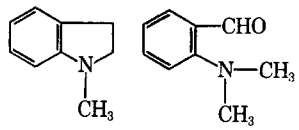


I II III IV



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EXPERIMENTAL

All melting points were taken in open capillaries and are uncorrected.

Determination of the Number of Oxygen Atoms Needed for a Known Weight of the Organic Substance (10).—The organic substance (0.2 Gm.) was added to a known volume of perbenzoic acid in chloroform with known normality. The mixture was left for 6 hr. in a dark place with occasional shaking. Five milliliters of 10% KI solution as well as 2 drops of concentrated H_2SO_4 were added to the mixture. The liberated iodine was then titrated against sodium thiosulfate (0.1 *N*).

With N-Methylpyrrole (I)—The determination showed two oxygen atoms to be involved in the reaction.

N-Methyl Succinimide (II)—*N*-Methylpyrrole (2.0 Gm.) was added to 190 ml. 0.54 *N* perbenzoic acid in chloroform. The reaction mixture was left in a dark place for 12 hr. Then it was neutralized by sodium bicarbonate solution. The chloroform layer was then isolated, washed with water several times, dried over calcium chloride, and finally evaporated.

Two and one half grams of *N*-methylsuccinimide was left. Yield 92%. Crystallization from acetone as white needles gave m.p. 67–68°. [Lit. m.p. 66° (11).]

Anal.—Calcd. for $C_5H_7NO_2$: C, 53.09; H, 6.29; N, 12.30; O, 28.31. Found: C, 53.00; H, 6.18; N, 12.30; O, 28.30.

With N-Methyl-3-pyrroline (III)—The determination showed that one oxygen atom was involved with each mole of the compound.

N-Methyl-α-pyrrolidone (IV)—*N*-Methyl-3-pyrroline (2.0 Gm.) was added to 120 ml. of perbenzoic acid in chloroform 0.4 *N* (one oxygen atom). The whole mixture was left for 3 hr. in a dark place at room temperature, then neutralized by sodium bicarbonate solution. The chloroform layer was then isolated, washed with water, dried over calcium chloride, and evaporated.

A 1.9-Gm. (79.1%) quantity of *N*-methyl-α-pyrrolidone was collected as a colorless oil, b.p. 201°/740 mm. [Lit. 202°/760 mm. (12).]

Anal.—Calcd. for $C_5H_9NO.HCl$: C, 44.40; H, 7.40; N, 10.36; O, 11.84. Found: C, 44.38; H, 6.99; N, 10.30; O, 11.81.

With N-Methylindoline (V)—The determination showed one oxygen atom was consumed in the reaction.

o-Dimethylaminobenzaldehyde (VI)—*N*-Methylindoline (2.0 Gm.) was added to 104 ml. 0.29 *N* perbenzoic acid in chloroform (one oxygen atom). The reaction mixture was left at room temperature in a dark place for 24 hr. with occasional shaking, then neutralized by solution of sodium bicarbonate. The chloroform layer was then separated, washed with water several times, dried over calcium chloride, and finally evaporated.

A 1.8-Gm. (81%) quantity of *o*-dimethylaminobenzaldehyde was collected at 141/30 mm. as yellow oil. [Lit. 142°/30 mm. (13).]

Anal.—Calcd. for $C_9H_{12}N_2O$: C, 60.10; H, 6.66; N, 15.55. Found: C, 59.87; H, 6.29; N, 15.49.

REFERENCES

(1) Maye, P., and Reid, S. T., *Chem. Ind.*, **35**, 1576 (1962); through *Chem. Abstr.*, **58**, 3379(1963).
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(3) Spiro, V., and Madonia, P., *Gazz. Chim. Ital.*, **86**, 101(1956); through *Chem. Abstr.*, **50**, 15529(1956).
(4) Witkop, B., and Patinck, J. P., *J. Am. Chem. Soc.*, **73**, 713(1951).
(5) Witkop, B., and Fieldler, H., *Ann.*, **55B**, 91(1947).
(6) Swern, D., *Chem. Rev.*, **45**, 1(1949).
(7) McDonald, R. N., and Schwab, P. A., *J. Am. Chem. Soc.*, **85**, 820(1963).
(8) Williamson, K. L., and Johnson, W. S., *J. Org. Chem.*, **26**, 4563(1961).
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(10) Vogel, A. I., "Practical Organic Chemistry Including Qualitative Organic Analysis," Longmans, London, England, 1957, p. 808.
(11) Labzzuto, G., *Gazz. Chim. Ital.*, **63**, 266(1933); through *Chem. Abstr.*, **27**, 3926(1933).
(12) Tafel, J., and Wassermann, O., *Ber.*, **40**, 2839(1907).
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By JEFFREY C. SPITZER and CORNELIUS STEELINK

The sesquiterpene lactone, matricin, has been isolated from *Artemisia caruthii* and is shown to be the precursor of chamazulene, previously reported in the steam distillate of this species.

THE COMMON SAGE brush *Artemisia caruthii* Wood, var. *Wrightii* (A. Gray), yields a blue oil upon steam distillation or upon simple brewing as a "folk" tea. The oil has been identified (1) as chamazulene (I). Because of the occasional use of the plant as a folk medicine by the Indians of Northern Arizona, and because of the wide distribution of sesquiterpene lactones in the *Artemisia* species, the authors were prompted to investigate the sesquiterpene lactone precursors of chamazulene (pro-chamazulenogens) in this species.

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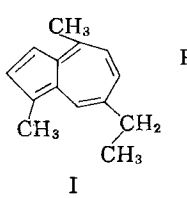

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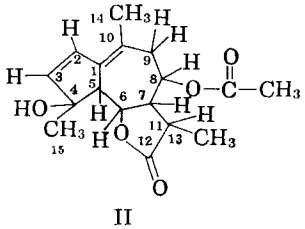

II

Received November 1, 1966, from the Department of Chemistry, University of Arizona, Tucson, AZ 85721
Accepted for publication December 30, 1966.
Abstracted in part from a thesis submitted by Jeffrey C. Spitzer to the University of Arizona, Tucson, in partial fulfillment of Doctor of Philosophy degree requirements.

EXPERIMENTAL

All melting points were taken in open capillaries and are uncorrected.

Determination of the Number of Oxygen Atoms Needed for a Known Weight of the Organic Substance (10).—The organic substance (0.2 Gm.) was added to a known volume of perbenzoic acid in chloroform with known normality. The mixture was left for 6 hr. in a dark place with occasional shaking. Five milliliters of 10% KI solution as well as 2 drops of concentrated H_2SO_4 were added to the mixture. The liberated iodine was then titrated against sodium thiosulfate (0.1 *N*).

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REFERENCES

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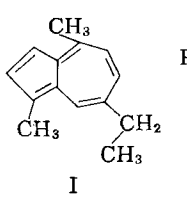

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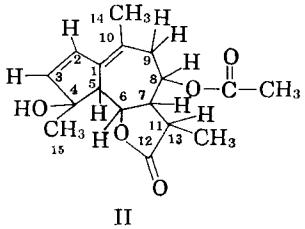

II

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dilute mineral acid. Matricin is concentrated in the bitter leaves of the plant and exhibits its maximum concentration during July. By September, it is almost undetectable in the plant. It has been previously found only in *Matricaria chamomilla* (2); this is the first report of its occurrence in the *Artemisia* species.

EXPERIMENTAL

Dried ground leaves of *A. caruthii* (collected from the Graham Mountain region of Arizona at elevations above 6000 ft.) were soaked in chloroform overnight. The green slurry was filtered, evaporated to dryness, and taken up in 100 ml. of 95% ethanol. The addition of 300 ml. of water precipitated a tar. The supernatant liquid was filtered through a mat of diatomaceous earth[1] and activated carbon.[2] The filtrate was then shaken with chloroform, and the chloroform layer concentrated to a gum (yield, 1.1%). Preparative thin-layer chromatography (1 mm. thick Silica Gel G; solvent: 90:1 chloroform–methanol) proved to be the most efficient method for separating the prochamazulenogen from other lactonic materials. Methanol elution of the band at R_f 0.22 from a number of TLC plates yielded enough tan colored material for characterization.

The following physical constants were entirely consistent with structure II and with a sample of authentic matricin,[3] m.p. 152–156°. $[\alpha]_D^{30}$ −122° ($CHCl_3$); $\lambda_{max.}$ 242 mμ (ε, 11,200); ν, 3600 cm.$^{-1}$ (OH), 1780 cm.$^{-1}$ (γ-lactone), 1747 cm.$^{-1}$ (acetoxy). The NMR assignments are shown in Table I. Although the evidence is not unequivocal, one would deduce from the NMR data that 5, 6, and 7 protons are all *trans*.

In addition to the above evidence, co-chromatography of the prochamazulenogen and authentic matricin in four different solvent systems showed no differences. Matricin polymerizes readily on exposure to light or upon moderate heating; this creates some problems in handling.

Since the original interest in the plant was motivated by reports of its use as a folk medicine, the authors submitted certain fractions for physiological analysis. The chloroform extract (matricin-rich) exhibited minor depressant activity with cats.[4] Chamazulene, whose precursor is matricin, showed some antispasmodic activity. It also produced urination and emesis in cats, when given intraperitoneally.

TABLE I—NMR ASSIGNMENTS

p.p.m.[a]	Multiplicity	Assignment	No. Protons
1.36	2	C-13	6
1.43	1	C-14	
1.90	1	C-15	11 or 12
2.14	1	OAc	
2.0–3.3	...	C-5,7,9,11, —OH	
4.12	3	C-6	1
4.9	...	C-8	1
5.94	2	C-3	1
6.33	2	C-2	1

[a] Relative to tetramethylsilane.

CONCLUSION

The labile sesquiterpene matricin (II) has been found to be the precursor of the blue oil, chamazulene (I), in *A. caruthii*. This is the first report of the occurrence of matricin (II) in an *Artemisia* species; the taxonomic implication of this type of lactone in plants is discussed elsewhere (3). The activity of I as an emetic is interesting. The Navajo and Hopi Indians use *A. caruthii* as an emetic in cleansing ceremonies (4). Although chamazulene (I) does not exist in the plant as such, it can be obtained from matricin (II) by the simple steam distillation or tea-brewing process. The conversion of II to I is not only a remarkable example of dehydration and dehydrogenation, but is also the basis of an effective folk medicine.

REFERENCES

(1) Steelink, C., and Penunuri, E., *J. Pharm. Sci.*, **51**, 598(1962).
(2) Cekan, Z., Herout, V., and Sorm, F., *Chem. Ind.*, 1234(1956).
(3) Steelink, C., and Spitzer, J. C., *Phytochem.*, **5**, 357 (1966).
(4) Wright, B. A., Museum of Northern Arizona, Flagstaff, Ariz., unpublished data.

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.
[2] Marketed as Nuchar by Braun Chemical Co.
[3] The authors are indebted to Professor Z. Cekan, Czechoslovak Academy of Science, for this sample of matricin.
[4] The authors are indebted to Dr. Bernard Loev, Smith Kline & French Laboratories, Philadelphia, Pa., for carrying out these analyses.

Method of Estimating Relative Absorption of a Drug in a Series of Clinical Studies in Which Blood Levels Are Measured After Single and/or Multiple Doses

By JOHN G. WAGNER

Previously published methods of estimating relative absorption of a drug are primarily applicable to crossover studies in which a panel of subjects is administered one or more test formulations and a readily available form of the drug. The method described provides estimates of average relative absorption of a drug administered to different panels of subjects in a series of clinical trials in which blood levels are measured after either single and/or multiple doses of the drug. The method incorporates corrections for differences in dosage, body weight or surface area, and intra- and inter-subject variation in half-life of the drug. The method is illustrated by results of calculations made from 167 sets of serum concentrations of tetracycline hydrochloride activity including 112 sets obtained following administration of tetracycline to children and 55 sets obtained following administration of tetracycline to adults.

WAGNER AND NELSON (1) summarized seven methods of estimating per cent availability. These methods are primarily applicable to a crossover study in which a panel of subjects are administered one or more test formulations and a readily available form of the drug. Frequently, however, one may wish to compare the relative absorption of a drug in a series of clinical trials performed with different panels of subjects at different times. In such a case one must make corrections for differences in dosage, body weight, and intra- and inter-subject variations in half-life of the drug. The method to be described allows such corrections to be made and also allows comparison of blood levels observed after a single dose and during a dosage interval at the equilibrium state after multiple doses of drug administered at uniform time intervals.

EXPERIMENTAL

Following single doses of drug the basic material balance equation[1] as given by Wagner *et al.* (2) is:

$$F \cdot D = V \cdot K \cdot \int_0^\infty C(t)\,dt \qquad \text{(Eq. 1)}$$

where F is the fraction of the dose which is absorbed, D is the dose, V is the apparent volume of distribution of the drug, K is the first-order rate constant for over-all loss of drug from the body, and the integral represents the area under the blood (serum or plasma) concentration curve from zero to infinite time.

Substitution of $0.693/t^1/_2$ for K, where $t^1/_2$ is the half-life, and rearrangement gives

$$\frac{\int_0^\infty C(t)dt}{D \cdot t^1/_2} = \frac{F}{0.693V} \qquad \text{(Eq. 2)}$$

If $C(t)$ is expressed in micrograms per milliliter, D in milligrams per kilogram of body weight, t and $t^1/_2$ is similar units, then V will be in units of liters per kilogram of body weight which is equivalent to fraction of body weight, and F is dimensionless. The dose, D, could also be expressed in milligrams per square meter of body surface area in which case V will be in units of liters per square meter of body surface area.

Similarly, following multiple doses of a drug one can derive (2) the relationship

$$\frac{\int_{t_1}^{t_2} C_\infty(t)}{D \cdot t^1/_2} = \frac{F}{0.693V} \qquad \text{(Eq. 3)}$$

where the integral represents the area under the blood (serum or plasma) concentration curve during a dosage interval at the steady state after multiple doses of drug given at uniform time intervals, τ, such that $\tau = t_2 - t_1$, and F is the fraction of each dose which is absorbed; the other symbols have the same meanings as indicated above.

The half-lives, $t^1/_2$, may be estimated in the usual manner from the terminal $C(t)$, t values after a single dose of drug or following the last dose of a series of doses given at uniform time intervals; in the latter case, it may or may not be necessary to include values beyond the end of the last dosage interval. The infinite area of Eq. 2 may be estimated from the equation

$$\int_0^\infty C(t)dt = \int_0^T C(t) + \frac{\hat{C}_T}{K} \qquad \text{(Eq. 4)}$$

where $\hat{C}_T$ is the estimated serum concentration at the last sampling time, T, following the single dose of drug, the integral represents the area under the blood (serum or plasma) concentration curve from zero time to time T, and K is defined in Eq. 1. The integrals $\int_0^T C(t)dt$ and $\int_{t_1}^{t_2} C_\infty(t)dt$ may be estimated by trapezoidal rule from observed serum concentrations measured at appropriate time intervals after dosing. The value of $\hat{C}_T$ is obtained by substituting T for t in the equation of the least squares line used to estimate K.

The method of estimating relative absorption of a

Received October 13, 1966, from the Medical Research Division, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication November 23, 1966.

The author expresses his appreciation to Dr. W. T. Sokolski and co-workers, Control Division of The Upjohn Co., for microbiological assays; Dr. I. J. Martens, Kalamazoo, Mich., Dr. J. F. Crosby, Richmond, Va., and Dr. K. Sunada, Wheatridge, Colo., who supervised the clinical trials; Dr. J. B. Lawson and Dr. R. E. Damiano, Medical Research Division, The Upjohn Co., who monitored the clinical trials; and Mark Becker, The Upjohn Co., who wrote digital computer programs based on Eqs. 2, 3, and 4.

[1] Some data may fit a model for which $\int_0^\infty C(t) \neq \frac{F.D.}{V.K.}$. In these cases, the method proposed is not applicable.

TABLE I—SUMMARY OF HALF-LIVES AND AREA/DOSE × HALF-LIFE RATIOS ESTIMATED FROM SERUM CONCENTRATIONS OF TETRACYCLINE HYDROCHLORIDE ACTIVITY (MICROBIOLOGICAL ASSAY) OBSERVED IN SEVERAL CLINICAL STUDIES AFTER SINGLE ORAL DOSES OF TETRACYCLINE HYDROCHLORIDE

Study	Formulation	Subj.	Type of Subjects	Av. Dose Of Tetracycline HCl, mg./Kg.	Dose of Prepn.	Half-Life, hr. Av.	Half-Life, hr. Range	Ratio of Area 0 → ∞ (mcg. × hr./ml.)[f] / Dose (mg./Kg.) × Half-Life (hr.) Av.	Ratio Range
1	Tetracycline and novobiocin[c] drops (exptl. formula)	19	Children 13–155 lb.	9.5	2.5 ml./15 lb. body wt.	5.24	2.87–10.3	1.12	0.50–1.87
2	Tetracycline and novobiocin[c] half-strength capsules (exptl. formula)	20	Children 37–120 lb.	8.6	1, 2, or 3 capsules	4.76	2.68–8.70	1.19	0.24–1.60
3	Novobiocin and tetracycline[d] suspension	34	Children 19–100 lb.	9.0	5 ml./15 lb. body wt.	5.31	2.06–9.93	1.12	0.59–1.98
4	Novobiocin and tetracycline[d] suspension	39	Children 17–100 lb.	9.0	5 ml./15 lb. body wt.	4.99	2.31–7.32	1.23	0.76–2.76
5 and 6[a] pooled	Tetracycline and novobiocin[c] flavored granules (exptl. formula)	20	Adults 120–204 lb.	7.0	20 ml.	7.08	3.84–18.0	1.13	0.51–2.20
7–12[b] pooled	H.F.C. Tetracycline,[e] 250 mg.	35	Adults 116–220 lb.	7.3	2 capsules	6.28	1.91–13.8	1.09	0.34–2.33

[a] Each study employed 10 subjects. Results of both studies were pooled. [b] In these six studies the same subjects were employed several times. The averages are based on 35 *different* subjects' data. [c] Marketed as Panalba KM by The Upjohn Co., Kalamazoo, Mich. [d] Marketed as Albamycin-T by The Upjohn Co., Kalamazoo, Mich. [e] Marketed as Panmycin by The Upjohn Co., Kalamazoo, Mich. [f] It is the author's experience that similarity in average ratios is not improved when surface area rather than body weight is used as the variable.

drug in a series of clinical studies utilizes calculation of the area/dose × half-life ratios shown in the left-hand side of Eqs. 2 and 3 for each subject administered a given dosage form in each study. The average value of the ratio for the panel of subjects administered a given dosage form of the drug in each study is calculated. If the assumption is made that the average apparent volume of distribution (expressed as a fraction of body weight or liters per $M.^2$ of body surface area) is the same from panel to panel of subjects, then the average ratios are indices of relative absorption efficiency.

RESULTS

The method has been applied by the author to estimate relative absorption efficiencies of several antibiotics administered in different dosage forms to different panels of subjects in dozens of clinical trials. As an example, results are presented of half-lives and area/dose × half-life ratios estimated from serum concentrations of tetracycline hydrochloride activity (in micrograms per milliliter) observed in several clinical trials in which children and adults were administered various novobiocin-tetracycline combinations and tetracycline hydrochloride alone in capsules. Results are summarized in Table I. Although there was wide variation in milligram dosage, body weight, and estimated half-life in these studies, the average area/dose × half-life ratios are amazingly similar. Appropriate statistical tests indicated that the difference between any pair of the averages of the ratios was not significant at the 5% level of confidence. If one assumes that the average volume of distribution expressed as a fraction of body weight was constant, these data indicate that the average efficiency of absorption of tetracycline was not significantly different in the six groups of subjects listed in Table I.

REFERENCES

(1) Wagner, J. G., and Nelson, E., *J. Pharm. Sci.*, **53**, 1392 (1964).

(2) Wagner, J. G., Northam, J. I., Alway, C. D., and Carpenter, O. S., *Nature*, **207**, 1301(1965).

Determination of 17α-Ethynylestradiol-3-methyl Ether in Tablets by a Colorimetric Assay

By ARVIN P. SHROFF and RICHARD E. HUETTEMANN

A colorimetric method has been developed for assaying 17α-ethynylestradiol-3-methyl ether in tablets. The method is based on the formation of a colored complex with phenol–sulfuric acid reagent. This complex exhibits an absorption maximum at 550 mμ, obeys Beer's law, and is stable for a reasonable length of time. Optimum conditions for the color formation in the presence of a nonphenolic steroid have been determined and are reported herein.

ESTROGENS in combination with progestational agents have come into prominence as a contraceptive composition in the last decade. The most frequently used estrogen in these combinations is 17α-ethynylestradiol-3-methyl ether (mestranol). A rapid and sensitive colorimetric determination of mestranol in a tablet combination with norethindrone[1] stored under a variety of conditions is the subject of this paper.

Steroidal estrogens have been assayed by a number of methods. Of these ultraviolet spectrophotometric (1, 2) and spectrofluorometric (3–5) analyses have been preferred by a number of authors. The lack of sensitivity of the ultraviolet method of assay on aged tablets was shown by this laboratory (2).

Recently ion-exchange (6) and paper chromatographic methods (7) have also appeared in the literature. These methods are sensitive but they lack the specificity required in the presence of other steroids for a rapid analytical method. Specificity can be imparted to these methods through prior isolation of the estrogens by other techniques. However, because of the number of manipulations required, varied results may be obtained. Schulz (8) has discussed the difficulties of assaying mestranol by a paper chromatographic method.

Gas-liquid chromatography has also been considered to be a desirable technique (2, 8–10). It offers quantitation with concurrent fractionation. The GLC process developed for assaying mestranol by this laboratory (2), although efficient, was relatively slow for routine assay purposes.

A colorimetric technique was resorted to because of its rapidity and reproducibility. Estrogens have been determined by colorimetric methods using 2,6-dibromoquinone chlorimide (11) and various diazotized amines (12–18) as the color-producing reagents. The official U.S.P. procedure for the determination of estrone, estradiol, and ethynyl estradiol, is based on the Kober reaction.

In this investigation the color development of mestranol was carried out with a phenol–sulfuric acid reagent. The color formation was stable over a reasonable period of time and no interference by nonphenolic steroids was observed. As a result of these studies with extraction procedures and different commercial phenols, a rapid, accurate, and sensitive colorimetric procedure for mestranol in estrogen–progestin tablets was developed. A comparison of the results from the ultraviolet, gas-liquid chromatographic, and colorimetric methods is also presented.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used to determine the absorbance values and a Cary model 11 recording spectrophotometer was used to obtain the spectrum in Fig. 1.

Reagents and Solutions—Freshly distilled phenol, methylene chloride, sulfuric acid (1:1), and methylcyclohexane (spectroquality) are used.

Phenol–Sulfuric Acid Solution—Add 40.0 Gm. of phenol to a 400-ml. beaker containing 100 ml. of 1:1 sulfuric acid solution. Bring the mixture to a boil with continuous stirring and until homogeneous. Store in a volumetric flask and in a dark place.

Mestranol Standard Solution—Weigh accurately 10.0 mg. of mestranol into a 100-ml. volumetric flask and dilute to mark with methylene chloride.

Procedure—Place a number of tablets equivalent to about 400 mcg. of mestranol in a 29.5 × 80-mm. vial. To this add 4.0 ml. of distilled water and shake until the tablets disintegrate. Add 4.0 ml. of methylcyclohexane and shake for 30–60 min. Centrifuge for approximately 2 min. and transfer 1.0 ml. of clear methylcyclohexane solution to a 21 × 50-mm. vial. Also transfer 1.0 ml. of the standard mestranol solution into another 21 × 50-mm. vial. Evaporate the solvent in each vial over a steam bath and under a stream of nitrogen. To each vial add 5.0 ml. of phenol–sulfuric acid solution. To a third 21 × 50-mm. vial add 5.0 ml. of phenol–sulfuric acid solution to serve as a reference. Shake the three vials thoroughly and place them in a 55° water bath for 5 min. Allow the solutions to come to room temperature in a dark place (about 15 min.) and determine the absorbance of the standard and the sample at 550 mμ, using the contents of the third vial as the reference solution.

DISCUSSION

The absorbance spectrum of mestranol resulting from the reaction with phenol–sulfuric acid is shown in Fig. 1. It exhibits broad absorption maxima at 470 mμ and 550 mμ. The stability of the color as measured at 550 mμ depended upon the phenol used. For example, a reagent made with May and Baker phenol gave a stable color over a period of 6 hr., whereas other reagents made from different phenols

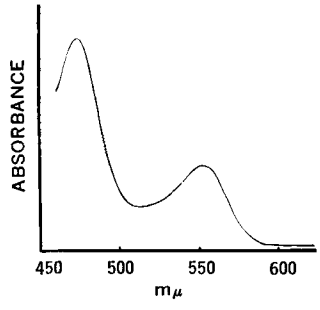

Fig. 1—Visible absorption spectrum of mestranol treated with phenol–sulfuric acid reagent.

Received November 3, 1966, from the Division of Organic Chemistry, Ortho Research Foundation, Raritan, NJ 08869
Accepted for publication January 19, 1967.

[1] This combination is marketed as Ortho-Novum by Ortho Pharmaceutical Corp., Raritan, N. J.

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Lot	Storage Conditions	Original Granulation U.V. Assay, mcg./Tab.	U.V. Assay After Storage, mcg./Tab.[a]	Colorimetric Assay, mcg./Tab.	GLC Assay[a]
A	6 wk. 100/80	50.7	39.1	50.3	51.4
B	3 mo. 100/80	51.3	42.8	50.1	47.2
C	6 mo. 37°	80.0	71.7	77.8	84.3
D	7.5 mo. R.T.	49.9	43.6	48.8	51.6
E	12 mo. R.T.	56.0	51.2	58.7	55.1
F	13 mo. R.T.	51.5	45.6	50.6	52.2
G	18 mo. R.T.	57.6	48.9	56.5	56.0

[a] Data obtained from *Reference 2*.

did not give so stable a color. However, in all instances the color intensity remained constant for a sufficient length of time (20–30 min.) to allow recording of the spectrophotometric readings.

The relationship between absorbance at 550 mμ and the quantity of mestranol was found to obey Beer's law. The standard deviation for accuracy and precision was found to be ± 1.6% and ± 1.52%, respectively.

The specificity of the method for mestranol in the presence of norethindrone and other tablet excipients is demonstrated by the results shown in Table I. The excipients used in the formulation of the tablets were lactose, cornstarch, magnesium stearate, and polyvinylpyrrolidine. The data in Table I include several samples of formulation stored under various conditions of temperature and humidity. A previous study (2) indicated that mestranol did not undergo any decomposition under these conditions. Hence, the selectivity and sensitivity of this colorimetric method appeared adequate for performing routine studies.

Table I compares the data obtained by the ultraviolet and gas chromatographic methods (2) with the present colorimetric results. It is evident that the color assay compares favorably with the gas chromatographic assay and is superior to the ultraviolet method. The mechanism by which phenol–sulfuric acid reacts with mestranol and other estrogens to give colored products is under investigation.

SUMMARY

A quantitative colorimetric procedure using phenol–sulfuric acid has been developed for the analysis of mestranol in estrogen–progestin tablets. The accuracy and precision of this method are ±1.6% and ±1.52%, respectively. The nonphenolic steroid and tablet excipients do not interfere in the determination.

REFERENCES

(1) Klein, S., James, A., and Tuckerman, M., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 314(1960).
(2) Shroff, A. P., and Grodsky, J., *J. Pharm. Sci.*, **56**, 460 (1967).
(3) Slaunwhite, W. R., Engle, L. L., Olmsted, P. C., and Carter, P. J., *J. Biol. Chem.*, **191**, 627(1951).
(4) Boscott, R., *Nature*, **162**, 577(1948).
(5) Huttenrauch, R., and Keiner, I., *Pharmazie*, **20**, 242 (1965).
(6) Sjostrom, E., and Nykanen, L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 321(1957).
(7) Kadin, H., Ugolini, M. S., and Roberts, H. R., *J. Pharm. Sci.*, **53**, 1313(1964).
(8) Schulz, E. P., *ibid.*, **54**, 144(1965).
(9) Talmage, J. M., Penner, M. H., and Geller, M., *ibid.*, **54**, 1194(1965).
(10) France, J. T., and Knox, B. S., *J. Gas Chromatog.*, **4**, 183(1966).
(11) Gibbs, H. D., *J. Biol. Chem.*, **72**, 649(1927).
(12) Schmulowitz, J. J., and Wylie, H. B., *J. Lab. Clin. Med.*, **21**, 210(1935).
(13) Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.*, **134**, 319(1940).
(14) Bender, A. E., and Wilson, A., *Biochem. J.*, **41**, 423 (1947).
(15) Mitchell, F. L., and Davies, R. E., *ibid.*, **56**, 690 (1954).
(16) Libermann, S., *J. Clin. Invest.*, **31**, 341(1952).
(17) Rehm, C. R., and Smith, J. B., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 386(1960).
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Communications

Identity of Columbianadin and Zosimin

Sir:

Recently, Nikonov and Baranauskaite (1, 2) reported the isolation of a purportedly new coumarin, zosimin, from the roots of *Zosimia absinthifolia* (Vent) Link. This compound analyzed for the formula $C_{19}H_{20}O_5$ and, on alkaline saponification, yielded *cis*-1,2-dimethylacrylic acid (*i.e.*, tiglic acid) and a hydroxylactone, zosimol (m.p. 156–158°), with the formula $C_{14}H_{14}O_4 \cdot {}^1/_2CH_3OH$. It was recognized that zosimin was isomeric with columbianadin (3, 4), but the apparent divergence in optical activities between the two coumarins and the corresponding hydroxylactones led the authors to the conclusion that they were different and that zosimin was, in fact, a new coumarin. Their decision apparently was confirmed by the fact that zosimin yielded tiglic acid on alkaline saponification. The identity of the hydroxylactone was ascertained by Perel'son *et al.* (5) by both spectral and chemical studies, thus eliminating the several other possible structural candidates [*e.g.*, marmesin (6), lomatin (7), 3′-hydroxy-3′,4′-dihydroxanthyletin (8), *etc.*]. On this basis it was concluded originally that zosimin

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REFERENCES

(1) Klein, S., James, A., and Tuckerman, M., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 314(1960).
(2) Shroff, A. P., and Grodsky, J., *J. Pharm. Sci.*, **56**, 460 (1967).
(3) Slaunwhite, W. R., Engle, L. L., Olmsted, P. C., and Carter, P. J., *J. Biol. Chem.*, **191**, 627(1951).
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TABLE I—ROTATIONS OF COMPOUNDS

Compd.	M. p.	Specific Rotation in $CHCl_3$
Zosimin	119–120° (1, 2)	$[\alpha]_D^{16}$ +272° (c, 1.03)
Columbianetin angelate (*i.e.*, columbianadin)	117–119° (3)	$[\alpha]_D^{16}$ +237.8° (c, 1.0)[a]
	121–122° (3)	$[\alpha]_D^{21}$ +234.5° (c, 1.0)[a]
	118.5–119° (4)	$[\alpha]_D^{26}$ +230.5° (c, 1.0)[a]
		$[\alpha]_D^{26}$ +227° (c, 2.8) (4)
Columbianetin tiglate	107–108° (3)	$[\alpha]_D^{16}$ +250.1° (c, 1.0)[a]
		$[\alpha]_D^{21}$ +246.5° (c, 1.0)[a]
		$[\alpha]_D^{26}$ + 243.9° (c, 1.0)[a]
Zosimol	156–158° (1, 2)[b]	$[\alpha]_D^{16}$ +209.4° (c, 0.74)
Columbianetin	164.5–165° (3)	$[\alpha]_D^{16}$ + 165.1° (c, 1.0)[a]
	162.8–163.3° (4)	$[\alpha]_D^{21}$ +164.5° (c, 1.0)[a]

[a] Measurements carried out on a Perkin-Elmer model 141 polarimeter. [b] The discrepancy between this melting point and that of columbianetin is apparently due to ½ mole of methanol of crystallization in contrast to the anhydrous form of columbianetin.

was the tiglate ester of 2′-*tert*-hydroxyisopropyl-2′,3′-dihydrofuro-4′,5′:8,7-coumarin, (*i.e.*, columbianetin tiglate).[1]

Previous studies (3) had encompassed the synthesis of both the angelate (m.p. 117–119°) and tiglate (m.p. 107–108°) esters of columbianetin, and it was apparent that the tiglate ester was not identical with zosimin on the basis of the melting point discrepancy. Since the reported melting point of zosimin (m.p. 119–120°) was so similar to that of columbianetin angelate, (*i.e.*, columbianadin), it seemed reasonable to suspect that they were one and the same. Furthermore, previous experience in this laboratory with a very similar alkaline saponification of columbianadin (3) indicated that tiglic acid was the product instead of the angelic acid that might have been expected. Such a facile conversion of angelic acid to tiglic acid, however, is not unexpected (10).

The difference in specific rotation reported by us for columbianadin $\{[\alpha]_D^{27}$ + 26.5° (c 1.0, dioxane)$\}$ and that obtained for zosimin $\{[\alpha]_D^{16}$ + 272° (c 1.03, $CHCl_3$)$\}$ appears to be one of the principal reasons for deciding on the nonidentity of the two coumarins. However, it is well known that the specific rotation of a given compound can vary widely when using different solvents. When we took the rotations of columbianadin, columbianetin tiglate, and columbianetin in chloroform in order to obtain a direct comparison with zosimin and zosimol (see Table I), the results showed that the apparent wide discrepancy in specific rotations between columbianadin and zosimin as well as between columbianetin and zosimol was substantially eliminated with identical solvents. Nevertheless, the difference in rotation of columbianetin tiglate and angelate was not great enough to make a decision for the identity of either ester with zosimin. The approximately 34° and 44° higher rotation of zosimin and zosimol over columbianetin angelate and columbianetin, respectively, however, suggested an instrumental difference rather than a real difference since these were the only two structural possibilities in each case.

In assessing the evidence for and against the identity of columbianadin with zosimin it was obvious that only a direct comparison of the two compounds would provide an unequivocal answer. Fortunately, Dr. Nikonov kindly supplied several milligrams of zosimin which enabled such a comparison. Infrared spectra in Nujol mull showed the two to be identical in every respect and, furthermore, no depression of melting point was observed in a mixed melting point determination. The optical rotation of Nikonov's sample of zosimin was $[\alpha]_D^{20}$ + 221.2° (c 1.0, $CHCl_3$). This was substantially lower than that reported by him and is in reasonable agreement with that of columbianadin from two independent laboratories (3, 4). It is, therefore, safe to conclude that columbianadin and zosimin are identical.

[1] More recently, Nikonov (9) has suggested that the difference between zosimin and columbianadin may be in an optically isomeric relationship between columbianetin and zosimol since the 4.05 τ position, in the nuclear magnetic resonance spectrum, of the methine proton found by them in zosimin is contradictory to a tiglate formulation (3).

(1) Nikonov, G. K., and Baranauskaite, D. I., *Khim. Prirodnykh Soedinenii*, **1**, 220(1965).
(2) Nikonov, G. K., and Baranauskaite, D. I., *Chem. Natl. Prod.*, **1**, 169(1966).
(3) Willette, R. E., and Soine, T. O., *J. Pharm. Sci.*, **53**, 275(1964).
(4) Nielsen, B. E., and Lemmich, J., *Acta Chem. Scand.*, **18**, 1379(1964).
(5) Perel'son, M. E., Nikonov, G. K., Pek, G. Yu., and Scheinker, Yu. N., *Dokl. Akad. Nauk SSSR*, **159**, 154(1964).
(6) Mookerjee, A., *Current Sci. India*, **12**, 209(1943).
(7) Soine, T. O., and Jawad, F. H., *J. Pharm. Sci.*, **53**, 990(1964).
(8) Hata, K., and Sano, K., *Tetrahedron Letters*, (No. 14) 1461(1966).
(9) Nikonov, G. K., personal communication, February 20, 1967.
(10) Buckles, R. E., Mock, G. V., and Locatell, L., Jr., *Chem. Rev.*, **55**, 659(1955).

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Received November 16, 1966.
Accepted for publication March 1, 1967.

This investigation was supported in whole by grant HE07101 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

Comparative Utility of the Silyl Ether and Acetate Ester Derivatives of Ethinyl Estradiol for Its Quantitative Gas Chromatographic Determination

Sir:

A recent paper (1) in *J. Pharm. Sci.*, comparing the U.S.P. method with a gas chromatographic determination of ethinyl estradiol, contains several allusions to our paper on the gas chromatographic assay of the estrogen (2), claiming certain advantages for the choice of the trimethyl silyl ether derivative over our use of the acetate derivative. We should like to offer a rebuttal on several of these comparisons.

Boughton, Bryant, Ludwig, and Timma (1) inferred from our work that, "The ethinyl estradiol appears to have been uncontaminated with other steroids . . . ." Since the extraction procedure reported by these authors is essentially identical with ours, one would hardly expect interference in one procedure and not the other. In point of fact, however, the samples on which we reported contained 10 to 20 times the concentration of ethinyl estradiol of an experimental progestational steroid, which resulted in no interference.

The authors (1) further claimed that evaporation of acetic anhydride necessary in our procedure introduces a possible source of error, presumably circumvented by substitution of silylation in their procedure for acetylation in ours. This contention is contradicted by comparison of our reported precision of $\pm 1.7\%$ with the standard deviations of $\pm 1.5\%$ found for the silylation method, which are not statistically different.

The statement that ". . . the authors wished to analyze for at least 50% less ethinyl estradiol than Talmage, Penner, and Geller had analyzed, and for our purposes the acetate derivative did not give sufficient response on the chromatograph" (1) is perhaps the most misleading. We did not find it necessary to use maximum sensitivity in our procedure; using the acetate derivative, sensitivity could easily have been increased by a factor of 10. We have chromatographed unmodified ethinyl estradiol, its silyl ether, and its acetate ester at the same concentration using instrumental conditions previously described (2). The chromatograms shown in Fig. 1 indicate that little sensitivity gain is afforded by use of the silyl ether in preference to the acetate ester derivative; however, it is interesting to note the increase in retention time with increase in molecular weight.

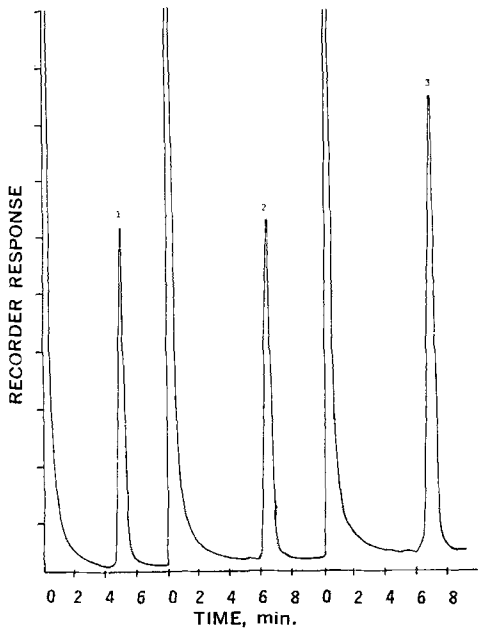

Fig. 1—Comparative chromatogram. Key: 1, ethinyl estradiol; 2, ethinyl estradiol, acetate ester; 3, ethinyl estradiol, silyl ether.

The results shown in Fig. 1 indicate that ethinyl estradiol can be determined by gas chromatography without formation of derivatives. We were aware of this during our work with the acetate ester, but found better precision using the derivative. Subsequent to this work, however, it was determined that the unmodified ethinyl estradiol can be estimated with good precision and accuracy if the column is saturated with the estrogen before quantitative samples are run. We interpret this as saturating the active sites on the column, thus making them unavailable for irreversible adsorption.

(1) Boughton, O. D., Bryant, R., Ludwig, W. S., and Timma, D. L., *J. Pharm. Sci.*, **55**, 951(1966).
(2) Talmage, J. M., Penner, M. H., and Geller, M., *ibid.*, **54**, 1194(1965).

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Estimation of Rate Constants for Absorption and Elimination from Blood Concentration Data

Sir:

Wagner (1) discussed estimation of the rate constant for elimination from the slope of the descending part of an apparently linear semilogarithmic plot of concentration of drug in blood plasma against time. He pointed out that such a graphical estimate may be lower than the true value of the rate constant. Also, since the estimate of the absorption rate constant depends upon the value of the elimination rate constant, the estimated value of the absorption rate constant may be in error too. It is the purpose of this communication to show that this difficulty may be overcome by methods of digital computation, although Krüger-Thiemer and Eriksen (2) recently reported that it could not. The problem with the data of Krüger-Thiemer and Eriksen (2) was that blood samples were not taken long enough following oral administration.

Several examples were presented by Wagner (3, 4). One example (4) was the fitting of serum concentrations of tetracycline hydrochloride activity observed in a subject following a single oral dose of a capsule of tetracycline HCl[1] 250 mg. The semilogarithmic plot gave an estimate of 0.130 hr.$^{-1}$ for the elimination rate constant. The residuals, plotted semilogarithmically, gave an estimate of 0.810 hr.$^{-1}$ for the absorption rate constant. Use of these preliminary estimates with an iterative digital computer program gave a least squares fit (using equal weights) with a 57.6% reduction in variance; the rate constant for elimination changed to 0.149 hr.$^{-1}$ and the rate constant for absorption changed to 0.716 hr.$^{-1}$. If one assumes the least squares estimates are the "best" values, then the graphical estimate of the absorption rate constant was 13.1% higher, and the graphical estimate of the elimination rate constant was 12.8% lower than the "best" value, respectively. The latter is of the same order of magnitude as in the simulation example of Wagner (1).

Another example is the fitting of average serum concentrations of erythromycin activity observed in a panel of 12 adult male volunteers following oral administration of 500 mg. of erythromycin activity in tablet form. Preliminary graphical estimation, shown in Fig. 1, gave an estimate of 0.899 hr.$^{-1}$ for the absorption rate constant, 0.393 hr.$^{-1}$ for the elimination rate constant, and 0.58 hr. for the lag time. An iterative digital computer program gave a least squares fit (with weighting according to the reciprocal of the average serum concentrations) with a 73.2% reduction in variance; the absorption rate constant changed to 0.708 hr.$^{-1}$, the elimination rate constant changed to 0.444 hr.$^{-1}$, and the lag time changed to 0.602 hr. If one assumes the least squares estimates are the "best" values, then the graphical estimate of the absorption rate constant was 27.0% higher and the graphical estimate of the elimination rate constant was 11.5% lower than the "best" value, respectively. The least squares fit is shown in Fig. 2.

These two examples are representative of a more general phenomenon. Least squares fitting of all observed serum or plasma concentrations of a set almost always results in an increase of 10 to 15% in the magnitude of the elimination rate constant and a decrease in the magnitude of the absorption rate constant compared with estimates obtained graphically using semilogarithmic paper. This implies that, in such cases, the trend line of the terminal data points of the semilogarithmic plot is slightly curved and not linear as one assumes when estimating the elimination rate constant by the graphical method. This is illustrated by Fig. 3, in which the least squares curve is drawn through the data points on semilogarithmic paper.

This underestimation of the elimination rate constant can be anticipated from the equation for plasma or serum concentration, namely:

$$\text{concn.} = (\text{constant})\,(e^{-k_2 t} - e^{k_1 t}) \quad \text{(Eq. 1)}$$

where $k_1 > k_2$. The observations of concentration underestimate $e^{-k_2 t}$ by the amount $e^{-k_1 t}$; this bias is greater for smaller values of t. A line drawn through the terminal points of a semilogarithmic plot of concentration against time will have successively greater bias to the left. This bias causes the line to have a smaller slope than it should have, thus underestimating k_2, the elimination rate constant.

We have assumed in the above discussion that the smallest rate constant estimated is the elimination rate constant and the larger rate constant is the absorption rate constant. This is usually the case but, in rare instances, the assignments could be reversed. We also assume that the estimate of the smallest rate constant by least squares fitting of all the data points is a more valid estimate than the estimate obtained

[1] Marketed as Panmycin Hydrochloride by The Upjohn Co., Kalamazoo, Mich.

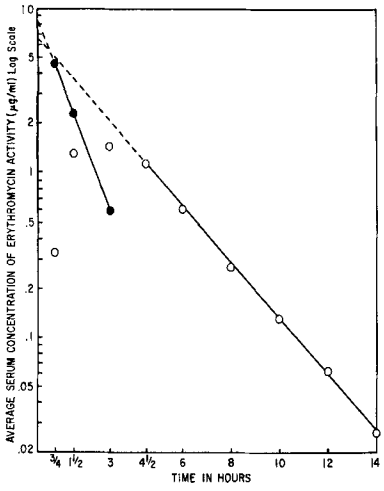

Fig. 1—Illustrates graphical estimation of the rate constants. The terminal linear segment gave an estimate of 0.393 hr.$^{-1}$ for the elimination rate constant. The residuals gave an estimate of 0.899 hr.$^{-1}$ for the absorption rate constant. The intersection of the two lines gave an estimate of 0.58 hr. for the lag time. Key: ○, observed; ●, residuals.

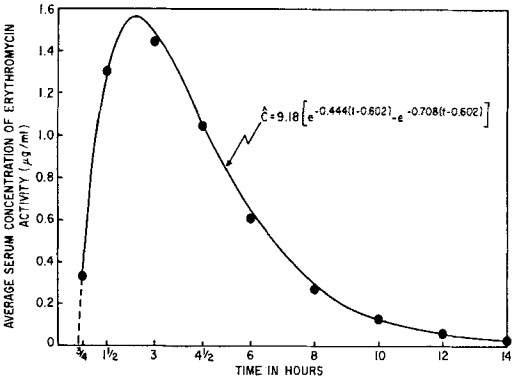

Fig. 2—The black circles are the observed average serum concentrations of erythromycin. The line was drawn by substituting values of time into the equation shown on the figure. The equation was the result of least squares fitting of the observed serum concentrations with a digital computer. Weighting was according to the reciprocals of the average serum concentrations.

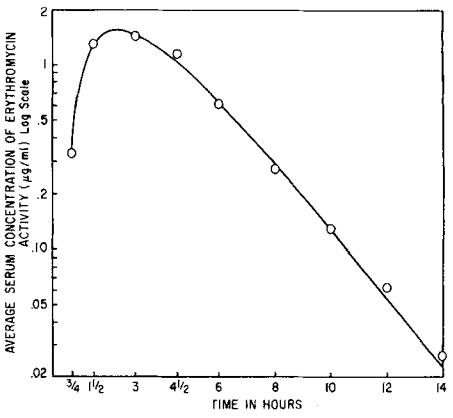

Fig. 3—The circles are the observed average serum concentrations of erythromycin. The line drawn through the points was obtained by substituting values of time into the equation shown on Fig. 2. It should be noted that the line drawn through the terminal six points is slightly curved compared with the straight line in Fig. 1.

graphically from only some of the points. Since most estimates of elimination rate constants reported in the literature were obtained by the latter method, we believe the reported rate constants are lower than the "best" values, and the corresponding half-lives are higher than the "best" values.

(1) Wagner, J. G., *J. Pharm. Sci.*, **52**, 1097(1963).
(2) Krüger-Thiemer, E., and Eriksen, S. P., *ibid.*, **55**, 1249(1966).
(3) Wagner, J. G., *Can. J. Pharm. Sci.*, **1**, 55(1966).
(4) Wagner, J. G., *Clin. Pharmacol. Therap.*, **8**, 201(1967).

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Received January 31, 1967.
Accepted for publication March 22, 1967.

The authors are grateful to C. G. Chidester and H. M. Murray, Control Division, The Upjohn Co., for microbiological assays.

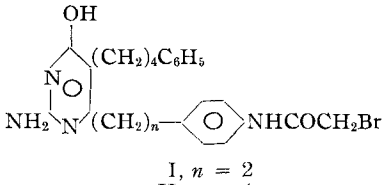
Irreversible Enzyme Inhibitors LXXXVIII. Differential Irreversible Inhibition of Dihydrofolic Reductase from Different Species

Sir:

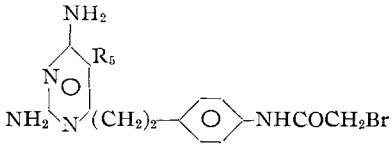
A variety of 2,4-diaminoheterocycles, known to be reversible inhibitors of dihydrofolic reductase, are sufficiently tissue- or species-specific to be useful chemotherapeutic agents (1, 2). Examples are amethopterin for leukemia (3), 5-(*p*-chlorophenyl)-2,4-diamino-6-ethylpyrimidine (4), and 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine (5) as antimalarials; 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine as an antibacterial (6); and 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-(2′-methyltetramethylene)-*s*-triazine as an anthelmintic (7, 8). The selectivity of amethopterin on certain tissues can be attributed to a difference in the efficiency of the folic acid active-transport system (9). The selectivity of the remaining compounds is most probably due to differences in binding by the aryl groups to the hydrophobic bonding region of dihydrofolic reductase (2, 10–12); these compounds appear to enter cells by passive diffusion (13, 14).

Although numerous 2,4-diaminoheterocycles of the type that enter cells by passive diffusion have been screened in a variety of tumors by the CCNSC, none have shown sufficient selectivity to warrant medical use; thus, to date, differences in the hydrophobic bonding region of dihydrofolic reductases from normal and tumor tissue have not been sufficiently great to be exploitable. Since with active-site-directed irreversible inhibitors an extra dimension of specificity can be obtained which is not present with reversible inhibitors (15–17), such irreversible inhibitors might exhibit more selectivity than reversible inhibitors. That selective irreversible inhibition can be obtained with inhibitors of the dihydrofolic reductases from different sources is the subject of this paper.

Compounds I–IV were evaluated as reversible and irreversible inhibitors of the dihydrofolic reductases from pigeon liver and *E. coli* B; the results are presented in Table I. The 2-amino-4-pyrimidinols (I and II) inactivated both enzymes at rates not differing more than a factor of 2 when compared at equal concentrations of reversible enzyme-inhibitor complex—the rate-determining species (16, 18). In contrast, the 2,4-diaminopyrimidine (III) analog of the 2-amino-4-pyrimidinol (I) showed no inactivation of either enzyme.



I, $n = 2$
II, $n = 4$



III, R = $-(CH_2)_4C_6H_5$
IV, R = $-C_6H_4Cl$-*p*

Although the 2,4-diamino-5-*p*-chlorophenylpyrimidine derivative (IV) was equally effective as a reversible inhibitor of both enzymes, irreversible inhibition showed dramatic selectivity; only the *E. coli* B enzyme was inactivated.

Also noteworthy is the comparative irreversible inhibition of the *E. coli* B dihydrofolic reductase with III and IV; the structural difference resides only in the hydrophobic bonding group at the 5-position, yet IV inactivated the enzyme, but III did not. Apparently the nature of binding of the hydrophobic bonding group (R_5) to the enzyme can change the position of the terminal leaving group, thus effecting the juxtaposition of the leaving group to the enzymic nucleophilic group—as previously predicted (12, 19).

The search for specificity with irreversible inhibitors between dihydrofolic reductases from selected animal tumors and a normal tissue, such as liver, from the same animal is currently being pursued.

(1) Jukes, T. H., and Broquist, H. P., in "Metabolic Inhibitors," Hochster, R. M., and Quastel, J. H., eds., Academic Press Inc., New York, N. Y., 1963, pp. 481–534.
(2) Hitchings, G. H., and Burchall, J. J., *Advan. Enzymol.*, **27**, 417(1965).
(3) Delmonte, L., and Jukes, T. H., *Pharmacol. Rev.*, **14**, 91(1962).
(4) Hitchings, G. H., *Clin. Pharmacol. Therap.*, **1**, 570 (1960).
(5) Crowther, A. F., and Levi, H. A., *Brit. J. Pharmacol.*, **8**, 93(1953).
(6) Roth, B., Falco, E. A., Hitchings, G. H., and Bushby, S. R. M., *J. Med. Pharm. Chem.*, **5**, 1103(1962).
(7) Roth, B., Burrows, R. B., and Hitchings, G. H., Abstracts from the 137th Meeting of the American Chemical Society, Cleveland, Ohio, 1960, p. 31 N.
(8) Baker, B. R., Lee, W. W., Skinner, W. A., Martinez, A. P., and Tong, E., *J. Med. Pharm. Chem.*, **2**, 633(1960).
(9) Werkheiser, W. C., *Proc. Am. Assoc. Cancer Res.*, **3**, 371(1962).
(10) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **53**, 1137(1964).
(11) Baker, B. R., Ho, B.-T., and Santi, D. V., *ibid.*, **54**, 1415(1965).
(12) Baker, B. R., and Ho, B.-T., *ibid.*, **55**, 470(1966).
(13) Wood, R. C., and Hitchings, G. H., *J. Biol. Chem.*, **234**, 2377, 2381(1959).
(14) Frenkel, J. K., and Hitchings, G. H., *Antibiot. Chemotherapy*, **7**, 630(1957).
(15) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(16) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, New York, N.Y., 1967.
(17) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714 (1964).

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASES FROM PIGEON LIVER AND *E. coli* B

No.	Reversible Inhibition, K_i (M)[a] Pigeon Liver	Reversible Inhibition, K_i (M)[a] *E. coli* B	Irreversible Inhibition[b] Pigeon Liver μM Concn. Inhib.	Pigeon Liver μM Concn. TPNH	Pigeon Liver % $E\cdots I$	Pigeon Liver Time, min.	Pigeon Liver % Inactivation	*E. coli* B μM Concn. Inhib.	*E. coli* B μM Concn. TPNH	*E. coli* B % $E\cdots I$	*E. coli* B Time, min.	*E. coli* B % Inactivation
I[c]	3×10^{-5}[d]	1×10^{-3}	40	0	57	12	50[d]	100	0	10	120	59
II[c]	4×10^{-5}[d]	5×10^{-4}	40	0	50	12	50[d]	50	0	10	120	22
III	7×10^{-9}[e]	1×10^{-7}	0.01	12[f]	59	60	0[e]	0.10	30[f]	50	120	0
IV	1×10^{-7}[e]	1×10^{-7}	0.25	12[f]	72	60	0[e]	0.10	30[f]	50	120	33

The technical assistance of Barbara Baine with these assays is acknowledged. [a] K_i was estimated (18) from the concentration of inhibitor necessary to give 50% inhibition in the presence of 6 μM dihydrofolate as previously described (10); K_m = 2.5×10^{-5} M was used for *E. coli* B (20) and 1×10^{-6} M for pigeon liver (18). [b] Inactivation at 37° and pH 7.4 was measured as previously described (21); for the assay of remaining *E. coli* enzyme, 60 μM each of dihydrofolate and TPNH were used to make the rate of reaction dependent on enzyme concentration. [c] All assays contained 10% *N,N*-dimethylformamide to aid solubility. [d] Data from *Reference 19*. [e] Data from *Reference 22*. [f] TPNH is required for optimum reversible complexing of 2,4-diaminoheterocycles, but not for 2-amino-4-hydroxy heterocycles; see Perkins, J. P., and Bertino, J. R., *Biochemistry*, **5**, 1005(1966).

(18) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(19) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **55**, 1417(1966).
(20) Burchall, J. J., and Hitchings, G. H., *Mol. Pharmacol.*, **1**, 126(1965).
(21) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(22) *Ibid.*, **4**, 31(1967).

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Received January 9, 1967.
Accepted for publication March 1, 1967.

This work was supported in part by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.
J. H. Jordaan wishes to thank the Atomic Energy Board, Republic of South Africa, for a fellowship.
Previous paper: Baker, B. R., and Lourens, G. J., *J. Pharm. Sci.*, to be published.

Revised Structure of Columbianin

Sir:

Columbianin is a naturally-occurring glycosidic coumarin isolated from *Lomatium columbianum* Math. and Const. (1) and from *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (2). Willette and Soine (1) suggested structure I for columbianin on the basis that, on acid hydrolysis, it yielded D-glucose and a tertiary alcohol (*i.e.*, columbianetin). The structure of the aglycone (II) was firmly established by spectral studies as well as by chemical degradative evidence and has since then been confirmed (3) and extended (4) by Nielsen and Lemmich to show that the absolute configuration is 8(*S*). Since no quantitative estimation of the D-glucose content was made in the earlier study and because the analytical data (*i.e.*, assuming water of crystallization) corresponded within limits for I and its tetraacetate, the structure assignment was considered adequate.

Recently, in connection with another study, we had occasion to examine the nuclear magnetic resonance (NMR) spectrum of acetylated columbianin and noted that the *O*-acetyl methyls were to be observed as four peaks at τ 7.92, 8.00, 8.04, and 8.07; whereas the *gem*-dimethyls of columbianetin were found as a singlet at τ 8.69. To our surprise, the peak ratio between the *O*-acetyl methyls and the *gem*-dimethyls was found to be 3.52:1 instead of the expected 2:1. This led us to re-examine the structural assignment for columbianin by means of additional analytical data as well as by further degradative studies.

An ebullioscopic determination on the acetate suggested the molecular weight to be about 800 and an acetyl analysis indicated 34.92%, both values being incompatible with the tetraacetate formulation. On the other hand, the results pointed strongly toward a disaccharide moiety in columbianin and a heptaacetate as the acetylated form. Furthermore, additional elemental analyses on the acetate derivative gave much closer correspondence with the values expected for $C_{40}H_{48}O_{21}$ than for the previously suggested $C_{28}H_{32}O_{13} \cdot 2H_2O$. The question of the presence of sugars other than D-glucose had been ruled out in the earlier work (1).

Partial hydrolysis of the glycoside was carried out using an ion exchange resin[1] in the acid cycle (5). Examination of the hydrolysate by paper chromatography was carried out using an ethyl acetate–pyridine–water mobile phase and developing the chromatogram by using the silver nitrate technique (6) for visualization of the

[1] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa.

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Received January 9, 1967.
Accepted for publication March 1, 1967.

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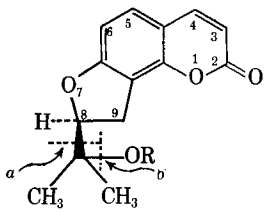

I, R=—D-Glucosyl
II, R=—H
III, R=—β-D-Gentiobiosyl

carbohydrate spots. Two spots were observed with one of these being identical to D-glucose in its R_f value. The R_f value of the other spot was consistent with that for disaccharides possessing a 1→6 linkage. Thus, the possibilities, virtually indistinguishable by paper chromatography, were isomaltose (α-linkage) and gentiobiose (β-linkage). The same partial hydrolysis carried out on a preparative scale led to the isolation of several milligrams of the crude disaccharide, $[\alpha]_D^{20}$ −6° (c 2.00, H_2O). Since the specific rotations (7) of the two possibilities are rather far apart—isomaltose, $[\alpha]_D^{24}$ +120° (H_2O); gentiobiose $[\alpha]_D^{20}$ +8.7° (H_2O)—the results strongly suggested gentiobiose as the disaccharide, with the small discrepancy probably being accounted for by extraction of impurities from the paper. Acetylation of the disaccharide provided gentiobiose octaacetate, m.p. 191–192°, $[\alpha]_D^{20}$ −1.86° (c 0.64, $CHCl_3$), identical with the authentic material m.p. 193°, $[\alpha]_D^{19.5}$ −5° (c 1.8, $CHCl_3$) (8) as shown by mixed melting point determination and comparison of infrared spectra.[2] These data indicate conclusively that columbianin is a gentiobioside of columbianetin. The question of whether the gentiobiose moiety is attached by an α- or β-linkage to the aglycone was examined by subjecting the glycoside to the action of β-glucosidase.[3] Enzymatic cleavage takes place stepwise from the nonreducing end of the molecule (9) and, in this case, one would expect to find D-glucose, a new monosaccharidic glucoside, and columbianetin as the products of hydrolysis if the linkage were β. On the other hand, if the glycosidic linkage were α, no columbianetin would be observed although the other two products would be expected. Paper and thin-layer chromatographic examination of the hydrolytic products showed no gentiobiose but did show spots corresponding to glucose and columbianetin (fluorescent) as well as a new fluorescent spot with chromatographic behavior indicating a lesser polarity than that of the original glucoside. This material has been isolated in a crystalline state with an indefinite melting point following softening at about 100° with slow effervescence, a behavior not uncommon to glycosides. Examination of this product by mass spectrometry confirmed the molecular weight of 408 for the $C_{20}H_{24}O_9$ monosaccharidic coumarin by virtue of the molecular ion. Furthermore, two major fragmentation peaks at *m/e* 187 and 229 are, very likely, representative of the coumarin portion of the molecule and represent $[C_{11}H_7O_3]^+$ and $[C_{14}H_{13}O_3]^+$ arising from fragmentation at *a* and *b*, respectively, in I. Complete characterization of this compound is being carried out presently and will be reported at a later date. Columbianetin was also obtained in a crystalline form and identified by mixed melting point and spectral comparison with authentic material. The fact that enzymatic cleavage has been accomplished to yield columbianetin as a final product identifies a β-linkage to the aglycone and, therefore, the structure of columbianin may be assigned as the β-gentiobioside of columbianetin (III).

(1) Willette, R. E., and Soine, T. O., *J. Pharm. Sci.*, **53**, 275(1964).
(2) Gupta, P. K., and Soine, T. O., *ibid.*, **53**, 1543(1964).
(3) Nielsen, B. E., and Lemmich, J., *Acta Chem. Scand.*, **18**, 1379(1964).
(4) *Ibid.*, **18**, 2111(1964).
(5) Smith, F., and Montgomery, R., "The Chemistry of Plant Gums and Mucilages," ACS Monograph Series No. 141, Rheinhold Publishing Co., New York, N.Y., 1959, p. 98.
(6) Hough, L., "Methods in Carbohydrate Chemistry," Whistler, R. L., and Wolfrom, M. L., eds., Academic Press Inc., New York, N.Y., 1962, vol. 1, p. 28.
(7) Hassid, W. Z., and Ballou, C. E., "The Carbohydrates," Pigman, W., ed., Academic Press Inc., New York, N.Y., 1957 pp. 492–493.
(8) Goldstein, I. J., and Whelan, W. J., "Methods in Carbohydrate Chemistry," Whistler, R. L., and Wolfrom, M.L., eds., Academic Press Inc., New York, N. Y., 1962, vol. 1, p. 315.
(9) Bailey, R. W., and Pridham, J. B., "Advances in Carbohydrate Chemistry," Wolfrom, M. L., ed., Academic Press Inc., New York, N.Y., 1962, vol. 17, p. 142.

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Received February 3, 1967.
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This investigation was supported by grant No. HE-07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[2] The authors acknowledge with gratitude the assistance of Dr. B. A. Lewis, Biochemistry Department, St. Paul Campus, University of Minnesota, St. Paul, in providing valuable guidance and authentic samples for comparison.
[3] Product of General Biochemicals, Chagrin Falls, Ohio.

REVIEWS

The Vitamin Co-factors of Enzyme Systems. By F. A. ROBINSON. Pergamon Press Inc., 44-01 21st St., Long Island City, NY 11101, 1966. ix + 896 pp. 15.5 × 23 cm. Price $25.

"The Vitamin Co-factors of Enzyme Systems" is a timely replacement of the author's earlier book, "The Vitamin B Complex," which was published in 1951. Most of the material from the earlier book has been incorporated in the present volume, in addition to a rather extensive collection of recent knowledge concerning the role of the B complex vitamins as enzyme co-factors.

Earlier books on vitamins were concerned principally with their importance in nutrition and deficiency syndromes, but during the past two decades, a considerable number of studies have been conducted to establish the enzyme co-factor function of the vitamin B complex and its role in metabolic processes. Robinson emphasizes this revolutionary new concept of vitamin B activity.

The volume is composed of an Introduction, a Conclusion, and nine chapters dealing with the B complex vitamins, including thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, lipoic acid, biotin, folic acid, and vitamin B_{12}. The chapters on folic acid, vitamin B_{12}, and lipoic acid are new additions; *p*-aminobenzoic acid, choline, and inositol are not included in the book because their co-factor function is obscure. Each chapter is divided into subsections for the convenience of the reader. The references which are cited at the end of each section are well selected, but include only those published up to 1963.

The author's discussion of the close inter-relationship between these co-factors and the fundamental basis for the need of these vitamins in proper and sufficient amounts in biological systems is inadequate. His relegation of this area to the 5-page conclusion section may lead the reader to overlook the dynamic aspect of these co-factors acting in biological systems as an interdependent group.

This book is well-organized, the facts are clearly presented, and it serves as an excellent reference source for information about the vitamin B complex.

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The Chemistry of Heterocyclic Compounds: Multi-Sulfur and Sulfur and Oxygen Five- and Six-Membered Heterocycles. Part I. By DAVID BRESLOW and HERMAN SKOLNIK. Interscience Publishers, a division of John Wiley & Sons, Inc., 605 Third Avenue, New York, N. Y. 10016, 1966. xxii + 610 pp. 15 × 22.5 cm. Price $33.00.

Part One of the twenty-first volume in the Weissberger series of "The Chemistry of Heterocyclic Compounds" maintains in quite adequate fashion the objective of this series to record the definitive chemistry of heterocyclic compounds. The authors' intent "to review the literature selectively and eclectically," establishing what is valid and denying insubstantial claims, has been followed throughout; an authoritative work has resulted.

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Boston, Mass.

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REVIEWS

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"The Vitamin Co-factors of Enzyme Systems" is a timely replacement of the author's earlier book, "The Vitamin B Complex," which was published in 1951. Most of the material from the earlier book has been incorporated in the present volume, in addition to a rather extensive collection of recent knowledge concerning the role of the B complex vitamins as enzyme co-factors.

Earlier books on vitamins were concerned principally with their importance in nutrition and deficiency syndromes, but during the past two decades, a considerable number of studies have been conducted to establish the enzyme co-factor function of the vitamin B complex and its role in metabolic processes. Robinson emphasizes this revolutionary new concept of vitamin B activity.

The volume is composed of an Introduction, a Conclusion, and nine chapters dealing with the B complex vitamins, including thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, lipoic acid, biotin, folic acid, and vitamin B_{12}. The chapters on folic acid, vitamin B_{12}, and lipoic acid are new additions; *p*-aminobenzoic acid, choline, and inositol are not included in the book because their co-factor function is obscure. Each chapter is divided into subsections for the convenience of the reader. The references which are cited at the end of each section are well selected, but include only those published up to 1963.

The author's discussion of the close inter-relationship between these co-factors and the fundamental basis for the need of these vitamins in proper and sufficient amounts in biological systems is inadequate. His relegation of this area to the 5-page conclusion section may lead the reader to overlook the dynamic aspect of these co-factors acting in biological systems as an interdependent group.

This book is well-organized, the facts are clearly presented, and it serves as an excellent reference source for information about the vitamin B complex.

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tion in an ever-increasing number of areas. Certainly, their use in pharmaceutical products as dispersing, emulsifying, solubilizing, and wetting agents has contributed to the formulation advances of the last two decades.

The subject matter is contained in 29 chapters and covers the organic chemistry (432 pages), physical chemistry (379 pages), and analytical chemistry (95 pages) of nonionic surfactants, with a final section of 73 pages devoted to the biology of these compounds. It is gratifying to note that several of the chapters have been contributed by authors from Schools of Pharmacy and the pharmaceutical industry.

Following an introductory chapter, the next twelve chapters deal with the organic chemistry of the numerous classes of nonionic surfactants; the preparative procedures, physical properties, and applications are described in each case. Inevitably, the overwhelming portion of this section is concerned with commercial surfactants. This results in a comprehensive coverage of the technical and patent literature pertaining to these compounds. Commercial trademarks abound in the text but are, annoyingly, omitted from the subject index. The average reader, not familiar with the chemical names of every commercial nonionic surfactant, may therefore have to search through no less than 27 tables scattered throughout the text in order to find a particular surfactant. The last chapter in this section discusses the synthesis of homogeneous nonionic surfactants and is a fitting introduction to the next nine chapters describing the physical chemistry of nonionics, since the more meaningful work in this area invariably requires the use of homogeneous compounds.

The chapters devoted to micelle formation, solubilization, detergency, and foaming are standard presentation of the subject matter. Unfortunately, the chapter supposedly dealing with the physical chemistry of emulsification is quite inadequate, especially in view of the comment, made in the introductory chapter, that one of the major applications of nonionic surfactants is in emulsion technology. Only 22 pages in the whole book are devoted to this topic with just over half a page allotted to the applications of nonionic emulsifiers. In contrast, the remaining chapters on surface films, the thermodynamics of micelle formation, the effect of nonionic surfactants on the stability of dispersions, and the configuration and hydrodynamics of the polyoxyethylene chain are excellent and add much to the value of this section of the book.

Four chapters comprise the third section which reviews the analytical chemistry of nonionic surfactants. Following an introductory chapter, the discussion turns to noninstrumental, instrumental, and separational methods of analysis. These chapters are written in a practical vein and succeed in presenting a logical methodology for achieving the separation and identification of this class of compounds.

The biological aspects of nonionic surfactants are dealt with in the final two chapters of the book. The first of these deals with the biological, pharmacological, and toxicological effects of nonionic surfactants on animals and humans. Much of this material, presented in a concise yet highly factual manner in less than 40 pages, is highly relevant to the contemporary practice of pharmacy. The final chapter is a necessarily brief account of the biodegradation of nonionic surfactants.

This then is a text covering a wide range of topics associated with nonionic surfactants. On the whole, the subject matter is well presented and the coverage adequate. The book is not written specifically for any one discipline and so no one in the pharmaceutical sciences is likely to find all the text of direct interest. By the same token, it will undoubtedly be of *some* interest to all those concerned with the science and technology of these compounds. While "Nonionic Surfactants" is not likely to receive a great deal of use as the personal copy of any one individual, it will be a valuable addition to any university or industrial pharmacy library.

Reviewed by James Swarbrick
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Lafayette, Ind.

NOTICES

Dynamics of Chromatography. Part I: Principles and Theory. By J. CALVIN GIDDINGS. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1965. xii + 323 pp. 15.5 × 23 cm. Price $11.50.

Chemical Carcinogenesis and Molecular Biology. By PASCALINE DAUDEL and RAYMOND DAUDEL. Interscience Publishers, a Division of John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1966. 159 pp. Price $7.

Anticancer Agents. By FRANCES E. KNOCK. Charles C Thomas, Publisher, 301–327 E. Lawrence Ave., Springfield, Ill., 1967. xix + 272 pp. 18 × 25.5 cm. Price $15.50.

Treatment of Psychiatric Outpatients. By R. R. KOEGLER and N. Q. BRILL. Appleton-Century Crofts, 440 Park Ave. So., New York, NY 10016, 1967. xvii + 223 pp. 14.5 × 21 cm. Price $6.95.

Handbuch der Kolorimetrie. Band III. Kolorimetrie in der Biologie, Biochemie und Medizin. 2. Teil. By B. KAKAC and Z. J. VEJDELEK. VEB Gustav Fisher Verlag, DDR, Jena, Villengang 2/PostshchlieBfach 176, Germany, 1966. 496 pp. 17 × 24.5 cm. Price Geb. MDN 49.60.

Arzneipflanzen. Part 24: *Aesculus hippocastanum L.* By F. AUSTER and J. SCHAFER. VEB Georg Thieme, Leipzig, Absatabteilung, DDR-Jena, Villengang 2/PostschlieBfach 176, Germany, 1966. 71 pp. 17 × 24 cm. Price MDN 6.75 Paperbound.

Hyaluronidase: An Annotated Bibliography. Part II. (1955–1966). By MYER M. FISHMAN. Technical Service Laboratories, River Edge, N. J., 1966. 88 pp. 21.5 × 27.5 cm. $5.00 Paperbound.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

June 1967 volume 56, number 6

Review Article

Potentiometric Determination of Dissociation Constants

By LESLIE Z. BENET and JERE E. GOYAN*

AT ONE TIME or another almost every pharmaceutical scientist wants to know the dissociation constant[1] for a particular drug. He might want to know whether his drug is in the ionic or nonionic form, if it will be sufficiently soluble in the gastrointestinal fluid, or how the gastric fluid might affect the rate of dissolution of the drug. He might wish to predict the stability or explain the kinetics of degradation for a dissociable drug in various buffer solutions. He might want to hypothesize a mechanism of action, predict the extent of binding, or examine complex formation. In all of these cases and in many others the pharmaceutical scientist needs to know the dissociation constant in order to solve his problem. His first reaction will be to "look it up" and if he is lucky he will find critically evaluated values in either "Dissociation Constants of Organic Acids in Aqueous Solutions" (1) or the companion volume for organic bases (2). However, in most cases he will be working with a drug for which the dissociation constant is not readily available, and unfortunately when dealing with drugs in biological systems at 37° the reliable data available are quite sparse.[2] He will then have to carry out an experimental determination, and will usually turn to the most readily available and easily operated instrument, the laboratory pH meter and its accompanying electrodes.

Potentiometric pH titration is by far the most convenient method for the determination of dissociation constants and, with care, this method can give the experimenter good reproducible results (pKa ± 0.03 units) for acids and bases having pKa values between 2.5 and 11 (errors due to residual junction potentials increase the error outside these limits).

The techniques used in carrying out the titration are standard and are well covered by Albert and Serjeant (4). The meter should be accurately calibrated with at least two standard buffers, the buffers and the solutions to be titrated should all be kept at a constant temperature, all solutions should be carbon dioxide free, the titration should be carried out under nitrogen, and the pH should be read for a large number of

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The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

[1] Whenever "dissociation constant" is used, it will refer to the *acid* dissociation constant.

[2] There is no universal relationship which will allow one to predict the dissociation constant of a compound at 37° when the more usually available value at 25° is known. The enthalpy of dissociation is a complex function of temperature and consequently the usual Van't Hoff type of plot is not useful. For example, the pKa (thermodynamic) for salicylic acid shows almost no change with values of 2.97 at 25° and 2.95 at 37°, while the values for barbital are 7.98 at 25° and 7.82 at 37°. Hall and Sprinkle (3) have found that the temperature coefficients for the nitrogenous bases between 0° and 40° follow a general pattern in that all become weaker bases as the temperature increases and that the depression in pKa per degree temperature rise becomes progressively larger as the basicity increases.

titrant additions. Up to this point, the procedure is unambiguous; however, the data must be interpreted and the dissociation constant calculated. There are a large number of possible ways in which the data may be treated; all are useful to some extent, but each method has its limitations. These methods, their usefulness, and their limitations are the subject of this review.

The pharmaceutical scientist and most other biological scientists are particularly interested in the aqueous dissociation constant, and this review will concentrate primarily in this area. However, since a great number of drugs are highly insoluble in the unionized form, dissociation constants are often determined in varying percentages of semiaqueous systems and these values are extrapolated to a value for water. These types of calculations will also be discussed. The sections dealing with aqueous and semiaqueous considerations will be applied to monoprotic species or to those polyprotic acids and bases for which the pKa values differ by at least 3 units. A third section of the review will be devoted to a short discussion of polyprotic species with overlapping pKa values.

AQUEOUS SOLUTIONS

Nonlinear Relationships—Nonlinear plots of pH *versus* titrant added are the most familiar and they may be obtained mechanically, utilizing an automatic titrator and a recorder. These plots are often used to determine the dissociation constant by what we shall call the half-neutralization method. This method is based on a corrupted form of the Henderson-Hasselbalch equation,[3]

$$p[H] = pKa^c + \log \frac{[base]}{[acid]} \qquad \text{(Eq. 1)}$$

where p denotes negative logarithm, [ ] concentration, and Ka^c is the stoichiometric dissociation constant. The equation utilizes the principle that when the concentration of the base (conjugate base if we are titrating an acid) equals the concentration of acid, the substance is half-neutralized. At this point the pH of the solution as read on the pH meter equals the pKa of the drug. However, assuming that the pH is equal to the pKa when an amount of strong titrant equal to one-half the concentration of the pure drug has been added can lead to erroneous results other than that due to assuming that the pH read on the meter is the negative logarithm of the hydrogen-ion concentration.[4]

Meites and Goldman (5–9) have examined the dilution and hydrogen-ion effect in relation to the inflection point of acid-base titration curves and have proposed some general equations to demonstrate the errors inherent in assuming that pH = pKa at half-neutralization. Let f be defined as the degree of neutralization of an acid. Where we are considering the titration of V_a^0 ml. of a C_a^0 solution

$$f = \frac{V_b C_b}{V_a^0 C_a^0} \qquad \text{(Eq. 2)}$$

of a weak monobasic acid with a C_b solution of a completely dissociated monoacidic base. Goldman and Meites (7) have derived the following equation to locate the point at which pH actually does equal pKa during potentiometric titrations:

$$f_{pKa} = \frac{1/2 - \{[K_a - (K_w/K_a)]/C_a^0\}}{1 + r\{[K_a - (K_w/K_a)]/C_a^0\}} \qquad \text{(Eq. 3)}$$

where f_{pKa} is the degree of neutralization when p[H] equals pKa, p[H] = negative logarithm of the hydrogen-ion concentration, pKa = negative logarithm of the apparent dissociation constant, K_w = the ion product of water and,

$$r = C_a^0/C_b \qquad \text{(Eq. 4)}$$

From Eq. 3 it can be seen that f_{pKa} will coincide with half-neutralization only when $K_a = (K_w)^{1/2}$. Substantial errors will result from taking pKa to be equal to pH at $f = 1/2$ if K_a is widely different from $(K_w)^{1/2}$ and this will be especially true if the concentrations of the reagents are small.

As an example, consider the titration of a 0.01 M solution of a weak acid ($K_a = 1 \times 10^{-3}$) by a 1.0 M solution of strong base. Putting these values into Eq. 3, $f_{pKa} = 0.40$. If the initial concentration of the weak acid were 0.001, the titration would begin at a pH higher than the pKa (as indicated by the fact that $f_{pKa} < 0$), and the hydrogen-ion concentration at "half-neutralization" would give a value of 2.8×10^{-4}, which would correspond to an error in the pKa of greater than 0.5 unit. It should be noted in Eq. 3 that r, the ratio of the concentration of solution being titrated to the concentration of titrant, also affects the f_{pKa}. For example, if the

[3] In 1908, Henderson (21) showed that the hydrogen-ion concentration of a solution equals the dissociation constant of a weak acid-base substance times the ratio of the concentration of the acid to the concentration of the base. It was not until the next year that Sørensen (22) introduced the term pH. As best as we can determine, Hasselbalch added his name to the Henderson equation by being the first (23) to combine Sørensen's definition with the equation. In any case, it is clear from the early work that all the values were in terms of concentration units.

[4] The pH meter does not give an exact measure of the hydrogen-ion activity. Instead we have an operational definition of the practical pH value and a standard scale fixed by one or more standard buffer solutions whose *assigned* pH values are at least formally consistent with the thermodynamic properties of a solution and the definition of what the activity of a single ion should be. However, for purposes of our discussion, we shall assume that the pH values read on the pH meter are the negative logarithm of the hydrogen-ion activity. For a detailed discussion of this point, consult the work of Bates (36).

TABLE I—LOCATIONS OF f_{pKa} IN WEAK ACID–STRONG BASE TITRATIONS AFTER GOLDMAN AND MEITES (7)

K_a	f_{pKa}[a]	f_i
1×10^{-2}	0.364	0.111
3×10^{-3}	0.456	0.360
1×10^{-3}	0.485	0.463
1×10^{-4}	0.498	0.496
1×10^{-5}	0.4998	0.499
1×10^{-7}	0.5000	0.5000

[a] The values of f_{pKa} were computed from Eq. 3 for $C_a^0 = 0.1$, $r = 1$, and $K_w = 1 \times 10^{-14}$. The values of f_i were computed as described by Meites and Goldman (5) and correspond to the points where the slopes of the titration curves are at a minimum.

0.01 M solution of acid were titrated with 0.01 M strong base, the f_{pKa} would drop to 0.364. This is due to a greater dilution effect, and gives a good illustration of the principle behind the usual instructions calling for a concentrated solution of titrant.

Very often, a variation of the "half-neutralization" method may be used where the pKa is taken to be equal to the pH at the inflection point of the sigmoid titration curve. Once again, this only gives a correct pKa when $K_a = (K_w)^{1/2}$, otherwise f_i (degree of neutralization at the inflection point) precedes f_{pKa} and the divergence increases as acid strength increases. Table I shows a comparison of some of the data of Goldman and Meites (7). The values of f_i are calculated by an iterative procedure applied to a very complex equation[5] similar to Eq. 16 in *Reference 5*.

In an attempt to overcome some of the errors inherent in utilizing the half-neutralization method, especially those due to the fact that the concentration of hydrogen ions is not negligible, as compared to the concentration of acid or base for fairly strong acids (pKa 2–4) and bases (pKa 10–12), Parke and Davis (11) introduced a different nonlinear method for determining dissociation constants. A survey of the pharmaceutical literature shows that until the past few years most dissociation contants were determined by methods which were modifications of that described by Parke and Davis.

These authors used hydrogen-ion binding curves to determine the apparent dissociation constants for a variety of mono- and polyprotic acids and bases. Identical volumes of sample solution and blank were titrated with a strong titrant over the pH range 2–12. The curves of these two titrations were plotted on a graph having pH as the abscissa and the amount of titrant added as the ordinate. A third curve, the difference curve, was then drawn with the units of the ordinate being the differences in milliliters of titrant required for the sample and blank to reach the same pH. Previously prepared transparent masks on which the standard curve has been drawn and the point of inflection marked are then fitted to the difference curve. Since "any titratable group produces an inflection having the same shape regardless of its position on the pH scale, the apparent dissociation constant will be the pH at the point of inflection" (11). In addition to the curve for exactly one equivalent of hydrogen ion bound per mole of sample, other masks for 1.1, 0.90, 0.75, 0.50, and 0.25 equivalent per mole were also included for use with samples of unknown purity or molecular weight.

Garrett (12) has made an analysis of the theoretical basis for the Parke and Davis method. He has shown that for dissociation constants in the range 4–10, the procedure has little advantage over the traditional technique of determining apparent pK's at half-neutralization. He also notes that for dissociation constants in the range 2–4, the technique is invalid, especially for estimates of stoichiometry, due to errors arising in the subtraction of volumes.

Stokes (13) has developed an ingenious graphical method to determine the pH value at the equivalence point, the pH for the pure weak acid, pure conjugate base, pH at $f = 0.5$, and the pH at the inflection points. His treatment is based upon: (*a*) formulating a degree of neutralization in terms of one parameter which is so defined that a weak acid is first neutralized with excess base, then back titrated with strong acid; (*b*) the use of dimensionless quantities throughout the calculations; (*c*) the use of hyperbolic functions; and (*d*) the use of logarithmic diagrams of the type favored by Sillén (14) and other Scandinavian workers, for the solutions of the equations. His procedures are similar to those proposed by Sillén, with the important difference that the preparation of only two permanent graphs is sufficient for all cases of a single monoprotic weak acid–base substance titrated with strong acid or base, in any solvent and at any temperature for which K_w is known. At high enough concentrations of a weak acid–base substance the titration curve will have three inflection points, one each at the acid and basic equivalence point, and one at half-neutralization. All of these points may be determined graphically, if the "high enough" concentrations are attainable physically.

Stokes further points out that in those cases

[5] In trying to solve Eq. 16 (*Reference 5*) the reader should be aware that the definitions for two of the terms are erroneous. Consult *Reference 10* for the corrections.

where the three inflection points do exist, it is possible to obtain both the pKa and the concentration of the weak acid or base without the use of any standardized strong acid or base solutions. Roe (15), commenting on this point in a review article, suggests that this opens up the possibility for the development of a rather unique automatic titrator. The use of Stokes' method for the determination of pKa values is unfortunately limited by manipulative difficulties.

Linear Relations—In 1958, Joseph (16) described the advantage of bringing the sigmoid form of titration curves based on the mass action law into a linear form by logarithmic transformation. This transformation was applied particularly to the Henderson-Hasselbalch equation and Joseph constructed a semilogarithmic plot as well as a slide rule for estimation of the pK values. Druckrey (17) has pointed out that he had previously proposed a similar logarithmic transformation of the mass action law which allowed linear representation on a prepared log-log paper (18). These methods have the advantage of ease in plotting and in visualizing the ratio of unionized to ionized concentration. Joseph (19) has also pointed out that when linear logarithmic plots are prepared for polyprotic species, the diagram clearly indicates the distribution of electrical charge over the molecule as a function of pH. Although linear logarithmic plots are easy to construct and helpful in visualizing the ratio of acid to base concentrations, they offer no improvement in the accuracy of dissociation constant determinations as compared to the methods listed under *Nonlinear Relationships*. The linear methods listed above are based on the corrupted Henderson-Hasselbalch equation and like the half-neutralization method, do not correct for the concentration of hydrogen and hydroxide ions, the dilution effect, or activities. Setnikar (20) has recently used a variant of the Druckrey method (17, 18) to determine the apparent dissociation constants of bases with limited solubility. For this type of determination, the Druckrey method has a real advantage in that initially a plot of pH *versus* degree of neutralization will be linear until the solubility of the conjugate species is exceeded and then the plot will degenerate from linearity. However, even if the solubility of the conjugate species is exceeded before half-neutralization, the initial linear portion of the plot may be extrapolated to the point where the degree of neutralization equals one-half (*i.e.* [acid] = [conjugate base]), and at that point the apparent pKa is equal to the pH.

Nonlogarithmic Linear Relationships—It should not be construed, from the statements above about the possible inaccuracy of methods based on the Henderson-Hasselbalch equation (Eq. 1), that the equation itself is inaccurate. Equation 1 is accurate, and it is only the substitution of approximations for the concentrations of the base and acid that lead to erroneous pKa^c values. The concentration of base at any time during a titration is given by the following equation:

$$[\text{base}] = C_b^0 - [X] + [M] + [H^+] - [OH^-] \quad \text{(Eq. 5)}$$

where C_b^0 = initial concentration of pure weak base,
$[X]$ = concentration of strong acid added,
$[M]$ = concentration of strong base added.

Likewise, the concentration of acid at any time during a titration is given by:

$$[\text{acid}] = C_a^0 + [X] - [M] - [H^+] + [OH^-] \quad \text{(Eq. 6)}$$

where C_a^0 is the initial concentration of pure weak acid. Substituting Eqs. 5 and 6 into the nonlogarithmic Henderson equation (see *Footnote 3*), we obtain:

$$[H^+] = K_a^c \frac{C_a^0 + [X] - [M] - [H^+] + [OH^-]}{C_b^0 - [X] + [M] + [H^+] - [OH^-]} \quad \text{(Eq. 7)}$$

Equation 7 is the general dissociation equation for a monoprotic species. Usually a titration involves the addition of a strong acid titrant to a solution of pure weak base, in which case C_a^0 and $[M]$ are dropped from Eq. 7, or the addition of a strong base titrant to a pure weak acid, in which case C_b^0 and $[X]$ are dropped from the equation. The derivation of Eq. 7 can be found in a number of publications (25, 43, 44).

Hofstee (26) has shown that titration curves can be based on linear nonlogarithmic forms of the equilibrium equation of a dissociation reaction. He stated that "from such curves, in contrast to those based on logarithmic transformations both the end point of the titration and the dissociation constant can be derived." This can be shown by letting Z equal the sum of all the known concentrations at any point in a titration.

$$Z = [X] - [M] - [H^+] + [OH^-] \quad \text{(Eq. 8)}$$

Therefore substituting Eq. 8 into Eq. 7, we get the general Eq. 9:

$$[H^+] = K_a^c \frac{C_a^0 + Z}{C_b^0 - Z} \quad \text{(Eq. 9)}$$

For the titration of a pure weak acid, $C_b^0 = 0$, Eq. 9 can be rearranged to the form:

$$Z = C_a^0 - (1/K_a^c)\, Z[H^+] \qquad \text{(Eq. 10)}$$

Therefore, a plot of Z *versus* $Z[H^+]$ would be a straight line with a slope which equals the negative reciprocal of the dissociation constant and an intercept which equals C_a^0.

For the titration of a pure weak base, $C_a^0 = 0$, Eq. 9 can be rearranged to the form:

$$Z = C_b^0 - K_a^c(Z/[H^+]) \qquad \text{(Eq. 11)}$$

Therefore, a plot of Z *versus* $(Z/[H^+])$ would be a straight line with a slope equal to minus the dissociation constant and an intercept of C_b^0.

Benet and Goyan (24, 25) have expanded on the advantages of this particular method: (*a*) good reproducible results can be determined using titration data from the pH ranges 1.5–4 and 9–11; (*b*) very weak concentrations of acids and bases which might not give clearly defined inflection points on a pH *versus* ml. titrant plot can give accurate results when [H] and [OH] corrections are included in the Z term for a nonlogarithmic plot. The advantages in being able to use low concentrations are (*i*) less problems will be encountered resulting from the precipitation of the unionized species, and (*ii*) the ionic strength and therefore the activity coefficients can be better controlled; (*c*) for a weak acid the titration may start at a pH already larger than the pKa without affecting the accuracy of the determination; (*d*) the line drawn in the nonlogarithmic plot serves as an estimate of the accuracy of the data. Any point which deviates greatly from the line immediately shows that the data at that point are invalid; and (*e*) one of the great advantages of nonlogarithmic titration curves is in the ability to determine the molarity of the solution being titrated without resorting to elegant drying techniques or an elemental analysis. This makes the method a valuable tool when determining the dissociation constant of a new monoprotic species.

Lanza and Mazzei (27, 28) have used a different form of nonlogarithmic linear plots to determine the end points of titrations. They report that in some cases they may detect end points at about tenfold lower concentrations than those required to get a utilizable inflection in a plot of pH *versus* ml. of titrant.

Solomons (29) has also used a straight-line relationship which is nonlogarithmic. Although his terms are different, he has essentially rearranged Eq. 10 into an equation that resembles the Lineweaver-Burk expression for describing enzyme–substrate dissociation (30).

$$1/Z = \frac{[H^+]}{K_a^c C_a^0} + \frac{1}{C_a^0} \qquad \text{(Eq. 10}a\text{)}$$

Equation 10*a* can be graphically represented as a straight line if $(1/Z)$ is plotted against $[H^+]$. The extrapolated line then cuts the $1/Z$ axis at $1/C_a^0$ and the $[H^+]$ axis at $-K_a^c$.

Not all authors recommend plotting the data even though they are using the general equation (Eq. 7). For example, Albert and Serjeant (31) recommend that the titrant should be added in 10 equal portions, each a tenth of an equivalent. Then using a variant of Eq. 7, the pKa is calculated at each of these points, and the average pKa is determined from the average of the 10 different K_a's. It seems that these authors are not using the equation to the greatest advantage, since they must put a predetermined value of either C_a^0 or C_b^0 into each calculation. This is emphasized by a quote from their book (31):

> One of the commonest errors in titrating with alkali is for the values, in the set of pK_a values, to show an upward trend as the titration progresses. This is usually caused by an impurity in the substance undergoing determination, so that not so much of it is present as had been supposed. By far the commonest and most troublesome impurity is water. To avoid this trouble, every substance submitted for determination of pK_a should be of analytical purity and dried under the same conditions that preceded its analysis.

Of course "this trouble" can easily be avoided by plotting the data according to Eq. 10.

Effects of Dilution—The work of Meites and Goldman (5–10), as described previously, deals with the dilution effects on the correspondence of pKa with the inflection points for sigmoid plots of pH *versus* ml. titrant (see Table I). Le Duigou and Lauer (32) have utilized the formulas given by Meites and Goldman (5, 10) to determine the pKa of the boric acid–mannitol complex. Conventional calculations resulted in a concentration-dependent "constant," but the inclusion of the dilution effect gave a true constant.

Leeson and Brown (33) have pointed out that the nonlogarithmic equations of Benet and Goyan (24, 25), Eqs. 10 and 11, are not truly general since the authors worked with concentrated titrant such that volume changes could be ignored. However, Leeson and Brown wished to work with volume changes of about 2–5% and proposed a variation of Eq. 10 using moles. According to their derivations, they would define a term similar to Eq. 8 as:

$$Z' = X - M - H^+ + OH^- \qquad \text{(Eq. 12)}$$

where H^+ = numbers of moles of hydrogen ion present in the solution,
M = numbers of moles of strong base added to the solution,

X = numbers of moles of strong acid added to the solution,
OH^- = numbers of moles of hydroxide ion present in the solution.

In this form, the numbers of moles of M or X can be determined by multiplying the number of ml. of titrant added times its normality divided by 1000. However, the number of moles of hydrogen and hydroxide ions must be obtained from the experimental pH data. First, the activity of hydrogen ions must be converted to a concentration value by methods described below. Then the numbers of moles of hydrogen ion can be found by Eq. 12*a*:

$$H^+ = \frac{V}{1000}[H^+] \qquad \text{(Eq. 12}a\text{)}$$

where V = volume of solution in ml. at any particular point in the titration (*i.e.*, original volume + volume of titrant added).

Deriving a general equation using moles according to the method of Leeson and Brown (33) results in the following:

$$[H^+] = K_a^c \frac{A^0 - Z'}{B^0 + Z'} \qquad \text{(Eq. 13)}$$

where A^0 and B^0 are the values for the numbers of moles of pure weak acid and base present at the beginning of the titration. Both A^0 and B^0 are constants throughout the titration.

For the titration of a pure weak acid, $B^0 = 0$, and Eq. 13 can be rearranged to the form:

$$Z' = A^0 - (1/K_a^c)\,Z'[H^+] \qquad \text{(Eq. 14)}$$

Therefore a plot of Z' *versus* $Z'[H^+]$ would be a straight line with a slope equal to the negative reciprocal of the dissociation constant and an intercept equal to A^0. The nonlogarithmic titration plot using the Leeson and Brown derivation now allows the investigator to use any concentration of titrant that he wishes. The use of this method limits the accuracy of the determination only to the abilities of the pH meter to read correct values and the investigator to convert activities to concentrations.

Activity Effects—"Although venous blood carries considerably more CO_2 than does the arterial blood, the buffers of the blood are so efficient that the pH of venous blood is more acid than that of arterial blood by only 0.01 to 0.03 unit, i.e., pH 7.40 vs. pH 7.43" (34). This is an interesting fact; the body through its complicated buffer systems is able to maintain the *activity* of hydrogen ions within the blood to a variance of about 0.03 pH units. For how many drugs do we know the dissociation constant at 37° within a variance of even ± 0.04 units?[6] In most cases we know an "apparent dissociation constant" at some particular ionic strength that may or may not be given. An "apparent dissociation constant" is one that is determined at half-neutralization, *i.e.*, when the concentration of titrant added equals one-half the original concentration of the weak acid or basic substance, the pH read on the meter equals the apparent dissociation constant. The apparent pKa is usually designated as pKa′ and is a hybrid of the stoichiometric (concentration) pK_a^c and the thermodynamic (activity) pK_a^T or simply pKa.

For the following dissociation we can define the various dissociation constants:

$$HY + H_2O \rightleftharpoons H_3O^+ + Y \qquad \text{(Eq. 15)}$$

$$K_a^T = \frac{(H_3O^+)(Y)}{(HY)} = \frac{[H_3O^+]\ \gamma_{H^+}[Y]\ \gamma_Y}{[HY]\ \gamma_{HY}} = K_a^c\left(\frac{\gamma_{H^+}\ \gamma_Y}{\gamma_{HY}}\right) \qquad \text{(Eq. 16)}$$

$$K_a' = \frac{(H_3O^+)\,[Y]}{[HY]} = K_a^c\,\gamma_{H^+} = K_a^T\left(\frac{\gamma_{HY}}{\gamma_Y}\right) \qquad \text{(Eq. 17)}$$

where ( ) denotes activity, [ ] concentration, and γ molar activity coefficient.

The charges on Y and HY have been omitted in Eqs. 15–17 so that the definitions may be general. It is usually assumed that the activity coefficient (γ) for an uncharged species is 1, so depending on whether we are titrating an acid or a base, Eqs. 16 and 17 may be simplified accordingly.

It may be seen from Eq. 17 that a correctly determined apparent or stoichiometric dissociation constant could readily be converted to a thermodynamic dissociation constant if the activity coefficient of the charged species can be determined. Unfortunately, it is unlikely that the apparent dissociation constant will be correct, as noted previously. However, assuming that the determination is correct (*i.e.*, that the effect of dilution and hydrogen ion has negligible effect on the divergence of f_{pKa} from f inflection) as for substances with pKa's in the 5–9 range, when and how should the activity correction be applied?

Albert and Serjeant (35) have also asked the rhetorical question: "When is it advisable to use activity corrections in calculating the results from a potentiometric titration?" They rec-

[6] For very few at any temperature. The editors of the two compilations of dissociation constants (1, 2) evaluate all determinations with a variance of greater than ± 0.04 pK units as "uncertain," and it is very difficult to find more than a few drugs without the "uncertain" label.

ommend that activity corrections be made: (*a*) if the pH is measured with an instrument calibrated in 0.01 pH units (or greater), and (*i*) the concentration lies between 0.01 and 0.1 *M*, or (*ii*) the hydrogen-ion or hydroxyl-ion concentration is comparable in magnitude to the stoichiometric concentration of the ionized species, or (*iii*) an extraneous salt is added to achieve "constant ionic strength." Also (*b*) if the pH is measured with an instrument calibrated in 0.005 pH units or less.

These are good rules, with a rationale behind each. For instance, (*b*) can be reread as saying that if you have an instrument that is capable of making good, accurate measurements, you should calculate and report a good, accurate dissociation constant. Criteria (*a*) (*i*) sets the limits for use on a less accurate instrument. A 0.1 *M* solution is taken as the upper limit since that is about the greatest concentration where a general equation describing ion interaction may be used to give valid activity coefficients.[7] The lower limit is included to prevent making calculations to a greater accuracy than the meter can feasibly be read. However, we would tend to be a bit more conservative and set the lower concentration limit at 0.005.

Criteria (*a*) (*ii*) is necessary when the hydrogen ion or hydroxide terms in Eqs. 8 or 12 are significant compared to the sum of other terms. Criteria (*a*) (*iii*) is necessary under the very common circumstances where a swamping concentration of a strong electrolyte, such as potassium chloride, has been added to the solution so that the ionic strength of the solution and the activity coefficients will remain constant throughout the titration. If this is not done, the ionic strength of the solution will change appreciably during the titration as titrant is added. For example, consider the titration of a 0.02 *M* solution of a monoprotic weak acid, HA, with a strong base, MOH. Initially this weak acid will be very slightly dissociated, let us say 3% dissociated; at this point in the titration the ionic strength of the solution would only be 0.0006. However, at half-neutralization, the solution would be 0.01 *M* in M^+ ions and 0.01 *M* in A^- ions. At this point the ionic strength would be 0.01. At the end of titration the ionic strength would have risen to 0.02 and thus, throughout the titration a different activity coefficient would have to be calculated after each addition of titrant. It is easy to see from this example that it would be preferable to add a swamping electrolyte and use a constant activity coefficient. However, since 0.1 *M* solutions are the upper limit for calculating reliable activity coefficients (*vide supra*) only 0.08 *M* KCl may be added, and the activity coefficient will still vary throughout the titration from 0.08 to 0.10. Therefore, in using the "conventional" sigmoid titration curves, we are faced with a paradox. We wish to use a low concentration of drug so that a truly swamping concentration of strong electrolyte may be added, but in using a low concentration of drug, we will have a less sharply defined inflection point and will increase the difference between f_{pKa} and f inflection as was pointed out by Goldman and Meites (*vide supra*). Therefore in order to get an accurate pKa from a nonlinear plot, a large concentration of drug should be used, and the activity coefficient should be calculated at the point of inflection.

Using the nonlogarithmic linear titration plots obtained from general Eq. 13, the problem disappears, since low concentration of drug will not impair the accuracy of the method.

Having decided to use activity coefficients, which value should be used? In order to answer this question we must briefly review the relation of activity coefficients to concentration. It is well known that when the activity coefficients of typical electrolytes are plotted against a function of the concentration (the square root of the ionic strength), the activity coefficients begin at 1, pass through a distinct minima, and then may even become greater than 1, and that these minima occur at different concentrations for each electrolyte.

In a simplified manner this may be explained as follows. At infinite dilution the electrolytes are completely dissociated and free to move; however, as the concentration is increased the ions come closer together and there is interionic attraction, which tends to draw ions together and render them less free to move. Therefore, the effective concentration or activity of ions are less than would be expected if complete dissociation took place.

It is well documented that many ions hydrate in water and that certain ions are hydrated to a greater extent than others (37). These hydrated water molecules are said to enter the coordination spheres of the ion and are essentially removed from the available free solvent. This decrease in available solvent seems to increase the effective concentration of the ions.

Actually both of these effects, the interionic attraction and the removal of solvent molecules, occur simultaneously.

[7] This is an unfortunate upper limit when examining drugs in biological systems, since we would, at least, like to know the activity coefficient for an ionizable drug at a sodium chloride concentration of 0.154 *M* (the isotonic concentration.)

At low concentrations the interionic attraction is the dominating factor affecting the activity coefficient; however, as the concentration of electrolyte is increased, more and more solvent water will be sequestered until this effect overcomes the interionic attraction effect and causes the activity coefficient to increase with increasing concentration. Obviously, the solvent sequestering effect will be different for different ions depending on how much water is coordinated to each ion and, therefore, no general relation can be made between activity coefficient and concentration for this effect.

However, Debye and Hückel (38–41) reasoned that in dilute solutions, only the interionic attraction would affect the activity coefficient and that the ions could be regarded as point charges. Starting with these assumptions, they derived what is known as the Debye-Hückel limiting law:

$$\log \gamma_i = -A(z_i)^2 \sqrt{\mu} \qquad \text{(Eq. 18)}$$

where γ_i = ionic activity coefficient,
A = a term made up of universal constants, the temperature, and the dielectric constant of the solvent,
μ = the ionic strength ($\mu = {}^1/_2 \Sigma c_i z_i^2$),
z_i = the charge of the ion.

According to the Debye-Hückel limiting law, all uni-univalent electrolytes at a given concentration should have the same activity coefficient, and experimental determinations show that this is true up to about 0.01 ionic strength (42). However, at an ionic strength of 0.01, the size of the ions begins to have an effect[8] on the interionic attraction and the limiting law must be extended:

$$\log \gamma_i = \frac{-A(z_i)^2 \sqrt{\mu}}{1 + a_i B \sqrt{\mu}} \qquad \text{(Eq. 19)}$$

where a_i = ion size parameter,
B = a constant dependent on the dielectric constant of the solvent and the temperature.

The above equation is considered accurate up to an ionic strength of 0.1 (45), and this sets the upper limit in concentration for determining accurate pKa values as quoted above from Albert and Serjeant.

Hückel (41) in 1925 extended the theory so as to apply to higher concentrations:

$$\log \gamma_i = \frac{-A z_i^2 \sqrt{\mu}}{1 + a_i B \sqrt{\mu}} + C\mu \qquad \text{(Eq. 20)}$$

where C is a constant dependent on the nature of *all* the ions in the solution. This equation has been shown to fit activity data with high precision[9] to about 1.0 M solutions (46).

However, now there are two unknown constants, a_i and C, which must be "guessed" before a calculation of γ_i can be made. Since it is difficult enough making one guess, Eq. 19 limited to ionic strengths no greater than 0.1 has been the equation of choice (25, 35). Benet and Goyan (24, 25) used the single ion size parameters which Kielland (47) determined from theoretical considerations. Kielland presents ion size parameters and individual activity coefficients for 130 inorganic and organic ions. He estimates a 9-Å. parameter for hydrogen ion, 3.5-Å. for hydroxide ions, and 3-Å. for potassium and chloride ions. The 9-Å. value for hydrogen is especially important, since the conversion of hydrogen-ion activity to concentration is the necessary step before any of the generalized equations presented previously may be used.

Leeson and Brown (33), for their calculations, used "the more familiar, experimentally obtained, $\gamma_\pm$ values for HCl as listed in Lewis and Randall" (48). Leeson and Brown make a comparison between the thermodynamic pKa's calculated using Kielland's activity coefficient and the Lewis and Randall mean ionic activity coefficient. They found that the Kielland activity coefficient for acetic acid gave a pKa value of 4.74, while the Lewis and Randall activity coefficient gave a pKa value of 4.76. The literature value for acetic acid may be taken as 4.76 (49, 50).

Leeson and Brown go on to point out that the intercept of the plot of the data for the two different calculations (using Eq. 14 and determining A^0) is not markedly influenced by the choice of the activity coefficient. It is rather the ionization constant (or slope) that reflects the difference. A plot of pK_a^c *versus* $\sqrt{\mu}$ demonstrates that both sets of data are essentially linear. These latter plots were extrapolated to infinite dilution to evaluate the thermodynamic ionization constants reported above. "These results," Leeson and Brown conclude, "indicate that the pK_a obtained with both values [Kielland, and Lewis and Randall] are consistent with the literature. Therefore, since the two sets of the data are the same, the type of activity coefficient

[8] The size of the ion includes its sequestered water of hydration and is defined as the ion size parameter.

[9] The Debye-Hückel equations (Eqs. 18–20) should actually read $\log f_i = \ldots$ since f_i is the activity coefficient when concentrations in moles per liter are used. The activity coefficient γ_i is associated with molality. However, this difference can be ignored for dilute solutions. For example, the two activity coefficients for univalent electrolytes differ by only 0.0004 for 0.1 M NaCl.

TABLE II—SPECIFIC ION-INTERACTION CONSTANTS AFTER GUGGENHEIM AND TURGEON (54)

Electrolyte	B_{MX} at 25° C.
HCl	0.23
NaCl	0.13
NaI	0.18
NaOAc	0.20
NaOH	0.05
KCl	0.09
KI	0.13
KOAc	0.23
KOH	0.11

employed for calculations appears to be a matter of personal choice."

The authors agree with Leeson and Brown that the Lewis and Randall values may be used for hydrogen ion activity coefficients when either KCl or NaCl is used as a swamping electrolyte and the ionic strength is less than one-tenth. There are sound reasons for this, based on the work of Bronsted and Guggenheim.

In 1922 Bronsted (51) proposed that there be a specific ion interaction term in the Debye-Hückel equation dependent on *which* two ions were involved. In 1935, Guggenheim (52) suggested that a standard value of a_i in the vicinity of 3 Å. would improve the empirical success of the Bronsted type of equation without introducing any serious complications. This yields for a simple M-X electrolyte the following equation:

$$\log \gamma_{\pm} = \frac{-A\,|z_+ z_-|\,\sqrt{I}}{1 + \sqrt{I}} + B_{MX} m \quad \text{(Eq. 21)}$$

where m = molality of MX,
B_{MX} = a constant at a particular temperature which characterizes the interaction between cation M and anion X, a specific ion-interaction constant,
I = ionic strength using molal concentrations.

Values for the coefficient B_{MX} for numerous electrolytes in aqueous solution have been determined (52–54) from experimental data and have been tabulated (54). The values derived from freezing point measurements relate to 0°. Those derived from EMF measurements and isopiestic measurements relate to 25° and other temperatures. A few pertinent values are listed in Table II.

As an example, a 0.05 M solution of HCl calculated by Eq. 21 gives a mean ionic activity coefficient $\gamma_{\pm}$ of 0.829 compared to the Lewis and Randall (48) experimentally determined value of 0.830. Using the specific ion-interaction constants, $\gamma_{\pm}$ values may be computed for solutions up to 0.1 M with an estimated accuracy of the order of 0.5% (55), which is certainly sufficient for most purposes.

The Guggenheim type of equation may also be used to calculate the ionic activity coefficient for a specific ion in a solution containing a mixture of electrolytes (56, 57). The equation for a particular cation M′ of charge $z_{M'}$ is:

$$\log \gamma_{M'} = \frac{-A z_{M'}^2 \sqrt{I}}{1 + \sqrt{I}} + \Sigma_X B_{M'X} m_X \quad \text{(Eq. 22)}$$

where each specific ion-interaction constant is multiplied by the molality of the anion involved (m_X) and summed. No terms are present for other cations.

Now consider the titration of a 1×10^{-3} aqueous solution of acidic drug HD at 25° where the ionic strength has been adjusted to 0.05 by the addition of KCl.[10] Calculating the activity coefficient for hydrogen ions using Eq. 22, gives the following:

$$\log \gamma_{H^+} = \frac{-.509\ (1)^2\ \sqrt{.05}}{1 + \sqrt{.05}} + B_{HCl}\,(.0495) + B_{HD}\,(.0005) \quad \text{(Eq. 23)}$$

The value for B_{HCl} can be obtained from Table II. The value for B_{HD} is unknown, but the third term on the righthand side of the equation is insignificant and can be dropped, so the γ_{H^+} will be identical to the one calculated above for a 0.05 M solution of HCl. Equation 22 should hold for the titration of any acid or base and thus the Lewis and Randall mean activity coefficient for HCl would be the correct value to use for the hydrogen-ion activity coefficient.

However, the Lewis and Randall values would be incorrect for use in calculating γ_{HD^+}, γ_{OH^-}, and γ_{D^-}. The activity coefficients for hydroxide ion could be calculated from the Guggenheim equation for X′, a particular anion (56, 57).

$$\log \gamma_{X'} = \frac{-A z_{X'}^2 \sqrt{I}}{1 + \sqrt{I}} + \Sigma_M B_{MX'} m_M \quad \text{(Eq. 24)}$$

where m_M is the molality of the cation involved. Using a swamping electrolyte, this will be the molality of the cation. We cannot calculate the activity coefficients for D^- or HD^+ (negatively and positively charged drug molecules) since there are no specified interionic–attraction coefficients for drugs. In these cases, we must still make a judicious guess as to which ion-size parameter we should put into the Debye-Hückel equation. However, it seems reasonable to expect that we could make a much better guess of the ion-interac-

[10] The convention usually followed is to add that amount of swamping electrolyte which will give the desired ionic strength at half-neutralization of the drug.

tion coefficient for most drugs using the pharmaceutical data for sodium chloride equivalents and cryoscopic properties (58–60) and isopiestic measurements (61) available for almost all acidic and basic drugs in use. One of us is presently examining the reliability of specific ion-interaction coefficients determined from the above-cited data (62).

Summary

For the potentiometric determination of dissociation constants in aqueous solution, the authors recommend the following on the basis of the foregoing review of the literature. Use the most accurate pH meter available and the techniques suggested by Albert and Serjeant (4). Use a concentration of drug between 5×10^{-4} and 1×10^{-3} M, add a swamping electrolyte such as KCl to adjust the ionic strength to 0.05. Take about 15 readings of pH after addition of titrant. Convert pH as read on the meter to hydrogen ion activity using either the Lewis and Randall values for the activity coefficient (48) or those calculated by Eq. 23. Calculate hydroxide-ion concentration, if necessary, from the K_w for the temperature used (63) and the activity coefficient for hydroxide calculated for Eq. 24, or use the values tabulated by Harned and Owen (64) for the ionic activity function of water. Calculate the data according to the method of Leeson and Brown (33). Plot the data after making the appropriate rearrangement of Eq. 13, and determine the slope of the line and K_a^c. Now determine K_a^T by Eq. 16, using the activity coefficient for hydrogen ion calculated from Eq. 23 and the activity coefficient for the drug ion determined by Eqs. 23 or 24 or some judicious choice of an ion size parameter. At present, however, the most accurate yet burdensome method is to calculate pK_a^c at four or more ionic strengths and then plot pK_a^c *versus* $\sqrt{\mu}/1 + \sqrt{\mu}$ and extrapolate to infinite dilution. Following these procedures, it is estimated that an accurate pK_a^T may be determined within ± 0.03 units.

SEMIAQUEOUS SOLUTIONS

For a large number of pharmaceutical compounds, the uncharged species is so insoluble that it is very difficult to determine accurate dissociation constants by even the most sensitive methods previously described. Albert and Serjeant (65) have said:

> When a substance is poorly soluble in water, but highly soluble in a volatile solvent, it is natural to consider determining the ionization constant in a mixture of the two solvents, e.g., in 50% ethanol. This temptation should be resisted, for reasons which will shortly be discussed.

They go on to point out that Hall and Sprinkle (3) plotted pKa against per cent alcohol concentration (range 10%–97%) for 18 different amines and then extrapolated these "hockey-stick" shaped curves back to zero per cent. Albert and Serjeant correctly point out that these values are highly suspect especially when the amine was too insoluble to give values at 10 and 20%.

They also present a table of pKa values of aniline, methylaniline, and dimethylaniline in various percentages of ethanol (66) showing that in solutions of increasing ethanol percentage, aniline, and its *N*-methyl derivatives become weaker bases. They state, "But the effect of the alcohol is least on the unmethylated substance with this paradoxical result: although methylation increases the basic strength in 0% to 35% alcohol, in more highly alcoholic solutions it decreases the basic strength. Dilute dimethylformamide, methyl cellosolve and other glycol derivatives cause this type of trouble also." Actually, these results may not be troublesome at all, and they can be explained by more recent ideas, as will be discussed.

Finally, Albert and Serjeant comment that ". . . the widespread availability of ultraviolet spectrophotometers makes it possible to obtain the pK_a values of many sparingly soluble substances by special methods." However, there are a large number of substances, particularly amines, which are not suitable for spectrophotometric analysis, and thus we must accept the "temptation" and determine the pKa by titration in semiaqueous solvents.

The practice of determining pKa's in varying concentrations of alcohol and plotting these values *versus* per cent alcohol originated with Mizutani (67), who then extrapolated the plot to zero per cent alcohol to find the aqueous pKa. This procedure has been followed by a number of workers dealing with substances of pharmaceutical interest. Marshall (68) determined the dissociation constants of various antihistamines in ethanol–water mixtures. Edmonson and Goyan (69) used the same solvent combination while studying phenobarbital solubility. A methanol–water solvent was used by Chatten and Harris (70) to study phenothiazine and sympathomimetic amines. Garrett (71) measured the variation of apparent dissociation constants for several tetracycline antibiotics in dimethylformamide–water solvent and used the information in the assignment of pK_a' values to functional groups.

Theoretical Considerations—There is no doubt that an investigator can take a known

amount of a substance in a given mixture of nonaqueous solvent–water containing a known amount of ionic substances and reproduce previously reported pH readings using the usual glass electrode–calomel electrode and a pH meter which has been standardized in a specified manner. The problem is to relate this reading and dissociation constant determinations to values obtained in water.

The numerical values of activity coefficients are commonly assigned with reference to a value of one at infinite dilution of the solute in the particular solvent being studied (72). However, there must be some way of relating an activity coefficient in water to that in a solvent containing alcohol, methanol, dioxane, or other organic liquids miscible with water. The medium effect ${}_m\gamma_i$ is a measure of the free energy change on transfer of 1 mole of a substance i from a standard state in water to the standard state in the mixed solvent SH:

$$\gamma_i = ({}_m\gamma_i)({}_s\gamma_i) \qquad \text{(Eq. 25)}$$

where γ_i = activity coefficient of ion i in water,
${}_s\gamma_i$ = activity coefficient of ion i in solvent SH.

The activity coefficients γ_i and ${}_s\gamma_i$, which become unity at infinite dilution in water and in solvent–water mixtures, respectively, characterize the interionic and ion-molecule forces and other effects dependent on the concentration of the medium. The medium effect ${}_m\gamma_i$ reflects the differences in the electrostatic and chemical interactions of the substance i in the two solvents. Of these interactions, solvation is probably the most important when ions are transferred from one medium to another (72).

The equation for the EMF of a cell with a liquid junction yields the following equation:[11]

$$\text{pH} = -\log a_H + \bar{E} \qquad \text{(Eq. 26)}$$

where $\bar{E}_j$ = the liquid-junction potential expressed in pH units.

Now we may define $a_H{}^*$ as the hydrogen-ion activity referred to the standard state in the mixed solvent SH.

$$pa_H{}^* = -\log (m_H)({}_s\gamma_H) \qquad \text{(Eq. 27)}$$

Now combining Eqs. 25, 26, and 27, we obtain:

$$pa_H{}^* = -\log (m_H) - \log (\gamma_H) + \log ({}_m\gamma_H) = \text{pH} - \bar{E}_j + \log {}_m\gamma_H = \text{pH} - \delta \qquad \text{(Eq. 28)}$$

[11] Equation 26 is actually what we are measuring on the pH meter in aqueous solutions. The pH read equals the negative log of the activity of hydrogen ion plus the residual liquid-junction potential. We attempt to cancel out this last term by standardizing the meter with a buffer solution that has the same $\bar{E}_j$ as the solution we are measuring. In practice, these $\bar{E}_j$'s do cancel out quite well for buffers and solutions between pH 3 and 11 in water (73).

where δ is written for $\bar{E}_j - \log {}_m\gamma_H$ and is a constant for a medium of a given composition. Now, $pa_H{}^*$ can be related to the dissociation equilibrium (Eq. 29) in a semiaqueous medium, by Eq. 30:

$$HA + SH \rightleftharpoons A + SH_2{}^+ \qquad \text{(Eq. 29)}$$

$$p({}_sK_a) = pa_H{}^* - \log \frac{m_A}{m_{HA}} - \log \frac{{}_s\gamma_A}{{}_s\gamma_{HA}} \qquad \text{(Eq. 30)}$$

where ${}_sK_a$ is the thermodynamic dissociation constant of acid HA in mixed solvent SH. This equation is just the Henderson-Hasselbalch equation using thermodynamic values for solvent SH.

The values of ${}_s\gamma_i$ can be calculated by the extended Debye-Hückel equation allowing for the effect of changes in dielectric constant:

$$-\log {}_s\gamma_i = \frac{(1.82455 \times 10^6)\, z_i{}^2 \sqrt{\mu}}{(DT)^{3/2}\,[1 + 50.2904\,(DT)^{-1/2} a_i \sqrt{\mu}]} \qquad \text{(Eq. 31)}$$

where D = dielectric constant,
T = temperature in degrees Kelvin,
a_i = the ion size parameter.

Determination of Dissociation Constants (${}_sK_a$) in Semiaqueous Solvents—With the foregoing equations, it is now possible to determine $p({}_sK_a)$ if $pa_H{}^*$ can be determined at a number of points during a titration. The simplest way to determine $pa_H{}^*$ values experimentally would be to apply tabulated corrections to the pH numbers read on a pH meter which had been standardized with aqueous buffer solutions in the usual way (74), *i.e.*, using Eq. 28, $pa_H{}^* = \text{pH} - \delta$. Ong, Robinson, and Bates (75) have carried this correction a step further and applied it to the "seeming" pKa,[12] which is defined as:

$$\text{pKa}'' = \text{pH} - \log \frac{m_A}{m_{HA}} - \log \frac{{}_s\gamma_A}{{}_s\gamma_{HA}} \qquad \text{(Eq. 32)}$$

where pH is the meter reading at any point during the titration, the meter having been standardized with aqueous buffers. Now at any point in the titration we may subtract Eq. 30 from Eq. 32, with the result:

$$\text{pKa}'' - p({}_sK_a) = \text{pH} - pa_H{}^* = \delta \qquad \text{(Eq. 33)}$$

The term δ (Eq. 28) contains two values, the residual junction potential and the medium effect, and is identical with the quantity derived by Bates, Paabo, and Robinson (76) from a comparison of the EMF of cells with and without liquid junctions.

Ong, Robinson, and Bates (75) have calculated the values for acetic acid and anilinium ions in varying weight percentages of methanol (0–70%)

[12] The authors have chosen the term "seeming" pKa so as not to confuse this term with the apparent pKa defined by Eq. 17.

TABLE III—VALUES OF THE CONSTANT $\delta \equiv (\bar{E} - \log {}_m\gamma_H)$ IN METHANOL–WATER[a] AT 25°C. AFTER ONG *et al.* (75)

Wt. % methanol	0	10	20	30	40	50	60	70
δ	0	0.01	0.02	0.04	0.07	0.11	0.15	0.13

[a] A similar set of values could be compiled for ethanol–water mixtures from the data of Bates *et al.* (76), Butbezahl and Grunwald (80), and Gelsema and de Ligny (81) [cited by Bates, Paabo, and Robinson (76) and Bates (36)] although the agreement is not so good as that for methanol–water solutions. (For tabulation of these data, see *Reference 36*, p. 224.)

by determining the pK″ values and subtracting them from the $p({}_sK_a)$ values of Shedlovksy and Kay (77) for acetic acid and the values of Bacarella *et al.* (78) for anilinium. Ong *et al.* then compared their calculated δ values with those obtained from the EMF of cells with and without liquid junctions (76) and to the values obtained by de Ligny and Rehback (79) who also used cells with and without liquid junctions. The agreement for δ values among these workers is good and the recommended values for each weight percentages of methanol are presented in Table III. The table only contains values up to 70% since ionic association may be considered minimal only up to 68 wt.% methanol.

Thus δ may be subtracted from the "seeming" pK″ value to give the true p_sK_a value since δ is independent of the solute composition, provided that the total ionic strength is not high and the pH is neither very high nor very low (the same conditions which were placed on determinations in aqueous solutions). Finally, Ong *et al.* (75) point out that they and Bacarella *et al.* (78) used glass electrodes, while Bates *et al.* (76) and de Ligny and Rehback (79) used hydrogen electrodes.

> The general correlation which all the authors obtained shows . . . that the standard potential of the glass electrode is independent of solvent composition in water–methanol mixtures as, indeed, it should be if the electrode is behaving as a reversible hydrogen electrode. Furthermore, the general agreement shows that there is little dependence of the liquid junction potential on the type of junction used; de Ligny and Rehback (79) formed the junction at the end of a tube with a capillary opening, Bates, Paabo, and Robinson (76) used sintered disks, while porous fibers were used in this work (75).

Previous to the above work, Bates *et al.* (76) felt that the possibility of a shifting asymmetry potential when the glass electrode was transferred from an aqueous medium to an alcoholic medium was sufficient reason to use standard solutions of known pa_H*, having the same solvent composition as the solution to be titrated. Bates (82) has tabulated the values for some methanol–water (76, 83–85) and ethanol–water (76, 81) buffer systems. Recently pa_H* values have been reported for a number of other buffers in methanol–water solvents at varying temperatures: (*a*) tris(hydroxymethyl)aminomethane and its hydrochloride (Tris buffers) (86); (*b*) ammonia–ammonium chloride (87); and (*c*) 4-aminopyridine and its hydrochloride (88). Paabo *et al.* (89) have also resolved the discrepancies in the standardization of the data obtained with silver–silver chloride electrodes in methanol–water solvents, and were able to report mean ionic activity coefficients for hydrochloric acid to three decimal places in varying percentages of water–methanol solvents at 25°.

Relation of Dissociation Constants in Mixed Solvents to Dissociation Constants in Water—As was stated earlier, Mizutani originated the practice of plotting pKa″ *versus* per cent alcohol and extrapolating back to zero per cent to find pKa′ in water. Although this is a logical approach, there are no theoretical relationships to predict how much the plot will curve at various percentages, and if the titrated substance is not soluble in 10 or 20% alcohol, the extrapolation back to 0% may be highly suspect.

Let us now examine what has to be determined in order to develop a theoretical method for converting $p_sK_a^T$ to pKa^T. Consider the dissociation of acid HA in solvent SH and in water:

$$HA + H_2O \rightleftharpoons A + H_3O^+ \qquad \text{(Eq. 34)}$$

$$HA + SH \rightleftharpoons A + SH_2^+ \qquad \text{(Eq. 29)}^{13}$$

$$K_a = \frac{(m_A\gamma_A)(m_{H^+}\gamma_{H^+})}{(m_{HA}\gamma_{HA})} \qquad \text{(Eq. 35)}$$

$${}_sK_a = \frac{(m_{A_s}\gamma_A)(m_{H^+_s}\gamma_{H^+})}{(m_{HA_s}\gamma_{HA})} \qquad \text{(Eq. 36)}$$

Recalling Eq. 25 and dividing Eq. 35 by Eq. 36, we derive Eq. 37:

$$\frac{K_a}{{}_sK_a} = \frac{({}_m\gamma_H)({}_m\gamma_A)}{({}_m\gamma_{HA})} \qquad \text{(Eq. 37)}$$

Taking the log of both sides of Eq. 37, we find that $p({}_sK_a) - pKa$ equals the log of the term on the righthand side of Eq. 37. Therefore, in order to calculate the aqueous dissociation constants from the experimentally determined nonaqueous values, we must be able to determine the medium effect. As stated earlier, the medium effect for an ion reflects the differences in the electrostatic and chemical interactions of the ion with the two solvents (72).

[13] Where we use SH_2^+ to designate the hydronium ion in a mixed solvent.

The standard free energy of transfer of a gram ion of a single ionic species i from water to another solvent SH is given by

$$\Delta G_i^0 = RT \ln {}_m\gamma_i \qquad \text{(Eq. 38}a\text{)}$$

This change in free energy is made up of an electrostatic part, ΔG_{el}, which can be estimated by the Born equation (90) and a nonelectrostatic part which accounts for the specific chemical interactions between the ions and the solvent. At present, there is no known way to characterize all of the nonelectrostatic effects, but since the electrostatic effects will predominate, the predictions of the Born equation are of some interest (91).

The change in free energy arising from charging effects in the process of transferring an ion from water to solvent SH as described by the Born equation is:

$$\Delta G_{el}^0 = \frac{Nz_i^2e^2}{2r}\left(\frac{1}{D_{SH}} - \frac{1}{D_w}\right) \qquad \text{(Eq. 38)}$$

where N = Avogadro's number,
z_i = charge on the ion,
e = the electronic charge,
r = the radius of the ion in the two solvents where the ion is considered a sphere,
D_{SH} = dielectric constant of solvent (SH in water).

Assuming that only electrostatic effects are taking place, substituting Eq. 38a into Eq. 38 and inserting numerical values for the constants at 25° results in Eq. 39:

$$\log {}_m\gamma_i = \frac{121.6z_i^2}{r}\left(\frac{1}{D_{SH}} - 0.0128\right) \qquad \text{(Eq. 39)}$$

Now substituting Eq. 39 into the logarithmic form of Eq. 37, we derive Eq. 40:

$$\mathrm{p}({}_sK_a) - \mathrm{pKa} = 121.6\left(\frac{1}{r_{H+}} + \frac{z_A^2}{r_A} - \frac{z_{HA}^2}{r_{HA}}\right)\left(\frac{1}{D_{SH}} - 0.0128\right) \qquad \text{(Eq. 40)}$$

Now assuming that the radii of all the ions are equal (*i.e.*, $r_{H^+} = r_A = r_{HA}$), it is possible to make the following predictions. For an acid such as acetic acid (where A is negatively charged and HA has no charge), a plot of $\mathrm{p}({}_sK_a)$ *versus* $1/D_{SH}$ (*e.g.*, dissociation constants determined in solutions of varying alcohol percentages) should be a straight line with a positive slope. For an acid such as anilinium chloride (where HA is positively charged and A has no charge) a plot of $\mathrm{p}({}_sK_a)$ *versus* $1/D_{SH}$ should be a straight line with zero slope. It is found [*e.g.*, (66) and (92)] that p_sK_a values for monoprotic acids do indeed increase markedly as the dielectric constant of the solvent decreases. However, the p_sK_a values for cationic acids (HA^+) do not stay constant as predicted above, but usually decrease, pass through a minimum, and then increase at high concentrations of organic solvent.

Unfortunately, the Born treatment often fails. For example, if the data of Shedlovsky and Kay (77) or Bacarella *et al.* (78) for acetic acid in methanol–water is plotted with the above coordinates, the resulting line will be far from straight. Equation 40 also predicts that an acid should have the same dissociation constant in two different solvent mixtures having the same dielectric constant. However, both Speakman (93) and Dondon (94) observed that aqueous dioxane acted as if it had a higher effective dielectric constant than aqueous alcohol of the same actual dielectric constant. In other words, the values of dissociation constants of several acids in aqueous dioxane were reported to be higher than those determined in aqueous alcohol with the same dielectric constant. Yasuda (95) suggests that the usual way of defining K_a assumes that the activity of water is constant, as in the following equilibrium, K_a is defined according to Eq. 41,

$$HA + H_2O \rightleftharpoons H_3O^+ + A \qquad \text{(Eq. 34)}$$

$$K = \frac{(H_3O^+)(A)}{(HA)} \qquad \text{(Eq. 41)}$$

while he proposes a new dissociation constant dependent on the activity of water, which we shall call K_I.

$$K_I = \frac{(H_3O^+)(A)}{(HA)(H_2O)} \qquad \text{(Eq. 42)}$$

Yasuda reasons thus:

> In aqueous solutions K_a may be used, for (H_2O) is nearly constant. But this is not true in mixed solvents containing a limited amount of water. Due consideration should be paid to the fact that the activity of water in mixed solvents changes with the molar fraction of organic solvents. Therefore, the constant K_I defined by Eq. 42 should be used for the estimation of the dissociation of an acid in mixed solvents, in place of K_a defined by Eq. 41.

Yasuda justified ignoring the activity of the nonaqueous portion of the solvent, by the fact that water has an exceptionally high proton affinity and must be included in the equilibrium calculations, whereas the alcohol or dioxane proton affinities are insignificant. Recalculating the work cited above (77, 78, 94, 95), Yasuda plots pK_I *versus* $1/D$ and finds that the anomalies mentioned above disappear and the plot is linear for values of $1/D$ less than 0.02. It appears that at D values greater than 50, the nonelectrostatic terms in the free energy of transfer are no longer negligible.

One month after the publication of Yasuda's paper (95), Shedlovsky (96) presented a similar equation to the Trieste Symposium on Electrolytes; however, his work had a more complete theoretical derivation. Shedlovsky also considered the data of Grunwald *et al.* (78, 92) and Shedlovsky and Kay (77), particularly the dissociation of acetic acid in water–methanol and water–ethanol solvents.

Shedlovsky points out that on dissolving acetic acid in water or in alcohol, the following sequence of events occurs: first, a "force linkage" forms between the acid and solvent molecules; second, a proton shifts from the acid-carboxyl group to a bound solvent molecule forming an ion pair; third, the ion pair dissociates. These steps are shown in the following equations and the over-all dissociation constant for each solvent, K_H and K_R are derived.

$$HA + H_2O \rightleftharpoons HA \cdot H_2O \qquad \text{(Eq. 43)}$$

$$HA \cdot H_2O \rightleftharpoons H_3O^+ \cdot A^- \qquad \text{(Eq. 44)}$$

$$H_3O^+ \cdot A^- \rightleftharpoons H_3O^+ + A^- \qquad \text{(Eq. 45)}$$

$$HA + ROH \rightleftharpoons HA \cdot ROH \qquad \text{(Eq. 46)}$$

$$HA \cdot ROH \rightleftharpoons RH_2O^+ \cdot A^- \qquad \text{(Eq. 47)}$$

$$RH_2O^+ \cdot A^- \rightleftharpoons RH_2O^+ + A^- \qquad \text{(Eq. 48)}$$

$$K_{43}K_{44}K_{45} = \frac{[H_3O^+][A^-]\,\gamma^2}{[HA][H_2O]} = K_H \qquad \text{(Eq. 49)}$$

$$K_{46}K_{47}K_{48} = \frac{[RH_2O^+][A^-]\,\gamma^2}{[HA][ROH]} = K_R \qquad \text{(Eq. 50)}$$

where it is assumed that the activity coefficients of all charged species are identical and that the activity coefficients of uncharged species are one.

The experimentally determined ionization constant ${}_sK_a$, however, depends on both kinds of hydrogen ions.

$${}_sK_a = \frac{[A^-][H_3O^+ + H_2OR^+]\,\gamma^2}{[HA]} = K_H[H_2O] + K_R[ROH] \qquad \text{(Eq. 51)}$$

Now Shedlovsky employs Bjerrum's theory of ionic association (97–99), to show that $H_2OR^+ \ll H_3O^+$, assuming that Eqs. 45 and 48 are controlled by simple coulombic forces. Therefore:

$$K_H = B_H \exp.(-b) \qquad \text{(Eq. 52)}$$

$$K_R = B_R \exp.(-b) \qquad \text{(Eq. 53)}$$

where $b = \left(\frac{e^2}{DkTa}\right)$ in which e is the electronic charge; D the dielectric constant; k is the Boltzman constant; T is the Kelvin temperature; and a is the average ionic diameter for the anion and cation (the distance of closest approach); B is a constant containing e, D, k, T, N (Avogadro's number), r the actual average distance between ions, and the appropriate K values (*e.g.*, B_H contains $K_{43}K_{44}$).

Shedlovsky further assumes that a is a constant over the entire range of solvent composition within a given solvent system. Then

$${}_sK_a[e^b] = B_H[H_2O] + B_R[HOR]$$

Now Shedlovsky points out that Eqs. 43 and 46 are for dipole–dipole interactions and Eqs. 45 and 48 are for ion pair dissociations. He feels that for acetic acid the magnitude of these interactions and dissociations should be approximately the same in both water and alcohol (*i.e.*, $K_{43} = K_{46}$ and $K_{45} = K_{48}$). However, the proton rearrangements, Eqs. 44 and 47, should take place much more readily in water than in alcohol so that K_{44} should be considerably greater than K_{47} and therefore B_H should be much greater than B_R. Therefore, Eq. 51 will reduce to Eq. 54 when Eqs. 52 and 53 are substituted into Eq. 51, along with the simplifying assumption $B_H \gg B_R$.

$${}_sK_a = B_H e^{-b}[H_2O] \qquad \text{(Eq. 54)}$$

This is essentially the same assumption made by Yasuda (95), except that the Shedlovsky equation can now be rearranged to:

$$p_sK_a + \log[H_2O] = \left(\frac{e^2}{(2.303)akT}\right)(1/D) - \log B_H \qquad \text{(Eq. 55)}$$

so that a plot of $p_sK_a + \log[H_2O]$ *versus* $1/D$ should be linear. But Shedlovsky's derivation shows that the slope of the line may be used to measure a, the average ion size of the cation and anion.

In closing this section, let us return to Albert and Serjeant's comment (65) concerning the variation of p_sK_a for aniline, methylaniline, and dimethylaniline in varying percentages of ethanol (66). A plot of $p_sK_a + \log[H_2O]$ *versus* $1/D$ for each of these three compounds are fairly linear below a value of $1/D = 0.02$. However, the three lines cross each other (*i.e.*, they have different slopes) giving rise to what Albert and Serjeant refer to as "paradoxical results." However, as shown, this may only be a result of different ionic sizes, and further work might elucidate these results.[14]

Summary

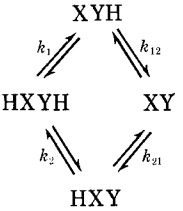
As has been reviewed above, there is presently no completely satisfactory method for converting p_sK_a values to pKa values. Therefore, although good accuracy can be realized in determining p_sK_a

[14] It should be emphasized that a direct correlation with Shedlovsky's equations cannot be derived, since here we are dealing with a protonic acid instead of an uncharged acid. However, an analysis could be made corresponding to the recent work of Paabo, Bates, and Robinson (87), who considered the variation in p_sK_a for ammonia as compared to Tris.

values, this accuracy cannot be carried over to the aqueous dissociation constants. The authors would recommend that semiaqueous titrations be carried out in methanol–water systems if possible, since there is an abundance of data concerning this solvent system. Procedures for determining p_sK_a need not differ from those in aqueous systems, except that Eq. 31 should be used for determining activity coefficients. The values of Ong *et al.* (75) appear to be sufficiently accurate, so that aqueous buffers may be used for standardizing the meter before titrations in a methanol–water system. In extrapolating p_sK_a values back to pKa in water, we recommend plotting $p_sK_a + \log [H_2O]$ *versus* $1/D$, as defined by Shedlovsky (96) and Yasuda (95). As long as p_sK_a values can be determined in solutions with dielectric constants greater than 50, these extrapolations (as per Yasuda and Shedlovsky) seem to give reasonable pKa^T values. Calculations of dissociation constants under these conditions should be accurate to about ± 0.06 pK units.

POLYPROTIC SPECIES

Whenever an acid has more than one ionizable hydrogen, the interpretation of potentiometric titration data becomes more complex. The authors will use the dissociation of a dibasic acid as shown in Scheme I to illustrate the problems,



Scheme I

where the lower case k is used to denote microconstants and the subscripts indicate the order of dissociation (*i.e.*, X is site 1, Y is site 2). Now the titration data will yield the two macroconstants defined as:

$$K_1^T = \frac{[(XYH) + (HXY)]\,(H)}{(HXYH)} = k_1 + k_2 \qquad \text{(Eq. 56)}$$

$$K_2^T = \frac{(XY)\,(H)}{[(XYH) + (HXY)]} = \frac{k_{12}k_{21}}{k_{12} + k_{21}} \qquad \text{(Eq. 57)}$$

If k_1 is much greater than k_2, k_2 and k_{21} will be a negligible pathway and:

$$K_1 = k_1,\ K_2 = k_{12}$$

Thus if K_1 is 1000 times or more larger than K_2, any of the methods outlined earlier may be used. (With polyprotic acids it is often possible to isolate one of the dissociations and determine it independently.)

However, some cases are still bound to occur where K_1 and K_2 overlap and the methods of determining their values differ somewhat from those previously discussed. Probably the oldest method is that due to Noyes (100). The equations are derived by use of the two dissociation equilibria (Eqs. 56 and 57 written in terms of concentration) and the equations for conservation of mass and charge. Where hydroxide-ion concentration may be ignored relative to the other terms in the equation for electroneutrality (*i.e.*, except for extremely weak acids and exp erimental points close to $f = 2$) he obtains:

$$K_1^c = \frac{B_1D_2 - B_2D_1}{A_1B_2 - A_2B_1} \qquad \text{(Eq. 58)}$$

$$K_2^c = \frac{A_1D_2 - A_2D_1}{B_1D_2 - B_2D_1} \qquad \text{(Eq. 59)}$$

Here $A_n = [M_n][H_n] + [H_n]^2 - [H_n][C^0]$

$B_n = 2[C^0] - [M_n] - [H_n]$

$D_n = ([H_n] + [M_n])[H_n]^2$

where [ ] denotes concentration, C^0 is the concentration of the acid being titrated, M is the total concentration of added alkali, and n is used to indicate individual points on the titration curve. Ordinarily, several pairs of data points are taken and the constants averaged to obtain the best estimate of K^c. These values may be converted to K^T values using the activity coefficients due to Kielland (47, 25). For the unusual case where the hydroxide-ion concentration is not negligible, see *Reference 100*. Another method of interest is that of Speakman (101). Using the same basic equations as above, he obtains:

$$\frac{(H)^2Pf_{XY}}{Rf_{HXYH}} = K_1^T\left[\frac{(H)Qf_{XY}}{Rf_{HXY}}\right] + K_1^TK_2^T \qquad \text{(Eq. 60)}$$

or

$$X = K_1^TY + K_1^TK_2^T \qquad \text{(Eq. 61)}$$

where $P = [M] + [H] - [OH]$

$Q = [C^0] - [M] - [H] + [OH]$

$R = 2[C^0] - [M] - [H] + [OH]$

and it is assumed $f_{HXY} = f_{XYH}$.

Thus in this method one uses the experimental data and activity coefficients in obtaining X and Y which are then plotted to obtain K_1^T and K_2^T.

Unfortunately, it is not possible to determine the microconstants from the macroconstants without one additional expression relating the various species (102). This subject is therefore outside the scope of this review, although several interesting papers have been published dealing

with medicinal agents (103–105). In the paper of Riegelman *et al.*, the values obtained for synephrine seem questionable since k_2 is less than k_{21}. Using the same data and equations with the Kielland values for activity coefficient (47), this anomaly disappears (pK_2 9.84 to 9.61, pK_{21} 9.69 to 9.71). This again demonstrates the value of working with reasonable estimates of activity rather than concentrations.

Summary

The determination of macroconstants requires the same degree of experimental care used with monoprotic acids. The data may be manipulated by either of the methods discussed to obtain K^T using the Kielland estimates for activity coefficients.

REFERENCES

(1) Kortüm, G., Vogel, W., and Andrussow, K., "Dissociation Constants of Organic Acids in Aqueous Solution," Butterworths, London, England, 1961.
(2) Perrin, D. C., "Dissociation Constants of Organic Bases in Aqueous Solution," Butterworths, London, England, 1965.
(3) Hall, N. F., and Sprinkle, M. R., *J. Am. Chem. Soc.*, **54,** 3469(1932).
(4) Albert, A., and Serjeant, E. P., "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962, chap. 2–3.
(5) Meites, L., and Goldman, J. A., *Anal. Chim. Acta*, **29,** 472(1963).
(6) *Ibid.*, **30,** 18(1964).
(7) Goldman, J. A., and Meites, L., *ibid.*, **30,** 28(1964).
(8) Meites, L., and Goldman, J. A., *ibid.*, **30,** 200(1964).
(9) Goldman, J. A., and Meites, L., *ibid.*, **30,** 280(1964).
(10) Meites, L., and Goldman, J. A., *ibid.*, **31,** 297(1964).
(11) Parke, T. V., and Davis, W. W., *Anal. Chem.*, **26,** 642(1954).
(12) Garrett, E. R., *J. Pharm. Sci.*, **52,** 400(1963).
(13) Stokes, R. H., *Australian J. Chem.*, **16,** 759(1963).
(14) Sillén, L. G., in "Treatise on Analytical Chemistry," Kolthoff, I. M., and Elving, P. J., eds., Interscience Publishers, New York, N. Y., 1959, part I, vol. 1, chap. 8.
(15) Roe, D. K., *Anal. Chem.*, **38,** 461R(1966).
(16) Joseph, N. R., *Science*, **128,** 1207(1958).
(17) Druckrey, H., *ibid.*, **129,** 1492(1959).
(18) Druckrey, H., *Arzneimittel-Forsch.*, **3,** 394(1953).
(19) Joseph, N. R., *Science*, **129,** 1493(1959).
(20) Setnikar, I., *J. Pharm. Sci.*, **55,** 1190(1966).
(21) Henderson, L. J., *J. Am. Chem. Soc.*, **30,** 954(1908).
(22) Sørensen, S. P. L., *Biochem. Z.*, **21,** 131(1909).
(23) Hasselbalch, K. A., *ibid.*, **78,** 112(1916).
(24) Benet, L. Z., and Goyan, J. E., *J. Pharm. Sci.*, **54,** 983(1965).
(25) *Ibid.*, **54,** 1179(1965).
(26) Hofstee, B. H. J., *Science*, **131,** 39(1959).
(27) Lanza, P., and Mazzei, I., *Ric. Sci. Rend. Sez. A*, **3,** 1227(1963).
(28) Lanza, P., and Mazzei, I., *J. Electroanal. Chem.*, **7,** 320(1964).
(29) Solomons, C. C., *J. Chem. Educ.*, **42,** 225(1965).
(30) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658(1934).
(31) Albert, A., and Serjeant, E. P., *op. cit.*, pp. 13, 28–40.
(32) Le Duigou, Y., and Lauer, K. F., *Anal. Chim. Acta*, **33,** 222(1965).
(33) Leeson, L. J., and Brown, M., *J. Pharm. Sci.*, **55,** 431(1966).
(34) Harper, H. A., "Review of Physiological Chemistry," 9th ed., Lange Medical Publications, Los Altos, Calif., 1963, p. 152.
(35) Albert, A., and Serjeant, E. P., *op. cit.*, p. 60.
(36) Bates, R. G., "Determination of pH," John Wiley & Sons, Inc., New York, N. Y., 1964, pp. v–vii, chap. 2.
(37) Hunt, J. P., "Metal Ions in Aqueous Solution," W. A. Benjamin, Inc., New York, N. Y., 1965, chap. 3.
(38) Debye, P., and Hückel, E., *Phys. Z.*, **24,** 185, 334 (1923).
(39) *Ibid.*, **25,** 97(1924).
(40) Debye, P., and Hückel, E., *Rec. Trav. Chim.*, **42,** 597 (1923).
(41) Hückel, E., *Phys. Z.*, **26,** 93(1925).
(42) Glasstone, S., "Textbook of Physical Chemistry," 2nd ed., Van Nostrand Co., New York, N. Y., 1946, p. 966.
(43) Fleck, G. M., "Equilibria in Solution," Holt, Rinehart, and Winston, Inc., New York, N. Y., 1966, chap. 4.
(44) Butler, J. N., "Ionic Equilibria—A Mathematical Approach," Addison-Wesley Publishing Co., Inc., Reading, Mass., 1964, chap. 5.
(45) Harned, H. S., and Owen, B. B., "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold Publishing Corp., New York, N. Y., 1958, p. 488.
(46) *Ibid.*, p. 432.
(47) Kielland, J., *J. Am. Chem. Soc.*, **59,** 1675(1937).
(48) Lewis, G. N., and Randall, M., "Thermodynamics," 2nd ed., revised by Pitzer, K. S., and Brewer, L., McGraw-Hill Book Co., New York, N. Y., 1961, p. 317.
(49) Harned, H. S., and Owen, B. B., *op. cit.*, pp. 676–677.
(50) Kortüm, B., Vogel, W., and Andrussow, K., *op. cit.*, p. 241.
(51) Bronsted, J. N., *J. Am. Chem. Soc.*, **44,** 877(1922).
(52) Guggenheim, E. A., *Phil. Mag.*, **19,** 588(1935).
(53) *Ibid.*, **22,** 322(1936).
(54) Guggenheim, E. A., and Turgeon, J. C., *Trans. Faraday Soc.*, **51,** 747(1955).
(55) Harned, H. S., and Owen, B. B., *op. cit.*, p. 516.
(56) Guggenheim, E. A., "Thermodynamics," 4th ed., North-Holland Publishing Co., Amsterdam, The Netherlands, 1959, p. 357.
(57) Lewis, G. N., and Randall, J., *op. cit.*, p. 346.
(58) Hammarlund, E. R., and Van Pevenage, G. L., *J. Pharm. Sci.*, **55,** 1448(1966).
(59) Hammarlund, E. R., Deming, J. G., and Pedersen-Bjergaard, K., *ibid.*, **54,** 160(1965).
(60) Hammarlund, E. R., and Pedersen-Bjergaard, K., *J. Am. Pharm. Assoc., Sci. Ed.*, **47,** 107(1958).
(61) Johnson, R. D., Goyan, F. M., and Tuck, L. D., *J. Pharm. Sci.*, **54,** 1176(1965).
(62) Benet, L. Z., and Ronfeld, R. A., to be published.
(63) Bates, R. G., *op. cit.*, p. 404.
(64) Harned, H. S., and Owen, B. B., *op. cit.*, pp. 752–754.
(65) Albert, A., and Serjeant, E. P., *op. cit.*, pp. 66–68.
(66) Gutbezahl, B., and Grunwald, E., *J. Am. Chem. Soc.*, **75,** 559(1953).
(67) Mizutani, M., *Z. Physik. Chem.*, **116,** 350(1925).
(68) Marshall, P. B., *Brit. J. Pharmacol.*, **10,** 270(1955).
(69) Edmonson, T. D., and Goyan, J. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **47,** 810(1958).
(70) Chatten, L. G., and Harris, L. E., *Anal. Chem.*, **34,** 149(1962).
(71) Garrett, E. R., *J. Pharm. Sci.*, **52,** 797(1963).
(72) Bates, R. G., *op. cit.*, pp. 189–190.
(73) *Ibid.*, pp. 58–61.
(74) *Ibid.*, p. 224.
(75) Ong, K. C., Robinson, R. A., and Bates, R. G., *Anal. Chem.*, **36,** 1971(1964).
(76) Bates, R. G., Paabo, M., and Robinson, R. A., *J. Phys. Chem.*, **67,** 1833(1963).
(77) Shedlovsky, T., and Kay, R. L., *ibid.*, **60,** 151(1956).
(78) Bacarella, A. L., Grunwald, E., Marshall, H. P., and Purlee, E. L., *J. Org. Chem.*, **20,** 747(1955); Bacarella, A. L., Grunwald, E., Marshall, H. P., and Purlee, E. L., *J. Phys. Chem.*, **62,** 856(1958).
(79) de Ligny, C. L., and Rehback, M., *Rec. Trav. Chim.*, **79,** 727(1960).
(80) Gutbezahl, B., and Grunwald, E., *J. Am. Chem. Soc.*, **75,** 565(1953).
(81) Gelsema, W. J., and de Ligny, C. L., unpublished measurements.
(82) Bates, R. G., *op. cit.*, pp. 226–227.
(83) Parks, R. L., Crockford, H. D., and Knight, S. B., *J. Elisha Mitchell Sci. Soc.*, **73,** 289(1957).
(84) de Ligny, C. L., Luykx, P. F. M., Rehback, M., and Wieneke, A. A., *Rec. Trav. Chim.*, **79,** 699, 713(1960).
(85) de Ligny, C. L., and Luykx, P. F. M., *ibid.*, **77,** 154 (1958).
(86) Woodhead, M., Paabo, M., Robinson, R. A., and Bates, R. G., *Anal. Chem.*, **37,** 1291(1965).
(87) Paabo, M., Bates, R. G., and Robinson, R. A., *J. Phys. Chem.*, **70,** 247(1966).
(88) Paabo, M., Robinson, R. A., and Bates, R. G., *Anal. Chem.*, **38,** 1573(1966).
(89) Paabo, M., Bates, R. G., and Robinson, R. A., *ibid.*, **37,** 462(1965).
(90) Born, M., *Z. Physik.*, **1,** 45(1920).
(91) Bates, R. G., *op. cit.*, pp. 193–196.
(92) Grunwald, E., and Berkowitz, B. J., *J. Am. Chem. Soc.*, **73,** 4939(1951).
(93) Speakman, J. C., *J. Chem. Soc.*, **1943,** 270.
(94) Dondon, M. L., *J. Chim. Phys.*, **54,** 290, 304(1957).
(95) Yasuda, M., *Bull. Chem. Soc. Japan*, **32,** 429(1959).
(96) Shedlovsky, T., in "Electrolytes," Pesce, B., ed., Pergamon Press, New York, N. Y., 1962, pp. 146–151.
(97) Bjerrum, N., *Kgl. Danske Vidensk. Selskab*, **7,** No. 9 (1926).
(98) Harned, H. S., and Owen, B. B., *op. cit.*, pp. 70–74.
(99) Fuoss, R. M., *Trans. Faraday Soc.*, **30,** 967(1934).
(100) Noyes, A. A., *Z. Physik. Chem.*, **11,** 495(1893); *cf.* Britton, H. T. S., "Hydrogen Ions," vol. 1, 4th ed., Chapman and Hall Ltd., London, England, 1955.
(101) Speakman, J. C., *J. Chem. Soc.*, **1940,** 855.
(102) Edsall, J. T., and Wyman, J., "Biophysical Chemistry," vol. 1, Academic Press Inc., New York, N. Y., 1958, chap. 9.
(103) Leeson, L. J., Krueger, J. E., and Nash, R. A., *Tetrahedron Letters*, **L18,** 1155(1963).
(104) Riegelman, S., Strait, L. A., and Fischer, E. Z., *J. Pharm. Sci.*, **51,** 129(1962).
(105) Rigler, N. E., Bag, S. P., Leyden, D. E., Sudmeier, J. L., and Reilley, C. N., *Anal. Chem.*, **37,** 872(1965).

Effect of Certain Nonsteroid Antirheumatic Drugs on Active Amino Acid Transport Across the Small Intestine

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Indomethacin (5 m*M*), phenylbutazone (5 m*M*), and salicylate (15 m*M*) prevent the active transport of the amino acid, L-tryptophan, across the small intestine of the rat. Aspirin (15 m*M*) appears to inhibit somewhat, but does not prevent, this process. Salicylate (15 m*M*) significantly inhibits the active transport of L-tryptophan across the hamster small intestine; aspirin (15 m*M*) has no inhibitory effect. These results are consistent with the relative effectiveness of these drugs as uncouplers of oxidative phosphorylation.

SALICYLATE and certain other nonsteroid anti-inflammatory drugs are potent uncouplers of oxidative phosphorylation (1). They may, therefore, be expected to interfere with the energy supply necessary for active intestinal transport processes (2, 3). Smith (3) has shown that 5 m*M* salicylate inhibits the active transport of glucose and water across the small intestine of the rat, and Quastel (4) has recently reported that 10 m*M* salicylate inhibits the active transport of phenylalanine by the guinea pig intestine. Since the oral administration of salicylate and other anti-inflammatory agents can yield appreciable concentrations of these drugs in intestinal fluids, it is desirable to assess the effect of these substances on active intestinal transport processes. It has been shown that modification of the phenolic group of salicylate causes a loss in uncoupling activity (5), and it became of interest therefore to determine if an inhibitory effect of salicylate may be reduced or circumvented totally by using aspirin. The present communication deals with the effects of salicylate, indomethacin, and phenylbutazone on the active intestinal transport of the neutral amino acid, L-tryptophan, with particular emphasis on the relative effects of salicylate and aspirin.

EXPERIMENTAL

Absorption of L-tryptophan across the rat small intestine was studied by the cannulated everted intestine method developed by Crane and Wilson (6). Male Sprague-Dawley rats, weighing about 250 Gm., were fasted overnight, but had access to drinking water at all times. The small intestine was removed under ether anesthesia and rinsed with Ringer's solution. The intestine was then sleeved onto a glass rod and everted carefully. Two segments of 10-cm. length (when stretched slightly by attaching an 8-Gm. weight and suspending the segment vertically) were obtained, distal ends were tied, and proximal ends were attached to the cannula of the apparatus described by Crane and Wilson (6). An equal number of first (from the proximal end) and second intestinal segments were used in each experiment. These segments will be referred to as proximal and distal segments, respectively. Each everted and cannulated intestine segment was suspended in 45 ml. of Krebs–Ringer–bicarbonate solution (pH 7.4) containing 2 m*M* L-tryptophan maintained at 37° and gassed continuously with a mixture of 95% oxygen and 5% carbon dioxide. The serosal (inner) solution also consisted of 2 ml. of Krebs–Ringer–bicarbonate solution containing 2 m*M* L-tryptophan. Drugs were added to both the mucosal and serosal solutions in equal specified concentrations. After 1 hr. of incubation, aliquots of both solutions were assayed. One of the two segments of intestine obtained from each rat was used for a drug experiment, while the other segment served as a control. Each set of drug and control experiments was carried out with an equal number of proximal and distal segments, except when an odd number of experiments were conducted.

Absorption of L-tryptophan across the hamster small intestine was studied by the everted sac method described by Wilson and DeCarlo (7). Three segments of small intestine, each approximately 6 cm. long, were removed under ether anesthesia from male golden hamsters weighing 100–150 Gm. Everted sacs were prepared and filled with 1 ml. of Krebs–Ringer–bicarbonate solution (pH 7.4) containing 2 m*M* L-tryptophan. The sacs were placed in 15 ml. of the same solution in 50-ml. capacity conical flasks. Both mucosal and serosal solutions contained drugs in equal specified concentrations. The flasks were flushed with a mixture of 95% oxygen and 5% carbon dioxide,

Received October 14, 1966, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication November 7, 1966.

This investigation was supported in part by grant R01 AM 08753-03 PET from the U. S. Public Health Service, Bethesda, Md.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

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TABLE I—ACTIVE TRANSPORT OF L-TRYPTOPHAN ACROSS THE SMALL INTESTINE OF THE RAT[a]

Mean Concn. Ratio,[b] Serosal/Mucosal			
All Expt.		Successful Expt.[c]	
Proximal Segments	Distal Segments	Proximal Segments	Distal Segments
1.31 (*13*)	1.40 (*13*)	1.43 (*9*)	1.54 (*9*)
All Segments		All Segments	
1.36 (*26*)		1.48 (*18*)	

[a] Cannulated everted segments of rat small intestine were incubated for 1 hr. at 37° in oxygenated Krebs–Ringer–bicarbonate solution (pH 7.4) containing 2 m*M* L-tryptophan. [b] Number of experiments listed in parentheses. [c] Concentration ratio of "controls" ≥1.20.

sealed, placed in a 37° water bath, and agitated moderately by means of a reciprocating shaker. Aliquots of the mucosal and serosal solutions were obtained after 1 hr. of incubation and were assayed.

The composition and method of preparation of Krebs–Ringer–bicarbonate solution are described elsewhere (8). Calcium chloride and magnesium sulfate had to be omitted in the indomethacin experiments due to the formation of precipitates; these salts were also excluded from the solution used in the corresponding control experiments. The drugs used in this study and their respective sources were: 2,4-dinitrophenol (Mann Research Laboratories); indomethacin (Merck Sharp and Dohme); phenylbutazone (Geigy Pharmaceuticals); aspirin (Mallinckrodt); sodium salicylate (Fisher Scientific Co.).

Tryptophan was determined by procedure *A* of the method of Spies and Chambers (9). Dinitrophenol, indomethacin, and phenylbutazone interfered with the tryptophan assay and had to be removed by extraction. For this purpose, an aliquot of the solution to be assayed was diluted with water to 3 ml., 0.5 ml. of 3 *N* hydrochloric acid was added, and the solution was extracted with 20 ml. of ethylene dichloride. The salicylate content of aspirin solutions was determined immediately after completion of the experiments by the method of Brodie *et al.* (10).

RESULTS AND DISCUSSION

The active intestinal transport of L-tryptophan has been studied in detail by Spencer and Samiy (11). This amino acid is transported by the monoamino-monocarboxylic acid transport system, which is responsible for the active transport of at least 14 amino acids (12). In the presently described investigation, L-tryptophan absorption was studied initially by the cannulated everted rat intestine method, using the occurrence of absorption against a concentration gradient (as evidenced by the magnitude of serosal/mucosal concentration ratios) as the criterion of active transport. The results of the experiments with L-tryptophan alone are shown in Table I. Some of the experiments did not yield serosal/mucosal concentration ratios significantly greater than unity; this appeared to be due to technical difficulties. However, there was an excellent correlation between the results obtained with the proximal and the distal intestine segments of any one animal. Using a serosal/mucosal concentration ratio of 1.2 as an arbitrary minimum value to establish active transport, only one out of 13 pairs of intestinal segments yielded dissimilar results in that one segment showed active transport while the other did not. Of the 13 pairs of experiments, nine pairs were designated as "successful" since the segments predesignated as the "control" (*i.e.*, either the proximal or the distal segment of each pair) yielded a serosal/mucosal tryptophan concentration ratio greater than 1.2.

The effects of various drugs on the active transport of L-tryptophan across the rat small intestine are shown in Table II. The results are listed both as the average of all experiments and as the average of the "successful" experiments. A 1 m*M* concentration of the classical uncoupling agent 2,4-dinitrophenol completely abolished active transport of L-tryptophan. Five millimolar indomethacin and 5 m*M* phenylbutazone, respectively, had the same effect. Fifteen millimolar salicylate prevented the active transport of L-tryptophan, while the same concentration of aspirin inhibited, but did not prevent, the active transport process.

To obtain more definitive data concerning the relative inhibitory effect of salicylate and aspirin on the active transport of L-tryptophan, additional experiments were carried out using the small intestine of hamsters. It has been pointed out recently that the hamster is a much more satisfactory experimental animal for active transport studies than is the rat (7). The results obtained were consistent with this statement since all the experiments were "successful" in that each control

TABLE II—EFFECT OF VARIOUS DRUGS ON ACTIVE TRANSPORT OF L-TRYPTOPHAN ACROSS THE SMALL INTESTINE OF THE RAT[a]

Drug	Concn., m*M*	Mean Tryptophan Concn. Ratio,[b] Serosal/Mucosal — All Expt. — With Drug	All Expt. — Without Drug	Successful Expt. — With Drug	Successful Expt. — Without Drug	Statistical Significance[c] of Difference, Drug *vs.* No Drug — All Expt.	Successful Expt.
2,4-Dinitrophenol	1	0.96 (*7*)	1.21 (*7*)	0.98 (*3*)	1.40 (*3*)	+	+
Indomethacin	5	0.92 (*6*)	1.41 (*6*)	0.93 (*4*)	1.56 (*4*)	+	+
Phenylbutazone	5	0.98 (*8*)	1.27 (*8*)	1.00 (*4*)	1.42 (*4*)	+	+
Phenylbutazone	10	1.01 (*5*)	1.26 (*5*)	1.00 (*4*)	1.30 (*4*)	+	+
Aspirin	10	1.16 (*16*)	1.26 (*16*)	1.23 (*7*)	1.47 (*7*)	−	−
Aspirin	15	1.19 (*13*)	1.27 (*13*)	1.20 (*7*)	1.42 (*7*)	−	+
Sod. salicylate	5	1.10 (*8*)	1.25 (*8*)	1.17 (*4*)	1.41 (*4*)	−	−
Sod. salicylate	10	1.16 (*11*)	1.35 (*11*)	1.24 (*9*)	1.40 (*9*)	+	−
Sod. salicylate	15	1.02 (*17*)	1.23 (*17*)	1.05 (*8*)	1.36 (*8*)	+	+

[a] Cannulated everted segments of rat small intestine were incubated for 1 hr. at 37° in oxygenated Krebs–Ringer–bicarbonate solution (pH 7.4) containing 2 m*M* L-tryptophan. [b] Number of experiments listed in parentheses. [c] Student's *t* test, significant differences $p < 0.05$.

segment yielded serosal/mucosal tryptophan concentration ratios appreciably greater than 1.2. The effects of salicylate and aspirin on L-tryptophan transport across the hamster small intestine are listed in Table III. Fifteen millimolar salicylate significantly inhibited the active transport of L-tryptophan, while the same concentration of aspirin had no inhibitory effect.

The relative inhibitory effectiveness of the drugs tested is consistent with their relative effectiveness as uncouplers of oxidative phosphorylation. Indomethacin and phenylbutazone are approximately equipotent but have greater activity than salicylate in uncoupling oxidative phosphorylation in rat liver mitochondria (1). The cytostatic effect of these agents, as determined by their inhibition of cellular proliferation of Ehrlich ascites in suspension cultures, is of the order: indomethacin > phenylbutazone > sodium salicylate > aspirin, while their inhibitory effect on glucosamine-6-phosphate synthesis in liver homogenates is phenylbutazone > indomethacin > sodium salicylate > aspirin (13). Aspirin has practically no inhibitory effect in the latter system. Similarly, up to 1.24 m*M* aspirin has no significant effect on the oxygen uptake of rat cerebral cortex slices, while salicylate concentrations as low as 0.06 m*M* have a significant effect (14). Brostaff *et al.* have shown that modification of the phenolic hydroxyl group of salicylate (*i.e.*, alteration of its position on the benzene ring, removal, methylation, or substitution) causes a loss of uncoupling activity (5). While the rapid *in vivo* hydrolysis of aspirin to the potent uncoupler salicylic acid has made it difficult to determine the uncoupling activity of aspirin as such, it is reasonable to expect that aspirin either has no or very little uncoupling activity. The results of the present study are consistent with this assumption in that aspirin has less inhibitory effect than salicylate on the active transport of L-tryptophan across the small intestine of the rat. Further, 15 m*M* aspirin does not inhibit L-tryptophan transport across the hamster small intestine, while the same concentration of salicylate has a pronounced inhibitory effect.

There was significant hydrolysis of aspirin to salicylic acid during the absorption experiments but, at least with hamster small intestine, the concentration of salicylate did not become high enough to reach inhibitory levels (Table IV). Aspirin hydrolysis appears to be due mainly to the presence of esterase(s) in the intestinal epithelium, since hydrolysis was appreciably reduced in the absence of intestinal segments or when the intestine had been placed in boiling water to destroy the enzymes. The presence of aspirin hydrolyzing esterase(s) in the intestinal epithelium, as well as in the gastric mucosa (15), is of considerable biopharmaceutic interest because it raises the possibility of appreciable aspirin hydrolysis during gastrointestinal absorption. In this connection it is significant that nonspecific esterase is present in all regions of the human gastrointestinal tract (16). Due to the limited capacity of enzyme systems located in the gastrointestinal mucosa, the extent of aspirin hydrolysis is probably more pronounced when the drug is administered in slowly absorbed form.

One can only speculate as to the clinical implications of the amino acid transport inhibiting effects observed in this study. Patients requiring anti-inflammatory therapy with salicylate, indomethacin, or phenylbutazone may be in nutritional deficiency for many reasons other than impaired absorption. On the other hand, the chronicity of anti-inflammatory drug therapy makes additional and more detailed studies of these effects highly desirable. A next logical step is to determine if the inhibitory effect of these anti-inflammatory agents depends on their presence in intestinal fluids, or if it persists for an appreciable time after exposure of the intestine to these drugs. This is presently being investigated and will be the subject of a future communication.

TABLE III—EFFECT OF ASPIRIN AND SALICYLATE ON ACTIVE TRANSPORT OF L-TRYPTOPHAN ACROSS THE SMALL INTESTINE OF THE HAMSTER[a]

	Mean Tryptophan Concn. Ratio, Serosal/Mucosal			
Drug	First Segment[b] (5 Sacs)	Second Segment (5 Sacs)	Third Segment (5 Sacs)	All Segments (15 Sacs)
None	1.42	1.46	1.42	1.43 ± 0.14[c]
15 m*M* Aspirin	1.36	1.66	1.66	1.56 ± 0.34
15 m*M* Sod. salicylate	1.18	1.16	1.11	1.15 ± 0.08[d]

[a] Everted sacs of small intestine from the golden hamster were incubated for 1 hr. at 37° in oxygenated Krebs–Ringer–bicarbonate solution (pH 7.4) containing 2 m*M* L-tryptophan. [b] Numbered from the pyloric end. [c] Standard deviation. [d] This value differs significantly ($p < 0.01$) from the control and the aspirin values.

TABLE IV—HYDROLYSIS OF ASPIRIN BY SMALL INTESTINE OF THE HAMSTER DURING 1-hr. INCUBATION AT pH 7.4 AND 37°

Conditions	No. of Expt.	Hydrolysis of Aspirin, %
Standard[a]	15	17.4 ± 2.1[c]
No intestine, no tryptophan	15	4.9 ± 0.4
Boiled intestine[b]	3	7.1
No intestine	5	6.3

[a] As described in *Footnote a* of Table III. [b] Intestine was immersed in boiling water for 5 min. [c] Standard deviation.

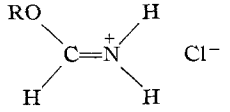
REFERENCES

(1) Whitehouse, M. W., *Nature*, **201**, 629(1964).
(2) Segal, S., *Ann. Internal Med.*, **62**, 847(1965).
(3) Smith, M. J. H., *Am. J. Physiol.*, **193**, 29(1958).
(4) Quastel, J. H., *Appl. Therap.*, **5**, 252(1963).
(5) Brostoff, D. V., Moses, V., and Smith, M. J. H., *J. Pharm. Pharmacol.*, **13**, 65(1961).
(6) Crane, R. K., and Wilson, T. H., *J. Appl. Physiol.*, **12**, 145(1958).
(7) Wilson, T. H., and DeCarlo, A., *ibid.*, **20**, 1102(1965).
(8) DeLuca, H. F., and Cohen, P. P., in "Manometric Techniques," 4th ed., Umbreit, W. W., Burris, R. H., and Stauffer, J. F., eds., Burgess Publishing Co., Minneapolis, Minn., 1964, pp. 131–133.
(9) Spies, J. R., and Chambers, D. C., *Anal. Chem.*, **20**, 30(1948).
(10) Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol. Exptl. Therap.*, **80**, 114(1944).
(11) Spencer, R. P., and Samiy, A. H., *Am. J. Physiol.*, **199**, 1033(1960).
(12) Saunders, S. J., and Isselbacher, K. J., *Gastroenterology*, **50**, 586(1966).
(13) Domenjoz, R., *Med. Pharmacol. Exptl.*, **14**, 321(1966).
(14) Fishgold, J. T., Field, J., and Hall, V. E., *Am. J. Physiol.*, **164**, 727(1951).
(15) Lutwak-Mann, C., *Biochem. J.*, **36**, 706(1942).
(16) Dawson, I., and Pryse-Davies, J., *Gastroenterology* **44**, 745(1963).

Detection of Some Formiminoesters and the Measurement of Their Rates of Formation Using NMR

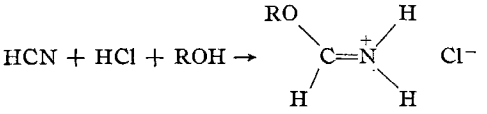
By JAY NEMATOLLAHI* and L. DALLAS TUCK

Formiminoesters have long been postulated as intermediates in the synthesis of formamidines and various heterocyclic compounds. The methyl, ethyl, and isopropyl homologs have been prepared by interaction of hydrogen cyanide with the appropriate alcohol in the presence of dry hydrogen chloride, and the progress of the formation was monitored by NMR at regulated probe temperatures from −25° to 0°. The rate of formation of formiminoesters depends principally on the concentration of hydrogen cyanide. The quartet in the NMR spectrum at a chemical shift near 8 p.p.m. arises from a single proton on a doubly bonded carbon, which is coupled to two nonequivalent protons. This is consistent with the structure:



The lifetime of the nitrogen-proton bond is of the order of 3 sec.

THE FORMIMINOESTER hydrochlorides have long been postulated as intermediates in the syntheses of formamidines and related compounds. These intermediates are presumed to form by interaction of hydrogen cyanide and dry hydrogen chloride with the appropriate alcohol. Pinner (1) established empirical formulas for several of these intermediates and proposed the structure shown in I.



I

Because of the extreme instability of these compounds under ordinary laboratory conditions, their properties have not been explored, and nothing is known about their rates of formation. The authors have confirmed the structure proposed by Pinner using NMR spectrometry and have studied the dependence of the rates of formation on the concentrations of the reactants and on temperature from −25° to 0°.

Received June 13, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication November 14, 1966.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported in part by a general research support grant FR 05453 from the Division of Research Facilities and Resources, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

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DISCUSSION

To explore the possibility of a reaction in the absence of hydrogen chloride, a mixture of liquid hydrogen cyanide and each of the alcohols was allowed to stand for 7 days at temperatures ranging from −10° to 25°. The NMR spectra showed that no reaction occurred during this period.

A series of experiments to test the dependence of the reaction on the concentration of hydrogen chloride proceeded as follows. To mixtures of hydrogen chloride with alcohol, ranging from a catalytic amount to 40% by weight, were added varying amounts of hydrogen cyanide. Unless the hydrogen chloride in the alcohol–HCl mixture exceeded 25% by weight, no reaction occurred based on the NMR spectra. At higher concentrations the reaction proceeded at a rate in which there was a minor dependence on HCl concentration. It was concluded that roughly a stoichiometric amount of hydrogen chloride is necessary in order for the reaction to proceed. From this result it would appear that an intermediate complex is formed which is structurally very close to the protonated imino ester which is the final product. More detailed studies on the effect of the concentration of HCl were not pursued because of the difficulties due to the extreme volatility of HCl under the conditions of these experiments.

Dry trifluoracetic acid was tried as a possible substitute for HCl gas. There was no interaction between HCN and alcohol in this solvent; the NMR spectrum showed only the rapid formation of esters of trifluoracetic acid. Unlike the case with HCl, there is no direct interaction between HCN and trifluoracetic acid (see below).

It is well known that HCN and HCl react to produce a solid from which *s*-triazine can be obtained (2). The product of this reaction does not occur in quantity detectable in the NMR spectrum when alcohol is present. Research on this product is continuing.

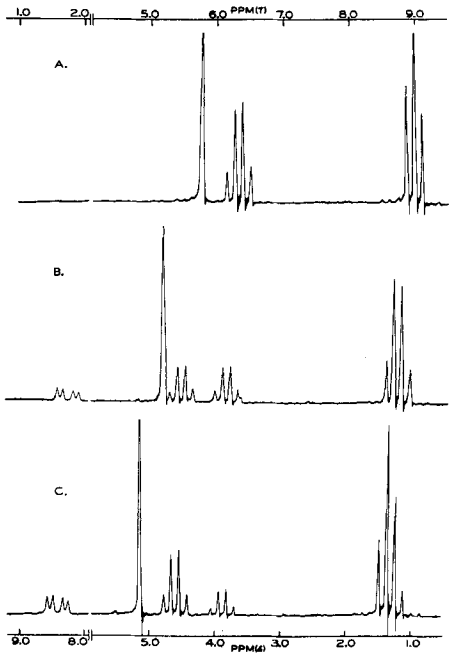

Fig. 1—NMR spectra during the formation of ethyl formimidate at −20°. Key: A, *0 min.;* B, *56 min.;* C, *94 min.*

As long as it remains liquid, the most useful measure of the extent of the formation of the formiminoesters is the integral of the NMR peaks of the imino ester described in a later paragraph. At a late stage of the reaction, a white crystalline solid is precipitated. The solid, which is insoluble in chloroform, reacts instantaneously with water. With dimethylsulfoxide it reacts to produce a brown solution containing incompletely dissolved material. It was desired to know whether the solid was the formiminoester hydrochloride, which would have a limited solubility in the alcoholic medium. The imino ester may react with alcohol in the mixture or with atmospheric water to produce ammonium chloride. Then

$$ROCH{:}NH_2^+Cl^- + 2ROH \rightarrow CH(OR)_3 + NH_4^+Cl^-$$
$$ROCH{:}NH_2^+Cl^- + H_2O \rightarrow HCOOR + NH_4^+Cl^-$$

In both cases soluble compounds with characteristic NMR spectra would be formed—in the former the orthoformate, in the latter, a formate ester. Neither of these compounds was found in the NMR spectrum. The infrared spectrum indicated that the solid was not ammonium chloride. Moreover, the effect of dimethylsulfoxide, together with NMR and I.R. evidence, showed that the solid was different from that observed by direct interaction of HCl and HCN.

NMR Spectrum—The progress of the reaction is illustrated by reference to the ethanol case, the other alcohols differing only in the details of the alkyl portions of the NMR spectrum. In Fig. 1, A, at zero time the triplet at $\delta = 1.0$ ($\tau = 9.0$) and the quartet at $\delta = 3.8$ arise, respectively, from the methyl and methylene portions of ethanol. The sharp singlet at 4.2 is due to HCN, and an additional peak due to mutually exchangeable alcoholic OH and HCl is found offscale to the left. Figure 1,B, shows after a lapse of time a new methylene quartet which grows at the expense of the original quartet. The transformation of the triplet into a quartet of the same over-all intensity is due to the overlap of the ethanol triplet with that of an ethyl group in the product. The fact that the new methylene and methyl multiplets are downfield (left) of ethanol indicates that in the product the ethyl group is associated with a functional group more electronegative than the alcoholic OH. At the same time a new quartet has appeared at $\delta = 8.0$, whose integral is exactly half of the integral of the new CH_2 quartet and is thus due to a single proton. Figure 1,C, shows a continuation of the same process as the product builds up at the expense of the reactants. There is a general downfield shift of the entire spectrum owing to changes in the solvent and acidic properties of the mixture. Table I summarizes the chemical shifts of the various alcohols and the corresponding esters.

TABLE I—CHEMICAL SHIFTS (p.p.m. FROM TETRAMETHYLSILANE) IN THE MIXTURE DURING THE FORMATION OF THE RESPECTIVE FORMIMINOESTER, AS INDICATED

	Methyl Formimidate	Ethyl Formimidate	Isopropyl Formimidate
HCN	3.9–5.4	4.3–5.0	4.1–4.9
Alcohol, αCH	3.0–3.3	3.7–4.1	3.8–4.4
βCH	...	0.9–1.2	1.0–1.2
Ester, αCH	3.9–4.0	4.4–4.8	4.6–5.2
βCH	...	1.1–1.5	1.2–1.35
N=CH—O	7.9–8.3	8.0–8.5	8.0–8.5

The feature of the spectrum which leads to important structural inference is the well-resolved quartet due to a proton on an sp^2 carbon at $\delta = 8$. This indicates the presence of two nonequivalent protons on the nitrogen *cis* and *trans* to the CH. The spin-spin splitting arising from the *cis* coupling is 5 c.p.s. and from the *trans* 14 c.p.s.

The separate lines are sufficiently well resolved that the lifetimes of the states may be calculated directly from their line width, about 2 c.p.s. The net lifetime T_2' is the magnetic lifetime determined by the spin-lattice interaction as modified by the chemical lifetime of the NH protons with respect to exchange (3). A spin-lattice relaxation time T_2 is obtained from the line width of the CH_3 line, and assuming that the same prevails for the NH, the exchange lifetime is found by the reciprocal difference, *viz.*,

$$1/\tau = 1/T_2' - 1/T_2 = 2\pi(\delta\nu_{CH} - \delta\nu_{CH_3}) \approx 3 \text{ sec.}$$

The relative amount of formiminoester formed was determined from the integral of an appropriate section of the NMR spectrum. The methyl singlets of the ester and alcohol were well separated, and their integrals were used as a measure of the extent of the methanol reaction. Because of overlap between the multiplets of the ethyl formimidate and the ethanol, as well as the HCN singlet, it was necessary to use the integral of the downfield peak of the methyl triplet as a measure of the ethanol reaction. In the isopropyl formimidate the integral of the methyl doublet was used. To establish a constant scale

which would cancel out instrumental fluctuations, the relative measure of the formimidate is taken as the ratio of the appropriate integral (see above) to the sum of the integrals of the peaks ascribed to the OH, NH_2, and HCl, which sum should remain constant during the reaction. For each series of measurements on a given mix, the relative concentration of formimidate was plotted against the time of measurement as shown for methyl formimidate in Fig. 2. During the early part of the experiment, the points in these plots describe straight lines, and the initial reaction rates calculated as the slopes are shown in Table II.

The results of the rate measurements of the formation of the methyl formimidate at −10° are the most complete and show a close proportionality with HCN concentration. Little can be said about the relationship of the rate with concentration of HCl or alcohol except the general comments found earlier in this paper. Not enough data were collected from the ethyl and isopropyl experiments to draw similar conclusions, but judging from the qualitative behavior and the time required for precipitation of the solid compound, the same approximate dependence on HCN and HCl prevails. Comparing the three alcohols, it is evident that for a comparable concentration of HCN the rate is higher for the higher homolog.

These temperature dependencies of the rates were used to determine the approximate values for the energy of activation. These are given in Table III, and their respective values seem to indicate a reasonable trend. As one might expect, the simpler structure has a lower energy of activation.

Because of the impossibility of independent adjustment of the concentration of the reactants in a system in which the solvent is itself a reactant, it is difficult to ascertain the dependency of the rate on the concentration of alcohol or HCl. Moreover, in these circumstances, mass action behavior is no longer the primary determinant of rate because of the importance of other variables of the mix, such as viscosity and specific solvation effects. On the other hand, it is the simplicity of the system, particularly the absence of a proton-containing solvent, which makes the study by NMR feasible.

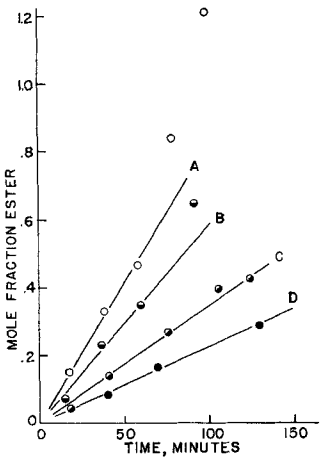

Fig. 2—Mole fraction of product during esterification of methanol at −10° calculated from the NMR integral. The straight lines yield the initial rates. The runs may be readily identified from the summary in Table II.

TABLE II—INITIAL RATES OF FORMATION OF FORMIMINO ESTERS

Temp.	Mole Fractions HCN	HCl	Alcohol	Rate, min.$^{-1}$	Rate/HCN
Methyl Formimidate					
−25	0.52	0.18	0.30	0.00052	0.001
−10	.60	.17	.23	0.0083	0.014
−10	.41	.25	.34	0.0059	0.014
−10	.27	.31	.42	.0035	0.013
−10	.46	.19	.35	.0022	0.048
0	.58	.15	.27	.015	0.026
Ethyl Formimidate					
−20	.53	.245	.22	.0028	0.005
−15	.545	.242	.213	.003	0.005
−10	.57	.222	.201	.0101	0.018
0	.70	.154	.141	Solid forms too fast to measure	...
Isopropyl Formimidate					
−10	.555	.265	.179	.0134	0.024
0	.64	.159	.204	.045	0.070
0	.59	.181	.229	.0275	0.047

TABLE III—ENERGIES (Kcal./mole) OF ACTIVATION OF THE FORMATION OF FORMIMINOESTERS

Methyl	26.4
Ethyl	34.4
Isopropyl	34.6

EXPERIMENTAL

Hydrogen chloride gas, dried by passing through concentrated sulfuric acid, was introduced into a weighed amount of alcohol cooled in a dry ice–acetone mixture. A sintered glass tip was used for the gas dispersion. Three hours were required to produce a solution of 25 to 30% HCl. The amount of hydrogen chloride was determined by the gain in weight. The solution was stored at −20°. The alcohol was dried using magnesium turnings and iodine.

Hydrogen cyanide from a tank was dried by passing through a tower containing $CaCl_2$ and $CaSO_4$ and condensed in an ice–salt mixture at −5°.

To a tared NMR tube was added about 0.5 ml. of HCN, which was then capped, cooled to −20°, and weighed. The tube was placed in a dry ice–acetone bath and to it was added 0.5 ml. of the cooled HCl–alcohol solution. The material was brought to the melting point of HCN just before placing it in the constant-temperature probe of the NMR spectrometer. The first spectrum was run immediately and then at time intervals varying from 5 to 30 min. The tube was not removed from the probe during the experiment.

The solid product remaining in the tube at the end of the experiment was filtered in a dry box under nitrogen atmosphere. The infrared spectrum of this solid was determined in KBr disks.

NMR spectra were run using a Varian A60 spectrometer with a temperature controlled probe. Infrared spectra were determined with a Beckman IR-8 spectrophotometer.

REFERENCES

(1) Pinner, A., and Klein, F., *Ber.*, **10**, 1889(1877); Pinner, A., *ibid.*, **16**, 352, 1643(1883).
(2) Smolin, E. M., and Rapaport, L., "*s*-Triazine and Derivatives," Interscience Publishers, Inc., New York, N. Y., 1959, pp. 6–10.
(3) Pople, J. A., Schneider, W. G., and Bernstein, H. J., "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 218 *ff*.

Binding of Cortisol and Its Degradation Products by Human Serum Albumin

By K. J. KRIPALANI and DONALD L. SORBY

The rate of degradation of the 17-dihydroxyacetone function of cortisol has been measured in a phosphate buffer system, pH 7.4 and ionic strength 0.16, at 15.0°, 25.0°, and 35.0°. The results correspond favorably with data in the literature for degradation of prednisolone under similar conditions. The binding of cortisol and its degradation products to human serum albumin was measured at 25.0° by an equilibrium dialysis procedure. The degradation products were found to be bound more strongly than was cortisol, but did not appear to compete with cortisol for binding sites on the protein molecule. In systems having a constant concentration of total corticosteroid, the apparent binding constant of total steroid increases as the fraction of the material present as degradation products increases. The results of this research are important to the design of experiments which attempt to study the binding of steroids containing the 17-dihydroxyacetone function to serum proteins.

INTERACTIONS with various serum proteins may potentially affect the distribution, metabolism, elimination, and therapeutic effects of a drug. Hence, interactions between drugs and proteins have been subjects for study by many investigators. In his extensive review, Goldstein (1) cites several hundred published studies of drug protein interactions. One particularly active area of interest has been the interactions which occur between various steroid hormones and serum proteins. Such interactions appear to be of major importance to the transport and distribution of these compounds. Cortisol, in particular, has received much attention. Sandberg *et al.* (2), Daughaday (3), Slaunwhite *et al.* (4), Brunkhorst and Hess (5), and Westphal (6), among others, have studied the interaction between cortisol and serum albumin. The literature pertaining to steroid–protein interactions has been reviewed by Daughaday (7).

Two plasma components appear to be involved in binding cortisol (8–10). At physiologic levels, cortisol is bound predominantly by an α globulin commonly termed "transcortin" or "corticosteroid binding globulin" (8, 10). Transcortin is present in relatively low concentrations, however, and rapidly becomes saturated in its binding ability if plasma cortisol concentrations rise much above normal physiologic levels. Serum albumin has a lesser affinity for binding cortisol than does transcortin but plays a major role in binding at elevated serum cortisol levels (11). Effects of cortisol concentration on its binding to plasma proteins have been discussed by Daughaday (7) and by Bush (12). For the most part, research on the steroid–plasma protein interaction to date has employed equilibrium dialysis procedures (7). Equilibration times have varied between 18 and 72 hr. One important criterion which must be met in such a study is that the solute molecules must be sufficiently stable so that chemical degradation does not occur to a significant extent during the equilibration period. Oesterling and Guttman (13) and Guttman and Meister (14) have studied prednisolone degradation. Their data show that the extent of degradation of the 17-dihydroxyacetone function might be significant during the average equilibrium dialysis experiment. While similar information pertaining to cortisol was not available, it was considered that it too should be subject to similar rates of degradation. This research was initiated to determine whether cortisol would undergo significant degradation under conditions of the equilibrium dialysis experiment and whether such degradation would significantly alter the results of

Received September 30, 1966, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122
Accepted for publication March 15, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Abstracted from a thesis submitted by K. J. Kripalani to the Graduate Division, University of California, San Francisco Medical Center, in partial fulfillment of Doctor of Philosophy degree requirements.
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protein binding experiments. Such information was needed for development of suitable experimental procedures for protein binding studies currently under way in this laboratory.

EXPERIMENTAL

Reagents and Materials—Phosphate buffer, pH 7.4, ionic strength 0.16, was prepared by dissolving 16.68 Gm. disodium phosphate, $Na_2HPO_4 \cdot 7H_2O$, and 2.209 Gm. monosodium phosphate, $NaH_2PO_4 \cdot H_2O$, in enough freshly boiled distilled water to make 1 L. Hydrocortisone was obtained from a commercial source and was recrystallized from isopropanol. The melting point was 217–219° (uncorrected). Tetramethylammonium hydroxide reagent was prepared by diluting 1 ml. of a 10% solution in water, obtained from Eastman Chemical Co., to 10 ml. with absolute ethanol. Triphenyltetrazolium chloride reagent was prepared by dissolving 1 Gm. of 2,3,5-triphenyltetrazolium chloride, obtained from Nutritional Biochemicals Corp., in enough absolute ethanol to make 100 ml. Crystalline human serum albumin[1] was purchased from Nutritional Biochemicals Corp. Information furnished by the supplier certified that this material was four times recrystallized and was 100% pure albumin by electrophoresis. The crystalline HSA was stored at −7° and was dried under vacuum over calcium chloride, to a constant weight before use. The molecular weight was assumed to be 69,000. All other solvents and chemicals employed at various stages of the experiment were reagent grade or comparable quality.

Thin-Layer Chromatography of Degradation Products—A 5×10^{-4} *M* solution of cortisol in phosphate buffer was prepared. Ethanol was used to facilitate dissolution of the cortisol and was present in the final sample at a concentration of 2%. The solution was stored at 25.0° for 10 days. An aliquot of the solution was then extracted with four 30-ml. portions of freshly distilled chloroform. The combined extracts were designated "extract I." The aqueous residue remaining after extraction was made strongly acidic with concentrated hydrochloric acid and was again extracted with four 30-ml. portions of chloroform. The combined extracts were designated "extract II." Both extracts I and II were concentrated *in vacuo* to 5 ml. Ten-microliter volumes of the concentrated extracts and of a freshly prepared solution of cortisol of identical concentration extracted in a similar fashion were spotted onto thin-layer chromatographic plates coated with Silica Gel G which had been activated at 110° for 1 hr. After drying, the plates were developed with a mixture consisting of 75 parts benzene, 50 parts acetone, and 0.2 part water. After drying, visualization of the spots was accomplished by first spraying with a solution consisting of equal parts of tetramethylammonium hydroxide reagent and triphenyltetrazolium chloride reagent. The plates were heated for 5 min. at 110°. Steroids reacting with the 2,3,5-triphenyltetrazolium chloride appeared as pink spots on the plate. After the pink zones were marked, the plates were sprayed with 70% w/w sulfuric acid and were heated at 110° for 15 min. All spots were then visible as brown-black areas.

Kinetic Experiments—Kinetic experiments were performed at 15.0°, 25.0°, and 35.0°. Flasks containing cortisol solutions of the appropriate concentration[2] in the phosphate buffer were placed in a thermostat maintained within ±0.05° of the desired temperature. These samples also contained 2% ethanol which was used to facilitate dissolution of the cortisol. At various time intervals, aliquots of the solutions were withdrawn and immediately assayed by a spectrophotometric procedure for their content of total steroid and by a colorimetric procedure for the amount of cortisol still possessing the 17-dihydroxyacetone function.

In the spectrophotometric procedure, the absorbance of the solution was measured at 248 mμ with a Beckman DU spectrophotometer. The apparent concentration of cortisol in the sample was calculated from the absorbance reading, using 16,000 as the molar absorption coefficient of cortisol in the buffer medium.

To determine the amount of steroid still possessing the 17-dihydroxyacetone function, the tetrazolium chloride procedure of Mader and Buck (15) was employed. The appropriate aliquot of the test solution was extracted with four 20-ml. portions of freshly distilled chloroform. The combined chloroform extracts were evaporated under vacuum to dryness. The residue was dissolved in 20 ml. absolute ethanol and 1 ml. each of triphenyltetrazolium chloride reagent and tetramethylammonium hydroxide reagent was added. The assay mixture was allowed to stand in the dark for exactly 30 min. One milliliter of glacial acetic acid was then added to quench further color formation. The absorbance of the mixture was determined with a Bausch & Lomb Spectronic 20 colorimeter at 485 mμ. Ten milliliters of buffer solution, treated in a similar fashion, served as a reagent blank for the procedure. The concentration of cortisol still retaining the 17-dihydroxyacetone function was determined by referring to a standard curve of absorbance *versus* concentration for freshly prepared cortisol solutions assayed in an identical fashion.

Spectrophotometric Estimation of Cortisol Binding—A spectrophotometric procedure was employed for preliminary experiments to determine whether degradation of the 17-dihydroxyacetone function of cortisol significantly affected binding to HSA. The method employed was adapted from one proposed by Klotz and Walker (16) and later used by Westphal (17) in his studies of steroid–protein interactions. Solutions of cortisol in the phosphate buffer, $3.86 \times 10^{-5}M$ and $7.88 \times 10^{-5}M$, were prepared. An aliquot of each solution was mixed with an equal volume of a 4×10^{-5} *M* HSA in phosphate buffer. The absorbance of each solution was measured at 248 mμ with the Beckman DU spectrophotometer. HSA, 4×10^{-5} *M*, diluted with an equal volume of phosphate buffer, served as the reference solution in the spectrophotometer.

The apparent molar extinction coefficient of cortisol ($\epsilon_{app.}$) in the presence of HSA was calculated from:

$$\epsilon_{app.} = \frac{1}{Cd} \log \left(\frac{I_0}{I} \right) \qquad \text{(Eq. 1)}$$

where C is the total molar concentration of cortisol,

[1] In this paper, human serum albumin is henceforth designated as HSA.

[2] See Table I for concentrations of cortisol employed in these experiments.

d is the path length of the cell, and log I_0/I is the absorbance of the mixture at 248 mμ.

The stock solutions of cortisol were then placed in a thermostat at 25.0°. During succeeding days, samples were withdrawn and mixed with freshly prepared HSA solutions. The ultraviolet absorbance was measured and the $\epsilon_{app.}$ was calculated as described.

Equilibrium Dialysis Experiments—The effect of cortisol degradation on the binding to HSA was quantitated by an equilibrium dialysis experiment (18). Eight-inch lengths of Visking cellulose dialysis casings (23/32 in. diameter) were immersed in distilled water and heated on a steam cone for 12 hr. The water was changed three times during this period. The inner lumen of each casing was then flushed with a running stream of distilled water. The casings were soaked an additional 0.5 hr. in distilled water and were then transferred to phosphate buffer solution for 6 hr. at room temperature. The casings were used immediately after this length of time.

Solutions of HSA, 4×10^{-5} M, in phosphate buffer were prepared immediately prior to each experiment. Octyl alcohol, 250 mg./L., was added to HSA solutions to prevent microbial attack. Octyl alcohol between 250 mg./L. and 1000 mg./L. did not interfere with the binding of cortisol by HSA.

Excess buffer solution was carefully expressed from the dialysis casings and a knot was tied in one end to make a small bag. A 10-ml. quantity of the HSA solution was pipetted into the bag and, finally, the open end was knotted. The bag was placed inside a 20 × 200-mm. glass-stoppered test tube containing 20 ml. cortisol solution of the appropriate concentration and age. The tubes were stoppered, dipped in paraffin, and placed on a rocker-type shaker completely immersed in a constant-temperature bath at 25.0°. Six different concentrations of cortisol solution between 5×10^{-5} M and 5×10^{-4} M were used in each experiment and duplicate samples were prepared for each concentration. A time period of 24 hr. was sufficient for attainment of diffusional equilibrium between the solutions inside and outside the dialysis bag. Three samples containing 20 ml. buffer solution in place of cortisol were included in each experiment as controls. There was negligible change in the volumes of solution inside and outside the dialysis sac during the course of the experiment. After 24 hr., the tubes were removed from the bath. The solution external to the dialysis bag was assayed as described previously.

Because cortisol was found to bind to the dialysis casing, parallel experiments were run to obtain corrections for "bag binding." Samples were prepared as described above except 10 ml. phosphate buffer was placed inside the bag instead of HSA solution. At the end of the dialysis experiment, the amount of steroid bound to the bag was calculated as the difference between the amount of compound remaining in solution and the amount originally present before equilibration. Ultraviolet spectrophotometric assay procedures were used to measure the steroid concentration in these experiments. A curve was prepared by plotting the amount of steroid bound by the dialysis bag *versus* the concentration of steroid not bound and served to provide correction values for the test samples containing protein.

To evaluate the effect of degradation of the 17-dihydroxyacetone function of cortisol on the binding of HSA, equilibrium dialysis studies were carried out using cortisol solutions which had been aged for periods of time sufficient to obtain a desired degree of degradation. A 5×10^{-4} M stock solution of cortisol in phosphate buffer was prepared by placing the necessary amount of cortisol in a volumetric flask nearly filled with phosphate buffer. The flask was placed on a shaking device in a constant-temperature room at 25° and agitated vigorously until dissolution of the cortisol was complete. This procedure was usually completed within a 6-hr. period. The solution was then made to volume with phosphate buffer and the flask was placed in a thermostat at 25.0° for the desired period of time. The age of the solution was taken as the time elapsed between first contact of the crystalline cortisol with the buffer solution and the end of the dialysis experiment. Dialysis experiments were performed at 25.0° using cortisol solutions aged for 48, 84, and 288 hr. After diffusional equilibrium was attained, two aliquots of the phase external to the dialysis bag were taken for assay. One portion was assayed by measuring the ultraviolet absorbance at 248 mμ. The second portion was assayed by the triphenyltetrazolium chloride method described above.

Calculations—*Total Steroid Bound*[3]—At equilibrium, the concentration of steroid in the volume outside the dialysis bag may be assumed to be equal to the concentration unbound inside the dialysis casing. The concentration of free steroid in the outside volume is given by the ultraviolet absorbance of this solution after equilibrium is established. The difference between the amount of free steroid in the total volume of the system and the amount of steroid initially placed in the test tube gives the amount of steroid bound. This figure also includes the amount of steroid bound to the dialysis bag. The correction for binding to the bag at any free steroid concentration was obtained from the bag binding correction curve described above.

Undegraded Cortisol Bound—The equilibrium concentration of undegraded cortisol in the volume outside the bag is measured by the triphenyltetrazolium assay method. The total concentration of undegraded cortisol can be obtained from assay of the stock solution at the end of the experiment. The difference between the amount of undegraded cortisol remaining free at equilibrium and the total amount theoretically present at this time gives the amount of cortisol which is bound.[4] A correction for bag binding was obtained by referring to the correction curve described above. It was assumed that the affinity for binding to the dialysis casing was the same for cortisol as for its degradation products.

Degraded Cortisol Bound—The data for the binding of degraded cortisol are calculated in the following manner. The equilibrium concentration of degraded cortisol remaining free in a given sample is obtained by calculating the difference between the equilibrium concentrations of total steroid and undegraded cortisol. In similar fashion, the amount of

[3] The term "steroid" is used here to denote all forms still possessing the steroid ring structure and includes cortisol plus its degradation products.

[4] It was assumed that the rate of degradation of cortisol is unchanged in the presence of HSA. In the actual case, the presence of HSA slows the rate of degradation somewhat (19). However, the difference is slight over the 24-hr. period encompassed by the equilibrium dialysis experiment.

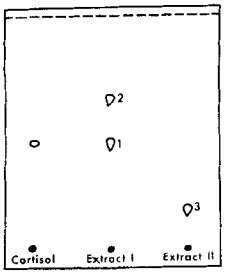


Fig. 1—Thin-layer chromatogram of extracts of a solution of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged for 240 hr. at 25.0°.

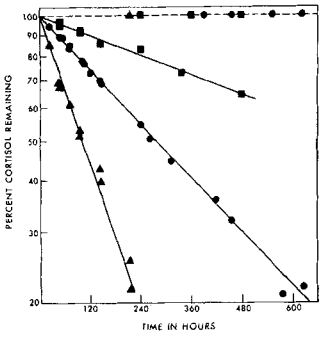


Fig. 2—Disappearance of cortisol from solutions in phosphate buffer, pH 7.4, μ = 0.16, at 15.0° (■), 25.0° (●), and 35.0° (▲), as measured by reaction with triphenyltetrazolium chloride (———) and by ultraviolet absorbance at 248 mμ (– – – –). (See also Table I.)

degradation products bound is calculated from the difference between the concentrations of total steroid bound and the concentration of cortisol bound.

RESULTS AND DISCUSSION

Figure 1 is a diagram of a thin-layer chromatogram of extracts from a cortisol solution aged at 25.0° for 10 days. The freshly prepared cortisol standard produced a spot reacting pink with triphenyltetrazolium chloride. Extract I gave two spots with 70% w/w sulfuric acid marked as 1 and 2 in Fig. 1. Spot 1 reacted with triphenyltetrazolium chloride and has the same R_f as the standard cortisol. It was considered to represent the cortisol remaining undegraded in the test sample. Spot 2 does not react with triphenyltetrazolium chloride and hence must represent a degradation product lacking the 17-dihydroxyacetone function. Extract II gave only one spot, marked 3 in Fig. 1, with sulfuric acid. Spot 3 does not react with triphenyltetrazolium chloride and hence must represent a second degradation product of cortisol also lacking the 17-dihydroxyacetone function. Under aerobic conditions, the degradation of the 17-dihydroxyacetone function of steroids has been reported to involve an oxidative cleavage to yield the corresponding etianic acid (20–22). Chulski and Forist (21) have investigated the effect of solid buffering agents on prednisolone in aqueous solution. Their paper chromatographic examination of the reaction mixture suggested that prednisolone was converted predominantly to a 17β-carboxylic acid and, to a lesser degree, to a 17-keto product. Since the compound represented by spot 3 corresponds to a product possessing acidic characteristics, it is likely that it represents a carboxyl group in place of the 17-dihydroxyacetone function.

It is likely that spot 2 represents a neutral product, probably a 17-keto compound similar to the one reported by Chulski and Forist for prednisolone (21).

TABLE I—RESULTS OF KINETIC STUDIES OF THE DEGRADATION OF THE 17-DIHYDROXYACETONE FUNCTION OF CORTISOL IN PHOSPHATE BUFFER, pH 7.4, IONIC STRENGTH 0.16

Temp., °C.	Apparent First-Order Rate Constant, $(hr.^{-1}) \times 10^3$	Half-Life, hr.	Activation Energy, Kcal./mole
15.0	0.896[a]	773	
	0.912	760	
25.0	2.46	282	
	2.69	258	
35.0	6.80	102	17.0[b]
	6.03	115	

[a] The first value at each temperature represents a solution having an initial concentration of 7.9×10^{-5} *M*, 5.8×10^{-5} *M*, or 7.6×10^{-5} *M*, respectively. The second value at each temperature represents a solution having an initial concentration of 4.1×10^{-4} *M*, 4.1×10^{-4} *M*, or 3.8×10^{-4} *M*, respectively. Rate constants were calculated by the method of least squares from experimental data arranged according to the equation, $\ln c/c_0 = -kt$, where c is the concentration of cortisol at any particular time t, c_0 is the concentration of cortisol when t is zero, and k is the apparent first-order rate constant. [b] Calculated from the Arrhenius equation using the method of least squares applied to the rate constant data.

The ultraviolet absorption by cortisol at 248 mμ is characteristic of the Δ^4-3-keto function of the A ring of the steroid nucleus. Under conditions employed in these experiments, the apparent concentration of cortisol determined by the ultraviolet absorption assay remains constant up to a period of 26 days at 25.0° and up to 9 days at 35.0° (see broken line in Fig. 2). This observation indicates that there is no significant degradation of the A ring of cortisol during the periods of time allowed for experiments in this study. The half-life for the photolytic degradation of the A ring of cortisol under ordinary fluorescent laboratory lighting at room temperature has been reported as 160 days (23). The stability of the A ring allows the ultraviolet absorption assay method to be used to show accurately the concentration of total steroid in aged solutions of cortisol. Inspection of Fig. 2 shows that even when considerable amounts of cortisol have degraded, the ultraviolet absorbance remains unchanged.

The results of kinetic studies of the degradation of cortisol in the phosphate buffer are summarized in Table I and Fig. 2. The degradation of the 17-dihydroxyacetone function follows apparent first-order kinetics at all concentrations and temperatures studied. Figure 3 shows an Arrhenius plot of the rate constant data. The activation energy calculated by the method of least squares from the rate constant data is 17.0 Kcal./mole. Data in Table I may be compared with those obtained by Oesterling and Guttman (13) for prednisolone and by Jensen and Lamb (24) for fluprednisolone. The former investigators reported a rate constant of approximately 0.07 $hr.^{-1}$ for degradation of the 17-dihydroxyacetone function of prednisolone in 0.1 *M* phosphate buffer at 70°.[5] Our data predict a value of 0.109 $hr.^{-1}$. The activation energy reported by Jensen and Lamb was 15.2 Kcal./mole. Their rate constant at 70° was reported as 0.0952 day^{-1} (0.004 hr^{-1}). Oesterling and Guttman have pointed out the strong de-

[5] The value for the rate constant was taken from Fig. 4 of the paper by Oesterling and Guttman (13).

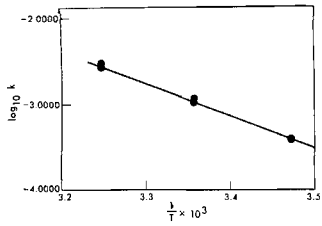


Fig. 3—Arrhenius plot of rate constants for degradation of the 17-dihydroxyacetone function of cortisol in phosphate buffer, pH 7.4, μ = 0.16.

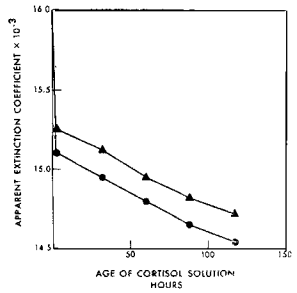


Fig. 4—Effect of aging in phosphate buffer, pH 7.4, μ = 0.16, on the apparent molar extinction coefficient of cortisol at 248 mμ when mixed with freshly prepared solutions of HSA, 2 × 10⁻⁵ M. Initial cortisol concentrations, 1.93 × 10⁻⁵ M (●) and 3.94 × 10⁻⁵ M (▲).

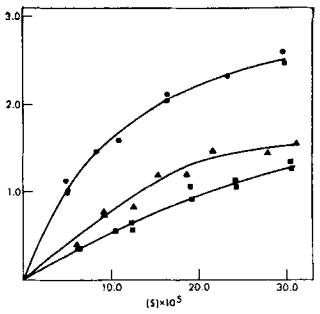


Fig. 5—Binding of total steroid by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), 84 hr. (▲), and 288 hr. (●).

pendence of the reaction upon the nature of the buffer components, particularly with respect to the content of trace metals in the system. Guttman and Meister (14) have shown the dependence of the reaction upon the hydroxyl-ion concentration. Thus the difference between the rate constants for the different compounds are not unexpected since conditions are not identical in the three different experiments.

Figure 4 shows the results of spectrophotometric studies concerning the effect of sample age on the apparent binding of cortisol by HSA. The large initial decrease in the apparent molar extinction coefficient at 248 mμ can be attributed to the binding interaction between cortisol and HSA (17). As Fig. 4 illustrates, there is a progressive decrease in the apparent molar extinction coefficient of these systems as the cortisol ages. This is indicative of either a greater amount of total steroid being bound or a change in the molar extinction coefficient of the steroid–protein complex. Due to uncertainty about the respective values of the molar extinction coefficients of the various possible steroid–protein complexes, the spectrophotometric method could not be employed to measure the magnitude of the interaction between cortisol or its degradation products and HSA.

Data from equilibrium dialysis studies of protein binding interactions are usually plotted according to:

$$\frac{1}{r} = \frac{1}{nK[S]} + \frac{1}{n} \qquad \text{(Eq. 2)}$$

where r is the average number of molecules of the particular steroid bound per molecule of HSA, n represents the maximum number of binding sites per molecule of HSA, K is the association constant for the interaction, and $[S]$ is the concentration of substrate molecules remaining free in solution when equilibrium between bound and unbound forms is established. This equation is applicable under the conditions that all binding sites have the same intrinsic association constant, K, and that, aside from a statistical factor, the free energy of binding to a particular site is independent of binding at other sites (25). A plot of $1/r$ *versus* $1/[S]$ will yield a straight line with $1/n$ as its intercept on the ordinate axis and $1/nK$ as its slope.

Equation 2 cannot be applied directly to data for aged solutions representing the binding of total steroid by HSA. In these systems, the steroid is present as three different compounds, each of which may bind to the protein with differing affinity. Since data expressing the concentration of each degradation product were not available, an expanded form of Eq. 2 was not employed. Figure 5 shows plots of r *versus* $[S]$ for the binding of total steroid by HSA.

From Fig. 5, it is obvious that the extent of interaction between steroid and HSA increases with the age of the steroid solution. This suggests that the degradation products either bind more strongly than does cortisol or that they are bound to sites not available to cortisol. In the 48-hr. solution, only about 11% of the total steroid is in the degraded form, while approximately 50% is degraded in the 288-hr. material. It is also interesting to note that application of Eq. 2 to the data yields linear plots (Fig. 6). From the slopes of these curves, one obtains values for nK as being 5,700 L./mole, 9,000 L./mole, and 30,000 L./mole at 48 hr., 84 hr., and 288 hr., respectively. Values of n calculated from the intercepts are 5, 5, and 3.6. As stated above, these values are without theoretical meaning; however, they do point out that failure to account for degradation as an experimental variable in these systems may easily go unrecognized. This is particularly true when the assay method employed is not sensitive to the presence of the 17-dihydroxyacetone function.

Figure 7 shows plots of data obtained for the binding of undegraded cortisol in the 48-hr. and 288-hr. samples. It appears that the presence of increased amounts of degradation products in the system at 288 hr. affects the binding of cortisol by HSA. The curves in Fig. 7 represent a visual fit of the data. From these curves, nK values of 4,500 L./mole and 8,900 L./mole are obtained at 48 hr. and 288 hr., respectively. The estimated value of n in each case is 5. Equations for the lines best fitting the experimental data were also calculated by the method of least squares. In this case, nK values were 4,080 L./mole and 10,429 L./mole at 48 hr. and 288 hr., respectively.[6] In this case, however, values for n were 5.3 and 2.1, respectively.

[6] Reciprocals of the 95% confidence intervals of the calculated slopes gave nK values ranging between 5952 and 3106 L./mole at 48 hr. and between 6579 and 26,316 L./mole at 288 hr.

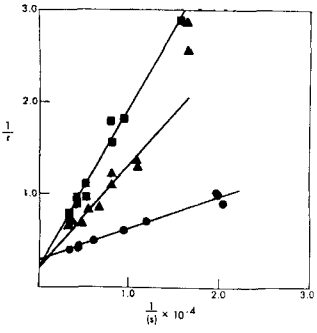


Fig. 6—Plot according to Eq. 2 of the binding of total steroid by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), 84 hr. (▲), and 288 hr. (●).

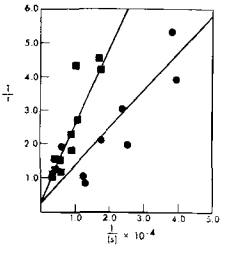


Fig. 7—Plot according to Eq. 2 expressing the binding of undegraded cortisol by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), and 288 hr. (●).

The values of the intercepts from the least squares calculations indicate that a competition for binding sites may exist between the degradation products and cortisol. This suggests that the affinity between the degradation products and certain sites on the HSA molecule is greater than the affinity of cortisol for the same sites.

This observation is consistent with the observations that the binding of total steroid increases in aged solutions of cortisol. In view of the scatter in the points for the 288-hr. data, there is considerable uncertainty in the value of n obtained from the intercept of the $1/r$ *versus* $1/[S]$ plot. Assigning such significance to the differences in n between 48-hr. and 288-hr. data must be done with caution. Unfortunately, even with repeated experiments, it was impossible to attain the degree of precision in the 288-hr. systems which would allow a more confident assessment of the situation.

Regardless of whether n remains at 5 or indeed is reduced to 2 in systems containing larger amounts of degradation products, it is of interest that the data at 288 hr. suggest that the affinity constant for the interaction between cortisol and HSA must also increase at least by a factor of two or more. This might be explained as being due to an actual difference between the affinity constant of cortisol for some sites as compared to others, especially if one accepts that the decrease in n actually does occur in the 288-hr. system. An alternative explanation would be that binding of the degradation products induces changes in HSA molecules which increase the affinity constant for interaction between cortisol and remaining free sites. Belman (26) has observed changes in the interaction between beryllium and bovine serum albumin when 1% dodecyl sulfate was present. Considerable evidence exists (27–30) that protein molecules are quite flexible and that the secondary and tertiary structure is easily deformed in the presence of certain other molecular species. Karush (27, 30) has suggested that serum albumins possess a number of combining regions, each of which is characterized by a high degree of configurational adaptability. He has also suggested that conformational changes in the protein resulting from binding of a first substrate molecule may induce a capacity for interaction with additional substrate molecules. It appears to be not impossible that an increased binding affinity of cortisol could result from the presence of increasing amounts of its degradation products also being bound by HSA. Brunkhorst and Hess (5) have observed changes in binding capacity caused by increasing concentrations of substrate in their investigations of the cortisol–HSA interaction.

Table II—Values of nK and $\Delta F°$ Reported in the Literature for the Interaction between Cortisol and Human Serum Albumin[a]

Temp., °C.	nK (L./mole) × 10^{-3}	$\Delta F°$ (Kcal./mole)	Ref.
5	5.0	−4.7	(2)
4	6.0	−4.8	(3)
4	2.3	−4.3	(3)
25	5.3[a]	...	(5)
25	4.9[a]	−5.0[b]	(5)
25	4.4[a]	...	(5)
25		−5.5	(31)

[a] The value for nK was dependent upon total concentration of cortisol used in the experiment. [b] Calculated from data given in Table II of *Reference 5*.

It is interesting to compare the results of this experiment with reports of other investigators for the cortisol–HSA interaction (Table II). While nK values in Table II agree favorably with the value obtained in this experiment for the 48-hr. sample, $\Delta F°$ for binding of cortisol in the 48-hr. sample is −4.0 Kcal./mole when n is taken as being 5. The lack of agreement in $\Delta F°$ values between Table II and this experiment is interesting in light of the fact that higher values similar to those in Table II can be obtained if one calculates this parameter from the plots of Fig. 6 for the binding of total steroid from solutions of cortisol aged for varying lengths of time. In such cases, $\Delta F°$ values of −4.1 Kcal./mole, −4.6 Kcal./mole, and −5.4 Kcal./mole are obtained at 48 hr., 84 hr., and 288 hr., respectively. Comparison of these values with those in Table II causes one to question in the experiments cited whether proper control was exerted to insure that cortisol degradation did not take place. Unfortunately, in these reports, no mention was made regarding control of the cortisol samples. Brunkhorst and Hess (5) employed a radiometric assay utilizing cortisol-4-^{14}C. Westphal (17, 31) employed ultraviolet spectrophotometric methods of assay. Neither method would be sensitive to the degradation phenomena reported here.

SUMMARY AND CONCLUSIONS

Data obtained in this research clearly show that the interaction between cortisol and HSA can only be studied by methods which take into account the degradation of the 17-dihydroxyacetone function of cortisol which can occur under usual experimental conditions. These results would also apply qualitatively to other steroids possessing the 17-dihydroxyacetone function.

This research also points out the necessity for considering stability factors in equilibrium dialysis experiments of this type. Competitive binding or other effects imparted by the presence of degradation products can significantly alter experimental results. In addition, since results of dialysis experiments are calculated from concentration differences before and after equilibration, disappearance of solute due to unrecognized chemical instability could be mistaken for binding by the protein and introduce further error into the experiment.

REFERENCES

(1) Goldstein, A., *Pharmacol. Rev.*, **1**, 102(1949).
(2) Sandberg, A. A., Slaunwhite, W. R., and Antoniades, H. N., *Recent Progr. Hormone Res.*, **13**, 209(1957).
(3) Daughaday, W. H., *J. Clin. Invest.*, **35**, 1428(1956).
(4) Slaunwhite, W. R., Rosenthal, H., and Sandberg, A. A., *Arch. Biochem. Biophys.*, **100**, 486(1963).
(5) Brunkhorst, W. K., and Hess, E. L., *ibid.*, **111**, 54 (1965).
(6) Westphal, U., and Ashley, B. D., *J. Biol. Chem.*, **234**, 2847(1959).
(7) Daughaday, W. H., *Physiol. Rev.*, **39**, 885(1959).
(8) Daughaday, W. H., *J. Clin. Invest.*, **37**, 511(1958).
(9) Upton, C. V., and Bondy, P. K., *Arch. Biochem.*, **78**, 197(1958).
(10) Slaunwhite, W. R., and Sandberg, A. A., *J. Clin. Invest.*, **38**, 384(1959).
(11) Beisel, W. R., DiRaimondo, V. C., and Forsham, P. H., *Ann. Internal Med.*, **60**, 641(1964).
(12) Bush, I. E., *Pharmacol. Rev.*, **14**, 317(1962).
(13) Oesterling, T. O., and Guttman, D. E., *J. Pharm. Sci.*, **53**, 1189(1964).
(14) Guttman, D. E., and Meister, P. D., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 773(1958).
(15) Mader, W. J., and Buck, R. R., *Anal. Chem.*, **24**, 666 (1952).
(16) Klotz, I. M., and Walker, F. M., *J. Am. Chem. Soc.*, **69**, 1609(1947).
(17) Westphal, U., Ashley, B. D., and Selden, G. L., *ibid.*, **80**, 5135(1958).
(18) Hughes, T. R., and Klotz, I. M., *Biochemical Methods*, **3**, 278(1956).
(19) Sorby, D. L., unpublished data.
(20) Velluz, L., Petit, A., and Berrett, R., *Bull. Soc. Chim. France*, **1947**, 123.
(21) Chulski, T., and Forist, A. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 553(1958).
(22) Caspi, E., Schmid, W., and Wittstruck, T. A., *Tetrahedron*, **16**, 271(1961).
(23) Hamlin, W. E., Chulski, T., Johnson, R. H., and Wagner, J. G., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 253(1960).
(24) Jensen, E. H., and Lamb, D. J., *J. Pharm. Sci.*, **53**, 402(1964).
(25) Klotz, I. M., in "The Proteins," vol. 1B, Academic Press Inc., New York, N. Y., 1953, chap. 8.
(26) Belman, S., *J. Am. Chem. Soc.*, **85**, 2154(1963).
(27) Karush, F., *ibid.*, **72**, 2705(1950).
(28) Kauzmann, W., *J. Cell Comp. Physiol., Suppl. 1*, **47**, 113(1956).
(29) Sogami, M., and Foster, J. F., *J. Biol. Chem.*, **238**, 2245(1963).
(30) Karush, F., *J. Phys. Chem.*, **56**, 70(1952).
(31) Westphal, U., *J. Am. Oil Chemists' Soc.*, **41**, 481 (1964).

Mechanistic Implication Between Quenching in Liquid Scintillation System and Photosensitivity with Respect to Energy Transfer

By C. T. PENG

A basic mechanism for energy transfer in photosensitive biological systems is postulated.

THE USE OF liquid scintillation system for radiation measurement has been widely accepted. Owing to its high counting efficiency, the internal sample method becomes the preferred mode of assaying radionuclides emitting β particles of low kinetic energy, particularly tritium, ^{14}C, ^{35}S, ^{45}Ca, *etc.* The introduction of a sample in the form of an organic compound into the liquid scintillation system frequently causes fluorescence quenching which manifests itself as a decrease in the counting efficiency of the system. The cause of quenching has been attributed to either a diminished light transmission owing to the color of the sample or a molecular interaction between the scintillator and the sample leading to thermal degradation of the excitation energy or a combination of both (1).

The liquid scintillation system is a sensitized fluorescent donor–acceptor system in which the solvent molecule, by virtue of its presence in an overwhelming number in the medium, absorbs the radiation energy and becomes excited; from the excited states, the solvent molecules may undergo de-excitation by fluorescence emission, nonradiative transition, or excitation transfer. In the presence of solute molecules which can accept the excitation energy from the donor solvent molecule, de-excitation can take place by emission of fluorescence of the solute. Because of the inherently greater fluorescence intensity, the spectrum of the solute characterizes that of the entire system. In the presence of a quencher, transfer of excitation energy is adversely affected. According to theory, energy transfer between unlike molecules is brought about by a resonance mechanism over long distances involving dipole–dipole interaction (2, 3) or dipole–quadrupole interaction (3) or by the exchange mechanism (3, 4) in which an overlap of the wave function of the molecules concerned is necessary.

Received October 3, 1966, from the Radioactivity Research Center and the Department of Pharmaceutical Chemistry, School of Pharmacy, San Francisco Medical Center, University of California, San Francisco, CA 94122

Accepted for publication November 7, 1966.

The author wishes to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

This research also points out the necessity for considering stability factors in equilibrium dialysis experiments of this type. Competitive binding or other effects imparted by the presence of degradation products can significantly alter experimental results. In addition, since results of dialysis experiments are calculated from concentration differences before and after equilibration, disappearance of solute due to unrecognized chemical instability could be mistaken for binding by the protein and introduce further error into the experiment.

REFERENCES

(1) Goldstein, A., *Pharmacol. Rev.*, **1**, 102(1949).
(2) Sandberg, A. A., Slaunwhite, W. R., and Antoniades, H. N., *Recent Progr. Hormone Res.*, **13**, 209(1957).
(3) Daughaday, W. H., *J. Clin. Invest.*, **35**, 1428(1956).
(4) Slaunwhite, W. R., Rosenthal, H., and Sandberg, A. A., *Arch. Biochem. Biophys.*, **100**, 486(1963).
(5) Brunkhorst, W. K., and Hess, E. L., *ibid.*, **111**, 54 (1965).
(6) Westphal, U., and Ashley, B. D., *J. Biol. Chem.*, **234**, 2847(1959).
(7) Daughaday, W. H., *Physiol. Rev.*, **39**, 885(1959).
(8) Daughaday, W. H., *J. Clin. Invest.*, **37**, 511(1958).
(9) Upton, C. V., and Bondy, P. K., *Arch. Biochem.*, **78**, 197(1958).
(10) Slaunwhite, W. R., and Sandberg, A. A., *J. Clin. Invest.*, **38**, 384(1959).
(11) Beisel, W. R., DiRaimondo, V. C., and Forsham, P. H., *Ann. Internal Med.*, **60**, 641(1964).
(12) Bush, I. E., *Pharmacol. Rev.*, **14**, 317(1962).
(13) Oesterling, T. O., and Guttman, D. E., *J. Pharm. Sci.*, **53**, 1189(1964).
(14) Guttman, D. E., and Meister, P. D., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 773(1958).
(15) Mader, W. J., and Buck, R. R., *Anal. Chem.*, **24**, 666 (1952).
(16) Klotz, I. M., and Walker, F. M., *J. Am. Chem. Soc.*, **69**, 1609(1947).
(17) Westphal, U., Ashley, B. D., and Selden, G. L., *ibid.*, **80**, 5135(1958).
(18) Hughes, T. R., and Klotz, I. M., *Biochemical Methods*, **3**, 278(1956).
(19) Sorby, D. L., unpublished data.
(20) Velluz, L., Petit, A., and Berrett, R., *Bull. Soc. Chim. France*, **1947**, 123.
(21) Chulski, T., and Forist, A. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 553(1958).
(22) Caspi, E., Schmid, W., and Wittstruck, T. A., *Tetrahedron*, **16**, 271(1961).
(23) Hamlin, W. E., Chulski, T., Johnson, R. H., and Wagner, J. G., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 253(1960).
(24) Jensen, E. H., and Lamb, D. J., *J. Pharm. Sci.*, **53**, 402(1964).
(25) Klotz, I. M., in "The Proteins," vol. 1B, Academic Press Inc., New York, N. Y., 1953, chap. 8.
(26) Belman, S., *J. Am. Chem. Soc.*, **85**, 2154(1963).
(27) Karush, F., *ibid.*, **72**, 2705(1950).
(28) Kauzmann, W., *J. Cell Comp. Physiol., Suppl. 1*, **47**, 113(1956).
(29) Sogami, M., and Foster, J. F., *J. Biol. Chem.*, **238**, 2245(1963).
(30) Karush, F., *J. Phys. Chem.*, **56**, 70(1952).
(31) Westphal, U., *J. Am. Oil Chemists' Soc.*, **41**, 481 (1964).

Mechanistic Implication Between Quenching in Liquid Scintillation System and Photosensitivity with Respect to Energy Transfer

By C. T. PENG

A basic mechanism for energy transfer in photosensitive biological systems is postulated.

THE USE OF liquid scintillation system for radiation measurement has been widely accepted. Owing to its high counting efficiency, the internal sample method becomes the preferred mode of assaying radionuclides emitting β particles of low kinetic energy, particularly tritium, ^{14}C, ^{35}S, ^{45}Ca, *etc.* The introduction of a sample in the form of an organic compound into the liquid scintillation system frequently causes fluorescence quenching which manifests itself as a decrease in the counting efficiency of the system. The cause of quenching has been attributed to either a diminished light transmission owing to the color of the sample or a molecular interaction between the scintillator and the sample leading to thermal degradation of the excitation energy or a combination of both (1).

The liquid scintillation system is a sensitized fluorescent donor–acceptor system in which the solvent molecule, by virtue of its presence in an overwhelming number in the medium, absorbs the radiation energy and becomes excited; from the excited states, the solvent molecules may undergo de-excitation by fluorescence emission, nonradiative transition, or excitation transfer. In the presence of solute molecules which can accept the excitation energy from the donor solvent molecule, de-excitation can take place by emission of fluorescence of the solute. Because of the inherently greater fluorescence intensity, the spectrum of the solute characterizes that of the entire system. In the presence of a quencher, transfer of excitation energy is adversely affected. According to theory, energy transfer between unlike molecules is brought about by a resonance mechanism over long distances involving dipole–dipole interaction (2, 3) or dipole–quadrupole interaction (3) or by the exchange mechanism (3, 4) in which an overlap of the wave function of the molecules concerned is necessary.

Received October 3, 1966, from the Radioactivity Research Center and the Department of Pharmaceutical Chemistry, School of Pharmacy, San Francisco Medical Center, University of California, San Francisco, CA 94122

Accepted for publication November 7, 1966.

The author wishes to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

Similar mechanisms may also be evident in biological systems. Aside from photosynthetic processes, the presence of dyes and certain drugs in biological systems causes sensitization to light, presumably as a result of a more efficient localization and utilization of the radiant energy. This report emphasizes the general aspect of energy transfer process in quenching in liquid scintillation system and by analogy extends the principle to energy transfer in photosensitive biological systems with an evaluation of the possibility of the use of the former as a means to elucidate the mechanism involved in the latter.

EXPERIMENTAL

The composition of the liquid scintillator used and the procedure of obtaining the concentration quenching curves have been previously described (5, 6). The quenching constant, q, was obtained from the initial portion of the concentration quenching curve which showed less than 50% reduction of the initial count rate of the sample by quenching.

The excitation sources were toluene-^{14}C and toluene-^{3}H. The ultraviolet excitation was carried out with an Aminco-Bowmann spectrophotofluorometer. The activating wavelength and the fluorescent wavelength were adjusted for each sample to give the maximum meter reading.

RESULTS AND DISCUSSION

Energy Transfer in Quenching in Liquid Scintillation System—The relative abundance of solvent and solute molecules in a liquid scintillator is given in Table I. In such system, toluene is the solvent, 2,5-diphenyloxazole, the primary solute, and 1,4-bis-2(5-phenyloxazolyl)benzene, the secondary solute. The use of a secondary solute is to shift the fluorescence spectrum of the scintillator system to longer wavelength to match the spectral response of the multiplier phototube (7), and its role of energy transfer between solute and solute in the liquid scintillation system need not concern our discussion. Dissipation of radiation energy in the scintillator system under these conditions causes the formation of the excited solvent molecules from which the excitation energy is transferred to the scintillator or the solute molecules which decay by fluorescence emission. In the presence of a quencher, this energy transfer between the excited solvent and the scintillator molecules is interrupted.

Energy transfer by molecular encounter is not so important a process as that by inductive resonance in a liquid scintillation system. The latter mechanism predicts energy transfer over distances as large as 50 to 100 Å. According to Förster (2), resonance transfer requires an overlapping of the absorption spectrum of the acceptor and the fluorescence spectrum of the donor. Since polyatomic molecules have broad spectra in solution, it is likely that coupled transitions can occur between unlike species of organic molecules. This may afford an explanation to the observed fact that addition of an organic compound to a liquid scintillation system frequently causes quenching.

TABLE I—COMPOSITION OF A LIQUID SCINTILLATOR

Compd.	Concn., mole/L.	Abundance Ratio	Av. Radius of Separation Between Adjacent Molecules, Å.
Solvent: Toluene	10.9	345	
Primary solute:			
2,5-Diphenyloxazole	3.16×10^{-2}	1 232	23.2
Secondary solute:			
1,4-bis-2(5-Phenyloxazolyl)benzene	1.36×10^{-4}	1	142

Based on quantum mechanical considerations involving dipole–dipole interaction between molecules, Förster concluded that the rate constant of excitation transfer from donor to acceptor molecules at thermal equilibrium is inversely dependent to the sixth power of the intermolecular distance. By assuming a sharp critical transfer distance with instantaneous excitation transfer for shorter and no transfer for longer distance, a simple exponential formula can be used to approximate the donor fluorescence yield. Thus:

$$\frac{n}{n_0} = \text{exp. } (-\alpha c/c_0) \qquad \text{(Eq. 1)}$$

where n is the fluorescence yield at quencher concentration c; n_0, that in the absence of the quencher; α, a constant; and c_0, the quencher concentration corresponding to the critical transfer distance R_0. Their interrelationship is given by the following equation:

$$c_0 = \frac{3000}{4\pi N R_0^3} \qquad \text{(Eq. 2)}$$

where N is Avogadro's number.

Equation 1 resembles an empirical equation derived by Peng (5, 8) for quenching correction in liquid scintillation counting, based on an observation by Kerr *et al.* (9) that quenching varies exponentially with the concentration of the quencher. Thus:

$$S_a = S_0 \text{ exp. } (-qc) \qquad \text{(Eq. 3)}$$

$$= S_0 \text{ exp. } (-c/c_0) \qquad \text{(Eq. 3}a\text{)}$$

where S_a and S_0 are the apparent specific count rates in the presence and in the absence of the quencher, respectively; c is the quencher concentration, and q, the quenching constant which equals $1/c_0$ or $0.693/c_{1/2}$. c_0 and $c_{1/2}$ are the concentrations of the quencher that reduce the count rate to e^{-1} and $1/2$ of its initial value, respectively. The two equations differ by a constant exponent α which was found to have a value of 1.42 (2), although the theoretical value should be 1.000. It may also be pointed out that c_0, the "relative" critical transfer concentration, can also be obtained from statistical consideration of a random interaction between the excited molecules and the quencher molecules according to poisson distribution with an average of one interaction between them. Such a consideration is

TABLE II—CRITICAL TRANSFER CONCENTRATION (IN ARBITRARY UNITS) AND QUENCHING INDEX OF VARIOUS COMPOUNDS (^{14}C β-EXCITATION)

Compd.	Critical Transfer Concn., c_0, moles/L.	Critical Transfer Distance,[a] R_0, Å.	Quenching Index
CH_3OH	14.18	3.06	1
$HCON(CH_3)_2$	5.2	4.25	1.41
CH_3COOH	1.41	6.70	2.22
CH_3COCH_3	.852	7.75	2.58
CD_3COCD_3[b]	.845	7.78	2.59
$CHCl_3$	.41	9.90	3.29
$CDCl_3$[b]	.43	9.75	3.24
CCl_4	.061	18.65	6.20
C_6H_5NCS	.055	19.35	6.43
Hyamine hydroxide	.0294	23.80	7.90
Benzophenone	.026	24.80	8.23
Benzil	.0055	41.70	13.90

[a] Calculated by Eq. 2. [b] The perdeuterated acetone and chloroform were obtained from New England Nuclear Corp., Boston, Mass.

analogous to that used in deriving the e^{-1} surviving fraction by the target theory in radiobiology (10).

The use of Eq. 3 to obtain c_0 or $c_{1/2}$ in a liquid scintillation system gives only relative values. It has been pointed out that $c_{1/2}$ value depends on the pulse height and consequently varies with the instrument settings of the spectrometer used for the measurement (5). But at given instrument settings, the $c_{1/2}$ values are quite reproducible. It is therefore feasible to use either q or its related values as an index of the quenching property of the compound, provided that the measurement is carried out simultaneously against a selected standard. This index, in order to eliminate variations in instrument settings, should be expressed as a ratio of q or its related value of the compound to that of the standard. Such a ratio would allow a comparison of the quenching properties of various compounds. The absolute value can be calculated once the true critical transfer concentration of the standard is known. Table II shows the arbitrary values of critical transfer concentration c_0 of various compounds and the ratios of their critical transfer distance to that of methanol. It may be noted that perdeuterated acetone and chloroform show insignificant difference in quenching property from their protonated forms indicating an absence of intermolecular isotope effect. The relative magnitude of the quenching index of benzophenone and benzil as given is of interest because they are known to form a donor–acceptor pair (11).

Deviation from Eq. 3 is frequently observed and is attributed to the presence in solution of molecular aggregates of fluorescent molecules which exhibit different quenching properties (6). Figure 1 shows the quenching curves of 2,5-diphenyloxazole in two different concentrations in toluene and in n-hexane with ultraviolet excitation and with varying amounts of carbon tetrachloride as quencher. The composite nature of the quenching curve in toluene can be represented by a summation expression of Eq. 3a. Thus:

$$S = \Sigma S_{0i} \exp.(-c/c_{0i}) \qquad i = 1, 2, 3, \ldots .$$

Since toluene is a very efficient solvent for energy transfer owing to the availability of π electrons, its replacement with n-hexane under similar conditions gives more linear quenching curves. From the linearity of the quenching curve it may be interpreted that in inefficient energy transfer systems there is generally an absence of excimer formation (12) or photoassociation of solute molecules or formation of molecular aggregates of similar nature which frequently occurs in liquid scintillation systems of efficient excitation transfer. Formation of dimers or molecular aggregates in solution has been reported for naphthalene (13) and 2,5-diphenyloxazole (14).

The relationship between the extent of quenching and the concentration of the quencher is frequently governed by the Stern-Volmer equation (15). In liquid scintillation system, Stern-Volmer plots for a number of quenchers studied were found to exhibit a nonlinear relationship over wide concentration ranges. A notable exception is the straight line obtained for hyamine hydroxide as shown in Fig. 2.

The nonlinearity of the Stern-Volmer plot may be interpreted as a result of energy transfer between the triplet state of molecules. Such interpretation is not only in accord with the findings of Terenin and Ermolaev (16) but also with the theoretical evaluation by Inokuti and Hirayama (4) of energy transfer by the exchange mechanism in sensitized luminescence. They demonstrated the constancy of the value of α as 1.00 in Eq. 1 in the approximation of the donor luminescence yield. Approximation with a Stern-Volmer type equation, $n/n_0 = (1 + \beta c/c_0)^{-1}$, would be less meaningful under similar circumstances because the β value which by definition of the equation should remain constant was found to vary with changing values of c/c_0.

Recent studies by pulse radiolysis of various solutions of binary mixtures of anthracene, biacetyl, naphthalene, *cis*- and *trans*-stilbene, *etc.*, indicate that the order of intermolecular energy transfer among these compounds may be related to their triplet energy level (17). Because of the low energy

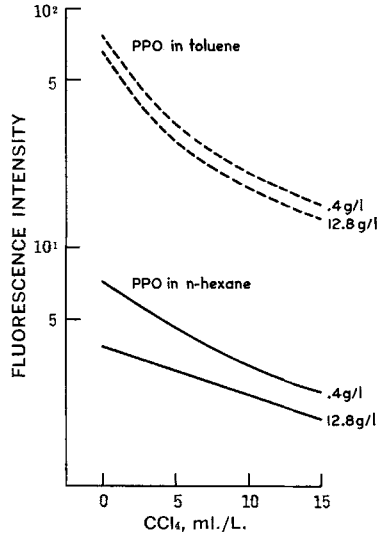


Fig. 1—Carbon tetrachloride quenching curves of 2,5-diphenyloxazole (PPO) solutions by U.V. excitation.

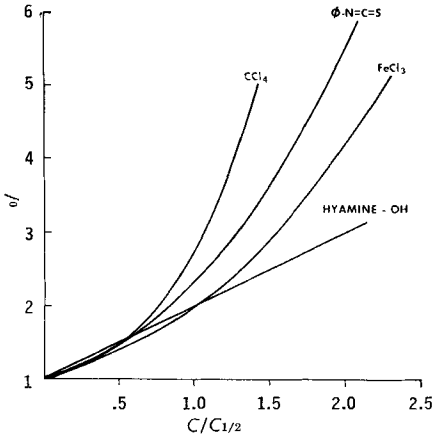


Fig. 2—Stern-Volmer plots for quenchers in liquid scintillation system. The quencher concentration on the abscissa is expressed in terms of $c_{1/2}$ for equivalent quenching effectiveness. The ordinate is the ratio of initial count rate to the quenched count rate.

level of the triplet state (E_T: 60.9 Kcal./mole) and an absence of the self-quenching effect, naphthalene has the property to suppress quenching in liquid scintillation systems (18) and to deactivate triplet benzophenone (E_T: 68.5 Kcal./mole) very efficiently (19). These observations constitute further evidence of support for our interpretation. The triplet–triplet energy transfer mechanism can also account for the observed change in the channels ratio which is a measure of the q values as the concentration of the quencher is increased, as shown in Fig. 2 of *Reference 20*. It is likely that at high concentrations, the molecules are in close proximity to each other, and consequently, energy transfer by the exchange mechanism becomes more probable.

Energy Transfer in Photosensitivity—Although the causation of hypersensitivity to light by the administration of drugs and dyes has been reported and exhaustively reviewed in the literature (21–24), the postulated mode of action has not been based on a consideration of energy transfer. As the conditions under which photosensitivity or photodynamic effect[1] occurs coincide in general with the conditions necessary for energy transfer, it is probable that a mechanism based on an intermolecular energy transfer of the radiant energy will be able to account inclusively for the observed facts such as the extremely dilute concentration of dyes that is sufficient to elicit a response, the diversity of the chemical structure of the active agents, the obligatory presence in the photosensitive biological system of compounds which can act as donors or sensitizers in energy transfer, the heavy atom effect, and the dependence of the end effect on light intensity.

A mechanism of energy transfer based on the consideration of similar processes in liquid scintillation system can be postulated to form a common basis for all observations on hypersensitivity to light; this consists of optical excitation of the sensitizer either directly or indirectly with intersystem crossing to the triplet state and formation of the triplet state of the acceptor from both the singlet and the triplet states of the sensitizer by energy transfer. The sequence of events may be represented by reactions *1*, *4*, *7*, *8*, and *10* in Scheme I. The intervening reactions represent a diversion of the excitation energy leading to a reduction of the quantum yield of the triplet acceptor. Once formed, the triplet acceptor will have a greater probability for participation in many biological reactions because of its long lifetime. In photodynamic action, the triplet acceptor can react with oxygen in its ground triplet state to yield the moleoxide according to reactions *12*, *13*, and *15*, similar to those postulated for photooxidation of anthracene (25) and other organic compounds (26).

Alternately, the peroxide can be formed by reaction with excited singlet oxygen molecule. The formation of singlet oxygen ($^1\Delta_g$) from the ground triplet state oxygen ($^3\Sigma_g^-$) is shown in Scheme II as involving the transfer of excitation energy either from singlet or from triplet, donor or acceptor molecules by the exchange mechanism or the triplet–triplet annihilation. In both instances, spin conservation is observed. The singlet oxygen will then react with the acceptor molecule or other oxidizable substances present in biological systems to form peroxides. This mode of reaction *via* singlet oxygen

1, $S + h\nu \rightarrow S^*$	Absorption of light
2, $S^* \rightarrow S + h\nu_f$	Emission of fluorescence
3, $S^* \rightarrow S$	Nonradiative transition
4, $S^* \rightarrow {}^3S$	Intersystem crossing to the triplet state
5, $^3S \rightarrow S + h\nu_p$	Emission of phosphorescence
6, $^3S \rightarrow S$	Nonradiative transition
7, $S^* + A \rightarrow A^* + S$	Energy transfer by resonance mechanism
8. $^3S + A \rightarrow {}^3A + S$	Energy transfer by the exchange mechanism
9, $A^* \rightarrow A$	Nonradiative transition
10, $A^* \rightarrow {}^3A$	Intersystem crossing to the triplet state
11, $^3A \rightarrow A$	Nonradiative transition
12, $A^* + O_2 \rightarrow {}^3AO_2$	Formation of the moleoxide
13, $^3A + O_2 \rightarrow {}^3AO_2$	Formation of the moleoxide
14, $^3AO_2 \rightarrow A + O_2$	Spontaneous decomposition of the moleoxide
15, $^3AO_2 + A \rightarrow AO_2 + A$	Formation of the stable peroxide

Scheme I

12a, A^* (or S^*) + $O_2(^3\Sigma_g^-) \rightarrow {}^3A$ (or ^3S) + $O_2(^1\Delta_g)$
Formation of singlet oxygen by the exchange mechanism

13a, ^3A (or ^3S) + $O_2(^3\Sigma_g^-) \rightarrow A$ (or S) + $O_2(^1\Delta_g)$
Formation of singlet oxygen by triplet–triplet annihilation

14a, $A + O_2(^1\Delta_g) \rightarrow AO_2$
Formation of the stable peroxide

Scheme II

[1] The term "photodynamic effect" is exclusively used for photosensitivity in the presence of oxygen because both oxygen and light are required for the hypersensitive response.

is in agreement with the evidence from recent photochemical studies (27, 28).

The importance of the triplet state in biological systems has been emphasized (29), especially for its long lifetime in which useful biological work can be done (30). The heavy atom effect which is uniquely linked to the triplet state has been observed in the killing of paramecia by fluorescein dyes as the efficacy decreases from erythrosin to eosin and to fluorescein (31). It is known that the presence of heavy atoms increases the rate of intersystem crossing leading to the population of the triplet state.

All sensitizers for photosensitive biological actions are fluorescent. The fluorescent state is an indication of the lifetime of the excited state of the molecule, as the fluorescence lifetime is usually approximately 10^{-8} sec. which is approximately 10^5 times longer than the lifetime for nonradiative transition. A long-lived state of the sensitizer is necessary to ensure a high probability of energy transfer. For effectiveness in excitation transfer, a maximum overlap of the absorption spectrum of the acceptor and the fluorescence spectrum of sensitizer is required. In riboflavin–cytochrome c system, the overlap integral for long distance resonance energy transfer has been evaluated (32).

The acceptor in photosensitive biological systems will be proteins, nucleic acids, and other normal tissue constituents. These are also fluorescent (33), and some even show phosphorescence. Protein phosphorescence was first observed in 1952 by Debye and Edwards (34). The triplet states of aromatic amino acids and proteins have also been observed and studied with electron spin resonance spectroscopy (35, 36). Together these observations lend credence to the formation of the triplet state by reactions *8* and *10*.

The postulated scheme for photosensitive action may also account for a lack of photosensitivity in many individuals by a quenching mechanism which diverts the excitation energy away from the target acceptor. Some of the strong quenchers such as carbon tetrachloride, pyridine, phenyl isothiocyanate, *etc.*, found in the liquid scintillation system are also toxicants (37). The exceptions are the carbonyl compounds. The presence of carbonyl groups, by their unique alignment in the condensed phase, gives the connective, skeletal, and keratinized tissues their luminescence (38). The strong interaction of the carbonyl group with the sensitizer would ensure a maximum utilization of the absorbed radiant energy. By the same principle, melanin or similar compounds may afford protection to photosensitive action by quenching since compounds with adjacent carbonyl groups such as benzil and biacetyl have been shown to be strong quenchers.

The parallelism in the basic mechanisms of energy transfer between a photosensitive biological system and a liquid scintillation system would allow the latter system to be used as a model for elucidation of the mechanism involved in the former, because the liquid scintillation system is more sensitive in detecting fluorescence quenching than the optical method. As already indicated, measurement in such a system gives only relative values. For absolute values, it needs to be standardized against reference compounds. To resolve color and chemical quenching in the system, a method by Ross (39) for color quenching may be used.

REFERENCES

(1) Peng, C. T., *Atomlight*, (No. 44) 1(1965).
(2) Förster, T., *Discussions Faraday Soc.*, **27**, 7(1959).
(3) Dexter, D. L., *J. Chem. Phys.*, **21**, 836(1953).
(4) Inokuti, M., and Hirayama, F., *ibid.*, **43**, 1978(1965).
(5) Peng, C. T., *Anal. Chem.*, **32**, 1292(1960).
(6) Peng, C. T., *Mol. Crystals*, to be published.
(7) Hayes, F. N., Ott, D. G., and Kerr, V. N., *Nucleonics*, **14** (No. 1), 42(1956).
(8) Peng, C. T., in "Liquid Scintillation Counting," Bell, C. G., Jr., and Hayes, F. N., eds., Pergamon Press, New York, N. Y., 1958, p. 198.
(9) Kerr, V. N., Hayes, F. N., and Ott, D. G., *Intern. J. Appl. Radiation Isotopes*, **1**, 284(1957).
(10) Fano, U., in "Radiation Biology," Hollaender, A., ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1954, vol. I, pp. 123–134.
(11) Bäckström, H. L. J., and Sandros, K., *Acta Chem. Scand.*, **14**, 48(1960).
(12) Stevens, B., *Nature*, **72**, 725(1961).
(13) Dammers-de Klerk, A., *Mol. Phys.*, **1**, 141(1958).
(14) Berlman, I. B., *J. Chem. Phys.*, **34**, 1083(1961).
(15) Stern, O., and Volmer, M., *Physik. Z.*, **20**, 183(1919).
(16) Terenin, A., and Ermolaev, V., *Trans. Faraday Soc.*, **52**, 1042(1956).
(17) Peng, C. T., and Dainton, F. S., unpublished data.
(18) Furst, M., and Kallmann, H., *Phys. Rev.*, **97**, 583 (1955).
(19) Porter, G., and Wilkinson, F., *Trans. Faraday Soc.*, **57**, 1686(1961).
(20) Peng, C. T., *Anal. Chem.*, **36**, 2456(1964).
(21) Blum, H. F., "Photodynamic Action and Diseases Caused by Light," Reinhold Publishing Co., New York, N. Y., 1941.
(22) Sollmann, T., "A Manual of Pharmacology," 8th ed., W. B. Saunders Co., Philadelphia, Pa., 1957, p. 201.
(23) Fitzpatrick, T. B., Pathak, M. A., Magnus, I. A., and Curwen, W. L., *Ann. Rev. Med.*, **14**, 195(1963).
(24) Spikes, J. D., and Glad, B. W., *Photochem. Photobiol.*, **3**, 471(1964).
(25) Livingston, R., in "Symposium on Photochemistry in the Liquid and Solid States," Daniels, F., ed., John Wiley & Sons, Inc., New York, N. Y., 1960, p. 76.
(26) Gollnick, K., and Schenck, G. O., *Pure Appl. Chem.*, **9**, 507(1964).
(27) Foote, C. S., and Wexler, S., *J. Am. Chem. Soc.*, **86**, 3880(1964).
(28) Wilson, T., *J. Am. Chem. Soc.*, **88**, 2898(1966).
(29) Reid, C., "Excited States in Chemistry and Biology," Butterworths, London, England, 1957, chap. 5, pp. 82–110.
(30) Becker, R. S., and Kasha, M., in "The Luminescence of Biological Systems," Johnson, F. H., ed., American Association for the Advancement of Science, Washington, D. C., 1955, p. 25.
(31) Giese, A. C., and Crossman, E. B., *J. Gen. Physiol.*, **29**, 193(1946).
(32) Karreman, G., and Steel, R. H., *Biochim. Biophys. Acta*, **25**, 280(1957).
(33) "Handbook of Chemistry and Physics," 41st ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1959–1960, p. 3002.
(34) Debye, P., and Edwards, J. O., *Science*, **116**, 143 (1952).
(35) Shiga, T., Mason, H. S., and Simo, C., *Biochemistry*, **5**, 1877(1966).
(36) Shiga, T., and Piette, L. H., *Photochem. Photobiol.*, **3**, 223(1964).
(37) Peng, C. T., unpublished data.
(38) Kallmann, H., Krasnansky, V. J., and Person, P., Abstracts. Second International Biophysics Congress, Abstr. No. 601, Vienna, Austria, 1966.
(39) Ross, H. H., *Anal. Chem.*, **37**, 621(1965).

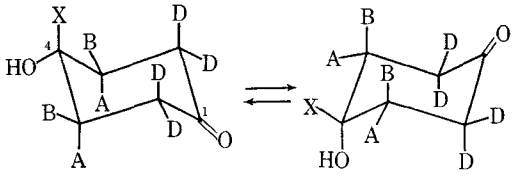
NMR Conformational Analysis of 4-Hydroxycyclohexanone and 1,4-Cyclohexanediols

Coupling Constants Averaging Induced by Conformational Equilibria and by Strong Coupling Effects—Role of Solvent

By WILLIAM F. TRAGER*, BERNARD J. NIST, and ALAIN C. HUITRIC

Significant differences are observed in the NMR spectra of *cis*-1,4-cyclohexanediol and 4-hydroxycyclohexanone measured in different solvents. For the diol the differences are shown to arise from averaging of coupling constants through strong coupling effects. The differences for the ketone result from a combination of the same factors plus differences in conformational equilibria. A discussion of the averaging of coupling constants due to conformational equilibria and due to strong coupling effects is given. The signal band width method for determination of conformational equilibria is shown to be independent of strong coupling effects.

FAILURE to recognize complications resulting from averaging of coupling constants due to strong coupling effects can lead to serious errors in the interpretation of NMR spectra. Early warnings concerning these complications were given by several authors (1–3). In two previous communications concerned with the conformational analysis of *cis*-1,4-cyclohexanediol (4) and 4-hydroxycyclohexanone (5) by NMR, the observation of solvent effects on chemical shifts leading to deceptively simple spectra, as well as solvent effects on conformational equilibria, has been reported. In both cases of solvent effects, the changes produced in the observed spectra are associated with averaging of coupling constants, but of different kinds. The studies were made possible by the use of selectively deuterated compounds. In the present paper the synthesis of the deuterated compounds is reported, and a discussion of averaging of coupling constants due to conformational equilibria and due to strong coupling effects is presented.



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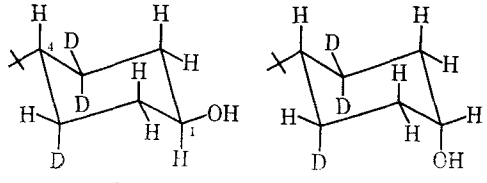
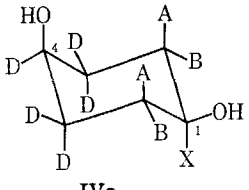
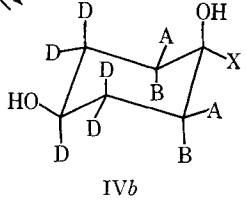


II III



IVa



IVb

DISCUSSION

In a mobile cyclohexane system such as I*a* and I*b* the observed coupling constants J_{XA} and J_{XB} represent the weighted time average values of what they would be in either pure I*a* or pure I*b*. That is, $J_{XA} = NaJ_{aa} + (1 - Na)J_{ee}$ and $J_{XB} = NaJ_{ae} + (1 - Na)J_{ea}$, where Na represents the mole fraction of the compound in conformation I*a*, J_{aa} and J_{ee} are axial–axial and equatorial–equatorial coupling constants, respectively. If the individual values of J_{XA} (J_{aa} in I*a*, and J_{ee} in I*b*) and J_{XB} (J_{ae} in I*a*, and J_{ea} in I*b*) in pure I*a* and I*b* are known, then the conformational position can be calculated from observed coupling constants (6) measured directly from the spectrum, providing that the first-order approximation is valid. This is not always the case because the situation is often complicated by strong coupling effects (1–3).

In an ABX system of the type X—C(A)—C(A)—B, where $J_{XA} \neq J_{XB}$, a first-order spectrum is possible providing the difference between the chemical shifts of A and B ($\nu_B - \nu_A$) is large compared to J_{AB}.

Received September 6, 1966, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication October 21, 1966.
The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.
* U. S. Public Health Service Predoctoral Fellow 5 FI-GM-18,507, 1962–1965.

As ($\nu_B - \nu_A$) approaches J_{AB} proton X is coupled to protons A and B behaving as a set, and individual values of J_{XA} and J_{XB} can no longer be obtained directly from the spectrum. In the limit where $\nu_B = \nu_A$, the signal of X yields a symmetrical triplet with separations equal to $(J_{XA} + J_{XB})/2$ even though J_{XA} and J_{XB} are not actually equal. The same applies to an $(AB)_2X$ system, such as 4-hydroxycyclohexanone-2,2,6,6-d_4 (I), or *cis*-1,4-cyclohexanediol-3,3,4,5,5-d_5 (IV) except that in the limit the signal of the X proton will now give a symmetrical pentuplet with line separations equal to $(J_{XA} + J_{XB})/2$. Thus both conformational equilibria and strong coupling effects tend to average coupling constants, but fortunately in an intrinsically different manner that permits separation of the two effects.

As stated above, the observed coupling constants, J_{XA} and J_{XB}, in a mobile cyclohexane in equilibrium between two chair conformations represent the weighted time average values of what they would be in each of the two alternate chair conformations. Hence, not only the individual magnitudes of J_{XA} and J_{XB} but also the sum of the coupling constants, and, therefore, the width of the signal of the X proton equal to $2(J_{XA} + J_{XB})$ is dependent upon the position of the equilibrium between chair conformations I*a* and I*b* and IV*a* and IV*b* (7–10). Changes in the width of the signal of the X proton reflect a change in the position of the equilibrium. On the other hand strong coupling effects will tend to average the individual values of J_{XA} and J_{XB} and thus cause changes in the pattern of the signal, but in this case the sum of the coupling constants remains constant. The important thing to recognize is that complications from strong coupling effects in systems such as (I*a* $\rightleftarrows$ I*b*) or (IV*a* $\rightleftarrows$ IV*b*) prevent the calculation of individual values of J_{XA} and J_{XB} by first-order approximation, but since the sum $2(J_{XA} + J_{XB})$ is not affected, the width of the signal of the X proton truly reflects the position of the equilibrium.

In the case of *cis*-1,4-cyclohexanediol-3,3,4,5,5-d_5 (IV) equal population of the two chair conformations is expected, and the individual values of observed J_{XA} and J_{XB} should be about 7 c.p.s. and 3.5 c.p.s., respectively (11, 12), providing that ($\nu_B - \nu_A$) is large with respect to J_{AB}. As the ratio $(\nu_B - \nu_A)/J_{AB} \rightarrow 0$, strong coupling effects become operative and J_{XA} and J_{XB} tend to average out. In the limit where $\nu_B = \nu_A$, the signal of the X proton will appear as a symmetrical pentuplet with line separations equal to $(J_{XA} + J_{XB})/2$, about 5 c.p.s., and the combined signals of the A and B protons will give a doublet with the same separation of about 5 c.p.s.

4-Hydroxycyclohexanone—The NMR study was done with 4-hydroxycyclohexanone-2,2,6,6-d_4 (I) in order to simplify the spectra. Spectra of I were obtained in three different solvents: pyridine, D_2O, and $CDCl_3$ (Fig. 1). Pyridine and $CDCl_3$ both yielded deceptively simple spectra while in D_2O the observed spectrum is more closely first order. The results are interpreted as being consistent with a rapid equilibrium between the two chair conformations (I*a* and I*b*) with the differences in the spectra resulting from solvent effects on conformation and/or solvent effects on chemical shifts.

Conformational preference in each solvent was calculated by the signal width method of Garbisch (7) or Feltkemp and Franklin (8–10) using the partially deuterated *trans* and *cis*-4-*tert*-butylcyclohexanol-3(axial),5,5-d_3, II and III, respectively, for conformationally homogeneous models. The advantage[1] of this method over the method employing individual coupling constants (6) is that the observed signal width of the X proton is dependent only upon the conformational equilibrium and not upon strong coupling effects (1–3). Moreover, since vicinal coupling constants in cyclohexane systems are generally much less sensitive than chemical shifts to remote structural changes, the chosen models are suitable for reasonable approximation of the conformational equilibrium by the signal width method but are entirely inadequate for calculation by the chemical shift method (13). That this is so becomes apparent from the fact that the chemical shift of the X proton in 4-hydroxycyclohexanone-2,2,6,6-d_4 (I) both in pyridine and $CDCl_3$ is downfield from the signal of H-1 in III in the corresponding solvent.

The *trans* reference model (II) gave $J_{aa} = 11.0$, $J_{ae} = 4.2$, and $J_{gem} = 12.5$ c.p.s.[2] in $CDCl_3$ and $J_{aa} = 10.7$, $J_{ae} = 4.4$, and $J_{gem} = 12.0$ c.p.s. in pyridine. Accurate values of J_{ea} and J_{ee} could not be obtained from the spectrum of the *cis* isomer (III), but the symmetrical pentuplet of H-1 indicates that $J_{ea} \simeq J_{ee}$ in III, since ($\nu_B - \nu_A$) is outside the boundary of serious strong coupling effects. The value of $2(J_{ea} + J_{ee})$ was 11.0 in $CDCl_3$ and pyridine, indicating that $J_{ea} \simeq J_{ee} \simeq 2.7$ c.p.s. at 37°. The signal width of H-1 in II, equal to $2(J_{aa} + J_{ae})$ was found to be 30.6 c.p.s. in $CDCl_3$ and 30.0 c.p.s. in pyridine.

The mole fraction *Na* of conformer I*a* in the mobile 4-hydroxycyclohexanone system can be calculated from the width of the signal of H-4 in the mobile system, and H-1 in the two conformationally homogeneous models II and III by the equation:

$$Na = \frac{2[J_{XA} + J_{XB}] - 2[J_{ae} + J_{ee}]}{2[J_{aa} + J_{ea}] - 2[J_{ae} + J_{ee}]} = \frac{W - W_a}{W_e - W_a}$$

where J_{XA} and J_{XB} refer to the mobile system; J_{aa}, J_{ae}, J_{ea}, and J_{ee} refer to the models II and III; W is the signal band width of H-4 in the mobile system, W_a and W_e[3] are signal width of H-1 in models III and II, respectively. The accuracy of the results will depend on the reliability of the chosen conformationally homogeneous models. In the present case, the models may not be ideal and the calculated mole fractions of conformer I*a* should be considered good approximations rather than absolute values; but the relative values in the different solvents are very reliable.

By utilizing $W_a = 11.0$ and $W_e = 30.0$ c.p.s. obtained in pyridine[4] and the measured value $W =$

[1] In nonfirst-order spectra it is possible to use the individual coupling constant method if the actual coupling constants can be calculated by complete analysis. Very often this is not possible because of overlapping of signals in the upper field region.

[2] Signs are not assigned, although it is generally understood that geminal coupling constants are of opposite signs to vicinal coupling constants.

[3] The symbols used are those of Feltkamp and Franklin (9). It should be noted that W_a refers to the width of the X proton when the substituent is axial and the X proton equatorial.

[4] The model compounds II and III are not sufficiently soluble for measurement in D_2O. The danger of error due to variations of coupling constants with solvent seems minimal because of the observation that the difference in width of the signal of H-1 in 4-*tert*-butyl-*cis*-4-hydroxycyclohexanol-3,3,5,5-d_4 measured in pyridine and 25% acetic acid in D_2O is only 0.5 c.p.s.

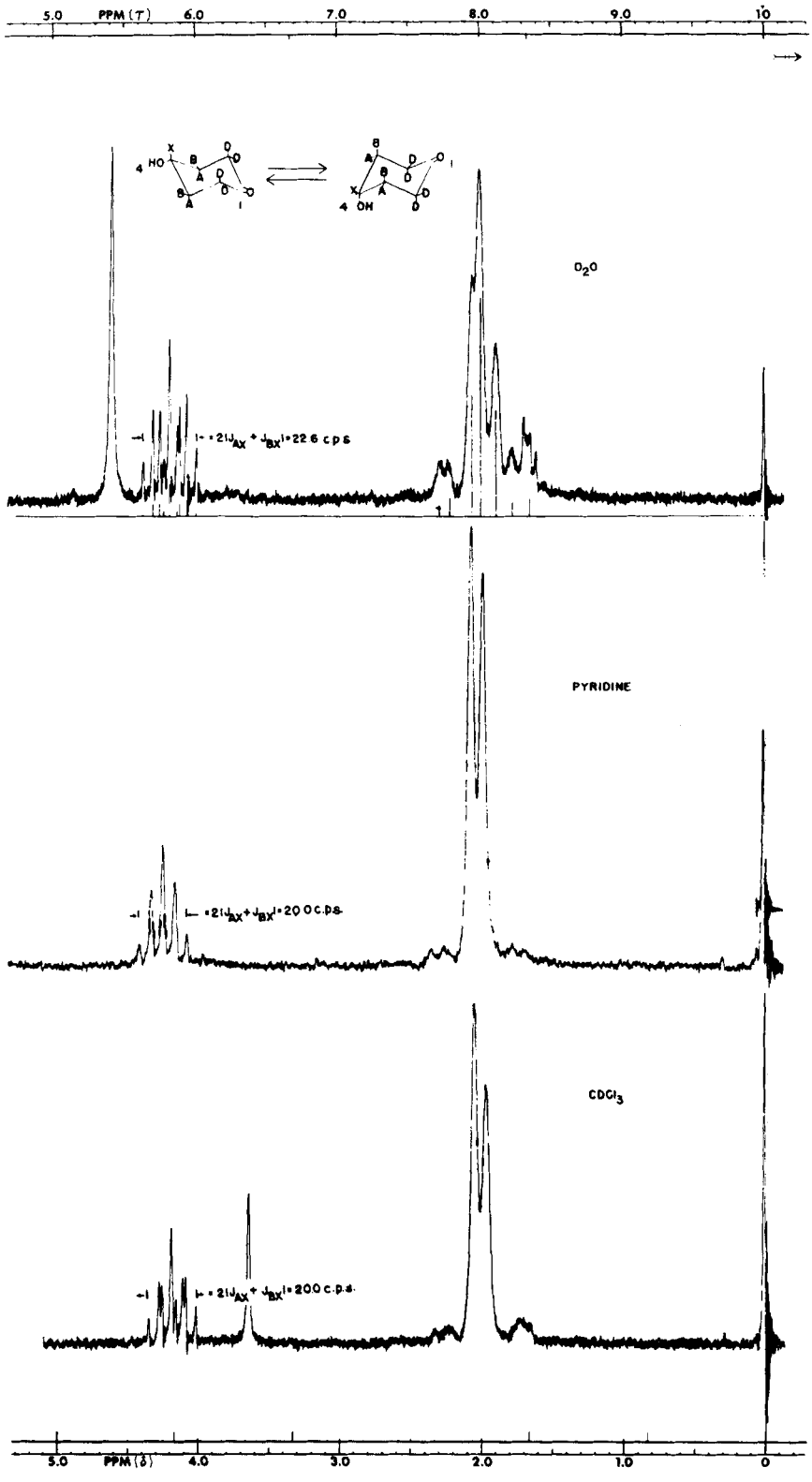


Fig. 1—NMR spectra of 4-hydroxycyclohexanone-2,2,6,6-d_4 in D_2O, pyridine, and deuterated chloroform at 60 Mc. and 37°. TMS and DSS were used as internal references in the organic solvents and D_2O, respectively. The calculated spectrum is shown beneath the observed spectrum in D_2O.

22.6 c.p.s. for the mobile system in D_2O, *Na* is calculated to be 0.61. This corresponds to a 61 ± 4% population of conformer Ia and a conformational free energy difference of 0.27 Kcal./mole for the

hydroxyl group in 4-hydroxycyclohexanone in D_2O The value W was found to be 20.0 c.p.s. in both pyridine and $CDCl_3$. Utilizing the respective W_e and W_a values this gives 47 ± 4% of conformer I*a* in pyridine and 46 ± 4% of conformer I*a* in $CDCl_3$. The apparent 3 to 4% predominance of conformer I*b* is probably not real and probably reflects the fact that models II and III are not exactly ideal. Moreover, a small contribution ($\simeq$ 1%) by nonchair flexible forms which would tend to narrow W is possible based on calculations of Allinger and co-workers (14), who predict that the ground state energy of the most stable flexible form of cyclohexanone and 4-methylcyclohexanone is about 3 Kcal./mole greater than the ground state of the chair conformation. Contribution of a boat form stabilized by intramolecular hydrogen bonding is argued against by the fact that the benzoate ester of 4-hydroxycyclohexanone-2,2,6,6-d_4 gives the typical pentuplet with signal width of 19.2 c.p.s. for the X proton and a doublet with separation of about 4.8 c.p.s. for the combined signals of the A and B protons in pyridine and in chloroform. There is no possibility of hydrogen bonding in the esters. Furthermore, Stolow (16) was unable to detect any evidence of intramolecular hydrogen bonding in 4-hydroxycyclohexanone by infrared studies in carbon tetrachloride.

The larger population of conformer I*a* in D_2O is substantiated by the following treatment. The spectrum in D_2O gives ν_X = 251; ν_B = 124.8; ν_A = 112.2, and J_{AB} = 13.5 c.p.s. The apparent coupling constants of J_{XA} = 7.0 and J_{XB} = 4.3 are erroneous because ($\nu_B - \nu_A$) = 12.6 is within the range of strong coupling complications. By utilizing the coupling constants obtained from II and III in pyridine the expected coupling constants of a rapid equilibrium between I*a* and I*b* with 61% of conformer 1*a* are calculated as:

$$J_{XA} = NaJ_{aa} + (1 - Na)J_{ee} = (0.61)(10.7) + (0.39)(2.7) = 7.6 \text{ c.p.s.}$$

$$J_{XB} = NaJ_{ae} + (1 - Na)J_{ea} = (0.61)(4.4) + (0.39)(2.7) = 3.76 \text{ c.p.s.}$$

When the observed chemical shift of ν_X = 251, ν_B = 124.8, ν_A = 112.2 c.p.s., observed J_{AB} = 13.5 c.p.s.[5] and calculated J_{XA} = 7.6 and J_{XB} = 3.76 c.p.s. were placed in the computer program of Wiberg and Nist (15), the resulting theoretical spectrum [reproduced under the experimental spectrum (Fig. 1)] gave apparent J_{XA} = 7.05 and J_{XB} = 4.3 and reproduced the experimental spectrum exactly.

The results thus indicate that the $-\Delta G_{OH}°$ value of the hydroxyl group in 4-hydroxylcyclohexanone is essentially zero in pyridine and $CDCl_3$, whereas it is 0.27 Kcal./mole in D_2O. The observed solvent effects on conformation are in agreement with the findings of Eliel (17), who postulated that the conformational equilibrium of the hydroxyl group in a cyclohexane system is a function of solvent where the $-\Delta G_{OH}°$ value is greater for proton donating solvents such as water or alcohols. A solvent effect on chemical shifts of the signals of the A and B protons of 4-hydroxycyclohexanone whereby ($\nu_B - \nu_A$) is smaller in pyridine than in $CDCl_3$ is indicated by the difference in the multiplicity but identical width of the signal of the X proton in the two solvents.

cis-1,4-Cyclohexanediol—The NMR spectrum of this compound obtained in pyridine was found to be very different from that obtained in D_2O (4). In order to simplify the spectra the deuterated *cis*-1,4-cyclohexanediol-3,3,4,5,5-d_5 (IV) was prepared and used for this study. In contrast to 4-hydroxycyclohexanone-2,2,6,6-d_4, it is pyridine which gives the approximately first-order spectrum, while D_2O gives the deceptively simple spectrum for IV (Fig. 2). The results are interpreted as being consistent with a rapid equilibrium between the two energetically equivalent chair conformations IV*a* and IV*b*, with differences in spectra arising from solvent effects on chemical shifts. The width of the signal of the X proton of IV in both pyridine and D_2O is 20.2 c.p.s., even though it appears as a seven-peak multiplet in the former solvent and symmetrical pentuplet in the latter. Calculation of the mole fraction of conformer IV*a* in the mobile *cis*-1,4-cyclohexanediol system utilizing the partially deuterated *trans* and *cis*-4-*tert*-butylcyclohexanol-3 (axial), 5,5-d_2, II and III, respectively, for conformationally homogeneous models and the signal width method of Garbisch (7) or Feltkamp and Franklin (8–10), leads to a 48 ± 4% population of conformer IV*a*. This result is in excellent agreement with expected 50% population of conformer IV*a* and attests to the reliability of the method.

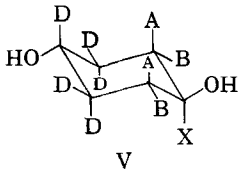
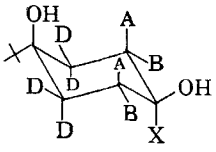
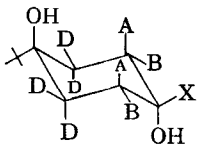
The measured values of J_{XA} and J_{XB} for IV in pyridine are 6.7 and 3.4 c.p.s., respectively. Utilizing the model compounds II and III, once again: $J_{XA} = (J_{aa} + J_{ee})/2 = (10.7 + 2.7)/2 = 6.7$ and $J_{XB} = (J_{ea} + J_{ee})/2 = (2.7 + 4.4)/2 = 3.6$ c.p.s., a result in good agreement with the observed couplings and one that should prevail in a system approaching first order. The line separation of the pentuplet in D_2O is 5.0 c.p.s., and this is in excellent agreement with the expected apparent coupling constant $(J_{XA} + J_{BX})/2 = (6.7 + 3.6)/2 = 5.1$ c.p.s. for a system under the influence of strong coupling in the limit where $\nu_A = \nu_B$. Thus, *cis*-1,4-cyclohexanediol-3,3,4,5,5-d_5 provides excellent experimental examples of the phenomenon of averaging of coupling constants due to conformational equilibria and averaging of coupling constants due to strong coupling effects. It also provides an excellent example of complications from strong coupling effects due to solvent effects on chemical shifts.

In an attempt to clarify the solvent effects on chemical shift and provide another method for checking the above results, the following selectively deuterated compounds were prepared: *trans*-1,4-cyclohexanediol-3,3,4,5,5-d_5 (V); 4-*tert*-butyl-*cis*-1,4-cyclohexanediol-3,3,5,5-d_4 (VI); and 4-*tert*-butyl-*trans*-1,4-cyclohexanediol-3,3,5,5-d_4 (VII) (Table I).

It should be possible, from the spectra of structures V, VI, and VII in a given solvent, to approximate the theoretical chemical shifts of the protons A and B in *cis*-1,4-cyclohexanediol-3,3,4,5,5-d_5 (IV) undergoing interconversion between conformations IV*a* and IV*b* in the same solvent. Compounds VI and VII were not sufficiently soluble in deuterium oxide, but it was found that the spectra of IV were identical when obtained from D_2O or from a mixture of 25% acetic acid in D_2O as solvent, a solvent mixture in which VI and VII were sufficiently soluble.

[5] The calculated spectrum is unaltered when J_{AB} is replaced by $-J_{AB}$.

TABLE I—CHEMICAL SHIFTS OF DEUTERATED COMPOUNDS PREPARED[a]

	Pyridine			D_2O or (D_2O–HAc)			Δ(Pyridine vs. D_2O)		
	ν_A	ν_B	ν_X	ν_A	ν_B	ν_X	a	o	x
 V	91.6	125.4	228.4	74.3	110.3	214.5	17.3	15.1	13.9
 VI	127.0	121.0	226.0	92.2	102.1	210.6	34.8	18.9	15.4
 VII	131.6	112.4	257.0	107.0	93.6	239.5	24.6	18.8	17.5

[a] Chemical shifts (from ABX analysis), ν c.p.s. from TMS internal reference at 60 Mc.

It was therefore assumed that the chemical shifts of protons A and B in VI and VII obtained in 25% acetic acid in deuterium oxide could be taken as representative of values that would prevail in pure D_2O. Additional supporting evidence for the validity of this assumption was obtained from the fact that the spectra of *trans*-diol V when obtained in pure D_2O or in 25% acetic acid in D_2O are essentially identical.

In order to approximate the average chemical shift of protons A and B in IV undergoing interconversion between conformations IV*a* and IV*b* in a given solvent it is necessary to have good approximations of the chemical shifts of A and B in each of the conformations IV*a* and IV*b* in that given solvent. Such approximations are possible from the spectra of V, VI, and VII if the assumption is made that the equatorial hydroxyl group on C-4 of V and the equatorial *tert*-butyl group on C-4 of structures VI and VII do not appreciably affect the chemical shifts of protons A and B in structures V, VI, and VII. If this assumption is correct, then the chemical shifts of A and B in conformation IV*a* are the same as those of A and B, respectively, in structure VI. In conformation IV*b*, A and B are best related to A and B, respectively, of structure V except that the hydroxyl group at C-1 of IV is axial in conformer IV*b* but equatorial on C-1 of V. A correction can be made for this from the relative chemical shifts of A in structure VI *versus* VII and likewise for B. From this information the calculated chemical shift for A in IV in D_2O–acetic acid mixture is 92.2/2 + (110.3 − 8.5)/2 = 97 c.p.s. and that of B is 102.1/2 + (74.3 + 14.8)/2 = 95.6 c.p.s. The observed doublet resulting from the combined signals of A and B is centered at 96.5 c.p.s.

The theoretical spectrum was obtained by means of the NMR computer program of Wiberg and Nist (15), using the calculated values of ν_B = 97 c.p.s., ν_A = 95.6 c.p.s., observed value ν_X = 225 c.p.s., and coupling constants of J_{AB} = 12.4,[6] J_{XA} = 6.7, and J_{XB} = 3.4 c.p.s. The theoretical spectrum so obtained is shown in Fig. 2. The agreement with the observed spectrum is excellent.

Similar treatment gave excellent agreement between the calculated and observed spectrum of IV in pyridine. In this case compound V was replaced by compound II as reference model.[7] The chemical shifts of the A and B protons of II in pyridine are 84 and 129 c.p s., respectively. The calculated chemical shifts of the A and B protons in the mobile system IV of ν_A = 124, ν_b = 105 c.p.s. are in excellent agreement with the experimental values of ν_A = 124.2 and ν_b = 104 c.p.s. The larger difference in the chemical shifts of the A and B protons of IV in pyridine compared to D_2O is in agreement with the observation (18) that the 1,3-diaxial deshielding effect of the hydroxyl group is larger in pyridine than in other solvents investigated, including the protic solvents methanol-d_4 and acetic acid.

EXPERIMENTAL

The NMR spectra were determined on a Varian A-60 spectrometer at 37° utilizing TMS as the internal standard for spectra determined in pyridine and $CDCl_3$ and DSS for spectra determined in

[6] The calculated spectrum is unaltered if J_{AB} is replaced by $-J_{AB}$.

[7] Compound V in pyridine exists as a mixture of the two chair conformers with about 15% of the diaxial form (unpublished results). By using Anet's (19) $-\Delta G_{OH}°$ value of 1.25 for the hydroxyl group in cyclohexanol in D_2O compound V is calculated to exist to the extent of 98% in the diequatorial conformation in D_2O.

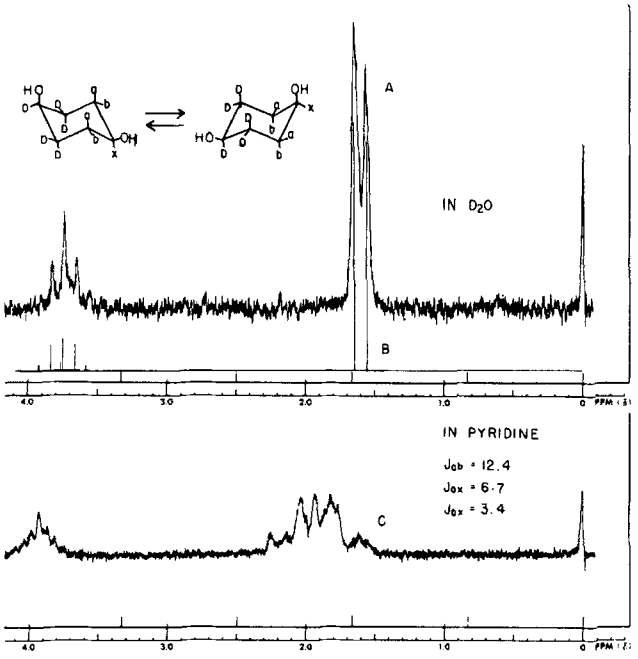


Fig. 2—NMR spectra of cis-*1,4-cyclohexanediol-3,3,4,5,5,-*d_5 *in* D_2O *and pyridine at 60 Mc. and 37°. TMS and DSS were used as internal references in pyridine and* D_2O*, respectively.* B *is the calculated spectrum using calculated chemical shifts as explained in the text.*

D_2O. The concentration of the sample solutions varied between 20 and 25% w/v. Melting points were determined on a Kofler hot-stage.

4-Hydroxycyclohexanone—This compound was prepared in a 27% over-all yield as follows. Commercially available 1,4-cyclohexanediol was converted to 4-hydroxycyclohexyl benzoate (42%) by the method of Jones and Sondheimer (20). The 4-hydroxycyclohexyl benzoate thus obtained was oxidized to 4-ketocyclohexyl benzoate (90%) by the general method of Brown and Garg (21), and finally 4-ketocyclohexyl benzoate was converted to 4-hydroxycyclohexanone (72%) by the method of Jones and Sondheimer (20).

4-Hydroxycyclohexanone-2,2,6,6-d_4 (I)—A solution of 4-hydroxycyclohexanone (25 Gm., 0.22 mole) in 99.8% D_2O (35 Gm., 1.8 moles) containing anhydrous potassium carbonate (0.30 Gm., 0.0022 mole) was allowed to stand at room temperature for 24 hr. The D_2O–water solution was removed from the partially deuterated 4-hydroxycyclohexanone by vacuum distillation. The receiver flask was cooled in a mixture of dry ice–isopropyl alcohol while the distilling flask was warmed in an oil bath (never above 50°). When all of the D_2O–water solution had been removed, the process was repeated twice and the product distilled under reduced pressure, b.p. 70° at 0.2 mm.

cis and trans-1,4-Cyclohexanediol-3,3,4,5,5-d_5 (IV and V)—A mixture of IV and V was obtained by lithium aluminum deuteride reduction of I in ether. The mixture thus obtained was separated by the method described by Brown and Zweifel (22) for a mixture of nondeuterated IV and V.

4-tert-Butyl-cis- and trans-1,4-cyclohexanediol—Anhydrous ether (500 ml.) was added to a 1-L. three-neck flask fitted with a mechanical stirrer, a 500-ml. additional funnel having a nitrogen inlet, a rubber stopper holding a low temperature thermometer, and a nitrogen outlet. A source of dry nitrogen was then fixed to the nitrogen inlet while the nitrogen outlet was fixed to a gas wash bottle containing mineral oil. The system was thoroughly flushed with dry nitrogen, and a slow stream of nitrogen passed through the system for the entire experiment. A solution (100 ml., 1.86 *M*) of *tert*-butyllithium (0.186 mole) in pentane was pipetted into the addition funnel. The pipet had been dried and helium gas blown through it just prior to use. The reaction flask was slowly immersed in a large Dewar flask containing a mixture of dry ice–isopropyl alcohol and the ether cooled to −72°. Vigorous stirring was initiated and the *tert*-butyllithium was added to the ether at such a rate as to maintain the temperature near −70°. The addition took approximately 30 min. A solution of 4-hydroxycyclohexanone (6.0 Gm., 0.0537 mole) in anhydrous ether (300 ml.) was transferred to the addition funnel and then added dropwise to the *tert*-butyllithium–ether–pentane solution, with vigorous stirring, over a period of 2 hr. The temperature was maintained at −72° throughout. Upon completion of the addition the reaction mixture was allowed to stir vigorously at −72° for another 24 hr., and then allowed to slowly warm to room temperature over a period of 4–5 hr. The reaction mixture was treated with 25 ml. of methanol followed by 75 ml. of water. The ethereal layer was separated and the aqueous layer extracted 3 times with ether. The ethereal extracts were combined, dried over anhydrous calcium sulfate, and the ether evaporated, yielding a mixture of 4-*tert*-butyl-*cis*- and *trans*-1,4-cyclohexanediol (5.4 Gm., 60%) and some unreacted 4-hydroxycyclohexanone. Analysis of the crude product by gas chromatography at 200° on a 5 ft. column of 20% basic Carbowax on Chromosorb indicated a 7.3 to 1.3 to 1.4 ratio of 4-*tert*-butyl-*cis*-1,4-cyclohexanediol to 4-*tert*-butyl-*trans*-1,4-cyclohexanediol to unreacted 4-hydroxycyclohexanone. Recrystallization from acetone yielded pure 4-*tert*-butyl-

cis-1,4-cyclohexanediol as large cubical crystals, m.p. 165.5–166°. After recovering most of the *cis* isomer from acetone the pure 4-*tert*-butyl-*trans*-1,4-cyclohexanediol was obtained by crystallization from *n*-heptane–isopropyl alcohol mixture, and finally from pure *n*-heptane as fine needles, m.p. 141.5–142°.

Anal.—Calcd. for $C_{10}H_{20}O_2$: C, 69.70; H, 11.70. Found: (*cis*) C, 70.21; H, 11.76; (*trans*) C, 70.08; H, 11.57.

4-tert-Butyl-cis- and trans-1,4-cyclohexanediol-3,3,5,5-d_4 (VI and VII)—Pure *cis* VI and *trans* VII were obtained by the addition of *tert*-butyllithium to I as described in the preceding section for the corresponding nondeuterated compounds.

1-tert-Butylcyclohexen-4-ol-2,6,6-d_3—A crystal of iodine and a boiling chip were added to a mixture of *cis* VI and *trans* VII (10.0 Gm., 0.0568 mole) contained in a 50-ml. flask fitted with a Dean-Stark water collector. The mixture was heated in an oil bath to 160° and the temperature maintained between 155 and 160° for approximately 15 min. At the end of this period the residual product was transferred into a large sublimator prior to solidification. The material was sublimated and the resulting crystalline product recrystallized from a mixture of methanol and water yielding 7.4 Gm. (80%) of product, m.p. 80–82°. Stolow and co-workers (23) report a m.p. of 86–86.5° for the corresponding nondeuterated compound.

trans and cis-4-tert-Butylcyclohexanol-3(axial),-5,5-d_3 (II and III)—The compounds were obtained by low pressure catalytic hydrogenation of 1-*tert*-butyl cyclohexen-4-ol-2,6,6-d_3 in purified acetic acid (24) with platinum oxide as catalyst according to the procedure described by Sauvage *et al.* (25) for a series of cyclohexenes. The calculated amount of hydrogen was picked up in 1.5 hr., when 8.0 Gm. of alkene was hydrogenated in 15 ml. of acetic acid with 0.8 Gm. of catalyst. After removal of the catalyst, water was added to the acetic acid solution to induce crystallization. Cooling and filtration yielded 7.2 Gm. (80%) of a mixture of II and III in ratio of 3 to 7 as determined by gas chromatography using a basic Carbowax column.

The *cis* and *trans* isomers were separated by column chromatography on neutral alumina using petroleum ether, mixtures of petroleum ether and benzene, and mixtures of benzene and diethyl ether. In a typical experiment 1.5 Gm. of the mixture was chromatographed on 30 Gm. of alumina to yield 1.1 Gm. of pure III, m.p. 80–82°, and 0.3 Gm. of pure II, m.p. 78–79°. This *cis* isomer (III) was eluted with the petroleum ether–benzene mixtures up to 100% benzene, and the *trans* isomer (II) was obtained with benzene and mixtures of benzene and diethyl ether up to 30% of ether. Winstein and Holness (26) have reported a m.p. of 78–79° for nondeuterated II and 81–82° for nondeuterated III.

REFERENCES

(1) Abraham, R. J., and Bernstein, H. J., *Can. J. Chem.*, **39**,216(1961).
(2) Musher, J. I., and Corey, E. J., *Tetrahedron*, **18**, 791 (1962).
(3) Schaefer, T., *Can. J. Chem.*, **40**, 1679(1962).
(4) Trager, W. F., Nist, B. J., and Huitric, A. C., *Tetrahedron Letters*, **1965**, 267.
(5) *Ibid.*, **1965**, 2931.
(6) Lemieux, R. U., and Lown, J. W., *Can. J. Chem.*, **42**, 893(1964).
(7) Garbisch, E. W., Jr., *J. Am. Chem. Soc.*, **86**, 1780 (1964).
(8) Feltkamp, H., and Franklin, N. C., *Ann. Chem.*, **683**, 55(1965).
(9) Feltkamp, H., and Franklin, N. C., *J. Am. Chem. Soc.*, **87**, 1616(1965).
(10) Feltkamp, H., and Franklin, N. C., *Angew. Chem. Intern. Ed.*, **4**, 774(1965).
(11) Huitric, A. C., Carr, J. B., Trager, W. F., and Nist, B. J., *Tetrahedron*, **19**, 2145(1963).
(12) Booth, H., *ibid.*, **20**, 2211(1964).
(13) Eliel, E. L., *Chem. Ind.*, 568(1959).
(14) Allinger, N. L., Allinger, J., and DaRooge, M. A., *J. Am. Chem. Soc.*, **86**, 4061(1964); Allinger, N. L., Blatter, H. M., Freiberg, L. A., and Karkowski, F. M., *ibid.*, **88**, 2999 (1966).
(15) Wiberg, K. B., and Nist, B. J., "The Interpretation of NMR Spectra," W. A. Benjamin, Inc., New York, N. Y., 1962.
(16) Stolow, R. D., *J. Am. Chem. Soc.*, **84**, 686(1962).
(17) Eliel, E. L., and Schroeter, S. H., *ibid.*, **87**, 5031 (1965).
(18) Huitric, A. C., Carr, J. B., and Trager, W. F., *J. Pharm. Sci.*, **55**, 211(1966).
(19) Anet, F. A. L., *J. Am. Chem. Soc.*, **84**, 1053(1962).
(20) Jones, E. R. H., and Sondheimer, F., *J. Chem. Soc.*, **1949**, 615.
(21) Brown, H. C., and Garg, C. P., *J. Am. Chem. Soc.*, **83**, 2952(1961).
(22) Brown, H. C., and Zweifel, G., *J. Org. Chem.*, **27**, 4708(1962).
(23) Stolow, R. D., Bonaventura, M. M., and Larsen, J. W., *ibid.*, **28**, 2862(1963).
(24) Eichelberger, W. C., and Lamer, B. K., *J. Am. Chem. Soc.*, **55**, 3633(1933).
(25) Sauvage, J. F., Baker, R. H., and Hudsey, A. S., *ibid.*, **82**, 6090(1960).
(26) Winstein, S., and Holness, N. J., *ibid.*, **77**, 562(1955).

Modified Cardenolides II

Synthesis, NMR Spectra, and Biological Activity of C-19 Halides and Sulfonates

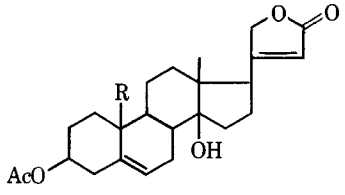
By MANFRED E. WOLFF and WINSTON HO

The synthesis of 19-halocardenolides by halide displacement of the corresponding 19-*p*-toluenesulfonate esters is described. The NMR spectra of the 19-substituted compounds indicate that the rotation of the 19- group is restricted and that one of the C-19 protons is shielded by the 5,6-double bond.

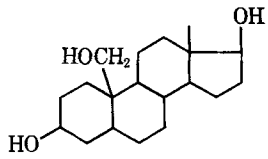
RECENT STUDIES (1) from this laboratory have allowed the formulation of a picture of steroid receptor interaction involving close spatial interaction of the 19-angular methyl group with a receptor surface. This concept was based on the effect on pharmacological activity of steroids with bulky C-19 substituents. It seemed useful to extend this sort of structure–function analysis to the cardiac aglycones.

It is known that the nature of the C-19 substituent in the aglycones has a profound effect on pharmacological activity (2). Thus, C-19 alcohols are more active than the corresponding aldehydes which are more active than the methyl compounds. These results have been obtained largely from naturally occurring glycosides; comparatively little has been done with respect to synthetic modification of the cardiac aglycones. Lingner *et al.* (3) prepared various derivatives of C-19 aldehydes and alcohols, including oximes, acetals, urethans, ethers, and esters. The nature of the substituent had a major effect on the oral activity of these compounds.

This paper describes the preparation, conformation, and structure–function relationships of C-19 halocardenolides.



I, R = CHO
II, R = CH_2OH
III, R = $CH_2OSO_2{\cdot}C_6H_4CH_3$
IV, R = $CH_2OSO_2CH_3$
V, R = CH_2Cl
VI, R = CH_2Br
VII, R = CH_2I



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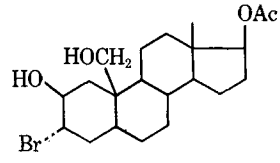
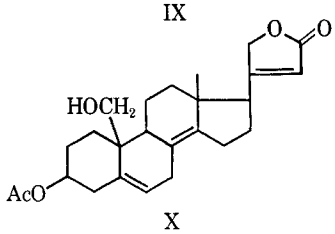


IX



X

DISCUSSION

Chemistry—Work by others (4–8) has resulted in the production of C-19 halo-Δ^5-steroids from the corresponding 19-tosylate or mesylate esters. It has been established that homoallylic carbonium ion intermediates are involved, since S_N2 displacements on saturated 19-tosylates fail. Nucleophilic displacement of 19-tosylates by chloride or bromide gives 19-halides, but other nucleophiles, such as hydroxide, alkoxide, or azide give 5,19-cyclo-6-substituted steroids. It has been concluded that the latter are the kinetically favored products, but the 6-halides are unstable and rearrange further to afford the 19-halides.

The starting material for the present work was monoanhydrostrophanthidin acetate (I), formed from strophanthidin by elimination of the elements of water (9). This material was originally formulated as a 14-ene (10) but Fieser and Goto (11) assigned the corrected Δ^5 structure (I) on the basis of rotations and a reinterpretation of the nature of products of basic isomerization. The reactions of II described in this study, as well as the NMR spectrum of products derived from I, fully support the reassigned structure.

Received September 2, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122

Accepted for publication October 25, 1966.

This research was supported in part by grant HE-09578 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

Previous paper: Wolff, M. E., Ho, W., and Katzung, B., *Chem. Ind. (London)*, **1965**, 1976.

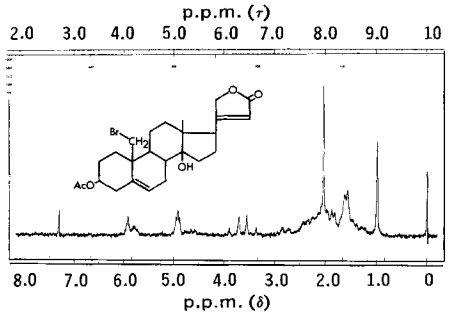


Fig. 1—NMR spectrum of 19-bromo-3β,14β-dihydroxycarda-5,20(22)-dienolide 3-acetate (VI).

Reduction of I with sodium borohydride in methanol solution gave the 19-alcohol (II). The tosylate (III) and the mesylate (IV) were prepared by treatment of II with the appropriate sulfonyl chloride in pyridine solution at 27°. The 19-halocardenolides (V, VI, and VII) were formed readily by the reaction of III and the appropriate lithium halide salt in boiling isopropanol solution.

NMR Spectra—An examination of the NMR spectra (Fig. 1) (Table I) of these C-19 modified steroids has revealed features which shed light on the conformation of the C-19 substituent under the conditions of measurement. Spectral features which did not vary include the 3β-acetate methyl resonance at 121–122 c.p.s., and the C-22 proton, seen centered at 353–354 c.p.s. as a singlet broadened by coupling to protons at C-21. The C-21 protons appear centered at 293–295 c.p.s. in a broadened triplet-like pattern owing to coupling to each other and to allylic coupling to the proton at C-22. The center of the "triplet" is actually the two central peaks of the AB system at C-21 and in a few spectra the satellite peaks can be discerned ($J_{AB} \cong 18$ c.p.s.). The side peaks in the "triplet" are actually due to allylic splitting (12) of the central peaks and $J_{21,22}$ is about 2 c.p.s., in agreement with an angle of about 90° between the plane of the double bond and each of the protons at C-21.

In the 19-halocardenolides (V, VI, and VII) as well as in the 19-hydroxy cardenolide (II), the two protons at C-19 produce an AB quartet having J_{AB} 11–13 c.p.s. which is typical of geminal coupling. Such a quartet could originate from restricted rotation of the 19-angular methyl group and also by selective shielding of one of the C-19 protons by the double bond. That restricted rotation alone does not account for the quartet is seen in the spectrum of the saturated compounds VIII (13) and IX (14), in which the C-19 protons produce broad singlets. The chemical shift of the C-19 protons in the simple saturated system (VIII) (235 c.p.s.), however, lies close to the computed position of one of the protons in II (231 c.p.s.). Moreover, chloride and hydroxyl exert deshielding effects of similar magnitude and it is noteworthy that in V also the low field peak is at 231 c.p.s. Thus, one of the peak positions is in the expected location for a proton of this type. The high field proton is at 215 and 214 c.p.s. in II and V, respectively. Molecular models of compounds II, V, VI, and VII show that the 19-substituent is most readily accommodated between the 2β- and 11β-hydrogens (Fig. 2), and thus the C-19 group is effectively locked into one position. Viewing the C-19 group from the top, this conformation places the lefthand hydrogen in a shielding environment due to the 5,6-double bond. The righthand hydrogen is more distant and appears to be in an area where neither shielding nor deshielding occurs. The spectrum of the doubly unsaturated compound X (15) was wholly in accord with this explanation. Models of X indicate that *both* C-19 hydrogens are located in a similar shielded environment and the spectrum indeed shows a single peak at 219 c.p.s. Thus, the difference in C-19 proton resonance positions between VIII and X is 16 c.p.s., the same value as found for $\delta_A - \delta_B$.

The tosylate (III) exhibits anomalous resonance from protons at C-18, C-6, and C-19. In all cases, the peaks occur at higher field than would be expected. That this shielding effect is due to the phenyl ring rather than the sulfonate group is shown by the spectrum of the mesylate (IV), in which no such anomalies occur.

Since the shielding due to the phenyl ring is seen in three widely separated points (C-6, C-18, and C-19), only the conformation shown in Fig. 3, in which the phenyl ring overlies the steroid, can account for the effect. From this it is also clear that it is the lefthand proton at C-19, rather than the righthand proton, which is shielded by the double bond at C-5, since in III, in which the righthand proton is clearly shielded, $\delta_A - \delta_B$ becomes *smaller*, rather than larger.

Biological Activity—The biological activity of the synthetic cardenolides was determined essentially by the method of Chen (16). Briefly, cats were anesthesized by intraperitoneal administration of 2 ml./Kg. of a freshly prepared aqueous solution containing in each ml. 30 mg. of α-chloralose and 250 mg. of urethan. The femoral vein was injected at 3-min. intervals with 0.5–1.0 ml. of 47.5% alcohol containing 0.2 mg. of drug. These doses

Table I—NMR Spectra of C-19 Substituted Steroids

Compd.	C-6-H($W_{1/2}$)	C-18-H	C-19-H δ_A	C-19-H δ_B	J_{AB}	$\delta_A - \delta_B$	C-21-H	C-22-H	C-3 Acetate
II	348 (10)	57	215	231	12	16	293 (m)	353	121
III	341 (10)	48	240	248	11	8	293 (m)	353	121
IV	348 (10)	57	251	267	11	16	293 (m)	354	121
V	347 (10)	58	214	231	13	17	294 (m)	354	121
VI	347 (10)	59	209	225	12	16	295 (m)	354	121
VII	344 (10)	60	196	215	12	19	294 (m)	354	122
VIII	...	47	235	($W_{1/2}$5)	..	..	...	...	...
IX	...	49	228	($W_{1/2}$3)	..	..	...	...	...
X	...	53	219	($W_{1/2}$4)	..	..	...	...	...

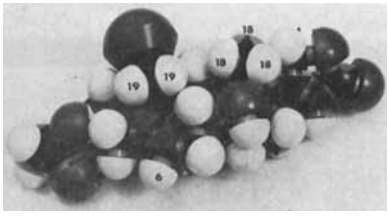


Fig. 2—Courtauld model of 19-iodo-3β,14β-dihydroxycarda-5,20(22)-dienolide 3-acetate (VII).

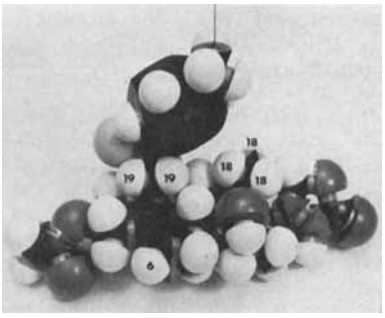


Fig. 3—Courtauld model of 3β,14β,19-trihydroxycarda-5,20(22)-dienolide 3-acetate 19-p-toluene sulfonate (III).

were designed to produce an end point at 30–60 min. Kg.[1] The effect of the drug was monitored by EKG readings.

Because of the lack of material, all compounds were assayed using only one animal. The lethal doses (mg./Kg.) are: III (1.6), V (0.4), and VI (1.5), but obviously no statistical analysis is possible. Arrhythmias typical of cardiotonic steroids were observed in the EKG in all cases. Although no quantitative evaluation can be made, it can nevertheless be concluded that these analogs are not inactive. Since both these compounds, with bulky β-face substituents, and A/B *cis* steroids, in which ring A is bent back, are active, it seems likely that the A ring is not in contact with the cellular receptor surface. This is supported by the activity of many other ring A and ring B modified cardenolide analogs which have been synthesized and tested in this laboratory (17), and is of particular interest in connection with the elegant work of Kupchan *et al.* (18). These workers found that strophanthidin 3-iodoacetate (SIA) irreversibly inhibits transport ATPase and concluded this was due to alkylation of the enzyme at the site where cardiotonic steroids bind. The present study, however, indicates that if transport ATPase is in fact the true receptor, the inhibition is due to exoalkylation (19) at a point removed from the active site. This is in harmony with the concept (20) of the steroid as a *haftgruppe* for the butenolide ring.

EXPERIMENTAL[2]

3β,14β,19-Trihydroxycarda-5,20(22)-dienolide 3-Acetate (II)—To a stirred suspension of 0.6 Gm. of I (9) in 50 ml. of tetrahydrofuran and 30 ml. of methanol, there was added 0.15 Gm. of sodium borohydride in 2 ml. of water. When the reaction was completed (1–2 hr., clear solution), 10 ml. of water was added and the solvent was evaporated under reduced pressure. More water was added, and the precipitated product was filtered, washed with water, and dried. There was obtained 0.55 Gm. of crude product, m.p. 245–252°. Recrystallization from acetonitrile twice gave 0.45 Gm. of crystals, m.p. 260–263°. Further recrystallization from the same solvent gave the analytical sample, m.p. 263–265°; $[\alpha]_D^{19}$ + 1° (c 1, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.34; H, 8.15.

3β,14β-19-Trihydroxycarda-5,20(22)-dienolide 3-Acetate 19-p-Toluenesulfonate (III)—A solution of 0.2 Gm. (0.00046 mole) of II and 0.2 Gm. of *p*-toluenesulfonyl chloride in 10 ml. of pyridine was kept at 27° for 48 hr. It was diluted with ice water and the resulting precipitate was collected and recrystallized from a mixture of methanol and a little acetonitrile to give 0.1 Gm. of colorless crystals, m.p. 155–157°. Further recrystallizations gave the analytical sample, m.p. 156–157°; $[\alpha]_D^{19}$ −41° (c 1, $CHCl_3$).

Anal.—Calcd. for $C_{32}H_{40}O_8S$: C, 65.77; H, 6.84. Found: C, 65.83; H, 6.52.

3β,14β-19-Trihydroxycarda-5,20(22)-dienolide 3-Acetate 19-Methanesulfonate (IV)—A solution of 0.4 Gm. (0.00092 mole) of II and 0.4 Gm. of methanesulfonyl chloride in 35 ml. of pyridine was kept at 27° for 48 hr. and worked up as described for III. Recrystallization from methanol gave the analytical sample, m.p. 145–146°; $[\alpha]_D^{20}$ −27° (c 1, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{36}O_8S$: C, 61.41; H, 7.14. Found: C, 61.36; H, 7.02.

19-Chloro-3β,14β-dihydroxycarda-5,20(22)-dienolide 3-Acetate (V)—A solution of 0.1 Gm. (0.00017 mole) of III and 0.1 Gm. (0.0023 mole) of lithium chloride in 12 ml. of isopropyl alcohol was heated under reflux for 3 hr. The solvent was evaporated under reduced pressure. The mixture was diluted with water and the resulting precipitate was collected and recrystallized three times from acetonitrile–methanol to give 0.05 Gm. of colorless crystals, m.p. 230–233°. Further recrystallizations gave the analytical sample, m.p. 242–244°; $[\alpha]_D^{19}$ −24° (c 0.6, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{33}ClO_5$: C, 66.87; H, 7.40. Found: C, 66.97; H, 7.36.

19-Bromo-3β,14β-dihydroxycarda-5,20(22)-dienolide 3-Acetate (VI)—A solution of 0.15 Gm. (0.00025 mole) of III and 0.15 Gm. (0.0017 mole) of lithium bromide in 15 ml. of isopropyl alcohol was heated under reflux for 2.5 hr. After evaporation of the solvent under reduced pressure, water was added to precipitate the product. Recrystallization from acetonitrile–methanol gave 0.06 Gm. of

[1] The authors are greatly indebted to Dr. Bertram Katzung and Mr. Charles Lee for help with the biological studies.

[2] Melting points were determined with a Thomas-Hoover apparatus equipped with a corrected thermometer. Infrared spectra were obtained with a Beckman IR-8 or Perkin Elmer 337 instrument. Microanalyses were performed by the Microanalytical Department, University of California, Berkeley, Calif. Optical rotations were obtained in a 0.5-cm. tube with a Rudolph photoelectric polarimeter. NMR spectra were obtained at a field strength of 60 Mc./sec. on samples in deuteriochloroform solution on a Varian A-60 or A-60A instrument, using tetramethylsilane as internal standard. Peak positions of AB quartets were computed with a punch card program on a Wyle calculator. When only small amounts of sample were available, or when the sample was poorly soluble in deuteriochloroform, a Varian C-1024 computer was used for time averaging.

product, m.p. 190–194°. The analytical sample had m.p. 195–197°; $[\alpha]_D^{19}$ −28° (c 1, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{33}BrO_5$: C, 60.85; H, 6.74. Found: C, 60.73; H, 6.72.

3β,14β-Dihydroxy-19-iodocarda-5,20(22)-dienolide 3-Acetate (VII)—A solution of 0.1 Gm. (0.00017 mole) of III and 0.1 Gm. (0.0007 mole) of lithium iodide in 20 ml. of isopropyl alcohol was heated under reflux for 2 hr. After evaporation of the solvent under reduced pressure, water was added to precipitate the product. Recrystallization twice from methanol gave 0.03 Gm. of product, m.p. 180–184°. Further recrystallizations from the same solvent gave the analytical sample, m.p. 184–185°; $[\alpha]_D^{19}$ −40° (c 0.6, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{33}IO_5$: C, 55.56; H, 6.16. Found: C, 55.79; H, 6.24.

REFERENCES

(1) Wolff, M. E., Ho, W., and Kwok, R., *J. Med. Chem.*, **7**, 577(1964).
(2) Fieser, L. F., and Fieser, M., "Steroids," Reinhold Publishing Co., New York, N.Y., 1959, pp. 800–808.
(3) Lingner, K., Irmscher, K., Küssner, W., Hotovy, R., and Gillissen, J., *Arzneimittel-Forsch.*, **13**, 142(1963).
(4) Djerassi, C., and Kielczewski, M. A., *Steroids*, **2**, 125(1963).
(5) Halpern, O., Vilotti, R., and Bowers, A., *Chem. Ind. (London)*, **1963**, 116.
(6) Halpern, O., Crabbé, P., Cross, A. D., Delfín, I., Cervantes, L., and Bowers, A., *Steroids*, **4**, 1(1964).
(7) Halpern, O., Delfín, I., Magaña, L., and Bowers, A., *J. Org. Chem.*, **31**, 693(1966).
(8) Tadanier, J., *J. Org. Chem.*, **31**, 2124(1966).
(9) Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **59**, 713(1924).
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(11) Fieser, L. F., and Goto, T., *J. Am. Chem. Soc.*, **82**, 1697(1960).
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(14) Wolff, M. E., and Morioka, T., *J. Org. Chem.*, **30** 2553(1965).
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(19) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
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The relative downfield shift of the protons in *1* can be accounted for as follows. The *a* protons (Table I), being farthest away from the nitrogen, are shifted downfield the least amount. The others are

product, m.p. 190–194°. The analytical sample had m.p. 195–197°; $[\alpha]_D^{19}$ −28° (c 1, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{33}BrO_5$: C, 60.85; H, 6.74. Found: C, 60.73; H, 6.72.

3β,14β-Dihydroxy-19-iodocarda-5,20(22)-dienolide 3-Acetate (VII)—A solution of 0.1 Gm. (0.00017 mole) of III and 0.1 Gm. (0.0007 mole) of lithium iodide in 20 ml. of isopropyl alcohol was heated under reflux for 2 hr. After evaporation of the solvent under reduced pressure, water was added to precipitate the product. Recrystallization twice from methanol gave 0.03 Gm. of product, m.p. 180–184°. Further recrystallizations from the same solvent gave the analytical sample, m.p. 184–185°; $[\alpha]_D^{19}$ −40° (c 0.6, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{33}IO_5$: C, 55.56; H, 6.16. Found: C, 55.79; H, 6.24.

REFERENCES

(1) Wolff, M. E., Ho, W., and Kwok, R., *J. Med. Chem.*, **7**, 577(1964).
(2) Fieser, L. F., and Fieser, M., "Steroids," Reinhold Publishing Co., New York, N.Y., 1959, pp. 800–808.
(3) Lingner, K., Irmscher, K., Küssner, W., Hotovy, R., and Gillissen, J., *Arzneimittel-Forsch.*, **13**, 142(1963).
(4) Djerassi, C., and Kielczewski, M. A., *Steroids*, **2**, 125(1963).
(5) Halpern, O., Vilotti, R., and Bowers, A., *Chem. Ind. (London)*, **1963**, 116.
(6) Halpern, O., Crabbé, P., Cross, A. D., Delfín, I., Cervantes, L., and Bowers, A., *Steroids*, **4**, 1(1964).
(7) Halpern, O., Delfín, I., Magaña, L., and Bowers, A., *J. Org. Chem.*, **31**, 693(1966).
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The relative downfield shift of the protons in *1* can be accounted for as follows. The *a* protons (Table I), being farthest away from the nitrogen, are shifted downfield the least amount. The others are

equidistant, but the *c* and *d* protons are on a carbon to which another carbon is attached and thus appear further downfield than the *b* protons. The presence of only one band for the *c* and *d* protons implies that their environment must be the same, which means that the nitrogen inverts rapidly causing the methyl group to spend half its time on each side of the ring. The methyl group gives a sharp singlet but the peaks for the methylene protons are complex. The *c* and *d* protons give rise to a triplet, while the *a* protons form a more complicated multiplet.

Introduction of oxygen giving *N*-methylpyrrolidine *N*-oxide (compound *2*) causes all protons to move downfield, the *a* proton by 25 c.p.s. and the *b*, *c*, and *d* protons by 60 c.p.s. The *a* protons are one atom farther away from the positively charged nitrogen atom and are deshielded less, as expected (3). However, the fact that there is only one band for the *c* and *d* protons is entirely unexpected, because the protons on the side of the O^- atom would be anticipated to be in a different environment from those on the side of the methyl group. The reason, apparently, is that the O^- and CH_3 group provide, by coincidence, essentially the same environment. This is reasonable because the negative charge on oxygen would oppose its electronegativity and the net effect would be close to that of a methyl group.

If this explanation is correct, then one would expect the chemical shifts for *N*-methylpyrrolidine methiodide (compound *3*), in which a methyl group has replaced the O^-, to come close to those for compound *2*, which they do, differing only by 3, 7, and 2 c.p.s., respectively. The signal for the methyl group in these two compounds is a sharp singlet, while those for the *a*, *c*, and *d* protons are complex multiplets.

Replacement of one of the methyl groups in compound *3* by a deuterium atom, giving compound *4*, results in multiplets for the *c* and *d* protons centered around 220 and 187 c.p.s. This is expected, for the environment around methyl will be different from that around deuterium, which has a lesser tendency to move protons downfield. This change has caused the *a* protons to move upfield by 8 c.p.s., the *b* protons by 14 c.p.s., one of the *c* and one of the *d* protons by 25 c.p.s., and the other *c* and *d* protons downfield by 8 c.p.s. It seems likely that the *c* and *d* protons on the same side as the methyl group give rise to the 187 c.p.s. signal, since they are more analogous to the methyl protons (with respect to the deuterium) than are the other *c* and *d* protons. This reasoning applies irrespective of what combination of inductive, magnetic anisotropy, or solvent effects may be involved.

Introduction of a pyridine ring into compound *1* gives nicotine (compound *5*) which has a sharp singlet at 127 c.p.s. due to the methyl group. There are multiplets centered at 119, 138, and 190 c.p.s. which correspond to 4, 1, and 2 protons, respectively. The signal at 119 c.p.s. can be assigned to the *a* protons, which are farthest from the pyrrolidine nitrogen. The multiplet at 138 c.p.s. was thought to arise from the *d* proton (and was indeed a triplet as expected for this proton), and the multiplet at 190 c.p.s. would then be due to the *c* protons. However, an argument against this assignment is that a calculation of shielding and deshielding effects (4) according to the method discussed by Bible (5), following Johnson and Bovey (6), for any possible conformation of nicotine does not put the *d* proton into a region of shielding and thus could not account for its movement upfield to 138 c.p.s. from its position at 150 c.p.s. in *N*-methylpyrrolidine.

To shed further light on this point, the spectrum of nicotine was examined both at 100 Mc./sec. and at 220 Mc./sec. in benzene.[1] Under these conditions, the multiplet centered at 190 c.p.s. in D_2O was resolved into a triplet and doublets of a triplet. The triplet at 187 c.p.s. can thus be assigned to the *d* proton while the two *c* protons give rise to the triplet at 138 c.p.s. and the multiplet at 192 c.p.s., respectively. This assignment has the advantage that the signal for the *d* proton is now in a region where the calculations place it, as is that for the *b* protons, and the *c* proton opposite the methyl group can be placed in a position consistent with its signal at 138 c.p.s. However, the calculations do not place the other *c* proton far enough downfield by about 40 c.p.s. Nevertheless, this assignment gives rise to better over-all consistency than does the alternative one. The reasons the calculations do not hold in the case of one of the *c* protons may be due to the method (6) having been developed for benzene rather than pyridine, plus the fact that the proton in question has the pyrrolidine nitrogen interposed between it and the pyridine ring for the conformations considered. This would be expected to alter the action of the pyridine ring currents on this proton that take place directly through space.

The Stuart-Briegleb model for L-nicotine shows considerable steric hindrance if the methyl group is placed on the same side of the pyrrolidine ring as the pyridine moiety, and demethylation studies on nicotine (2) indicate the methyl group and pyridine ring to be on opposite sides of the pyrrolidine ring.

The above assignment of the bands for nicotine means that the introduction of the pyridine ring in position 2 of *N*-methylpyrrolidine has shifted the *a* protons downfield by 12 c.p.s., the *c* proton on the same side as the methyl group down by 42 c.p.s., and the *d* proton by 37 c.p.s., while the *b* protons are displaced upfield by 10 c.p.s. and the *c* proton opposite the methyl group by 12 c.p.s.

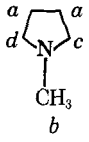
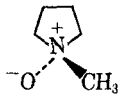
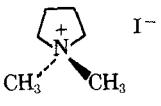
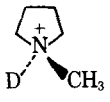
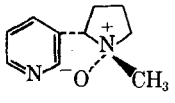
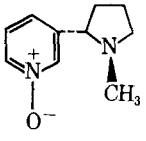
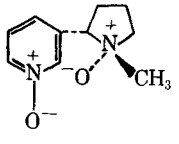
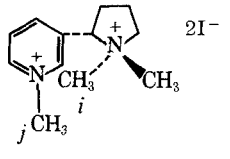
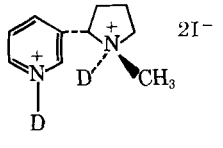
Placing an oxygen on the pyrrolidine nitrogen gives nicotine *N*-oxide (compound *6*) and the bands are assigned as indicated in Table I. The downfield shift, caused by the change from *5* → *6*, is one of 23 c.p.s. for the *a* protons, 57 c.p.s. for the *b* protons, 36 for the *c* proton on the same side as the methyl group, 90 for the *c* proton opposite methyl, and 94 for the *d* proton. The comparable changes in going from *1* → *2* were 25 c.p.s. for the *a* protons and 60 c.p.s. for the *b*, *c*, and *d* protons. The large effect on the *c* proton opposite the methyl group may be due to the oxygen being interposed between it and the pyridine ring, thus reducing the shielding effect of the ring currents which originally moved this proton upfield in nicotine.

Introduction of an oxygen on the pyridine nitrogen, giving compound *7*, leaves the signals for the protons of the pyrrolidine ring almost unchanged. The largest shift (9 c.p.s.) is that of the *c* proton opposite the methyl group, *i.e.*, the proton closest in space to the pyridine ring with its (now positively charged) nitrogen.

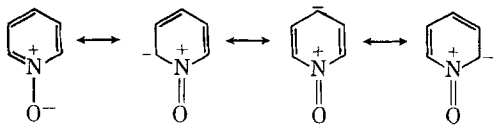
The movement of the protons on the pyridine

[1] The authors are indebted to Dr. N. S. Bhacca, Varian Associates, for these spectra.

TABLE I—CHEMICAL SHIFTS OF PROTONS, IN C.P.S., OF *N*-METHYL PYRROLIDINE, NICOTINE, AND ANALOGS RELATIVE TO SODIUM 2,2-DIMETHYL-2-SILAPENTANE-5-SULFONATE (DSS) IN DEUTERIUM OXIDE, MEASURED AT 60 MC./SEC.

		a	*b*	*c*	*d*	*e*		*g*	*h*	*i*	
1		107	137	150	150						
2		132	197	210	210						
3		135	190	212	212						
4		127	176	187 220	187 220						
5		119	127	192 138	187	446	470	507	507		
6		142	184	228	281	448	486	517	519		
7		115	129	192 147	192	456	460	494	496		
8		145	185	227	282	459	477	504	511		
9		155	196	231	305	495	531	540	550	177	270
10		148	176	214 231	291	495	531	540	545		

ring in *7* compared with *5* are interpretable in terms of the resonance forms expected in pyridine *N*-oxide (7). These put negative charges (Scheme I) on the *h*, *f*, and *g* carbon atoms which correspond to the 2, 4, and 6 positions, and protons attached to these carbons move upfield by 11, 10, and 13 c.p.s. while the *e* proton, attached to a carbon which has a relative positive charge, is moved downfield by 10



Scheme I

c.p.s. The 3 position on the pyridine ring will likewise be relatively positive, and this is another reason for the downfield shift of the *d* proton attached to the adjacent carbon in *7* compared to *5*.

Conversion of compound *7* to nicotine *N,N'*-dioxide (compound *8*) caused downfield shifts of all aliphatic protons. The *a*, *b*, and *c* protons moved by 30, 56, and 90 c.p.s., respectively, while the *c* proton on the same side as the methyl group moved by 35, and the other *c* proton by 80 c.p.s. All of these values are in good agreement with the comparable change from nicotine to the *N*-oxide, *6*. The positions of the *a*, *b*, *c*, and *d* protons are almost identical in the *N*-oxide (*6*) and the *N,N'*-dioxide (*8*), showing that an oxygen added to the pyridine nitrogen is virtually without effect on the position of the signals for the pyrrolidine protons.

When an additional methyl group is placed on both nitrogens in nicotine, giving compound *9*, all signals move downfield due to the greater effect of a positive charge on nitrogen alone in contrast to a positive charge on nitrogen tempered by a negative charge on the adjacent oxygen. The shifts in the proton signals from compound *8* → *9* are twice as great for the *d* proton (which is closest to the pyridine ring) as for the other protons in the pyrrolidine ring, and the *f* and *h* protons (which have both a relative positive charge due to resonance, and are nearest the pyrrolidine ring) are displaced most in the pyridine ring. The downfield location of the *j* protons compared with the *i* protons results from the greater electronegativity of pyridine compared to pyrrolidine, and to the *j* protons being in or near the plane of the pyridine ring while the *i* protons are above it.

Dipole moments were measured and compared with the theoretical moments for various conformations of (L)-nicotine, *5*; (L)-nicotine *N'*-oxide, *7*; (L)-nicotine *N*-oxide, *6*; and (L)-nicotine *N,N'*-dioxide, *8*. The values used in calculating the theoretical moments are given in Table II, and the observed and theoretical calculated moments for the different configurations and conformations in Table III.

The four extreme conformations of nicotine are shown in Fig. 1. The observed moment for nicotine is close to that for rotation between conformations I and II. It is not quite so close to that for conformation IV. The models indicate that rotation is relatively free between either I and II, or III and IV, but would be prevented between other combinations. If, therefore, the molecule existed in conformation IV, there seems no reason why it should not rotate between III and IV, in which case

TABLE II—VALUES USED IN CALCULATING THEORETICAL DIPOLE MOMENTS

Compd.	$\mu_{obs.}$ at 25° C.	Solvent
Trimethylamine-*N*-oxide[a]	5.03 D.[b] (9)	Dioxane
Pyridine	2.22 D. (10)	Dioxane
	2.21 D. (11)	Benzene
Pyridine-1-oxide	4.32 D. (12)	Dioxane
N-Methylpyrrolidine	1.34 D. (13)	Dioxane
	0.83 D.	Benzene

[a] As an approximation of the moment expected for *N*-methylpyrrolidine *N*-oxide. [b] Debye units.

TABLE III—OBSERVED AND CALCULATED DIPOLE MOMENTS FOR NICOTINE AND RELATED COMPOUNDS[a]

Conformation	$\mu_{obs.}$	F.R.[b]	$\mu_{calcd.}$
5, (L)-Nicotine (13)			
I	2.64 D. (20° C.	2.68 D.	2.97 D.
II	in benzene)		2.34
III			1.73
		2.29	
IV			2.73
I[c]			1.84
		1.70	
II[c]			1.55
III[c]			2.73
		2.48	
IV[c]			2.19
7, (L)-Nicotine N'-oxide, m.p. 50–52° C.			
I	4.89 ± .03		5.52
		5.04	
II			4.52
III			3.51
		4.40	
IV			5.13
I[c]			3.65
		3.46	
II[c]			3.24
III[c]			5.14
		4.70	
IV[c]			4.23
6, (L)-Nicotine N-oxide, m.p. 171.5–173° C. (sealed capillary)			
I	4.77 ± .06		6.44
		5.98	
II			5.49
III			5.02
		5.78	
IV			6.44
I[c]			4.07
		3.71	
II[c]			3.30
III[c]			6.34
		5.72	
IV[c]			5.02
8, (L)-Nicotine N,N'-dioxide, m.p. 183–183.5° C. (sealed capillary)			
I	6.11 ± .07		8.10
		7.40	
II			6.63
III			5.85
		7.07	
IV			8.10
I[c]			4.05
		3.46	
II[c]			2.47
III[c]			8.12
		7.07	
IV[c]			5.85

[a] In Debye units (D.); measured at 25° C. in dioxane unless otherwise noted. [b] F.R., free rotation between conformations of a configuration. [c] Conformations with the methyl group toward the pyridine ring, which were ruled out by demethylation studies and from space-filling models.

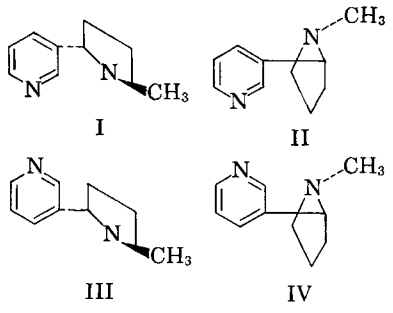


Fig. 1—Four extreme conformations of nicotine. In conformations II and III, the hydrogen on carbon 3 of the pyrrolidine ring is behind H-2 of the pyridine ring, while in conformations I and IV, it is behind H-4 of the pyridine ring. Thus I and IV, and II and III, are not identical since the pyridine ring is inverted. Conversion of conformation I into II, or of III into IV, is achieved by rotating the pyrrolidine ring. Conformation II cannot be converted into III except by breaking the carbon-carbon bond between the rings, rotating one ring, and reassembling.

the calculated moment would be considerably below that observed. Since the conformations with the methyl group next to the pyridine ring are ruled out by demethylation data and space-filling models, they need not be considered further. The most likely structure for nicotine appears to be that of a rotamer between conformations I and II.

The data for (L)-nicotine *N'*-oxide lead to the same conclusion, namely, rotation between conformations I and II for its structure. This is reasonable since the two molecules have the same structural features at the ring junction in the various conformations. The fact that the observed moment is 0.15 D. below that calculated for free rotation suggests that it spends a longer time in conformation II with the lower moment, which would be favored on a dipole interaction basis. With (L)-nicotine *N*-oxide, models indicate steric hindrance alters conformations I and III but not II and IV. In conformation I, an oxygen placed on the pyrrolidine nitrogen interferes with the hydrogen at carbon 2 of pyridine, and does not permit the downward-pointing hydrogen at carbon 3 of the pyrrolidine

TABLE IV—DIPOLE MOMENTS OF NICOTINE AND RELATED COMPOUNDS[a]

Nicotine N,N'-dioxide	ω_2	ϵ_{12}	v_{12}	
	0.0	2.227845	.970628	
m.p. 183–183.5° C.	0.398975	2.237857	.970408	
μ = 6.11 ± .07 D.	0.701764	2.244934	.970408	
mol. wt. 194.23	ϵ_1 2.227930	v_1 .970601	P_{2_0} 808.083	
	α 24.391123	β −.325828	P_{E_2} 44.457	
Nicotine N-oxide				
	0.0	2.227327	.970628	
m.p. 171.5–173° C.	0.986434	2.243208	.970408	
	1.092887	2.244589	.970408	
μ = 4.77 ± .06 D.	1.963564	2.258226	.969858	
mol. wt. 178.23	2.479078	2.268238	.969638	
	ϵ_1 2.227043	v_1 .970741	P_{2_0} 502.085	
	α 16.310666	β −.424233	P_{E_2} 36.801	
Nicotine N'-oxide				
	0.0	2.227413	.970628	
m.p. 50–52° C.	1.056411	2.246143	.970518	
	1.427574	2.252530	.970298	
μ = 4.89 ± .03 D.	1.837944	2.260211	.970188	
	3.290192	2.284292	.969858	
mol. wt. 178.23	3.707915	2.291024	.969748	
	ϵ_1 2.227949	v_1 .970674	P_{2_0} 534.794	
	α 17.130637	β −.247928	P_{E_2} 45.424	
N-Methylpyrrolidine	ω_2	ϵ_{12}	v_{12}	
	0.0	2.239750	.970298	
b.p. 79.5°/754.4 mm. Hg	2.498532	2.245692	.970958	
	3.877602	2.248533	.971289	
μ = 1.34 ± .02 D.	5.759391	2.253183	.971840	
	6.850177	2.256714	.972282	
mol. wt. 85.15	9.013237	2.261622	.972945	
	ϵ_1 2.239509	v_1 .970226	P_{2_0} 65.195	
	α 2.444263	β .294738	P_{E_2} 28.234	
N-Methylpyrrolidine				
	0.0	2.270061	1.143639	
b.p. 79.5°/754.4 mm. Hg	1.885702	2.271266	1.143639	
	4.727287	2.273075	1.143792	
μ = 0.83 ± .001 D.	8.188559	2.275227	1.143945	
	11.531298	2.277294	1.144098	
mol. wt. 85.15	14.922103	2.279447	1.144251	
Benzene				
	ϵ_1 2.270080	v_1 1.143597	P_{2_0} 40.111	
	α .627504	β .043219	P_{E_2} 25.996	

[a] Measured in dioxane at 25° C. unless otherwise noted.

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EXPERIMENTAL

The dielectric constants were measured with a WTW Dipolmeter model DM-01 with a DF L-2 4-ml. cell with a thermostated jacket maintained at a temperature of 25.00° ± 0.03°. The P_{E_2} values are molar refractions calculated from refractive indices of the solutions measured with the D sodium line at 25.00° ± 0.03°. Densities were measured with a pycnometer of approximately 2-ml. capacity. Solvents were refluxed over sodium twice and distilled twice from a 1-M. column. Dipole moments were calculated using the Halverstadt-Kumler (8) method, for which programs were developed for use on both the IBM 1620 and 1401 computers. The results are given in Table IV.

REFERENCES

(1) Karrer, P., and Widmer, R., *Helv. Chim. Acta*, **8**, 364 (1925); Bijvoet, M., Peerdeman, A. F., and Van Bommel, A. J., *Nature*, **168**, 271(1951).
(2) Craig, J. C., Mary, N. Y., Goldman, N. L., and Wolf, L., *J. Am. Chem. Soc.*, **86**, 3866(1964).
(3) Jackman, L. M., "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, New York, N. Y., 1959, p. 56.
(4) Roberts, J. D., "Nuclear Magnetic Resonance," McGraw-Hill Book Co., New York, N. Y., 1959, pp. 20–21.
(5) Bible, R. H., Jr., "Interpretation of NMR Spectra," Plenum Press, New York, N. Y., 1965, p. 19.
(6) Johnson, C. E., Jr., and Bovey, F. A., *J. Chem. Phys.*, **29**, 1012(1958).
(7) Katritzky, A. R., and Lagowski, J. M., *J. Chem. Soc.*, **1961**, 43.
(8) Halverstadt, I. F., and Kumler, W. D., *J. Am. Chem. Soc.*, **64**, 2988(1942).
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(10) Leis, D. G., and Curran, B. C., *J. Am. Chem. Soc.*, **67**, 79(1945).
(11) Cumper, C. W. N., and Vogel, A. I., *J. Chem. Soc.*, **1956**, 3621.
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Methodology and *In Vitro* Studies

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Received April 25, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122
Accepted for publication October 5, 1966.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
This investigation was supported in part by research funds from the American Arthritis Foundation, Northern California Chapter, and training grant GM 0728 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.
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(9) Hackett, N., and LeFevre, R. J. W., *J. Chem. Soc.*, **1961**, 2612.
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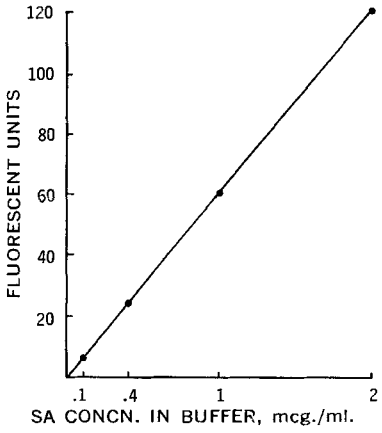

Fig. 1—Standard curve for salicylic acid in phosphate buffer (0.1 M, pH 7) using the Aminco Bowman spectrophotofluorometer. The activating wavelength was 315 mμ (uncorrected) and the emission wavelength was 420 mμ (uncorrected). (See Footnote 1.)

the reaction to follow zero-order kinetics. High dilution of the blood and plasma may have been required for the analytical approach utilized, but extrapolation back to undiluted blood cannot be made with certainty. These circumstances make it difficult to apply the results to an *in vivo* system.

The object of this paper is to report a method of studying ASA hydrolysis in virtually undiluted blood and plasma (90 vol. %) and at therapeutically significant concentrations (below 15 mcg./ml.).

EXPERIMENTAL

Rate Study—The subjects were four healthy males and one healthy female, ranging in age from 26 to 42 years. Blood was drawn from the antecubital vein into a heparanized syringe. The experiment was started soon after drawing the blood and lasted in most instances for 1 hr. All rate studies were carried out with U.S.P. acetylsalicylic acid which we found to contain less than 0.1% free salicylic acid (SA).

The blood sample was divided into two parts. One part was used for the study of the hydrolysis of ASA in whole blood. The other was centrifuged at 2200 RCF for 20 min., and the separated plasma was then used for the study of the hydrolysis of ASA in plasma. From each 10 ml. of blood or plasma, 1 ml. was removed for a blank analysis. Then 1 ml. of ASA in isotonic NaCl solution was added to the remainder, resulting in a 10% dilution of the blood. Two initial concentrations of ASA (13 mcg./ml. and 6.5 mcg./ml.) in the blood or plasma were used. The rate studies were carried out at 37°, and in most cases data points were obtained at 5, 10, 20, 30, 45, and 60 min.

Spectrophotofluorometric Assay—One-milliliter samples of blood or plasma were withdrawn from the test medium, mixed immediately with 1 ml. isotonic NaCl solution, and extracted with 5 ml. ether containing oxalic acid (8 mg./ml.). The extraction with acidified ether serves several functions: (*a*) the oxalic acid partitions into the aqueous phase rendering it acidic to a pH of approximately 2; (*b*) the esterases in the blood and plasma are immediately inactivated; (*c*) whole blood mixes well with the ether before clumping occurs; and (*d*) under these pH conditions this volume of ether virtually completely extracts the ASA and SA. These phenomena were verified by preliminary experiments using spectrophotometric and spectrophotofluorometric measurements.

The sample of ether and blood or plasma was centrifuged and 3 ml. of ether was withdrawn and extracted with 3 ml. phosphate buffer (0.1 *M*, pH 7). Nitrogen gas was bubbled through the buffer for 2 or 3 min. to remove residual ether, since the presence of ether affects the SA fluorescence in the buffer.

The fluorescence of salicylic acid in pH 7 phosphate buffer was determined using the Aminco-Bowman spectrophotofluorometer by activating at a wavelength of 315 mμ (uncorrected) and reading the emission at 420 mμ (uncorrected).[1] (ASA is not fluorescent under these conditions.) The slits were placed in the following order: 1/2, 1/16, 1/8, 1/8, 1/16, 1/8, and 3/16, respectively.

Buffer solutions containing both ASA and SA from blood or plasma samples were analyzed fluorometrically for free SA within 2 to 3 hr. after extraction. At pH 7 and room temperature, ASA hydrolysis proceeds relatively slowly with a half-life of approximately 3 days (3). However, when heated to 100° hydrolysis is very rapid with a half-life of approximately 10 min. The buffer solutions were heated at 100° in stoppered tubes for 1.5 hr., which completely hydrolyzes the intact ASA, and analyzed for total SA (*i.e.*, that liberated by hydrolysis of ASA and the free SA).

The fluorescence of each sample was determined at three dilutions. First, 1.0 ml. of undiluted sample was read; then 1.0 ml. of buffer was added and the sample read again; finally, another 1.0 ml. of buffer was added and the sample read again. This was done so that quenching corrections could be applied. The recovery of ASA and SA was greater than 98%, with somewhat better recovery of ASA than of SA due to its more favorable partition characteristics.

RESULTS AND DISCUSSION

Spectrophotofluorometric Assay—Figure 1 is a plot of fluorescence against various concentrations of SA in pH 7 phosphate buffer. The fluorescence reading of SA is linear with concentration over the range of concentrations studied. The concentrations used in this standard curve were the same magnitude as those encountered in the actual experiments after the dilution involved in the extractions.

The lower limit of detection of SA using the Aminco-Bowman spectrophotofluorometer is approximately 1.0 ng./ml. However, the blank readings obtained from blood and plasma are equivalent to a 25 ng./ml. of SA and remain constant before and after heating. The lowest amount of

[1] The Aminco-Bowman spectrophotofluorometer used in this study was not standardized to precise wavelength, and the values of excitation reported are probably 5 to 10 mμ too high.

free SA determined was at least 125 ng./ml. However, this is a false evaluation of the accuracy of the method, since the relative reproducibility of the readings is important and the total method is a difference method requiring an assessment of the relative error of the reading of the free salicylate and the total salicylate. It was the authors' experience that there was an over-all error of approximately 1 to 2% in the procedure. Therefore, the authors would not be able to discriminate accurately less than 2 parts of ASA in the presence of 100 parts of SA regardless of the concentrations.

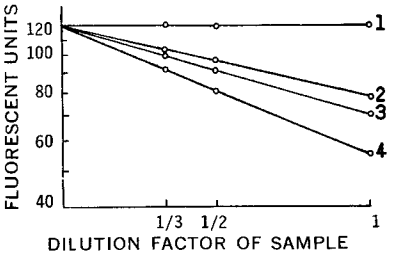

Fig. 2—Semilogarithmic plots illustrating the method of quenching correction. Key: 1, a standard buffer solution of SA; 2, 3, and 4, standard buffer solutions of SA shaken with ether containing quenching components from blood.

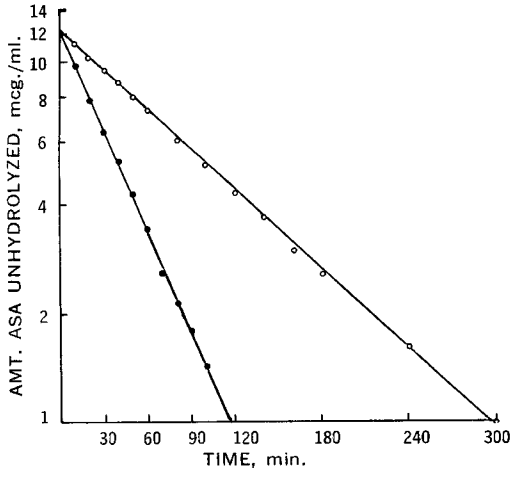

Fig. 3—Semilogarithmic plots of the hydrolysis of ASA carried out for 100 min. with blood and 300 min. with plasma. Initial ASA concn. was 13 mcg./ml. Key: ●, blood; ○, plasma.

It was found that the SA fluorescence in whole blood extracts was highly quenched. This was true of plasma extracts also but to a much lesser extent. A quenching correction procedure was developed in this laboratory analogous to that developed by Peng (4) for quenching corrections in scintillation counting of radioactive samples. It was observed that quenching obeys the Beer-Lambert law. A 1.0-ml. sample of the quenched solution was diluted in three increments and the fluorescence readings corrected for dilution (*i.e.*, if diluted $^1/_2$ the reading is multiplied by 2, if diluted $^1/_3$, by 3, *etc.*). The readings were plotted on semilogarithmic paper with the abscissa as the dilution factor and the ordinate as the corrected fluorescence reading. A line was drawn through these points and the intercept on the ordinate was taken as the true fluorescence in the absence of quenching components from the blood. If a standard buffer solution of SA in the absence of quenching components from the blood is diluted and read by this procedure, there is no change in the fluorescence reading. This is shown in Fig. 2 as the horizontal line along with three samples representing blood extractions with acidified ether subsequently shaken with the standard buffer solution of SA. Each sample varied in the degree of quenching as shown by the different slopes obtained. This is apparently a function of the amount and manner of shaking in the ether extraction of blood which can release varying amounts of quenching components. In assessing the true value by extrapolation, it was found that the method was reproducible with a range of ±1% of the standard.

Rate Data—It is apparent from Fig. 3 that the hydrolysis of ASA in blood and plasma appears to obey pseudo first-order kinetics for more than three half-lives. A summary of all the half-lives obtained, including repeated determinations at different times with the same subject, is shown in Table I. Figure 4 shows typical plots of hydrolysis data at initial ASA concentrations of 13 and 6.5 mcg./ml. in blood and plasma from three subjects

TABLE I—HALF-LIVES OF ASA HYDROLYSIS IN MINUTES AT 37° C. IN 90 VOL. PER CENT HUMAN BLOOD AND PLASMA

Subject	13 mcg./ml. Initial ASA Concn. Blood	13 mcg./ml. Initial ASA Concn. Plasma	6.5 mcg./ml. Initial ASA Concn. Blood	6.5 mcg./ml. Initial ASA Concn. Plasma
A	38.5	62.5		
B	35	56	27.5	80
	30.5	72		
	28	68		
C	33	70	28	65
	32	68.5	31	70
	27	67		
D	31	57	30.5	63
	31.5	58	31	68
	30	67		
	32.5	80		
E	35	65		
Mean ± S.E.	32.0 ± 0.91	65.9 ± 1.98	29.6 ± 0.77	69.2 ± 2.96

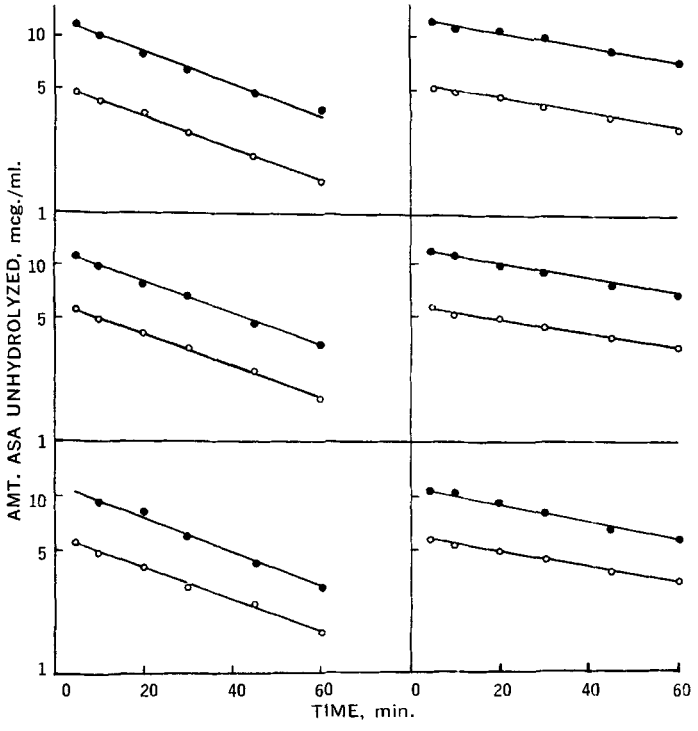

Fig. 4—Typical plots of hydrolysis of ASA in blood (left) and plasma (right) from three subjects. Hydrolysis was followed for 60 min. at two initial levels of ASA in both the blood and plasma. Key: ●, 13 mcg./ml.; ○, 6.5 mcg./ml.

It is to be noted that while the half-lives in these systems are not exactly reproducible, they are reasonably close indicating that the hydrolysis in this concentration range obeys first-order kinetics. However, this system is first order only at constant enzyme concentration. Since the blood and plasma were diluted reproducibly in each experiment, the differences in hydrolysis rates in repeated experiments in the same subject might be explained in terms of day-to-day variations of enzyme concentration in the blood.

Table I shows an average value of approximately 30 min. for the half-life of hydrolysis in whole blood and slightly over 1 hr. in plasma. It is important to note that the studies carried out in this investigation are at the levels of ASA that might be expected in the body from normal doses (5).

However, the results do not agree with the recent report of Morgan and Truitt (2), wherein they studied the hydrolysis of ASA in thirtyfold dilutions of serum and blood and at a concentration of 2.4×10^{-3} *M* ASA (433 mcg./ml.), a level 33 times the concentrations used in this study. Their reported average rate constants for ASA hydrolysis at 25° are 4.74×10^{-2} hr. ($t_{1/2}$ = 14.7 hr.) and 11.7×10^{-2} hr.$^{-1}$ ($t_{1/2}$ = 5.9 hr.) for serum and blood, respectively. They also showed that at 37° the rate is about twice that at 25°. These half-lives can only be compared to our own by linearly correcting for enzyme dilution by dividing by 30 and by 2 to correct for temperature. If we adopt such a correction procedure, even though Morgan and Truitt used a 3.3% serum concentration, we obtain half-lives of approximately 15 min. and 6 min for serum and blood, respectively. These are approximately 1/4 to 1/5 the estimates of the half-lives for hydrolysis obtained in the present study. Edwards (6) studied the hydrolysis of ASA in guinea pig plasma from 1 to 50 vol. %. Below approximately 15%, there was considerable departure from linearity.

The observation that the hydrolytic rate in whole blood is about twice as rapid as in plasma suggests the presence of higher concentrations of the hydrolyzing enzyme(s) or additional enzyme(s) in the red blood cells or in the leucocytes. Red blood cells are known to possess a high content of acetylcholinesterase. A recent review (7) includes a discussion of esterases found in leucocytes.

SUMMARY

An assay for acetylsalicylic acid is reported based on a spectrophotofluorometric assay of the free and total salicylate content. Using this assay the hydrolytic rate of acetylsalicylic acid in 90 vol. % human whole blood and plasma at levels of 13 and 6.5 mcg./ml. was studied. The hydrolysis was found to obey pseudo first-order kinetics. The mean half-lives of hydrolysis were 32 ± 0.91 min. (mean ± S.E.) for blood and 65.92 ± 1.98 min. for plasma at the 13 mcg./ml. level. At the 6.5 mcg./ml. level the values were 29.6 ± 0.77 min. for blood and 69.2 ± 2.96 min. for plasma.

REFERENCES

(1) Lester, D., Lolli, G., and Greenberg, L. A., *J. Pharmacol. Exptl. Therap.*, **87**, 329(1946).
(2) Morgan, A. M., and Truitt, E. B., *J. Pharm. Sci.*, **54**, 1640(1965).
(3) Edwards, L. J., *Trans. Faraday Soc.*, **48**, 696(1952).
(4) Peng, C. T., *Anal. Chem.*, **32**, 1292(1960).
(5) Leonards, J. R., *Proc. Soc. Exptl. Biol. Med.*, **110**, 304 (1962).
(6) Edwards, L. J., *J. Chem. Soc.*, **1952**, 4114.
(7) Cline, M. J., *Physiol. Rev.*, **45**, 674(1965).

Determination of Acetylsalicylic Acid and Salicylic Acid in Plasma

By MALCOLM ROWLAND and SIDNEY RIEGELMAN

A specific and sensitive method has been developed for the determination of acetylsalicylic acid and salicylic acid in plasma. Both of these compounds are extracted from acidified plasma with ether. A portion of the ether extract is concentrated, reacted with a silanizing reagent in carbon disulfide, and the silyl derivative of acetylsalicylic acid is analyzed by gas–liquid chromatography using dibutyl maleate as the internal standard. Another portion of the ether extract is extracted with phosphate buffer and the salicylate read spectrophotofluorometrically. Only 0.5 ml. or less of plasma is required for analysis and one can readily determine 0.4 mcg. acetylsalicylic acid/ml. in the presence of 100 mcg. salicylic acid/ml.

IN THE PAST, whole blood and plasma acetylsalicylic acid (ASA) levels have been calculated as the difference in the salicylic acid (SA) concentrations before and after hydrolysis of an aqueous extract of the sample (1–3). Such methods have large errors when SA is present in vast excess of ASA, a situation which often occurs in practice. Recently, ASA has been separated from SA by a paper chromatographic method (4) or by selective extraction (5), and assayed spectrophotofluorometrically after subsequent hydrolysis of ASA to SA.

Direct determinations of ASA have been made using gas–liquid chromatography (GLC) (6, 7) but these have been confined to tablet formulations. Crippen *et al.* (8) have chromatographed ASA as its methyl ester, while Horii *et al.* (9) have separated a large number of aromatic acids, including SA, as their trimethylsilyl derivatives. However, in none of the above methods has ASA been determined specifically in blood or plasma. A specific and sensitive method for the determination of ASA and SA in plasma, requiring only 0.5 ml. sample, is now presented. The ASA is determined directly by GLC as its trimethylsilyl derivative and the SA is determined spectrophotofluorometrically.

EXPERIMENTAL

Materials—The majority of materials used were analytical grade from Baker Chemical Co. The ASA sample from Merck contained less than 0.1% SA as shown by spectrophotofluorometric assay. Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), and most gas chromatographic materials were obtained from Applied Science Laboratories.

Received December 27, 1966, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication April 4, 1967.

This investigation supported in part by a grant-in-aid from the Glenbrook Laboratories, New York, N.Y.

The authors acknowledge the technical assistance of Mr. George King. Malcolm Rowland thanks the Welcome Foundation for a travel grant.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

Apparatus—An F & M model 700 gas chromatograph, with a flame ionization detector, model 810 electrometer, 220 power proportioning temperature controller, and a Hewlett-Packard 7127 A recorder, was used for ASA determinations. An Aminco-Bowman spectrophotofluorometer, model 8210, was used for SA assays.

Analytical Procedure

Reagents—(*a*) Fifty per cent w/v potassium fluoride in distilled water. (*b*) Heparin solution 1000 units /ml. (free of preservatives). (*c*) Distilled ether, prepared daily. (*d*) Distilled carbon disulfide, prepared daily. (*e*) Five per cent w/v potassium hydrogen sulfate in distilled water. (*f*) Hexamethyldisilazane (HMDS). (*g*) Trimethylchlorosilane (TMCS). (*h*) Dibutyl maleate standard solution, 1 mcg./ml. ether. (*i*) Carbon disulfide solution; each ml. CS_2 containing 2 μl. silanizing reagent (20 parts HMDS/1 part TMCS). (*j*) Phosphate buffer (0.1 *M*), pH = 7.0.

Collection and Storage of Samples—A 5-ml. sample of whole blood was placed immediately into a centrifuge tube containing 50 μl. heparin solution and 50 μl. potassium fluoride solution, the tube being kept in an ice bath. The blood was gently mixed and kept in the ice bath for 2 min. (final blood temperature = +2°). The blood was then centrifuged, and the plasma pipetted off and stored in dry ice until required for analysis.

Analysis—Plasma (0.05–0.5 ml.) was pipeted into a centrifuge tube containing 0.5 ml. potassium bisulfate solution (which acted as an acidifying medium and adjusted the pH to 2.0), extracted with 7 ml. of ether, and then centrifuged.

ASA Assay—A 5-ml. aliquot of the above ethereal extract was pipetted into a centrifuge tube containing 1 ml. dibutyl maleate standard (1 mcg./ml. ether). The combined ethereal solutions were concentrated on a water bath at 42° to about 50 μl. Carbon disulfide reagent (1 ml.) was then added to the solution which was then once again concentrated on a water bath at 60° to about 50 μl. Approximately 1 μl. of the concentrate was injected for analysis into the chromatograph. The peak height ratio of ASA derivative to dibutyl maleate was measured and the concentration of ASA determined by reference to a calibration curve. Freshly prepared ASA aqueous standards (1–10 mcg./ml.) were assayed in the same manner and run with each batch of plasma samples.

The column was 100–120 mesh Gas Chrome Q onto which was coated 3% DC QF-1, and packed into a 7-ft., 1/8-in. o.d. stainless steel tube. The op-

erating conditions were as follows: column temperature, 125°; injection block and detector temperatures, 200° and 180°, respectively; nitrogen pressure, 40 lb./sq. in.; hydrogen pressure, 16 lb./sq. in.; air pressure, 20 lb./sq. in.

SA Assay—A 1-ml. aliquot of the above ethereal extract of the acidified plasma was re-extracted with 5 ml. phosphate buffer (pH 7.0) in a centrifuge tube. The sample was centrifuged, excess ether sucked off, and nitrogen blown through the phosphate buffer for 30 sec. to remove the dissolved ether (which modified the spectrophotofluorometric readings). The solutions were then read on the spectrophotofluorometer, activation wavelength, 315 mμ. (uncorrected); emission wavelength, 420 mμ (uncorrected).[1] Fluorescent measurements of standard solutions of SA in phosphate buffer (0.1–1.0 mcg./ml.) were made at the same time. In a number of samples the phosphate solutions were heated for 1 hr. at 100° to hydrolyze the ASA to SA, and the total SA was then measured.

Calibration Curves—The following concentrations of ASA and SA were added to plasma obtained from a subject who had not taken any drugs during the previous week (mcg. ASA/ml.–mcg. SA/ml.: 0/0, 0.4/100, 1.0/80, 2.0/40, 4.0/20, 10/10, and 20/4.0). The samples of plasma were then assayed for ASA and SA by the above methods. With ASA, the peak height ratio of ASA to dibutyl maleate was plotted against the concentration of ASA; and with SA, the fluorescence was plotted against the concentration of SA.

RESULTS AND DISCUSSION

Inhibition of the Hydrolysis of ASA in Whole Blood—The hydrolysis of ASA in whole blood at 37° is very rapid, $t_{1/2}$ = 30 min. (10). Therefore, it is very important to inhibit the enzymatic hydrolysis after withdrawal of blood samples; otherwise, appreciable error in the estimation of ASA occurs. Normally the hydrolysis has been stopped by addition of whole blood into acid. However, this also hemolyzes the red blood cells and this was to be avoided since the interest was in assaying ASA plasma levels.

Initially, the concentration of free[2] and total[2] SA was determined, by the method of Harris and Riegelman (10), in blood samples taken at 2, 5, 10, and 15 min. after the addition of 1 ml. of solution containing 200 mcg. ASA/ml. of normal saline to 9 ml. of freshly drawn heparinized blood. The blood was left out at room temperature. The above experiment was repeated with the addition of 5 mg. potassium fluoride/ml. blood (100 μl. of 50% KF per 10 ml. blood) prior to mixing with the ASA solution. No hemolysis of the blood occurred when using this amount of potassium fluoride. The hydrolysis of ASA was also determined when blood was maintained in an ice bath (+1°) both with and without the addition of 5 mg. KF/ml. blood. The appearance of free SA, rather than the disappearance of unchanged drug, was used to calculate the degree of hydrolysis. The results, given in Table I, indicate that while fluoride and cold each decrease hydrolysis, the combination of both is best.

Table I—The Effect of Temperature and Potassium Fluoride on the Hydrolysis of Acetylsalicylic Acid in Whole Blood

Conditions	% ASA Hydrolyzed					
Time, min.:	2	5	10	15	20	60
Room temp.	3.5	9.6	13.6	21	...	...
Room temp. + KF (5 mg./ml.)	1.5	1.8	7.0	11	...	...
Ice bath (+1°)	...	3.7	...	8.6	...	...
Ice bath (+1°) + KF (5 mg./ml.)	...	0.8	0.8	...	0.85	1.1

In a separate experiment, the effect of centrifugation on the hydrolysis of ASA was investigated. Fresh heparinized blood containing 20 mcg. ASA/ml. and 5 mg. KF/ml. was cooled in an ice bath and divided into four parts. Each sample of whole blood was immediately assayed for free and total SA and the remainder of each sample was centrifuged for 3 min., the buckets of the centrifuge being previously cooled in a refrigerator. Each centrifuged sample was then shaken and assayed for free and total SA. It was found that there was no increase in the degree of hydrolysis of ASA when centrifuged under these conditions. Therefore, since the total time taken between withdrawal of the blood sample and the separation of the plasma is approximately 15 min., the hydrolysis of ASA was never greater than 2%.

Preparation of Sample for Analysis—Potassium hydrogen sulfate is a useful acidifying agent; a 5% solution has a pH of 1.6. The mixing of an equal volume of this solution with plasma gives a final pH of 1.9–2.2. Under these conditions extraction of ASA and SA with ether is quantitative, as shown by spectrophotofluorescent measurements.

Gas Chromatography—Marked difficulty was experienced in chromatographing free ASA or SA on isophthalic acid/Carbowax terephthalic acid and terephthalic acid/FFAP columns. In all cases a complex peak was obtained with both SA and ASA, while a symmetrical peak was obtained with benzoic acid. This phenomenon may be due to the decarboxylation of SA and ASA at the operating temperatures of 180° or more required for these columns.

A symmetrical peak of the ASA-trimethylsilyl derivative was obtained on SE-30, QF-1, and XE-60 columns. Although three derivatives of salicylic acid are possible with HMDS, only two derivative peaks were obtained, a major and a minor one. These were not investigated further, other than it was noted that they were obtained in the same ratio after repeated recrystallization of SA. The QF-1 column gave the best separation of the ASA and SA derivative peaks and thus was chosen for the analysis of ASA.

Carbon disulfide was chosen as the final reaction solvent since the flame ionization detector does not respond to this compound. The formation of the TMS derivative appeared to be almost spontaneous and quantitative in this solvent, since there was no increase in the peak height ratio of ASA–TMS derivative to internal standard before or after heating the reaction mixture for 1 hr. at 40°.

[1] Although ASA is extracted into the phosphate buffer, this compound does not fluoresce. Neither does ASA quench the fluorescence of SA since varying amounts of ASA did not affect the linearity of the SA calibration curve. However, it is important to read the SA fluorescence promptly since ASA does hydrolyze in phosphate buffer ($t_{1/2}$ at 25° approximately 2.5 days) liberating SA.

[2] The term free SA is used here to mean both protein bound and unbound SA, and total SA is the sum of the free SA and that produced by the hydrolysis of ASA to SA.

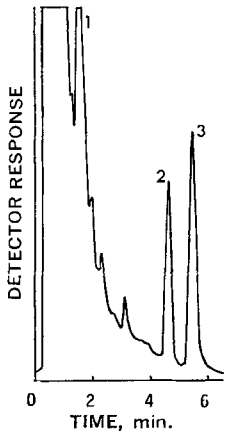

Fig. 1—Chromatogram of an extract of plasma from a subject who had taken an oral dose of acetylsalicylic acid. Key: 1, *salicylic acid–trimethylsilyl derivative* (T_r = *1.5 min.*); 2, *dibutyl maleate (marker;* T_r = *4.6 min.*); 3, *acetylsalicylic acid trimethylsilyl derivative* (T_r = *5.4 min.*).

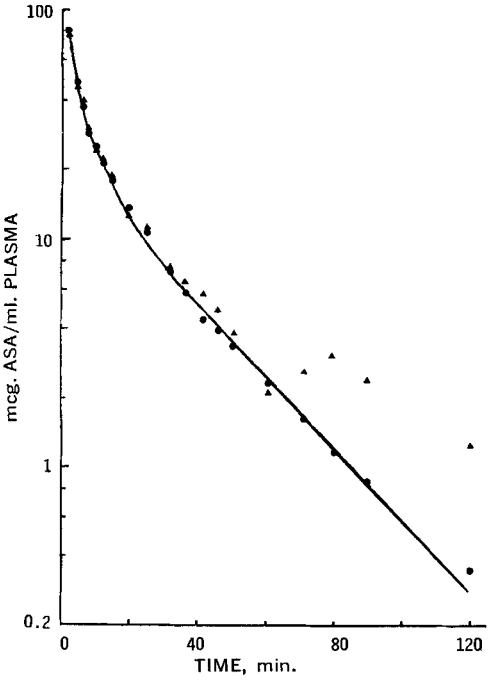

Fig. 2—Comparison of the gas–liquid chromatographic method (●) with the differential salicylic acid method (▲) for the determination of the acetylsalicylic acid plasma levels after the intravenous administration of acetylsalicylic acid.

A large number of compounds were screened as an internal marker. The most promising were methyl myristate, methyl tridecanoate, dimethyl phthalate, and dibutyl maleate; the last proved the best. Dibutyl maleate is soluble and stable in ether and carbon disulfide, does not react with HMDS, and gives a symmetrical peak close to the ASA–TMS derivative (Fig. 1).

Determination of SA in Plasma—Although SA gives a TMS derivative with HMDS, the drug was assayed spectrophotofluorometrically. The reasons for this were: (*a*) the retention time for the major SA–TMS peak was 1.5 min., which is on the solvent peak, rendering measurements of this peak height extremely difficult and inaccurate with concentrations of less than 10 mcg./ml. (*b*) Since generally SA concentrations are much larger than the ASA concentrations, constant changing of the attenuation switch would be necessary if both the SA and the ASA peaks are to be seen on the same scale. This demands constant attention of the analyst. On the other hand, the fluorescence method is sensitive and all samples can be analyzed in one batch.

Specificity, Sensitivity, and Reproducibility—Plasma from many subjects who had not ingested either ASA or SA were analyzed for these drugs. The GLC method for ASA was found to be specific, as no peak was ever observed, in these blank plasma samples, at the same retention time as the ASA–TMS derivative or that of the internal marker, dibutyl maleate. Also, neither salicylamide, which occasionally is formulated with ASA, nor the metabolite of SA, salicyluric acid, gave interfering peaks under the present assay conditions. The GLC method is quite sensitive as 0.2 mcg. ASA/ml. plasma could be readily demonstrated. The fluorescent method for SA determination is also very sensitive, and it is reasonably specific as no blank plasma sample gave a fluorescent reading greater than that equivalent to 0.5 mcg. SA/ml. plasma.

For reproducible results in the ASA determinations it was found necessary to saturate the column. This is readily achieved by rapidly injecting 4–5 samples and waiting about 15 min. for the column to clear. When this was carried out, the peak height ratio of standards were within 2% at the beginning and end of the run, and the calibration curve was linear from 0.4–20 mcg. ASA/ml. plasma. Also, samples of plasma with ASA added in concentrations of 1.0, 10, and 20 mcg./ml. were analyzed for ASA. (Seven samples were analyzed at each concentration.) The relative standard deviations (in per cent) of peak height ratio at 1, 10, and 20 mcg. ASA/ml. plasma were 1.8, 2, and 1.5%, respectively.

The SA calibration curve was linear from 2–100 mcg. SA/ml. plasma and the relative standard deviations (per cent) of the fluorescent readings at 5, 20, and 50 mcg. SA/ml. plasma were 1.7, 2.1, and 0.8%, respectively. (Seven samples were analyzed at each concentration.)

In addition, it was shown that both the ASA and SA calibration curves were unaffected by the presence of a large amount of the other drug, *e.g.*, with 0.4 mcg. ASA/ml. plasma the same peak height ratio was obtained in the presence or absence of 100 mcg. SA/ml. plasma. This further demonstrates the specificity of each of these assays.

Stability of ASA and SA in Plasma—Plasma samples, obtained from subjects who had taken ASA, were analyzed for ASA and SA. Analyses were made immediately, and 1, 2, 3, and 7 days after collection of the samples, and then once again after 30 days. All plasma samples were stored at dry ice temperature.

No loss of ASA or SA occurred in any samples stored up to 1 week. Further, several samples have been studied after 1 month or more of storage, and the variations of results were within the standard error of the method.

CONCLUSIONS

The present method for the simultaneous determinations of ASA and SA in plasma is simple and relatively rapid, since at least 20 samples can be analyzed in 5 hr. The stability of ASA under the sample and storage conditions enable determinations to be made at the analyst's convenience. The advantage of this method over the differential SA method for the determination of low concentrations of ASA in the presence of large amounts of SA is clearly shown in Fig. 2. Forty minutes after the administration of 650 mg. of ASA, the SA levels usually reach 40–50 mcg./ml., and shortly thereafter one must be able to determine as little as 0.4 mcg. ASA/ml. in the presence of the above vast excess of SA. It is only with a GLC method that the biological half-life of ASA can be accurately determined. Also, the recently proposed alternative method of Cotty *et al.* (5) may not be any more useful than the differential methods due to relative errors indicated in the published results. The methods presented in this paper are now being used to study the pharmacokinetics of ASA and SA in man and to investigate the influence of dosage forms on the absorption of ASA.

REFERENCES

(1) Leonard, J. R., *Proc. Expll. Biol. Med.*, **110**, 304 (1962).
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(9) Horii, Z., Makita, M., Takeda, I., Tamura, Y., and Ohnishi, Y., *Chem. Pharm. Bull.*, **13**, 636(1965).
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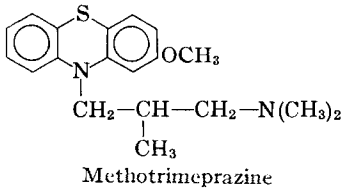
Estimation of Methotrimeprazine in Brain and Correlation of Brain Levels with Pharmacologic Activity

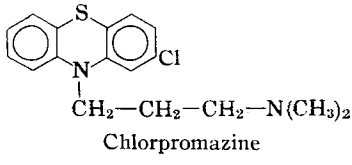
By ABDEL-HALIM M. AFIFI* and E. LEONG WAY

A method for estimating methotrimeprazine in brain by ultraviolet spectrophotometry was developed which is based on extracting the compound from alkalinized tissue with 1 per cent butanol in cyclohexane and determining its absorbance in acid at 250 mμ. A high degree of specificity for methotrimeprazine was conveyed to the procedure by subjecting the organic solvent extract of tissue to several buffer washes. Following intravenous injection of the compound in mice, methotrimeprazine was found to be metabolized rapidly during the first hour, after which its rate of disappearance slowed considerably. Brain levels were maximal at 1 hr. and were still appreciable at 4 hr. The onset and duration of pharmacologic effects as measured by a loss in coordinated motor ability varied directly with the concentration of drug in the brain, suggesting that the parent compound is principally responsible for mediating the response.

THE PHENOTHIAZINE DERIVATIVE, methotrimeprazine maleate (levomepromazine, and R.P. 7044),[1] until recently an investigational drug in the United States,[2] has been used for some time in Europe by psychiatrists as an alternative for chlorpromazine. The structures of the two agents are shown for comparison.



Methotrimeprazine



Chlorpromazine

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Received October 3, 1966, from the Department of Pharmacology, Schools of Medicine and Pharmacy, University of California Medical Center, San Francisco, CA 94122
Accepted for publication January 23, 1967.
This investigation was supported in part by grant GM-01839 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.
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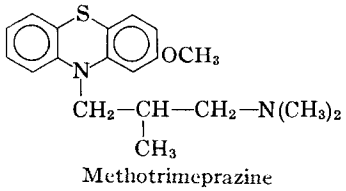

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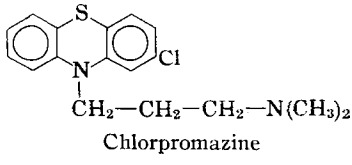

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depressant effects of other CNS agents. The analgetic effect of methotrimeprazine was noted by Sigwald *et al.* in the clinic (2).

More recently Lasagna *et al.* (3) reported that when injected methotrimeprazine appeared to be as effective as morphine, milligram for milligram, in the alleviation of postpartum pain, and that 15 mg. of methotrimeprazine gave as much relief as 75 mg. of meperidine in the management of pain during childbirth (4). The therapeutic potential of such a potent nonaddicting analgetic with minimal respiratory depressant properties prompted the study of the metabolism of the compound. The present report, using the mouse, is the first in a series concerning the biologic disposition of methotrimeprazine. A study in humans has been previously reported by Allgén *et al.* (5).

EXPERIMENTAL

Estimation of Methotrimeprazine in Mouse Carcass and Brain—The procedure used by Salzman and Brodie (6) for chlorpromazine was adapted and modified for methotrimeprazine. The method is based on determining the compound by ultraviolet spectrophotometry after its extraction from alkalinized tissue homogenate with a suitable organic solvent. The homogenate is obtained by mincing 1 part of tissue with 4 parts of 0.1 *N* H_2SO_4 in an electric blender.

Assay Procedure—Pipet 5 ml. of tissue homogenate into a 40-ml. glass-stoppered extraction tube; add 1 ml. of 10% NaOH and 25 ml. of cyclohexane containing 1% *n*-butanol. Shake for 5 min. and separate the two layers by centrifugation at 2500 r.p.m. Transfer 23 ml. of the organic phase to another 40-ml. extraction tube containing 10 ml. of 0.2 *M* phosphate buffer, pH 6.4. Shake for 5 min. and separate by centrifugation. Remove the buffer layer and add another 10 ml. of fresh buffer and repeat the washing process twice more. Transfer 20 ml. of the organic phase to a tube containing 4 ml. of 0.1 *N* H_2SO_4. Shake for 5 min., centrifuge, and remove the organic phase completely by aspiration. Transfer about 3 ml. of the aqueous acid phase to a quartz cell and determine its absorbance at 250 mμ in a Beckman DU spectrophotometer. The amount of methotrimeprazine recovered from a sample containing the drug is read from the corresponding standard curve derived from known amounts of methotrimeprazine added to each tissue homogenate.

Appraisal of Specificity of the Method—Countercurrent distribution (7) and partition behavior at varying pH values (8) were employed to characterize the ultraviolet absorbing material extracted from the carcasses of mice given methotrimeprazine.

Countercurrent Distribution—Two mice were injected intravenously with 15 mg./Kg. methotrimeprazine and sacrificed after 1 hr. The total carcass was diluted 1:5 with 0.1 *N* sulfuric acid and homogenized thoroughly in an electric blender. The homogenate was made alkaline with sodium hydroxide and extracted three times with 5 times its volume of cyclohexane containing 1% *n*-butanol. The extracts were pooled and divided into two parts. One part was used directly for countercurrent distribution and the other portion was washed three times with 0.2 *M* phosphate buffer, pH 6.4, before being subjected to countercurrent transfer. In each experiment an eight plate transfer separation was carried out in a system consisting of equal parts of 1% *n*-butanol in cyclohexane and 0.2 *M* phosphate buffer, pH 4, moving the lower aqueous phase. At the end of the distribution, 1 ml. of 10% sodium hydroxide was added to each tube. After shaking and centrifuging, an aliquot of the organic solvent was transferred to another tube containing 4 ml. of 0.1 *N* sulfuric acid, and the acid layer was analyzed for methotrimeprazine in the usual manner. A distribution curve was obtained by plotting the fraction of the total amount of methotrimeprazine present in each tube against the number of the tube, and the experimental distribution curve was fitted according to the method of Way and Bennett (9).

Partition Behavior—The alternative technique for appraising specificity of the procedure consisted of determining the distribution ratios of authentic methotrimeprazine in an organic solvent–water system at various pH values of the aqueous phase, and comparing them with those obtained for the extracted substance recovered from mice sacrificed after receiving methotrimeprazine. The carcass was homogenized as before in 4 parts of 0.1 *N* H_2SO_4, and after the addition of sufficient alkali, the homogenate was extracted with five times its volume of 1% *n*-butanol in cyclohexane. Duplicate 10-ml. aliquots of the organic solvent extract were washed three times with 0.2 *M* phosphate buffer of pH 6.4 and then equilibrated with 4 ml. of 0.1 *N* sulfuric acid. Other 10-ml. samples of the organic solvent extract were similarly washed and then equilibrated with equal volumes of 0.2 *M* phosphate buffer solutions of pH 7, 6, 5, or 4, respectively. After removal of the buffer phase, the organic solvent layer was shaken with 4 ml. of 0.1 *N* H_2SO_4. The organic solvent layer was discarded and the amount of methotrimeprazine in the acid aqueous phase was determined. The results were compared with those obtained from a similar treatment of authentic methotrimeprazine in aqueous solution.

Rate of Metabolism—The rate of disappearance of methotrimeprazine was studied in 23 intact mice. Methotrimeprazine, 15 mg./Kg., was injected into the tail vein of Swiss-Webster mice weighing 30 ± 5 Gm. The drug was dissolved in isotonic solution to give a concentration of 2 mg./ml. At fixed time intervals of 15, 30, 60, 120, 240 min., and 24 hr., at least two and generally four mice were individually homogenized and assayed for methotrimeprazine as described previously; the results are expressed as per cent of drug recovered.

Brain Concentrations of Methotrimeprazine—Mice were injected subcutaneously with methotrimeprazine and then sacrificed by decapitation at 15, 30, 60, 90, 120, 180, and 240 min.; two mice were used at each time interval. The brains were extirpated, weighed, diluted 10 times with 0.1 *N* sulfuric acid, and analyzed for methotrimeprazine in the usual manner.

Onset and Duration of Pharmacologic Effects—The time-response relationship of methotrimeprazine was assessed in mice in conjunction with brain concentration determinations. Two tests for central

nervous system activity were used: "analgesia," by the tail flick method of D'Amour and Smith (10); and coordinated motor ability, by the method of Dunham and Miya (11).

For analgetic activity, the reaction time for mice to remove their tails from a thermal stimulus, provided by a beam of light focused 1.5 cm. from the blackened tip of the mouse tail, was determined prior to drug injection and every 20 min. after subcutaneous administration of methotrimeprazine at doses of 2, 4, and 8 mg./Kg. A cut-off time of 5 sec. was used to prevent damage to the tail.

To test coordinated motor activity, the time an animal remained on a rotating rod was determined. One end of a wooden rod, 48 in. long and 1.2 in. in diameter, was connected to the motor shaft of an electric kymograph. The two ends of the rod were inserted into ball bearings supported by suitable clamps at a height of 28 in. above the table top to discourage animals from jumping off the roller. The speed selector was set so that the rod made 15 r.p.m. In order that multiple tests could be performed simultaneously, cardboard disks were placed along the rod at suitable distances to divide it into five compartments. Untreated animals after conditioning can maintain their balance on the rotating rod for at least 20 min. Performance trials were conducted on 10 mice at 15, 30, and then every 30 min. after subcutaneous injection of 4 mg./Kg. of the drug. The time each animal remained on the rotating rod was recorded up to a limit of 1 min. An animal remaining on the rod at the end of this observation period was assigned a relative activity of 1.0 and any perfomance value of less than 60 sec. was expressed as a fraction. The average relative activities and the mean deviations were calculated and plotted against time.

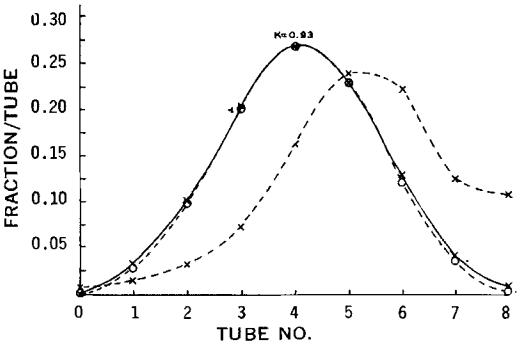

Fig. 1—Countercurrent distribution curves of the pooled extracts from the homogenized carcasses of two mice injected with methotrimeprazine.

$$System = \frac{0.01 \text{ n-butanol in cyclohexane}}{0.2 \text{ M phosphate buffer pH } 4}$$

Key: X---X, *unwashed solvent extract of mouse homogenate;* X—X, *washed solvent extract of mouse homogenate;* O---O, *theoretical.*

TABLE I—DISTRIBUTION RATIOS[a] OF AUTHENTIC METHOTRIMEPRAZINE AND OF APPARENT DRUG RECOVERED FROM MICE RECEIVING THE COMPOUND. THE SOLVENT PAIR CONSISTED OF 1% *n*-BUTANOL IN CYCLOHEXANE AND BUFFERS OF VARYING pH

Sample	pH 7	6	5	4
Added to water	0.98	0.93	0.73	0.35
Recovered from total mouse carcass	0.96	0.92	0.74	0.40

[a] $\frac{\text{solvent concentration post equilibration}}{\text{solvent concentration pre-equilibration}}$

RESULTS

Precision and Specificity of the Procedure—The application of single extraction techniques in conjunction with ultraviolet spectrophotometry appeared to be quite satisfactory for estimating methotrimeprazine in the mouse carcass or brain. Based on the standard curve obtained for methotrimeprazine extracted from water, the recovery of methotrimeprazine added to the tissue homogenates at concentrations ranging from 5 to 20 mcg. of drug per Gm. of wet tissues exceeded 90%. The blank correction was negligible, amounting to less than 0.1 mcg. of drug equivalent for brain and to less than 0.5 mcg. for the carcass.

The procedure was found to possess a high degree of specificity for methotrimeprazine. The countercurrent distribution experiments indicate that specificity is conveyed to the procedure by washing the solvent extract of tissue with a suitable buffer. As seen in Fig. 1, the distribution curve of the unwashed extract did not yield a curve with the distribution characteristics of a single substance. On the other hand, with the washed solvent extract of mouse homogenate, the experimental distribution curve with a maximum at tube 4 exhibited the characteristics of a single substance. With the latter curve, the calculated values of each tube for a partition ratio, K, of 0.93 yielded a theoretical curve which fitted almost perfectly with the experimental curve. When the K of methotrimeprazine added to a mouse homogenate was determined for the same system used in the countercurrent distribution, it was found to be 0.90. Thus, there is a strong evidence indicating that the substance characterized by countercurrent distribution is actually methotrimeprazine and that the introduction of a three-buffer washing removes interfering substances and renders the method highly specific for methotrimeprazine. The major fraction in the unwashed extract of total mouse homogenate appears to be biotransformation products and only about 30% of the ultraviolet absorbing material represents unchanged methotrimeprazine.

The partition behavior of biologically recovered methotrimeprazine at varying pH was also virtually identical to that of authentic methotrimeprazine. As can be noted in Table I, the distribution ratios of the recovered material in a solvent buffer pair system of varying pH were highly similar to those for authentic methotrimeprazine, and the probability for two different compounds to have the same distribution ratios under such rigidly defined conditions appears rather remote.

Rate of Metabolism in Intact Mice—Figure 2 represents a plot of the average per cent of the dose of methotrimeprazine recovered against the time interval after injection of the drug. The heavy line indicates the calculated mean recovery for each interval and the broken lines the range. Considerable varia-

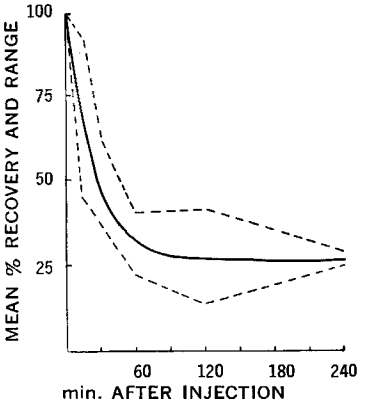

Fig. 2—Rate of metabolism of methotrimeprazine in intact mice after 15 mg./Kg. intravenously. Key: ———, mean obtained on two to four animals at six different time intervals; ----, range.

TABLE II—RESPONSE OF MICE TO THERMAL STIMULUS (TAIL FLICK METHOD)

Dose, mg./Kg.	Mice, No.	Reaction Time, sec., Mean ± m.d.— Time After Injection, min. 20	40	60
2	13	1.6 ± 0.3	2.1 ± 0.8	2.6 ± 1.1
4	14	2.5 ± 0.9	2.5 ± 0.9	2.7 ± 1.1
8	14	2.1 ± 0.9	2.6 ± 0.8	3.1 ± 1.1
15[a]	10	1.6 ± 0.3	1.9 ± 0.5	1.6 ± 0.3

[a] Values at 80, 100, 120, 140, 160, and 180 min. were, respectively, 2.3 ± 0.7, 2.5 ± 1.0, 2.0 ± 0.4, 1.7 ± 0.5, 1.7 ± 0.3, and 1.4 ± 0.2 sec.

tion in the rate of metabolism of methotrimeprazine was noted among the individual animals during the first 2 hr. The variation is less marked at 4 and 24 hr. after the drug injection. It can be noted that the initial part of the curve falls steeply and that after 60 min. about 70% of the injected dose has already disappeared. However, after the initial rapid decline the rate of disappearance of methotrimeprazine tends to level off, and the curve flattens out between 1 and 2 hr. After 24 hr., 7 and 8% methotrimeprazine was still recovered from two mice, respectively. Contamination of body levels by any drug in bladder urine was negligible since the amount of the drug present in the bladder urine at various time intervals in 8 of 23 mice was barely detectable, and the total 24-hr. excretion reflected less than 1% of the injected dose.

Methotrimeprazine "Analgesia"—Table II shows the mean values of the reaction time and their mean deviations obtained by injecting 2, 4, 8, and 15 mg./Kg. methotrimeprazine in 51 mice. The mean value of the control reaction time of the untreated mice was found to be 1.2 ± 0.04 sec.

While there appears to be a slight increase in the reaction time at the three dose levels and at 20, 40, and 60 min. after the administration of the drug, this increase is more apparent than real. The individual values varied considerably and the intensity of the responses correlated poorly with increasing doses of the compound. In fact with the highest dose, 15 mg./Kg. of methotrimeprazine, the reaction

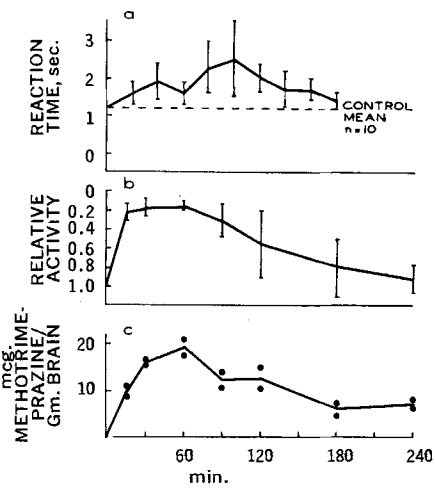

Fig. 3—Time response curves for mice receiving methotrimeprazine and assessed for (a) *"analgetic" activity (15 mg./Kg. dose);* (b) *loss of motor coordinating ability (4 mg./Kg. dose); and* (c) *brain levels of the drug (4 mg./Kg. dose).*

time was found to be almost the same or even less than that of lower dosages. Figure 3, a, represents the reaction time in seconds obtained with this dosage over a 3-hr. period with animals tested at 20-min. intervals. The mean deviations plotted in the same figure demonstrate the marked variation at each time interval. Despite the fact that little "analgesia" was noted, the mice appeared heavily sedated and exhibited little physical activity even at the lowest dose (2 mg./Kg.) of methotrimeprazine.

Coordinated Motor Ability—Figure 3, b, represents the average values of the relative activity of 10 mice when given 4 mg./Kg. of methotrimeprazine subcutaneously. It is apparent from the curve that the coordinated motor ability of the animals was reduced about 80% during the first hour. The mice gradually recovered, and by the end of the 4-hr. period there was only a 5% reduction in the relative activity of the mice. Subjectively, however, the animals still appeared very sedated and moved only when prodded.

Brain Levels of Methotrimeprazine—Figure 3, c, represents the concentration of methotrimeprazine as mcg./Gm. brain over the 4-hr. period after the administration of 4 mg./Kg. subcutaneously. The concentration in the brain increases gradually with time and reaches a peak level at 60 min. and then the drug concentration slowly declines. Significant amounts of methotrimeprazine were still present in the brain at the end of the 4-hr. period. The brain concentrations appeared to correlate with the coordinated motor ability of the mice to remain on the rotating rod.

DISCUSSION

The prolonged sedation and rather persistent tissue levels of methotrimeprazine observed in the mouse are in agreement with what is known concerning the duration of action and rate of metabolism of phenothiazine compounds in other animal species.

The psychotropic effects of chlorpromazine in humans have been reported to be maintained for 1 to 2 weeks after its discontinuation (5), and excretion of the drug in trace amounts in the urine may last for weeks (5, 12). Salzman and Brodie reported a relatively slow rate of biotransformation of chlorpromazine in dogs, and attribute the slow rate partly to extensive localization of the drug in various organs with consequent lesser immediate availability of the drug to metabolizing enzymes (6).

The studies on the organ distribution of methotrimeprazine in the rat (13) indicate that there is also rapid uptake of the drug by organ depots, especially parenchymatous tissues, and this is consistent with findings on most basic compounds (15). In the case of methotrimeprazine, organ levels of the drug may still be appreciable 12 hr. after its administration. Ultimately, however, the drug is extensively biotransformed. Less than 1% of the unchanged drug is excreted in the urine, but a variety of metabolic products, including sulfoxide metabolites, are detectable in varying amounts (13); similar findings have been reported for humans (5).

To what extent the biotransformation products of methotrimeprazine may contribute to the sum total of pharmacologic effects remains a question. The prolonged half-life of the drug indicates that adequate amounts of the original substance are available for eliciting drug effects. A reasonable degree of parallelism was noted for the curve for concentration of drug in the brain and that for coordinated motor ability. Such a correlation suggests that the parent substance is principally mediating the pharmacologic effects under observation, but whether loss of motor coordination is accompanied by analgesia cannot be answered by our experiments.

The failure to obtain a good correlation with "analgesia" resulted from the inability to establish a clear-cut response with methotrimeprazine by the test procedure. This is not surprising and was in part anticipated. Agents other than the narcotic analgetic type yield erratic effects in prolonging the tail reaction time to thermal stimulus and in our hands methotrimeprazine proved to be no exception to the rule. In view of the established effectiveness of the compound as a potent analgetic clinically (2–4), the tail flick procedure obviously would be a poor screening method for other compounds in this series. These findings are not consistent with an earlier report (14), and we have no explanation for the discrepancy. There is still clearly a need to establish a suitable test procedure for surveying non-narcotic type analgetics.

REFERENCES

(1) Courvoisier, S., Ducrot, R., Fournel, J., and Julou, L., *Compt. Rend. Soc. Biol.*, **151**, 2, 1387(1958).
(2) Sigwald, J., Henne, M., Bouttier, D., Raymondeaud, C., and Quetin, A., *Presse Med.*, **64**, 2011(1956).
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(4) DeKornfeld, T. J., Pearson, J. W., and Lasagna, L., *New Engl. J. Med.*, **270**, 391(1964).
(5) Allgén, L. G., Hellström, L., and Sant'Orp, C. J., *Acta Psychiat. Neurol. Scand., Suppl.*, **169**, 366(1963).
(6) Salzman, N. P., and Brodie, B. B., *J. Pharmacol. Exptl. Therap.*, **118**, 46(1956).
(7) Craig, L. C., and Craig, D., in "Technique of Organic Chemistry," Weissberger, A. A., ed., Interscience Publishers, Inc., New York, N. Y., p. 171.
(8) Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, **158**, 705(1945).
(9) Way, E. L., and Bennett, B. M., *ibid.*, **192**, 335(1951).
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(11) Dunham, N. W., and Miya, T. S., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 208(1957).
(12) Forrest, I. S., and Forrest, F. M., *Clin. Chem.*, **6**, 11 (1960).
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(15) Way, E. L., and Adler, T. K., "The Biologic Disposition of Morphine and Its Surrogates," World Health Organization, Geneva, Switzerland, 1962, pp. 90–92.

Effect of γ Radiation on Selected Pharmaceuticals

By LAWRENCE J. RASERO, JR.*, and DONALD M. SKAUEN

Aqueous solutions of chlorobutanol, theophylline, and sodium carboxymethylcellulose (CMC) were irradiated with γ radiation from a ^{60}Co source. Solutions were irradiated in glass and polyethylene containers. Chlorobutanol solutions were analyzed for appearance, pH changes, chlorobutanol degradation, and production of chlorine, acetone, and hydrogen peroxide. Theophylline solutions were analyzed for appearance, pH changes, theophylline degradation, and production of hydrogen peroxide. CMC solutions were analyzed for viscosity changes. All systems showed pronounced changes, even at low radiation levels.

THE ABILITY of γ radiation to destroy microorganisms without an appreciable temperature rise in the substrate offers an attractive means of sterilizing pharmaceuticals. The possibility of side effects must be considered, however, as γ radiation has the capacity to create highly reactive chemical species in any matter through which it passes. This can lead to an alteration of the physical properties of materials and to the production of numerous chemical changes.

Willis (1) and Proctor and Goldblith (2) have published review articles on the effects of radiation on pharmaceuticals. Although much valuable information has appeared in the literature, the majority of the published reports are confined

Received October 18, 1966, from the Pharmacy Research Institute, University of Connecticut, Storrs, CT 06268
Accepted for publication February 6, 1967.
This work was supported in part by funds from the Connecticut Research Foundation.
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REFERENCES

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to the study of the active ingredients in the system, and usually only indicate which products may or may not be successfully radiation sterilized. Reports concerning the effects of radiation on pharmaceutical adjuncts, as for example, preservatives or suspending agents, are few. Detailed studies showing degradation curves and degradation products are also lacking.

Few reports have been published which investigate the effects of the type container used in the systems studied. Hills and Johnson (3) have shown that the type container used in radiation studies may influence the final result. For irradiation of distilled water with γ radiation, they reported that the samples became more acid, an effect which was greater in polyethylene containers than in glass. They also noted that with dextrose and fructose solutions the coloration produced was more pronounced for the solutions packaged in glass containers.

The object of this investigation was to study the effect of γ radiation from a ^{60}Co source on aqueous solutions of chlorobutanol, theophylline, and sodium carboxymethylcellulose packaged in glass and polyethylene containers.

EXPERIMENTAL

Equipment—The irradiation facility used in this investigation was a ^{60}Co source.[1] A calibration certificate supplied by the company indicated a dose rate of 5.5×10^5 rad/hr. on May 17, 1962. The measuring technique was by ferrous chemical dosimetry. A check of the calibration was made using the ASTM Tentative Method of Test for Absorbed Gamma Radiation Dose in the Fricke Dosimeter (4).

A Beckman DU,[2] model 2400, spectrophotometer with attached Beckman DU power supply was used for colorimetric and ultraviolet analysis.

A Will 80–100 sec. 2-ml. Ostwald viscometer[3] was used for all viscosity determinations, except the 2% sodium carboxymethylcellulose (CMC) solution. For this CMC solution, the viscometer employed was a Haake Rotovisco,[4] with an attached Haake Ultra-Thermostat, type NBS.

A Beckman Zeromatic pH meter,[2] number 9500, with standard electrodes was used for all pH measurements.

Containers—Two types of containers were used in this investigation. One was a 4-oz., 0.95 d., amber polyethylene container.[5] The second type was a 4-oz. amber glass container.[6] Screw type lined, plastic caps were used for both containers.

PROCEDURES

The solutions were prepared, packaged in glass and polyethylene containers, irradiated for varying lengths of time, and analyzed within 24 hr. Irradiation was performed at room temperature. Air was not evacuated from the containers.

Analysis of chlorobutanol was by the colorimetric method of Rehm and Mader (5). Hydrogen peroxide was determined by the colorimetric method of Eisenberg (6). Acetone produced in chlorobutanol solutions was determined by the method of Greenberg and Lester (7). Chlorine was determined with *o*-tolidine reagent using the procedure described by Boltz (8). Theophylline was determined by the spectrophotometric method of Comer and Hiltz (9).

All viscosity determinations were made at 100° F. For the Ostwald viscometer 4-ml. aliquots of the solution were pipetted into the lower bulb of the viscometer, and allowed to stand 15 min. for the temperature to equilibrate. The liquid was then drawn into the second bulb and to a position about 5 mm. above the first timing mark. The time required for the meniscus to pass from the first timing mark to the second was then measured with a stopwatch.

The Haake viscometer was used to follow the change in the characteristic flow curve of the 2% CMC solution. The measurements were made at 100° F., using the MV-11 cup and bob system.

The increase in the viscosity of a polymer solution over that of the pure solvent can be expressed as specific viscosity (10)

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0}$$

where η = viscosity of the solution,
η_0 = viscosity of the pure solvent,
η_{sp} = specific viscosity.

If η_{sp}/concentration is plotted against concentration, the point of intercept at zero concentration is known as the intrinsic viscosity $[\eta]$. The intrinsic viscosity is related to the molecular weight by two constants (K and a) in the equation $[\eta] = KM^a$ (11), so any change in the intrinsic viscosity should be indicative of a change in the molecular weight.

For the molecular weight changes, a 0.25% solution of sodium CMC was irradiated for 0, 5, 10, and 20 min. Each sample was then diluted 5, 10, 50, and 100 times, and the viscosities were determined with the Ostwald viscometer, as described previously. The η_{sp} was then calculated and plots were made of η_{sp}/C *versus* C.

RESULTS AND DISCUSSION

Chlorobutanol Solutions—Visual observation of the chlorobutanol solutions showed the development of an amorphous, yellow precipitate during irradiation. No attempt was made to identify the composition of the precipitate.

Analysis for chlorine showed that no chlorine was produced in a 0.5% chlorobutanol solution irradiated up to 6.8×10^6 rads.

Measurement of pH changes showed that irradiated chlorobutanol solutions suffered severe drops in pH after very short periods of irradiation. Table I presents the change in pH of a 0.5% chlorobutanol solution. It can be seen that a dose of only 6.1×10^3 rads causes a pH drop of more than 1 unit. Measurements of irradiated distilled water

[1] Gammacell 200. Atomic Energy of Canada Limited, Commercial Products Division, Ottawa, Ontario, Canada.
[2] Beckman Instruments, Inc., Fullerton, Calif.
[3] Will Scientific, Inc., New York, N. Y.
[4] Brinkmann Instruments, Inc., Great Neck, N. Y.
[5] Monsanto Co., Packaging Division, Hartford, Conn.
[6] Armstrong Co., Lancaster, Pa.

showed no change in pH over the same dose range as the chlorobutanol solution. This indicates that the drop in pH is due to the degradation of the chlorobutanol. Similar pH drops have been reported by others for heated chlorobutanol solutions (12, 13).

TABLE I—pH OF 0.5% CHLOROBUTANOL SOLUTION

Total Dose, rads	pH
0	5.78
6.1×10^3	4.55
3.1×10^4	3.40
9.1×10^4	2.90
1.8×10^5	2.68
3.7×10^5	2.37

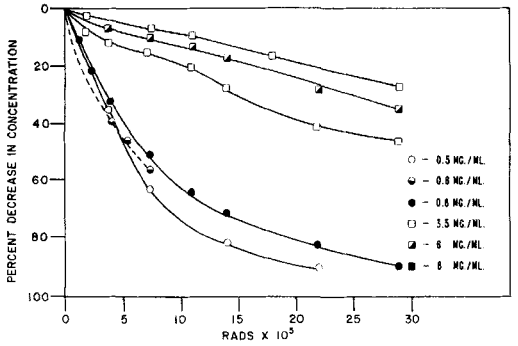


Fig. 1—Per cent decrease of chlorobutanol solutions.

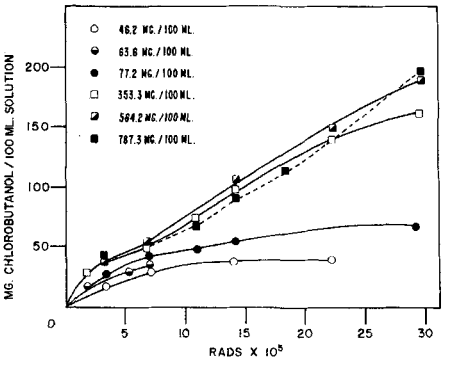


Fig. 2—Milligram loss curve for chlorobutanol solution.

Figure 1 shows the per cent decrease in irradiated chlorobutanol solutions, and Fig. 2 presents the total milligram loss for the same solutions. Figure 1 shows that those solutions containing a higher concentration of chlorobutanol have less per cent decomposition, and this appears to indicate that the more concentrated solutions are more stable to γ radiation. If, however, the total milligram loss is plotted for each solution of chlorobutanol, it is found that the total milligram loss at first increases as the concentration increases, until a limiting factor is reached. These results support the indirect action theory which states that in dilute aqueous solutions the major effect of radiation is on the water molecules, and the degradation of the solution is due to a secondary reaction. Since the number of water molecules remains relatively constant, the same amount of chlorobutanol should be degraded in each case. The apparent discrepancy which occurs in the chlorobutanol solutions of low concentration may be due to the fact that a large percentage of the chlorobutanol is degraded, and this in turn limits the total milligram loss.

The production of hydrogen peroxide in chlorobutanol solutions presents a most unusual result (Fig. 3). The data show that more concentrated solutions produce higher levels of hydrogen peroxide. However, in every instance the level of hydrogen peroxide increases rapidly, reaches a peak, and then suffers a reduction in concentration. The point of maximum hydrogen peroxide concentration occurs at about the time the precipitate becomes evident. One possible explanation for this unusual result is that the peroxide may be used up during formation of the insoluble material. It is impossible, however, to suggest appropriate mechanisms without knowing all the degradation products produced during irradiation.

Analysis of the data for acetone production (Fig. 4) shows that as the concentration of chlorobutanol increases, so does the level of acetone. The amount of acetone produced is less than would be expected if each mole of chlorobutanol degraded formed 1 mole of acetone.

For all the assay procedures used, there was no difference in results due to the container.

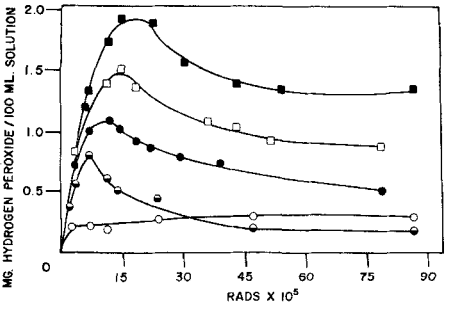


Fig. 3—Hydrogen peroxide levels of chlorobutanol solutions and distilled water. Key: ○, distilled water; ◒, 0.5 mg./ml.; ●, 1.5 mg./ml.; □, 2.5 mg./ml.; ■, 5.0 mg./ml.

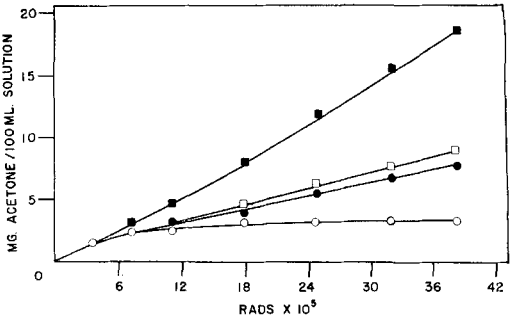


Fig. 4—Acetone levels of chlorobutanol solutions. Key: ○, 0.5 mg./ml.; ●, 1.5 mg./ml.; □, 2.5 mg./ml.; ■, 5.0 mg./ml.

TABLE II—pH OF THEOPHYLLINE SOLUTION (0.1 Gm./L.)

Total Dose, rads	pH
0	6.50
8.8×10^4	6.45
1.8×10^5	6.40
2.6×10^5	6.50
3.5×10^5	6.50
5.3×10^5	6.50
7.0×10^5	6.50
1.1×10^6	6.50
1.4×10^6	6.48
2.1×10^6	6.55

TABLE III—pH OF THEOPHYLLINE SOLUTION (5.0 Gm./L.)

Total Dose, rads	pH
0	6.58
3.9×10^6	6.40
7.7×10^6	6.39
1.2×10^7	6.42
1.5×10^7	6.49
2.0×10^7	6.40

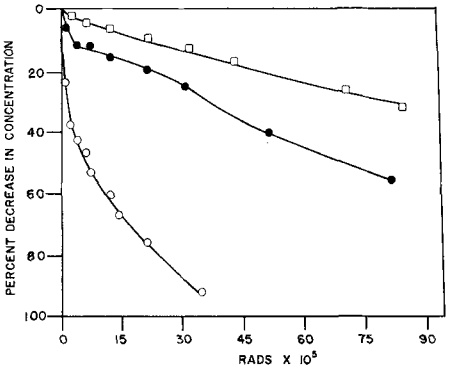


Fig. 5—Per cent decrease curve of theophylline solution. Key: ○, 0.1 Gm./L.; ●, 0.5 Gm./L.; □, 1.0 Gm./L.

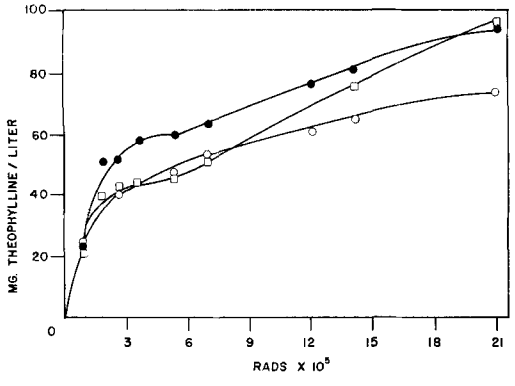


Fig. 6—Milligram loss curve for theophylline solutions. Key: ○, 0.1 Gm./L.; ●, 0.5 Gm./L.; □, 1.0 Gm./L.

Theophylline Solutions—Visual observations of theophylline solutions showed that irradiation causes a slight yellowing to develop in the solution. This yellow tint was more pronounced the higher the concentration of the solution, and the longer the irradiation time.

Unlike chlorobutanol solutions, the pH of theophylline solutions remain relatively constant, even after prolonged periods of irradiation (Tables II and III).

The degradation of theophylline solutions is very similar to chlorobutanol solutions (Figs. 5 and 6). The more concentrated solutions have less degradation, on a per cent basis, than the more dilute solutions. But, once again, it may be seen that the milligram loss for all solutions remains relatively constant. This, as explained previously, is an indication of an indirect effect on the theophylline molecule.

The curves for hydrogen peroxide levels (Fig. 7) are of a much simpler nature than those for chlorobutanol solutions. For more concentrated solutions of theophylline, there is a higher production

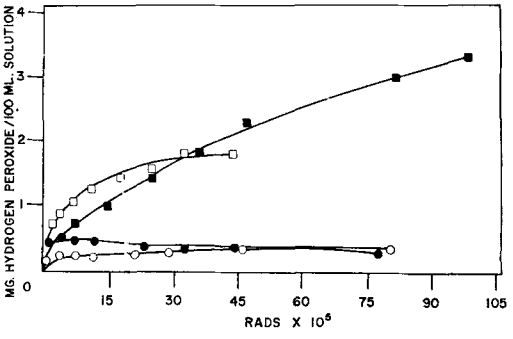


Fig. 7—Hydrogen peroxide levels of theophylline solutions. Key: ○, distilled water; ●, 0.1 Gm./L.; □, 0.5 Gm./L.; ■, 5.0 Gm./L.

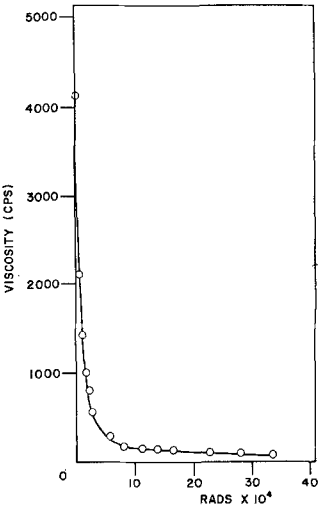


Fig. 8—Viscosity decrease of 0.5% sodium carboxymethylcellulose at 100° F.

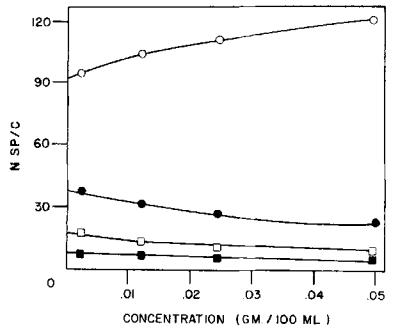


Fig. 9—Effect of irradiation on η_{sp}/C versus C curves for 0.25% sodium carboxymethylcellulose at 100° F. Key: ○, control; ●, 2.8 × 10⁴ rads; □, 5.7 × 10⁴ rads; ■, 1.1 × 10⁵ rads.

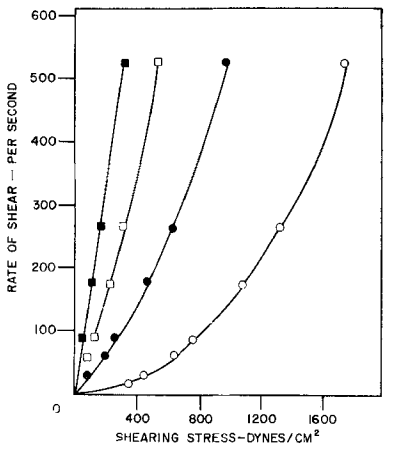


Fig. 10—Effect of irradiation on flow curves of 2% sodium carboxymethylcellulose at 100° F. Key: ○, control; ●, 2.8 × 10⁴ rads; □, 5.7 × 10⁴ rads; ■, 8.5 × 10⁴ rads.

of hydrogen peroxide, but there is no decrease in the level after a certain point, as was the case with chlorobutanol solutions.

There was no difference in effects due to the containers used for the theophylline solutions.

Sodium Carboxymethylcellulose Solutions (CMC)—Ostwald viscometer measurements of 0.125%, 0.25%, and 0.5% CMC solutions indicated that irradiation with γ rays causes a severe and immediate drop in viscosity. As little as 5.7 × 10^3 rads caused a decrease in viscosity of 48 to 66% for the solutions. Figure 8 presents the decrease for the 0.5% solution. The curves for the 0.125% and 0.25% solutions were very similar.

The results of intrinsic viscosity determination (Fig. 9) indicate a profound reduction in molecular weight. As discussed previously, the intrinsic viscosity is related to the average molecular weight.

Analysis of the total flow curve for a 2% CMC solution shows that irradiation causes the curve to change from a pseudoplastic flow curve to a nearly Newtonian system, after a dose of 8.5 × 10^4 rads (Fig. 10). This loss of pseudoplastic behavior is another indication of the reduction in length of the polymer chain which occurs during irradiation.

No container effect was noted for the CMC solutions.

CONCLUSIONS

Gamma irradiation of aqueous solutions of chlorobutanol, theophylline, and sodium carboxymethylcellulose caused severe degradation in all three systems.

These results indicate that γ irradiation is not a suitable method for sterilizing aqueous solutions of these products.

REFERENCES

(1) Willis, P. A., *Australasian J. Pharm., Suppl. 5*, **44**, S-50(1963).

(2) Proctor, B. E., and Goldblith, S. A., U. S. Atomic Energy Commission Report NYO-3337, 1952.

(3) Hills, P. R., and Johnson, R. A., United Kingdom Atomic Energy Authority Research Group Report AERE-R3750, 1961.

(4) *ASTM Standards, Part 11*, **1961**, 1326.

(5) Rehm, C. R., and Mader, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 621(1957).

(6) Eisenberg, G. M., *Ind. Eng. Chem., Anal. Ed.*, **15**, 327(1943).

(7) Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, **154**, 178(1944).

(8) Boltz, D. F., "Colorimetric Determination of Non-Metals," Interscience Publishers, John Wiley & Sons, New York, N. Y., 1958, p. 162.

(9) Comer, J. P., and Hiltz, W. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 287(1954).

(10) Martin, A. N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, p. 535.

(11) *Ibid.*, p. 536.

(12) Taub, A., and Luckey, W. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **32**, 28(1943).

(13) Nair, A. D., "The Kinetics of Degradation of Chlorobutanol," Thesis, College of Pharmacy, Graduate College, State University of Iowa, Ames, Iowa, 1958.

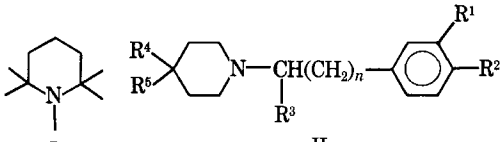
Preparation of Some *N*-Benzylpiperidines

By JOSEPH SAM and DOROTHY M. NOBLES*

A number of *N*-(*p*-substituted benzoyl)piperidines was prepared. The reduction of the amides with lithium aluminum hydride provided the corresponding *N*-(*p*-substituted benzyl)piperidines. Isobutyric anhydride and 3,4,5-trimethoxybenzoyl chloride were used to prepare several esters of *N*-(*p*-hydroxybenzyl)piperidines. Several of the compounds were tested in mice for their general behavioral effects and in dogs for pharmacodynamic activity.

EXTENSIVE INVESTIGATIONS have been conducted in a search for hypotensive agents and a number of useful compounds have been produced (1).

The need for agents with greater safety characteristics and different limits of tolerance prompted an investigation into the preparation of some *N*-benzylpiperidines (III). The hypotensive properties exhibited by 1,2,2,6,6-pentamethylpiperidine (I) and related compounds (2), and the observations of Somers and Handley (3) that 1-aralkyl-4,4-dialkylpiperidines (II) possess substantial hypotensive activity, added impetus to this work.



I

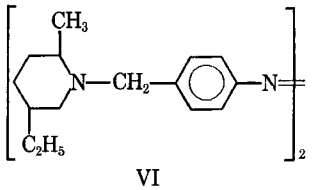
II

DISCUSSION

The synthesis of *N*-(*p*-substituted benzyl)piperidines (III) was accomplished primarily *via* reduction of the corresponding benzoylpiperidines (IV). (Scheme I.)

The intermediate *N*-(*p*-substituted benzoyl)-piperidines (IV) (Table I) were prepared by the reaction of a substituted benzoyl chloride with a substituted piperidine.

The reduction of IV with lithium aluminum hydride, with one exception, provided the corresponding benzyl derivatives (III) (Table II). The reduction of *N*-(*p*-nitrobenzoyl)-2-methyl-5-ethylpiperidine yielded the azo derivative (VI). The formation of azo compounds *via* lithium aluminum hydride reduction of nitro groups is not uncommon (4). The *N*-(*p*-aminobenzyl)-2-methyl-5-ethylpiperidine is obtained if, preceding lithium hydride reduction, the nitro group is reduced with palladium-carbon.



VI

The synthesis of *N*-(*p*-hydroxybenzyl)piperidines (Table II) ultimately was effected by the following methods: (*a*) demethylation of *N*-(*p*-methoxybenzyl)piperidines, (*b*) debenzylation of *N*-(*p*-benzyloxybenzyl)piperidines, (*c*) utilization of a modified Wallach reaction, and (*d*) reduction of *N*-(*p*-acetoxybenzoyl)piperidines.

Demethylation was accomplished using refluxing hydriodic acid in glacial acetic acid (5) and debenzylation was performed by hydrogenolysis with palladium-carbon.

A modified Wallach reaction (6) was utilized for the preparation of *N*-(*p*-hydroxybenzyl)piperidine. The maximum yield, however, never exceeded 10%. Increasing the length of reaction time did not increase the yield.

The *N*-(*p*-acetoxybenzoyl)piperidines were prepared by condensing *p*-acetoxybenzoyl chloride with an appropriately substituted piperidine and were employed without purification in subsequent lithium aluminum hydride reductions.

The esterification of the *N*-(*p*-hydroxybenzyl)-piperidines to V was accomplished using isobutyric anhydride and 3,4,5-trimethoxybenzoyl chloride. The esters of both of these acids are prevalent in the veratrum series (7). Table III lists the esters prepared.

Preliminary studies in mice indicated that the amides (Table I) in general exhibit weak central nervous system (CNS) depressant properties. Cheymol and associates (8) noted hypnotic properties of *o*-(piperidinothiocarbonyl)phenol. The amines (Table II) in general exhibited weak CNS stimulant activity. Preliminary studies of the esters (Table III) in dogs showed transient depressor responses.

EXPERIMENTAL[1]

p-Benzyloxybenzoyl chloride (9), *p*-acetoxybenzoyl chloride (10), and *p*-nitrobenzoyl chloride (11) were prepared according to reported procedures. Commercially available anisoyl chloride was utilized.

N-(p-Substituted Benzoyl)piperidines (Table I)—*Method A*—A cooled solution of 0.07 mole of the

Received February 6, 1967, from the Department of Pharmaceutical Chemistry, University of Mississippi, University, MS 38677

Accepted for publication March 14, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by Dorothy M. Nobles to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.

Supported in part by the Mississippi Heart Association and McNeil Laboratories, Fort Washington, Pa.

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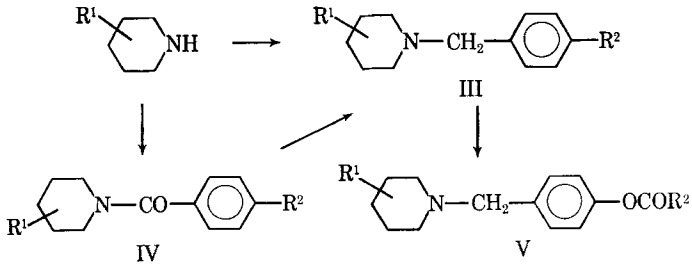
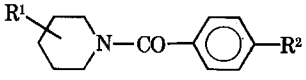
[1] All melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Infrared data were obtained on all compounds with a Perkin-Elmer model 137 G Infracord spectrophotometer using KBr pellets.



Scheme I

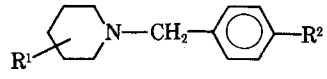
TABLE I—*N*-(*p*-SUBSTITUTED BENZOYL)PIPERIDINES



R_1	R_2	Method	M.p., °C. or B.p., °C./mm.	Yield, %	Molecular Formula	Anal., % Calcd. C	H	N	Found C	H	N
2-CH_3-5-C_2H_5	OCH_3	*A*	140–142/0.05	86	$C_{16}H_{23}NO_2$	74.0	8.7	5.3	73.9	8.9	5.7
2,6-di-CH_3	OCH_3	*A*	140–142/0.05	62	$C_{15}H_{21}NO_2$	72.9	8.5	5.7	72.7	8.5	5.5
2-CH_3	OCH_3	*A*	53–54[a]	24	$C_{14}H_{19}NO_2$	72.1	8.2	6.0	72.3	8.3	6.1
3-CH_3	OCH_3	*A*	52–53[a]	60	$C_{14}H_{19}NO_2$	72.1	8.2	6.0	72.0	8.0	6.0
4-CH_3	OCH_3	*A*	54–55.5[a]	90	$C_{14}H_{19}NO_2$	72.1	8.2	6.0	72.4	8.1	6.2
4-CH_3	$OCH_2C_6H_5$	*A*	105–107[b]	50	$C_{20}H_{23}NO_2$	77.7	7.4	4.5	77.8	7.4	4.6
2-CH_3-5-C_2H_5	NO_2	*B*	185/0.75	86	$C_{15}H_{20}N_2O_3$	65.2	7.3	10.1	65.3	7.3	11.0
2-CH_3-5-C_2H_5	NH_2	*C*	183–184.5[b]	96	$C_{15}H_{22}N_2O$	73.2	8.9	11.4	73.1	8.8	11.4
2-CH_3	OH	*D*	213–213.5[c]	60	$C_{13}H_{17}NO_2$	71.2	7.8	6.4	71.4	7.7	6.3

[a] Recrystallized from petroleum ether (30–60°). [b] Recrystallized from ethanol–water. [c] Recrystallized from ethanol.

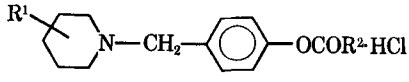
TABLE II—*N*-(*p*-SUBSTITUTED BENZYL)PIPERIDINES



R_1	R_2	Method	M.p., °C.	Recrystn. Solvent[a]	Yield, %	Molecular Formula	Anal., % Calcd. C	H	N	Found C	H	N
H	OH	*H, I*	254–255	Et–W	98, 5	$C_{12}H_{18}ClNO$[b]	63.3	8.0	6.1	63.2	8.3	6.2
2-CH_3	OH	*F, I*	191–193	Et–E	60, 67	$C_{13}H_{20}ClNO$[b]	64.7	8.3	5.8	64.5	8.3	5.8
3-CH_3	OH	*I*	163–165	A–E	53	$C_{13}H_{20}ClNO$[b]	64.6	8.3	5.8	64.5	8.4	5.8
4-CH_3	OH	*I, G*	171–172	A–M	75, 42	$C_{13}H_{20}ClNO$[b]	64.6	8.3	5.8	64.3	8.4	6.0
2-CH_3-5-C_2H_5	OH	*I*	191–193.5	A–M	75	$C_{15}H_{24}ClNO$[b]	66.9	9.0	5.2	66.1	8.8	5.5
2,6-diCH_3	OH	*I*	173–174	B	60	$C_{14}H_{22}ClNO$[b]	65.9	8.6	5.4	65.7	8.8	5.6
2-CH_3-5-C_2H_5	OCH_3	[c]	141/0.75 mm.[d]		23	$C_{16}H_{25}NO$	77.7	10.1	5.6	77.9	9.8	5.7
2-CH_3-5-C_2H_5	NH_2	[c]	113–114.5	Et–W	86	$C_{15}H_{24}N_2$	77.6	10.3	12.1	77.2	10.2	12.7
2-CH_3-5-C_2H_5	N=NC$_6$H$_4$-*p*-CH_2—R^3[e]	*E*[f]	82–84	M	33	$C_{30}H_{44}N_4$	78.3	9.6	12.2	78.2	9.6	12.2

[a] Et, ethanol; W, water; A, acetone; E, ether; M, methanol; B, 2-butanone. [b] Hydrochloride. [c] Prepared from the corresponding amide utilizing the procedure described by Sommers (12). [d] Boiling point. [e] R^3 = 2-ethyl-6-methylpiperidine. [f] From *N*-(*p*-nitrobenzoyl)-2-methyl-5-ethylpiperidine.

TABLE III—*N*-(*p*-ACYLOXYBENZYL)PIPERIDINES



R_1	R_2	Method	M.p., °C.	Recryst. Solvent[a]	Yield, %	Molecular Formula	Anal., % Calcd. C	H	N	Found C	H	N
H	$(CH_3)_2CH$	*J*	227–228.5	A–E	58	$C_{16}H_{24}ClNO_2$	64.6	8.1	4.7	64.5	8.1	4.7
2-Me	$(CH_3)_2CH$	*J*	179.5–180	B–E	95	$C_{17}H_{26}ClNO_2$	65.6	8.4	4.5	65.4	8.2	4.5
3-Me	$(CH_3)_2CH$	*J*	176–177	Ea	66	$C_{17}H_{26}ClNO_2$	65.6	8.4	4.5	65.6	8.5	4.6
4-Me	$(CH_3)_2CH$	*J*	202.5–203	Ea	93	$C_{17}H_{26}ClNO_2$	65.6	8.4	4.5	65.4	8.4	4.3
4-Me	3,4,5-triCH_3OC$_6$H$_2$	*K*	217–218	A–E	91	$C_{23}H_{30}ClNO_5$	63.4	6.9	3.2	62.8	6.6	3.4

[a] A, acetone; E, ether; B, benzene; Ea, ethyl acetate.

appropriate piperidine in 50 ml. of anhydrous ether was treated dropwise with 0.03 mole of anisoyl or *p*-benzyloxybenzoyl chloride. The reaction mixture was allowed to stand 15 min. and then treated with 25 ml. of ice water. The ether solution was washed with two 50-ml. portions of 10% sodium bicarbonate and two 50-ml. portions of 10% hydrochloric acid, respectively. The ether was evaporated and the residual material either distilled or recrystallized from a suitable solvent.

In the case of the *N*-(*p*-acetoxybenzoyl)piperidines, the final ethereal layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residual product was used in subsequent reduction reactions.

Method B—To a solution of 38 Gm. (0.3 mole) of 2-methyl-5-ethyl piperidine in 150 ml. of pyridine was added slowly 28 Gm. (0.18 mole) of *p*-nitrobenzoyl chloride. The reaction mixture was heated 45 min. on a steam bath. The solution was cooled and then washed with two 50-ml. portions of 10% sodium bicarbonate and two 50-ml. portions of 10% hydrochloric acid, respectively. The residual oil was extracted with ether, dried, and then distilled.

Method C—A solution of 18.6 Gm. (0.07 mole) of *N*-(*p*-nitrobenzoyl)-2-methyl-5-ethyl piperidine in 50 ml. of ethanol was hydrogenated with 10% palladium-carbon at 40 p.s.i. for 2 hr. The product was isolated in the usual manner.

Method D—To 7 Gm. (0.03 mole) of *N*-(*p*-methoxybenzoyl)-2-methyl piperidine was added 100 ml. of glacial acetic acid and 100 ml. of 57% hydriodic acid. The mixture was refluxed 6 hr., cooled, and treated with 100 ml. of water. The solution was decolorized with sodium bisulfite and extracted with two 50-ml. portions of ether. The ether was extracted with 50 ml. of 10% sodium hydroxide. The aqueous layer was separated and acidified with 10% hydrochloric acid. The acid solution was extracted with 50 ml. of ether. Evaporation of the ether yielded the product.

N-(p-Substituted Benzyl)piperidines (Table II)—*Method E*—The procedure outlined by Sommers (12) for lithium aluminum hydride reductions was modified and used. A stirred slurry of 18.2 Gm. (0.4 mole) of lithium aluminum hydride and 100 ml. of anhydrous tetrahydrofuran was treated dropwise with 0.1 mole of the amide in 50 ml. of anhydrous tetrahydrofuran. The mixture was stirred and refluxed 24 hr., cooled, and cautiously decomposed with 200 ml. of ethyl acetate. The reaction mixture was filtered and the solvent distilled. The residual oil was converted to the hydrochloride in the usual manner and recrystallized from the appropriate solvent.

Method F—To a solution of 20 ml. of 57% hydriodic acid in 20 ml. of glacial acetic acid was added 4.3 Gm. (0.02 mole) of *N*-(*p*-methoxybenzyl)-2-methylpiperidine and the whole refluxed 6 hr. The cooled solution was treated with 100 ml. of water, made alkaline with solid sodium bicarbonate, and extracted with ether. The ether layer was dried over anhydrous magnesium sulfate. The hydrochloride was prepared in the usual manner.

Method G—A solution of 29.5 Gm. (0.1 mole) of *N*-(*p*-benzyloxybenzyl)-4-methylpiperidine in 90% ethanol was hydrogenated with 10% palladium-carbon catalyst at 42 p.s.i. for 12 hr. The catalyst was removed by filtration and the ethanol distilled under reduced pressure. The oil was extracted with 50 ml. of 10% sodium hydroxide; the latter was washed with ether and then neutralized with solid carbon dioxide. The product was extracted with ether and converted to the hydrochloride in the usual manner.

Method H—Following a modified procedure of Staple and Wagner (6), 16 Gm. (0.2 mole) of piperidine and 4.6 Gm. (0.1 mole) of formic acid (90%) were heated on a steam bath for 30 min. The mixture was cooled, treated with 12.2 Gm. (0.1 mole) of *p*-hydroxybenzaldehyde, and thereafter heated in an oil bath at 110–120° for 14 hr. The mixture was cooled, treated with 100 ml. of 10% sodium bicarbonate, and extracted with ether. The hydrochloride was prepared in the usual manner.

Method I—The *N*-(*p*-acetoxybenzoyl)piperidines (prepared according to method *A*) were reduced with lithium aluminum hydride according to the procedure outlined in method *E* to *N*-(*p*-hydroxybenzyl)piperidines.

Esters of N-(p-Hydroxybenzyl)piperidine (Table III)—*Method J*—The procedure outlined by Behrend (13) and Hudson and Dale (14) was followed. A solution of 0.004 mole of the requisite *N*-(*p*-hydroxybenzyl)piperidine hydrochloride in 20 ml. of pyridine was treated with 3.2 Gm. (0.02 mole) of isobutyric anhydride and stirred 12 hr. The pyridine was distilled under reduced pressure and the residue was recrystallized from an appropriate solvent.

Method K—A modified procedure of Counsell and Soine (15) was employed. A solution of 1 Gm. (0.005 mole) of *N*-(*p*-hydroxybenzyl)-4-methylpiperidine in 25 ml. of anhydrous benzene was treated with 2.3 Gm. (0.01 mole) of commercial 3,4,5-trimethoxybenzoyl chloride and 2 Gm. (0.02 mole) of triethylamine. The mixture was refluxed 12 hr., cooled, and washed with 10% sodium hydroxide and water, respectively. The product was isolated in the usual manner and converted to a hydrochloride.

REFERENCES

(1) Page, I. H., *Clin. Pharmacol. Therap.*, **7**, 567(1966).

(2) Spinks, A., and Young, E. H. P., *Nature*, **181**, 1397 (1958); Lee, G. E., Wragg, W. R., Corne, S. J., Edge, N. D., and Reading, H. W., *ibid.*, **181**, 1717(1958); Bretherick, L., Lee, G. E., Hunt, E., Wragg, W. R., and Edge, N. D., *ibid.*, **184**, 1707(1959); Lutz, W. B., Lazarus, S., and Meltzer, R. I., *J. Org. Chem.*, **27**, 1695(1962); Robertson, J. E., Biel, J. H., and DiPierro, R., *J. Med. Chem.*, **6**, 381(1963).

(3) Somers, T. C., and Handley, G. J., *J. Med. Chem.*, **7**, 784(1964).

(4) Brown, W. G., *Organic Reactions*, **6**, 471(1951).

(5) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," 4th ed., John Wiley & Sons, Inc., New York, N. Y., 1959, p. 200.

(6) Staple, E., and Wagner, E. C., *J. Org. Chem.*, **14**, 559(1949).

(7) Krayer, O., and Acheson, G. H., *Physiol. Rev.*, **26** 383(1946).

(8) Cheymol, J., Giudicelli, R., Glasson, B., Chabrier, P., and Renard, S. H., *Therapie*, **8**, 237(1953); through *Chem. Abstr.*, **48**, 4702(1954).

(9) Cavillito, C. J., and Buck, J. S., *J. Am. Chem. Soc.*, **65**, 2140(1943).

(10) Kaufmann, A., and Luterbacher, A., *Ber.*, **42**, 3483 (1909).

(11) Vogel, A. I., "A Textbook of Practical Organic Chemistry," 3rd ed., John Wiley & Sons, Inc., New York, N. Y., 1956, p. 791.

(12) Sommers, A. H., Freifelder, M., Wright, H. B., and Weston, A. W., *J. Am. Chem. Soc.*, **75**, 57(1953).

(13) Behrend, R., and Roth, P., *Ann.*, **331**, 359(1904).

(14) Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, **37**, 1264(1915).

(15) Counsell, R. E., and Soine, T. O., *J. Pharm. Sci.*, **49**, 289(1960).

Constituents from *Gymnema sylvestre* Leaves II

Nitrogenous Compounds

By JOSEPH E. SINSHEIMER and HUGH M. MCILHENNY*

The leaves of *Gymnema sylvestre* were examined for the presence of alkaloids through the establishment of quaternary and nonquaternary nitrogenous fractions. A trace amount of an alkaloidal substance was isolated from a nonphenolic nonquaternary fraction and partially characterized. The plant bases choline, betaine, and adenine were isolated and identified in a combined yield of 0.04 per cent and would appear to be primarily responsible for the positive alkaloid tests previously reported for the leaves. The amino acids leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid were also detected. Of particular interest was the isolation (0.0003 per cent of leaf) and firm identification of trimethylamine oxide which has not been previously reported in plant material.

LEAF EXTRACTS of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) have an extensive history of pharmacological activity in Indian medicine (1–5) including use as an agent for the treatment of glycosuria and diabetes. It became the objective in these laboratories to examine the chemical nature of plant constituents with potential medicinal importance and to make them available for biological evaluation. The present study describes the isolation and identification of nitrogenous compounds during an investigation of dried *G. sylvestre* leaf for the reported presence (6, 7) of alkaloids.

EXPERIMENTAL

Dried *G. sylvestre* leaf processed during this investigation was commercially supplied by Prachi Gobeson Co., Calcutta, India, and in the later stages by the Himalaya Drug Co., Bombay, India.

Melting points were obtained with the Kofler micro hot stage and are corrected unless noted. An F&M carbon hydrogen nitrogen analyzer model 180 was used to follow nitrogen enrichment in alkaloid-containing fractions and as a deductive lead to structural characterization, but not to confirm a proposed structure. Spectra were determined on Perkin-Elmer models 137B and 337 infrared, Beckman DK-2A ultraviolet, Varian A-60 NMR, and Bendix-Time-of-Flight model 14 mass spectrometers. Neutral alumina, grade 1, Woelm, and silicic acid, 100 mesh (powder) (Mallinckrodt) were used for column chromatography. Silica gel G and alumina G supports and equipment supplied by Warner-Chilcott Laboratories were used for TLC. Continuous extractions of alkaloids were performed with a rotary film liquid-liquid extractor, Rinco VE-4000-x. The adjustments of pH were followed with a Beckman Zero matic pH meter.

Separation Methods

Methanol–Ammonia Extraction—A series of individual extractions was performed with portions (2.4–2.8 Kg.) of finely ground *G. sylvestre* leaf by maceration with methanol saturated with ammonia. The total maceration period was 10 days and included two fresh solvent exchanges. The combined extract was concentrated under reduced pressure to a thick, dark concentrate and diluted with an equal volume of 5% hydrochloric acid. Evaporation to original volume precipitated plant acids which were separated by filtration. The dark filtrate was continuously extracted with chloroform for 48–96 hr. to remove residual plant acids, pigment, and other nonalkaloidal impurities. The acidic aqueous system was made alkaline to pH 11.5 with cold 5 *N* sodium hydroxide and continuously extracted with chloroform for 48 hr. The chloroform extract was washed, dried over sodium sulfate, and evaporated to a brown nitrogenous semisolid, fraction A (0.023% of leaf), as the nonquaternary nonphenolic alkaloid fraction (positive Dragendorff's test). The alkaline solution after chloroform extraction was adjusted to pH 8 with cold 10% hydrochloric acid and continuously extracted with chloroform for 48 hr. The extract was separated, washed, dried over sodium sulfate, and reduced to a semisolid nitrogenous residue, fraction B (0.013% of leaf), as the nonquaternary phenolic alkaloid fraction (positive Dragendorff's test).

Aqueous layers after chloroform extraction were combined (representing 23.58 Kg. of leaf) and processed for the recovery of quaternary alkaloids. The system was concentrated to 8.2 L. in a flash evaporator (40°), adjusted to pH 2 with cold 10% hydrochloric acid, and refrigerated for 24 hr. After separation from an insoluble residue, the dark concentrate (9 L.) was treated with a saturated aqueous solution of Reinecke salt to completion of precipitation (3 L.). The reineckates were separated by filtration after chilling 24 hr. and washed with *n*-propanol (3.3 L.) to remove impurities. To the remaining solids on the filter was added acetone until the disappearance of color in

Received January 13, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication February 20, 1967.

Abstracted in part from a dissertation submitted by Hugh M. McIlhenny in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported by grant AM 06224 from the National Institutes of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.

The authors are indebted to Liggett and Myers Tobacco Co., Durham, N. C., through Dr. P. Manni, for providing NMR and mass spectrometric data and to Mr. Lee Below and Mr. Michael Donahue for technical assistance.

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Previous paper: Manni, P. E. and Sinsheimer, J. E., *J. Pharm. Sci.*, **54**, 1541(1965).

the filtrate (2.5 L.). The reineckates in acetone solution were diluted with an equal volume of methanol and applied to a column of ion-exchange resin[1] (1075 ml., 3 cm. × 126 cm.), flow rate ≤12 ml./min. Column effluent and washes of acetone–methanol (1:1) were collected until a negative reineckate test was given (6.5 L.) and concentrated under reduced pressure to a solid fraction of quaternary bases.

The crude bases were dissolved in 2 L. of water and passed through a column of resin[2] (250 ml., 2.5 cm. × 211 cm.), flow rate 8 ml./min. Column effluent was concentrated to dryness *in vacuo* (50–60°) to give 3.1 Gm. of a tan residue as fraction C. The column[2] had assumed a dark coloration and was therefore removed and slurried batchwise with water. The dark aqueous supernatant was separated by filtration, evaporated, and dried to yield 1 Gm. of a brown residue as fraction D. The washed resin[2] was returned to the column and eluted with 1 *N* hydrochloric acid until a negative reineckate test was given in the column discharge (2 L.). Eluate was concentrated to dryness *in vacuo* (50–60°) to give tan solids as fraction E.

Aqueous Extraction—A solution resulting from a hot aqueous percolate of 403 Gm. of defatted ground leaf was applied to a column containing 500 ml. of ion-exchange resin.[3] The percolate prior to application to the column was kept under a layer of toluene. The effluent was discarded, and the resin was slurried with water to remove precipitated plant acids. Elution with 2.5 L. of 1 *N* ammonium hydroxide gave six fractions. Fractions 1–3 (1.3 L. of eluate) were reduced to dryness, extracted with hot ethanol, and filtered collectively through a 5-Gm. column of alumina. The yellow filtrate and ethanol washings were combined (200 ml.) and evaporated to a brown residue (1.2 Gm.) giving positive ninhydrin and picrate tests.

A 0.885-Gm. sample was dissolved in 50 ml. of water and freed of impurities by extraction with ether (3 × 25 ml.). To the aqueous layer was added a saturated aqueous solution of picric acid to completion of precipitation. The picrate was recovered and dissolved in 130 ml. of 0.1 *N* hydrochloric acid. Extraction with ether (5 × 65 ml.) removed picric acid and left an aqueous layer which upon repeated concentrations from water *in vacuo* (50°) gave 49.3 mg. of a yellow hydrochloride powder, fraction F.

The supernatant remaining after picrate precipitation was concentrated to 45 ml. and diluted to 50 ml. with 1 *N* hydrochloric acid. Picric acid was extracted with ether (8 × 25 ml.), and the aqueous layer was reduced to a yellow gum, fraction G.

Purification and Characterization

Nonquaternary Alkaloids—A 2.67-Gm. quantity of nonquaternary nonphenolic residue fraction A (representing 13.18 Kg. of leaf) was freed of water-soluble impurities and successively extracted with portions of 2% hydrochloric acid until reineckate spot tests were negative. The extract was treated with a saturated aqueous solution of Reinecke salt until precipitation was completed (60 ml.). After a 16-hr. refrigeration period, the reineckate derivative was filtered and recovered in 50 ml. of acetone. Regeneration of amine hydrochlorides by the classical method (8) gave a residue which was converted to free base form in methanol on a column of ion-exchange resin.[1] Collection of effluent and 80 ml. of methanol wash gave a brown residue, 0.145 Gm., as the purified nonquaternary nonphenolic alkaloid fraction (0.001% of leaf). Alumina column chromatography gave 7.5 mg. of the major component 5[4] from chloroform eluates, as a brown solid, m.p. 60–70°.

Anal. (CHN analyzer)—C, 68.01; H, 8.41; N, 7.26.

The substance gave a positive Dragendorff's test, was homogeneous by TLC on alumina in 2% methanol in benzene (blue fluorescence), and was soluble in chloroform, ethanol, methanol, benzene and ether and insoluble in water. Neither hydrochloride nor 2,4-dinitrophenylhydrazone derivatives could be prepared. A picrate, exhibiting a crystalline sublimate at 212° and m.p. 260°, indicated a molecular weight of 221 for the conjugate base by a spectrophotometric (Zeiss model PMQ II) method (9). An infrared spectrum (neat) showed $\nu_{max.}$ 3400 cm.$^{-1}$, 2950 cm.$^{-1}$, 1650 cm.$^{-1}$. The ultraviolet spectrum revealed $\lambda_{max.}^{ethanol}$, 231 mμ (*a* 39.68), 250(sh) (24.63), 281 (14.10), 288 (18.35); $\lambda_{max.}^{0.1\ N\ HCl\text{-}ethanol}$ 250 mμ (*a* 27.79), 302–303 (16.48).

A 1-mg. sample of component 5 purified by silica gel chromatography with 5% ethanol in chloroform as eluant gave an NMR spectrum which showed τ 9.10, 9.01, 8.74, 8.16, 3.15. A Varian C-1024 time-averaging computer was used to enhance the signal-to-noise ratio. A mass spectrum revealed the following peaks in order of decreasing intensity: *m/e* 28, 32, 168, 43, 41, 55, 57, 29, 18, 69, 71, 83, 14, 84.

Betaine—Fraction C was reconstituted in 25 ml. of ethanol, filtered from an insoluble residue, and washed with ethanol (3 × 5 ml.). To the solution in an ice bath was added cold concentrated hydrochloric acid, dropwise, until precipitation of base hydrochloride solids was completed (3 ml.). Solids were removed by filtration and washed with portions of ether and dried in vacuum over calcium chloride to give 1.8 Gm. of crude base hydrochloride. TLC on silica gel in methanol–28% ammonium hydroxide (75:25) showed a single zone with a modified Dragendorff's reagent (10) and was similar to a reference sample of betaine hydrochloride prepared from betaine (Nutritional Biochemical). Repeated recrystallization from 95% ethanol deposited a white crystalline solid, m.p. 264–285° dec.

Anal.—Calcd. for $C_5H_{12}ClNO_2$: C, 39.09; H, 7.87; N, 9.12. Found:[5] C, 39.37; H, 8.01; N, 8.73.

An infrared spectrum (KBr) of the substance was identical with that of reference betaine hydrochloride, m.p. 254–265° dec. The plant compound formed a picrate, m.p. 186° [lit. (11) m.p. 180–181°], and chloroaurate, m.p. 207.5–208.5° [lit. (11) m.p. 200–209°], derivatives which were identical with similarly prepared samples of reference betaine

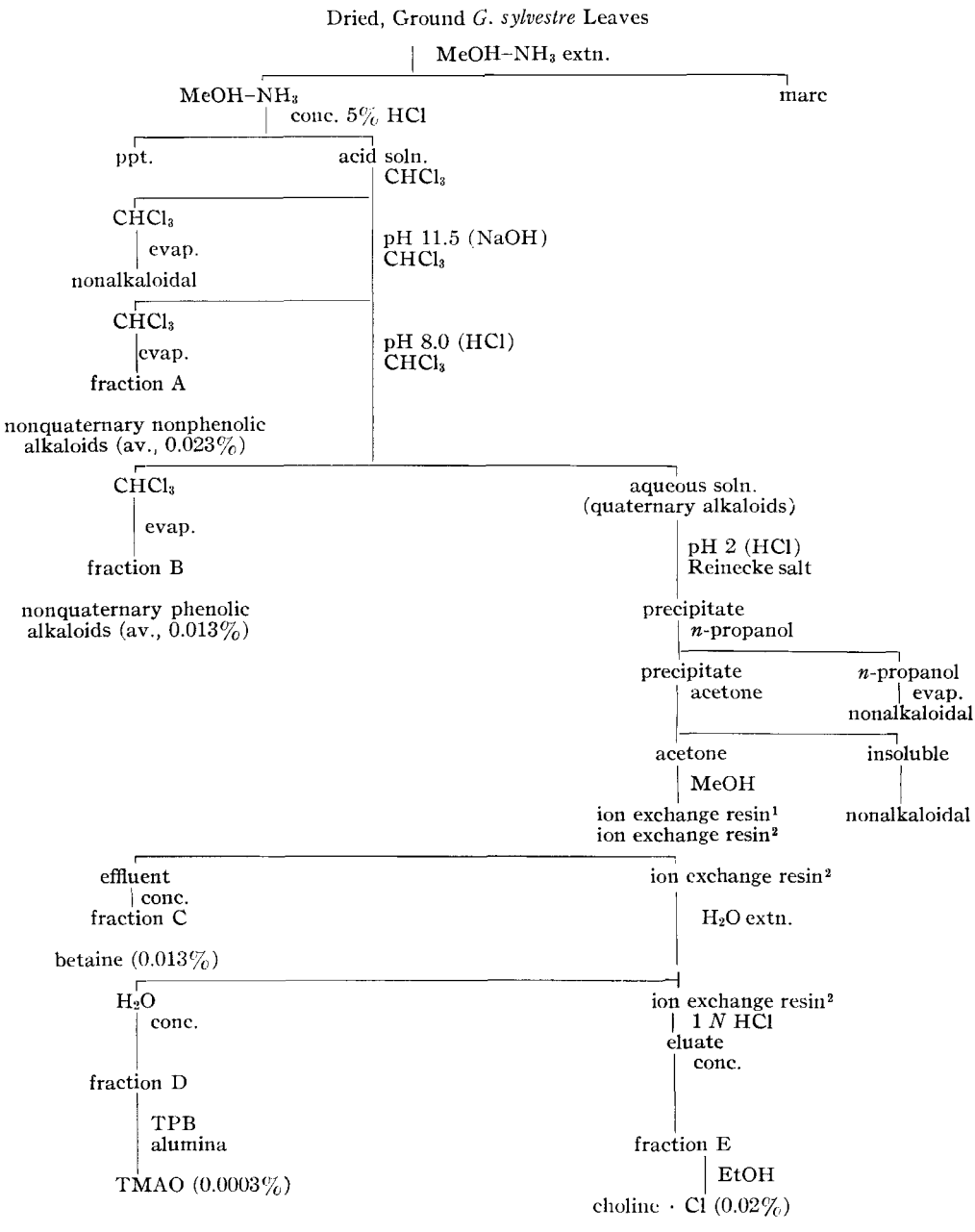
[1] Marketed as Amberlite IRA 401(OH^-) by Rohm and Haas Co., Philadelphia, Pa.

[2] Marketed as Amberlite IRC 50(H^+) by Rohm and Haas Co., Philadelphia, Pa.

[3] Marketed as Amberlite IR 120(H^+) by Rohm and Haas Co., Philadelphia, Pa.

[4] Designated component 5 according to its distribution as the fifth component from the origin on chromatograms.

[5] Performed by Micro-Tech Laboratories, Inc., Skokie, Ill.



Flow Sheet for Methanol–Ammonia Separation of Alkaloids of G. sylvestre

Scheme I

compounds, by melting point, mixed melting point, and infrared (KBr) analyses.

Trimethylamine Oxide—A 1% aqueous solution of fraction D was acidified to pH 2.5 with 5 *N* acetic acid, filtered, and treated with 70 ml. of 0.1 *M* sodium tetraphenylboron (aqueous). The precipitate was separated and converted to its chloride salts by passage through a column of ion-exchange resin.[6] Reduction of the effluent to dryness gave a residue which was fractionated by alumina column chromatography. Elution with 2% ethanol in chloroform (1.2 L.) gave 70.8 mg. of a hygroscopic white solid (0.0003% of leaf) which could be crystallized from ethanol–ether as needles, m.p. 80–81°. The addition of a 5% solution of oxalic acid dihydrate (0.6 ml.) to a 5.5% solution of the isolated compound in ethanol (0.5 ml.) gave crystals upon chilling. Recrystallization from ethanol gave needles (8.8 mg.), m.p. 119°, which showed a single peak in an NMR (D_2O) spectrum, τ 8.12.

Anal. (CHN analyzer)—C, 36.40; H, 7.05; N, 7.99.

Reference trimethylamine oxide (TMAO) di-

[6] Marketed as Amberlite IRA 401(Cl⁻) by Rohm and Haas Co., Philadelphia, Pa.

hydrate (K & K Laboratories) showed an infrared spectrum (KBr) that was superimposable with the isolated substance and gave an oxalate, which was identical with the unknown oxalate by mixed melting point, infrared (KBr), and NMR analyses.

Anal. (reference TMAO oxalate)—Calcd. for $C_3H_9NO \cdot C_2H_2O_4$: C, 36.36; H, 6.67; N, 8.48. Found:[7] C, 36.55; H, 6.80; N, 8.31.

A picrate derivative, m.p. 208.5–209.5° dec., indicated a molecular weight of 72 for the conjugate base of a monopicrate, by a spectrophotometric (Zeiss model PMQ II) method (9). This picrate derivative was subsequently shown to be identical in melting point, mixed melting point, and infrared (KBr) to reference TMAO picrate.

Residues from chromatographic fractions 4 and 5 of the aqueous extraction procedure (see under *Aqueous Extraction*) representing 1.3–2.0 L. of 1 *N* ammonium hydroxide eluate, were extracted with hot ethanol, combined, and filtered over 2 Gm. of alumina. The colorless filtrate and ethanol washings were collected (50 ml.) and evaporated to a white scale. Thin-layer chromatography on alumina G in methanol–carbon tetrachloride–acetic acid (10:30:1) revealed a zone (iodine vapors) that corresponded to that of trimethylamine oxide when developed side by side. Response to modified Dragendorff's reagent (delayed orange coloration) was the same with each sample.

Choline—Fraction E was separated from considerable inorganic chloride content by reconstituting the fraction in 200 ml. of ethanol and concentrating to a small volume. The dark supernatant was decolorized[8] and concentrated to dryness *in vacuo* (60°) which left a yellow semisolid (0.02% of leaf). A 0.500-Gm. quantity of crude base chloride was chromatographed over alumina to yield 0.211 Gm. which was recrystallized from ethanol–ether to give 0.177 Gm. of needles, m.p. gradual dec. above 250°. Paper chromatography in three solvent systems (10, 12) gave R_f values and characteristic color responses with modified Dragendorff's and Levine Chargaff reagents consistent with reference choline chloride (Eastman recrystallized). The infrared spectra (KBr) of plant base chloride and authentic choline chloride were similar. Chloroaurate derivatives of the plant compound and reference choline were identical with m.p. 230.5–232.5°[9] and by mixed melting point and infrared (KBr) analyses. The melting point of tetraphenylborate derivatives of the plant isolate, reference choline chloride, and a mixture of the two were identical at 227–229°. [Lit. (14) m.p. 219–221° uncorrected.] The infrared spectra (KBr) of these derivatives as well as a reineckate from the isolated plant base were identical with the spectra of the corresponding derivatives from reference choline chloride.

Adenine—Ether addition to a concentrated methanolic solution of fraction F afforded a flocculent precipitate which dried to 33 mg. of an off-white powder (0.008% of leaf), m.p. 280–292° dec., uncorrected. It gave a weakly positive Dragendorff's test for alkaloids, did not form a reineckate, and yielded a picrate, m.p. 284–288° dec., uncorrected, and picrolonate, m.p. 256–259°. An infrared spectrum (KBr) of the powder was similar to that of adenine·HCl·$3H_2O$. A picrate derivative, m.p. 284–288° dec., uncorrected [lit. m.p. 279–281° (15), 292° (16)], was prepared from reagent adenine sulfate (Nutritional Biochemical) and converted to adenine hydrochloride, m.p. 280–292° dec., uncorrected, in the same manner as was the amine picrate of plant origin. A reference sample of adenine picrolonate, m.p. 256–259° [lit. (15) m.p. 265°], was prepared from the hydrochloride. Mixed melting points of reference picrate and picrolonate derivatives of adenine were not depressed with the corresponding derivatives of plant origin; the infrared spectra (KBr) of reference adenine hydrochloride, picrate, and picrolonate were superimposable with the respective samples derived from the plant compound.

Amino Acids—Fraction G (positive ninhydrin and negative picrate tests) as a 0.5% aqueous solution was applied successively to two ion-exchange resin columns.[2, 10] No basic or acidic amino acids could be recovered from these columns. However, the final effluent passing through both columns was reduced to a yellow semicrystalline residue, 0.545 Gm. (0.23% of leaf), as the purified neutral amino acid fraction. The residue was examined by paper (17) and silica gel thin-layer (18) chromatography with a series of naturally occurring reference L-amino acids. Agreement of R_f values in three paper chromatographic systems and a two-dimensional thin-layer pattern with reference mixtures run in parallel and in admixture revealed the presence of leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid as the major amino acid constituents of the plant fraction.

RESULTS AND DISCUSSION

The methanol–ammonia extraction for alkaloids from *G. sylvestre* (Scheme I) gave residues of nonquaternary nitrogenous substances. After nitrogen enrichment by acid exchange resin adsorption and binary phase extraction, TLC indicated that both phenolic and nonphenolic fractions contained only small amounts of a complex mixture of alkaloid positive constituents (0.001% of leaf) and considerable nonalkaloidal impurities. Thus, interest was limited to the isolation of the major alkaloid of the nonquaternary nonphenolic fraction A. Several milligrams of the base (5×10^{-6}% of leaf) were made available by alumina column chromatography as a brown solid, m.p. 60–70°. The fluorescent substance formed a picrate from which a molecular weight of 221 was estimated for the base by a spectrophotometric method (9). A mass spectrum did not substantiate this value and showed a peak of highest mass at *m/e* 168, suggesting the presence of a stabilized fragment ion from the intact molecule. The base had ultraviolet absorption characteristics of an aniline derivative, particularly the indoline chromophore (19), but exhibited a bathochromic shift in acid and lacked the features of aromatic structure from infrared and mass spectrometric data. NMR and mass spectra

[7] Performed by Spang Microanalytical Laboratories, Ann Arbor, Mich.

[8] The decolorizing agent was Norit, American Norit Co., Jacksonville, Fla.

[9] This melting point was increased to 245–250° when rate of heating was increased to 30°/min. [Lit. (13) m.p. 250–255° with rapid heating.]

[10] Marketed as Amberlite IR-4B(OAc⁻) by Rohm and Haas Co., Philadelphia, Pa.

indicated significant aliphatic nature. Efforts to isolate sufficient quantities of the alkaloid substance for further characterization are in progress.

The major alkaloid-positive constituents of *G. sylvestre* were detected in quaternary base fractions and were indicated as the biological amines choline and betaine (combined yield, 0.03%) by chromatography and infrared data. The compounds were isolated by reineckate precipitation and separated by an exchange resin technique. Firm identification was established by analysis of choline as reineckate, tetraphenylborate, and chloroaurate derivatives, and betaine as chloroaurate and picrate derivatives.

During the separation process of choline and betaine, minor amounts of a third alkaloid-positive component were isolated (0.0003% of leaf). The substance was identified as trimethylamine oxide (TMAO) by NMR, infrared, molecular weight, and elemental analyses, and by agreement of oxalate and picrate derivatives with reference samples. The oxalate of TMAO has not been described previously in the literature. However, the ease with which a crystalline, nonhygroscopic compound with sharp melting point was obtained makes trimethylamine oxide acid oxalate an excellent derivative for characterization of TMAO.

Aqueous leaf extractions removed an alkaloid-positive substance which was separated by adsorption on a strong acid exchanger and isolated as an insoluble picrate derivative. Conversion to the amine hydrochloride (fraction F) and precipitation gave a compound (0.008% of leaf) which was similar to adenine·HCl·$3H_2O$ by infrared analysis. A picrate of reference adenine sulfate was processed in the same manner to give a hydrochloride with similar chromatographic and melting point characteristics as the plant product. Their infrared spectra were superimposable. Picrate and picrolonate derivatives of both reference and plant samples of adenine were identical by melting point and infrared analysis. The occurrence of compounds such as adenine in plants and their relationship to nucleic acids is discussed by Markham (20).

The supernatant remaining after precipitation of adenine from aqueous extracts contained a series of neutral amino acids (0.23% of leaf). The possibility that an uncommon amino acid of potential pharmacological significance (*e.g.*, as a hypoglycemic agent) might reside in this fraction prompted the characterization of major constituents by paper and thin-layer chromatographic methods. By agreement of R_f values with reference mixtures in a series of solvent systems, the amino acids leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid were detected. A two-dimensional thin-layer chromatogram confirmed these results.

Identification of the protoalkaloids (21), choline and betaine, and the pseudoalkaloid (22), adenine, offers an explanation for Webb's report of positive alkaloid tests from 1% hydrochloric acid extracts of *G. sylvestre* leaves (7). Phosphomolybdic acid, the only reagent in Webb's survey that showed appreciable alkaloid content, affords heavy precipitates with betaine, choline, and adenine. Minor amounts of complex alkaloid mixtures in nonquaternary plant fractions exclude *G. sylvestre* as a true alkaloid-bearing plant in taxonomic terms, which require an accumulation of 0.01% alkaloid from dried tissue (23). Hooper's original alkaloid-positive extract (6) most likely contained this group of trace nonquaternary alkaloidal substances. The level of occurrence of all nitrogenous compounds found in *G. sylvestre* and the chemical identity of major constituents show essentially no correlation with pharmacological activity of the plant.

The occurrence of TMAO in dried leaves was reaffirmed when direct aqueous extractions, which minimized artifact production by chemical and bacterial influences, gave chromatographic evidence for the presence of the oxide. The natural occurrence of TMAO in plant tissue has not been previously described in the literature. However, the biogenesis of TMAO in biological systems has been discussed by Guggenheim (24). Also, evidence has recently been presented for the ability of alfalfa (25) and *Chenopodium vulvaria* (26) seedlings to convert radioactive trimethylamine to TMAO *in vivo*, while inherent oxidative mechanisms are proposed for the biogenesis of amine oxides in the seeds of *Piptadenia* species (27). It would be of particular interest to examine fresh *G. sylvestre* leaves for the natural occurrence of TMAO, and to explore the conversion of trimethylamine to TMAO in this plant.

REFERENCES

(1) Mhaskar, K. S., and Caius, J. F., *Indian Med. Res. Mem.*, **16**, 1(1930).
(2) Mukerji, B., *J. Sci. Ind. Res. (India), Suppl.*, **16A**, 6(1957).
(3) Chopra, R. M., "Indigenous Drugs of India," 2nd ed., U. N. Dhur, Calcutta, India, 1958, p. 336.
(4) Gupta, S. S., *Indian J. Med. Sci.*, **17**, 501(1963).
(5) Gupta, S. S., and Variyar, M. C., *Indian J. Med. Res.*, **52**, 200(1964).
(6) Hooper, D., *Pharm. J.*, **17**, 867(1887).
(7) Webb, L. J., *Australia Commonwealth Sci. Ind. Res. Organ. Bull.*, **268**, 1(1952).
(8) Kapfhammer, J., and Bischoff, C., *Z. Physiol. Chem.*, **191**, 179(1930).
(9) Cunningham, K. G., Dawson, W., and Spring, F. S., *J. Chem. Soc.*, **1951**, 2305.
(10) Bregoff, H. M., Roberts, E., and Delwiche, C. C., *J. Biol. Chem.*, **205**, 565(1953).
(11) Werle, E., in "Modern Methods of Plant Analysis," vol. 4, Paech, K., and Tracey, M. V., eds., Springer-Verlag, Berlin, Germany, 1955, p. 599.
(12) Levine, C., and Chargaff, E., *J. Biol. Chem.*, **192**, 465(1951).
(13) Werle, E., *op. cit.*, p. 559.
(14) Marquardt, P., and Vogg, G., *Z. Physiol. Chem.*, **291**, 143(1952).
(15) "Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 20.
(16) "Dictionary of Organic Compounds," vol. 1, 4th ed., Oxford University Press, New York, N. Y., 1965, p. 42.
(17) Smith, I., "Chromatographic and Electrophoretic Techniques," vol. 1, Interscience Publishers, Inc., New York, N. Y., 1960, pp. 82–100.
(18) Brenner, M., Niederwieser, A., and Pataki, G., "New Biochemical Separations," James, A. T., and Morris, L. J., eds., Van Nostrand Co., London, England, 1964, pp. 136–141.
(19) Scott, A. I., "Interpretations of the Ultra-Violet Spectra of Natural Products," The Macmillan Co., New York, N. Y., 1964, pp. 297–303.
(20) Markham, R., in "Modern Methods of Plant Analysis," *op. cit.*, p. 246.
(21) Ackermann, D., *Abh. Dtsch. Akad. Wiss. Berl. Kl. Chem., Geol. Biol.*, **7**, 1(1956).
(22) Hegnauer, R., *ibid.*, **7**, 10(1956).
(23) Swain, T., "Chemical Plant Taxonomy," Academic Press Inc., New York, N. Y., 1963, pp. 397–399.
(24) Guggenheim, M., "Die Biogenen Amine," 4th ed., S. Karger, New York, N. Y., 1951.
(25) Baker, J. R., Struempler, A., and Chaykin, S., *Biochim. Biophys. Acta*, **71**, 58(1963).
(26) Cromwell, B. T., and Richardson, M., *Phytochemistry*, **5**, 735(1966).
(27) Fish, M. S., Johnson, N. M., and Horning, E. C., *J. Am. Chem. Soc.*, **77**, 5892(1955).

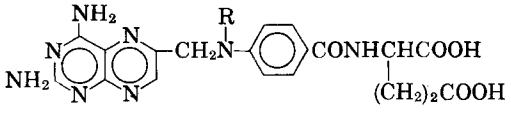
Irreversible Enzyme Inhibitors LXXXVI

Hydrophobic Bonding to Dihydrofolic Reductase VIII. Substituted-1-aryl-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines

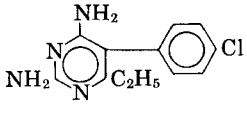
By B. R. BAKER, BENG-THONG HO, and GERHARDUS J. LOURENS

A series of 1-phenyl-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines (IV) bearing a phenylalkyl group on the *m*- or *p*-position of the 1-phenyl moiety was synthesized for enzymic evaluation with dihydrofolic reductase. The largest increment in binding was observed with a *m*-phenylbutyl group (XIV) which gave a fortyfold increment in binding; activity was not increased by substitution of one or two chlorines on the terminal phenyl group. The most active compound (XIV) was complexed one-third as well as the potent aminopterin, that is, XIV had 95 per cent of the free energy of binding shown by aminopterin. The potential use of XIV and related compounds for treatment of tumors with an impaired active-transport system for folic acid and aminopterin is discussed.

AMETHOPTERIN (I) has been useful for the treatment of leukemia in children for a number of years (1); unfortunately, only about 20% of the cases respond, and then only a temporary remission occurs. Amethopterin (I) and aminopterin (II) pass through a cell wall by active-transport (2–8), presumably by the same active-transport system used by a cell for folic acid. By use of tritiated amethopterin, Bertino (9) has been able to correlate the 20% of patients responding to amethopterin with ability of the patients' leukemic cells to take up the drug; the 70% showing no response to amethopterin had virtually no uptake of amethopterin in their leukemic cells.



I, R = CH_3
II, R = H



III

Citrovorum factor (5-formyl-tetrahydrofolic acid, CF) is used as an antidote for amethopterin toxicity, since CF can be utilized by a cell in place of tetrahydrofolic acid—the product of the reaction catalyzed by folic reductase which is blocked by amethopterin; presumably CF also enters the cell by the active-transport system used for the uptake of folic acid. It should therefore be theoretically possible to treat those leukemic patients with an impaired leukemic active-transport system with a potent folic reductase inhibitor that enters the cell by passive diffusion; by pre- and post-treatment with the antidotal CF, those cells with an impaired active-transport system would take up less CF and therefore not be protected as well by CF. Folic reductase inhibitors, such as 2,4-diaminopyrimidines of the pyrimethamine type (III), enter cells by passive diffusion (2, 10); presumably 4,6-diamino-1,2-dihydro-*s*-triazines of type VII (Table I) also enter cells by passive diffusion. If an inhibitor of the 4,6-diamino-1,2-dihydro-*s*-triazine type could be found that approached the potency of amethopterin for blocking folic reductase, it would be useful to investigate such a passive transport inhibitor of folic reductase in conjunction with CF treatment. Such an approach has been previously suggested (11); the results of a search for such potent inhibitors is the subject of this paper.

DISCUSSION

Both amethopterin (I) and aminopterin (II) have a strong pH dependence as inhibitors (12, 13), the optimum inhibition being close to pH 6 (13). The far less inhibition at pH 5.5 or 6.5 is dramatic (13), but the difference in inhibition between pH 6.5–7.4 is only nominal. Since few if any cells attain a pH of 6.0, a more reasonable comparison of inhibitors with aminopterin should be made at pH 6.5–7.4; the authors have selected pH 7.4 primarily because almost all previous work was at this pH. Aminopterin (II) (Table I) requires a concentration of 1×10^{-9} *M* for 50% inhibition in our system at pH 7.4.

The most potent compounds of the passive diffusion type observed on the pigeon liver enzyme are the 3-chloro (V) and 3,4-dichloro (VI)-1-phenyl-*s*-

Received January 5, 1967, from the Department of Chemistry, University of California, Santa Barbara, CA 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication March 6, 1967.

This work was supported by grants CA-06624 and CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

G. J. Lourens thanks the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition scholarship.

Previous paper: Baker, B. R., Lourens, G. J., and Jordaan, J. H., *J. Heterocyclic Chem.*, **4**, 39(1967). Previous paper on hydrophobic bonding to dihydrofolic reductase: Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 470(1966).

triazines (Table I); these two compounds are still $^1/_{10}$ to $^1/_{15}$ so effective as aminopterin (II), but about 7–9 times more effective than the parent 1-phenyl-*s*-triazine (IV). It was noted in earlier studies that substitution of benzyl (VIII) or phenethyl (IX) on the 3-position (14) or benzyl (X) at the 4-position (15) of the 1-phenyl group gave three- to sixfold enhanced binding. Therefore, a study of longer aralkyl groups at the 3-position was initiated first. The phenylpropyl (XIII), phenylbutyl (XIV), and phenylamyl (XV) were all more potent than the phenethyl (IX) group at the 3-position of the 1-phenyl moiety; optimum activity appeared to be at phenylbutyl (XIV), which was fortyfold more potent than the parent 1-phenyl-*s*-triazine (IV) and approached the potency of aminopterin (II) within a factor of three.

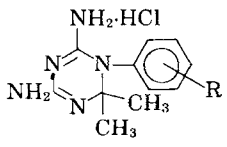
That there was some hydrophobic bonding by a 3-alkyl group was indicated by the 3-methyl (XVII) and 3-*n*-butyl (XII) groups, the latter being 3 times as effective as the parent 1-phenyl-*s*-triazine (IV). The presence of this hydrophobic bonding area near the 3-position (15) of the 1-phenyl group was also indicated by evaluation of the more polar anilinomethyl (XVI) and *n*-propoxy (XVIII) groups; the 3-anilinomethyl group (XVI) was about threefold less effective than the 3-phenethyl group (IX), while the 3-*n*-propoxy group (XVIII) was fiftyfold less effective than the 3-*n*-butyl group (XII).

Since phenyl binding to a receptor can sometimes be increased by halogenation (for example, VI *versus* IV) halogen derivatives of the 3-phenylbutyl group of XIV were investigated; no increased binding was observed by substitution of 3,4-dichloro (XIX), 2,4-dichloro (XX), 2,6-dichloro (XXI), or 2-chloro (XXII) substituents, but the binding was within a factor of two of the parent phenylbutyl group (XIV).

The 4-position of the 1-phenyl triazine was then investigated. 4-Phenethyl (XXIII) was as good an inhibitor as 3-phenethyl (IX) and the 2,4-dichlorophenylbutyl group at the 3-position (XX) and 4-position (XXIV) were equivalent. Therefore, XXV was synthesized to see if the ninefold increment in binding seen by introducing a 3-chloro group (V) on on the parent phenyl triazine (IV) would be additive with the 4-phenylbutyl side chain; that this additivity was not achieved can be seen by comparison of XXV and XXVI.

Although the most potent compound (XIV) in this study is less potent than aminopterin (II) by a factor of three, XIV is still sufficiently potent to warrant animal studies on tumor lines with an impaired active-transport system for amethopterin (I) (6). It can be estimated from the concentration of aminopterin required for 50% inhibition at pH 7.4 that $K_i \cong 1 \times 10^{-10}$ *M*, or a free energy of binding of 14 Kcal./mole. Since XIV only binds threefold less than aminopterin, the difference in binding energies is about 0.7 Kcal./mole. Therefore, at pH 7.4 XIV has about 95% of the free energy of binding of aminopterin.

Table I—Inhibition of Dihydrofolic Reductase[a] by



Compd.	R	mμ*M* Concn. for 50% Inhibition[b]
IV	H	110[c]
V	3-Cl	12[c]
VI	3,4-Cl_2	15[d]
VII	4-Cl	440[c]
VIII	3-$CH_2C_6H_5$	19[c]
IX	3-$(CH_2)_2C_6H_5$	24[c]
X	4-$CH_2C_6H_5$	40[e]
XI	4-C_4H_9-*n*	64[e]
XII	3-C_4H_9-*n*	30
XIII	3-$(CH_2)_3C_6H_5$	5.9
XIV	3-$(CH_2)_4C_6H_5$	2.7
XV	3-$(CH_2)_5C_6H_5$	7.1
XVI	3-$CH_2NHC_6H_5$	66
XVII	3-CH_3	78[e]
XVIII	3-OC_3H_7-*n*	1500
XIX	3-$(CH_2)_4C_6H_3Cl_2$-3,4	3.7
XX	3-$(CH_2)_4C_6H_3Cl_2$-2,4	4.9
XXI	3-$(CH_2)_4C_6H_2Cl_2$-2,6	4.4
XXII	3-$(CH_2)_4C_6H_4Cl$-2	6.1
XXIII	4-$(CH_2)_2C_6H_5$	17
XXIV	4-$(CH_2)_4C_6H_3Cl_2$-2,4	5.3
XXV	3-Cl-4-$(CH_2)_4C_6H_5$	9.3
XXVI	4-$(CH_2)_4C_6H_5$	10
II	(Aminopterin)	1.0[f], 0.86[g]

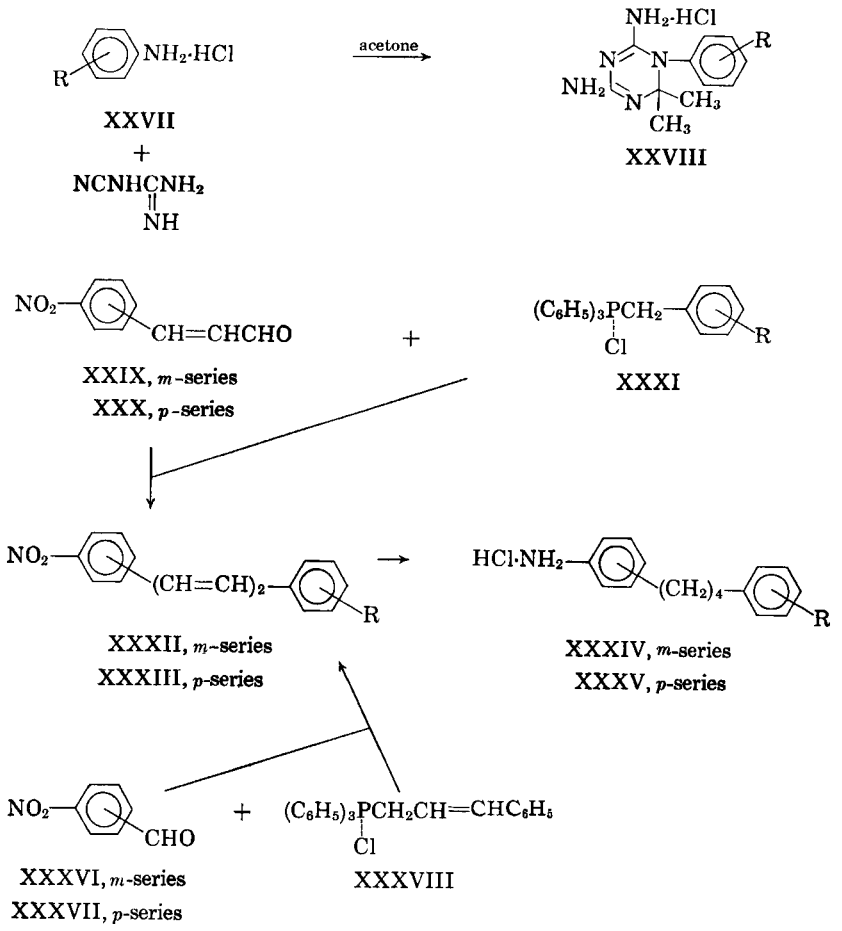
The technical assistance of Maureen Baker, Barbara Baine, and Karen Smith is acknowledged. [a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver prepared and assayed with 6 μ*M* dihydrofolate and 12 μ*M* TPNH at pH 7.4 as previously described (21). [b] Inhibitors were dissolved in 1 m*M* hydrochloric acid and dilutions were made with the same solvent. Solutions of some inhibitors at concentrations below 0.1 μ*M* lost appreciable inhibitory activity after 1–2 hr. at room temperature; since more concentrated solutions in the range of 0.1–1.0 m*M* were stable, the loss of inhibitor in highly dilute solution may have been due to adsorption on the glass. Consistent results were obtained by frequent dilution from a master solution of inhibitor at 0.31 m*M* so that solutions below 0.1 μ*M* were not aged for more than 30 min. [c] Data from *Reference 14*. [d] Data from *Reference 22*. [e] Data from *Reference 15*. [f] Data from *Reference 12*. [g] Data from *Reference 23*

CHEMISTRY

Synthesis

All of the substituted-1-phenyl-*s*-triazines (XXVIII) (Tables I and V) were synthesized by the three-component method of Modest (16), where the appropriate arylamine hydrochloride (XXVII) was condensed with cyanoguanidine in acetone. The synthesis of the requisite *m*- or *p*-aralkylanilines could be divided into three classes: (*a*) those where the alkyl was tetramethylene, (*b*) those where the alkyl was trimethylene or pentamethylene, and (*c*) miscellaneous.

The *m*-(XXXIV) and *p*-phenylbutylanilines (XXXV) were prepared by catalytic reduction of the appropriate 1,4-diphenylbutadiene (XXXII or XXXIII); the dienes were synthesized by the Wittig reaction. If the aralkyl group contained no other substituent, as in series *a*, the 1,4-diphenylbutadiene (XXXII*a* and XXXIII*a*) were conveniently synthesized by condensation of the Wittig reagent from cinnamyl chloride (XXXVIII) with the appropriate isomer of nitrobenzaldehyde (XXXVI or XXXVII). When the aralkyl had halogen substituents (series *b–e*), the appropriate benzyl chloride was converted to the Wittig reagent



Series:

a, R = H
b, R = 3,4-Cl_2
c, R = 2,4-Cl_2
d, R = 2,6-Cl_2
e, R = 2-Cl

Scheme I

(XXXI), then condensed with the *m*-isomer (XXIX) or *p*-isomer (XXX) of nitrocinnamaldehyde. In many cases, the 1,4-diphenylbutadienes melted over a wide range; since these crude materials gave good yields of 1,4-diphenylbutanes (XXIV, XXV) on hydrogenation, they were probably mixtures of *cis*- and *trans*-isomers. The yields obtained by the Wittig reaction were far better than those obtained by the older Meerwein and Perkin reactions. (Scheme I.)

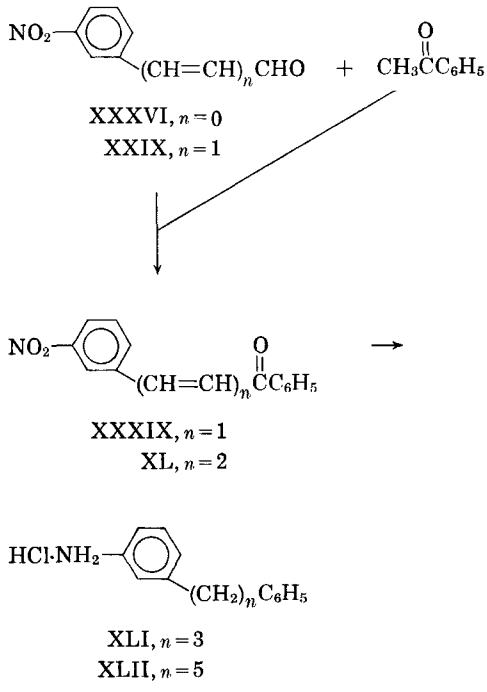
The second class of *m*-aralkylanilines containing an odd number (3 or 5) of methylene groups was synthesized in two steps. Condensation of *m*-nitrobenzaldehyde (XXXVI) or *m*-nitrocinnamaldehyde (XXIX) with acetophenone in aqueous ethanol containing sodium hydroxide gave the chalcones (XXXIX and XL) (17); catalytic reduction of the nitro, vinyl, and carbonyl groups with a palladium charcoal catalyst in glacial acetic acid containing ethanesulfonic acid (14) afforded the required *m*-aralkylanilines (XLI and XLII) in good yield. (Scheme II.)

In the miscellaneous class of 1-phenyl-*s*-triazines were the *m*-(*n*-butyl) (XII), the *p*-phenethyl (XXIII), *m*-anilinomethyl (XVI), and *m*-(*n*-propoxy) (XVIII) derivatives, which were synthesized from the known amines.

Methods

Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer.

(3,4-Dichlorobenzyl)triphenylphosphonium Chloride (XXXIb)—*Method A*—A solution of 18 Gm. (0.07



Scheme II

mole) of triphenylphosphine and 13.7 Gm. (0.07 mole) of α,3,4-trichlorotoluene in 36 ml. of benzene was refluxed with magnetic stirring for 15 hr.; within 15 min., the product began to separate. The product was collected on a filter and washed with benzene; yield, 21.3 Gm. (66%) of white crystals, m.p. 305–306°. Recrystallization of a sample from absolute ethanol–ether gave white crystals, m.p. 309–310°. $\nu_{max.}$ 1580, 1550 (C=C); 1430, 1105 cm.$^{-1}$ (P-C). (See Table II for additional data.)

1 - (m - Nitrophenyl) - 4 - phenyl - 1,3 - butadiene (XXXIIa)—*Method B*—To a stirred solution of 10.1 Gm. (24 mmoles) of XXXVIII (18) and 3.02 Gm. (20 mmoles) of *m*-nitrobenzaldehyde (XXXVI) in 50 ml. of reagent methanol was added 1.62 Gm. (30 mmoles) of solid sodium methoxide; a yellow solid immediately separated. After being stirred for 20 hr. at ambient temperature protected from moisture, the mixture was filtered. The product was washed successively with methanol, water, and methanol; yield, 2.0 Gm. (40%), m.p. 146–147°. The combined filtrate and washings deposited another 1.46 Gm. (total 69%) of product, m.p. 146–147°, after about 18 hr. at −15°. An analytical sample of unchanged melting point was obtained by recrystallization from methanol as yellow crystals; $\nu_{max.}$ 1600, 1560 (C=C); 1505, 1340 cm.$^{-1}$ (NO_2); $\lambda_{max.}$ (EtOH): 331, 345 mμ (inflection). (See Table III for additional data.)

This compound has been prepared by the Meerwein reaction in 12% yield and melting points of 146° (19) and 145° (20) have been recorded.

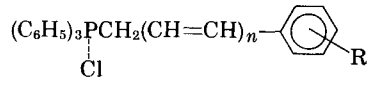
1 - (3,4 - Dichlorophenyl) - 4 - (m - nitrophenyl) - 1,3 - butadiene (XXXIIb)—*Method C*—Condensation of 0.885 Gm. (5 mmoles) of *m*-nitrocinnamaldehyde (XXIX) with 2.74 Gm. (6 mmoles) of XXXI*b* in methanol, as described under *Method B*, gave 0.900 Gm. (56%) of product, m.p. 112–124°, that was suitable for the next step. One crystallization from petroleum ether (b.p. 60–110°) gave 0.451 Gm. (28%) of yellow crystals, m.p. 129–136°. A second recrystallization afforded 0.315 Gm. (20%), m.p. 138–141°. $\nu_{max.}$ 1600, 1560 (C=C); 1520, 1345 cm.$^{-1}$ (NO_2); $\lambda_{max.}$ (EtOH) 338 mμ with shoulders at 325 and 353 mμ.

α-(m-Nitrobenzal)acetophenone (XXXIX)—To a stirred solution of 2.1 Gm. (0.055 mole) of sodium hydroxide in 20 ml. of water was added 10 ml. of ethanol and 5.2 Gm. (0.043 mole) of acetophenone. Then 6.5 Gm. (0.043 mole) of *m*-nitrobenzaldehyde was added in one portion; the temperature was maintained at 20–25° by occasional cooling. After being stirred an additional 2 hr., the thick mixture was kept at 4° for about 18 hr. The product was collected on a filter, then ground in a mortar with water; the solid was collected by filtration. The grinding with water in a mortar was repeated until the final washing was neutral. The product was then washed with cold ethanol; yield, 10.9 Gm. (100%), m.p. 143–144°. Recrystallization from ethanol afforded 7.81 Gm. (72%) of beige-colored crystals, m.p. 145–146°. $\lambda_{max.}$ (EtOH): 294 mμ; $\nu_{max.}$ 1650 (C=O); 1600, 1570 (C=C); 1525, 1340 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{15}H_{11}NO_3$: C, 71.1; H, 4.38; N, 5.53. Found: C, 71.0; H, 4.16; N, 5.41.

α-(m-Nitrocinnamal)acetophenone (XL)—By condensation of 0.841 Gm. (7 mmoles) of acetophenone with 1.24 Gm. (7 mmoles) of *m*-nitrocinnamalde-

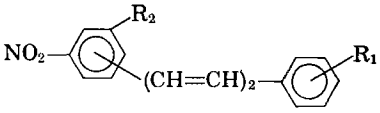
TABLE II—PHYSICAL CONSTANTS OF



Compd.[a]	n	R	M.p., °C.	% Yield	Anal. Calcd. C	Anal. Calcd. H	Anal. Found C	Anal. Found H
XXXI*b*	0	3,4-Cl_2	309–310[b]	66	65.6	4.40	65.3	4.54
XXXI*c*	0	2,4-Cl_2	248–249[c]	73				
XXXI*d*	0	2,6-Cl_2	257–262[d]	46	65.6	4.40	65.4	4.50
XXXI*e*	0	2-Cl	252–253[b]	71	70.9	5.00	70.9	5.06
XXXVIII	1	H	220–222[d,e]	92[d]				

[a] All compounds had infrared spectra in agreement with their assigned structures. [b] Recrystallized from absolute ethanol–ether. [c] Melting point of unrecrystallized product; Keaveney, W. P., and Hennessy, D. J., *J. Org. Chem.*, **27**, 1057(1962) have recorded m.p. 246–249° and a yield of 93%. [d] Recrystallized from methanol–ether. [e] McDonald and Campbell (18) have recorded m.p. 224–226° and a yield of 92%.

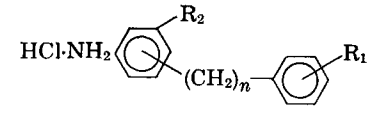
TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	Isomer	R_1	R_2	Method	% Yield	M. p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
XXXIIa	*meta*	H	H	*B*	69	146–147[b]	76.5	5.21	5.57	76.4	5.27	5.44
XXXIIb	*meta*	3,4-Cl_2	H	*C*	56[c]	112–124[c] 138–141[d]	60.0	3.46	4.38	60.3[d]	3.62	4.21
XXXIIc	*meta*	2,4-Cl_2	H	*C*	98[c]	110–121[c] 116–123[d]	60.0	3.46	4.38	59.9[d]	3.60	4.13
XXXIId	*meta*	2,6-Cl_2	H	*C*	90[c]	160–180[c] 187–188[e]	60.0	3.46	4.38	60.0[e]	3.61	4.19
XXXIIe	*meta*	2-Cl	H	*C*	31[c]	88– 93[c] 105–107[d]	67.2	4.23	4.90	67.1[d]	4.39	4.71
XXXIIIa	*para*	H	H	*B*	61[c]	128–165[c] 175–176[d,f]						
XXXIIIc	*para*	2,4-Cl_2	H	*C*	87[c]	155–158[c] 143–148[g]	60.0	3.46	4.38	60.3[g]	3.60	4.20
XLIII[h]	*para*	H	Cl	*B*	90[c]	93–138[c] 127–153[e]	67.2	4.23	4.90	67.1[e]	4.29	4.71

[a] All compounds had infrared and ultraviolet spectra in agreement with their assigned structure. [b] See *Method B* under *Experimental*. [c] Yield and melting point of mixed isomers suitable for further transformation. [d] Recrystallized from petroleum ether (b.p. 60–110°). [e] Recrystallized from ethyl acetate. [f] The lower melting isomer, m.p. 79–81°, was recovered from the filtrate in 27% yield. A m.p. of 181–182° and yield of 65% have been recorded for the higher melting isomer (18). [g] Recrystallized from 2-methoxyethanol. [h] The starting 2-chloro-4-nitrobenzaldehyde, m.p. 70–71°, was prepared by oxidation of 2-chloro-4-nitrotoluene in 24% over-all yield for two steps by the method used for synthesis of the isomeric 4-chloro-2-nitrobenzaldehyde; *cf.* Spalding, D. P., Moersch, G. W., Mosher, H. S., and Whitmore, F. C., *J. Am. Chem. Soc.*, **68,** 1596(1946). A lengthy alternate procedure has been previously described by Chardonnens, L., and Heinrich, P., *Helv. Chim. Acta*, **23,** 292 (1940) and Sharadamma, H. S., Kulkarni, S. N., Sattur, P. B., and Nargund, K. S., *J. Karnatak Univ.*, **1,** 61(1956); through *Chem. Abstr.*, **52,** 8083*i*(1958).

TABLE IV—PHYSICAL PROPERTIES OF



Compd.[a]	Isomer	*n*	R_1	R_2	Method	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
XXXIVa	*meta*	4	H	H	*D*	75	127–128[b]	73.4	7.70	5.34	73.2	7.77	5.26
XXXIVb	*meta*	4	3,4-Cl_2	H	*D*	98	Oil[c]						
XXXIVc	*meta*	4	2,4-Cl_2	H	*D*	78	129–130[b]	58.1	5.48	4.24	57.9	5.32	4.11
XXXIVd	*meta*	4	2,6-Cl_2	H	*D*	99	Oil[c]						
XXXIVe	*meta*	4	2-Cl	H	*D*	90	Amorphous[c]						
XXXVa	*para*	4	H	H	*D*	34	165–169[d]	73.4	7.70	5.34	73.4	7.93	5.14
XXXVc	*para*	4	2,4-Cl_2	H	*D*	88	155–156[e]	58.1	5.48	4.24	58.4	5.68	4.18
XLI	*meta*	3	H	H	*E*	89	118–119[b]	72.7	7.32	5.65	72.5	7.27	5.46
XLII	*meta*	5	H	H	*E*	88	137–138[b]	71.7[f]	8.13	4.93	71.9	8.11	5.02
XLIV	*para*	4	H	Cl	*D*	69	158–160[b]	64.9	6.46	4.73	65.0	6.58	4.87
XLV	*para*	2	H	H	*D*[g]	31	48– 49[h]						

[a] All compounds had infrared spectra in agreement with their assigned structures. [b] Recrystallized from isopropyl alcohol–ether. [c] The crude hydrochloride could not be crystallized and was therefore converted directly to the corresponding *s*-triazine in Table V. [d] The free base has been previously described (19). [e] Recrystallized from absolute ethanol–ether. [f] Hemihydrate. [g] The starting 4-nitrostilbene was obtained by the Meerwein method (19). [h] Free base from petroleum ether; a m.p. of 48° has been recorded by von Braun, J., Deutsch, H., and Koscielski, O., *Chem. Ber.*, **46,** 1511(1913). Considerable *p*-aminostilbene, m.p. 139–142°, insoluble in petroleum ether, was obtained as a by-product.

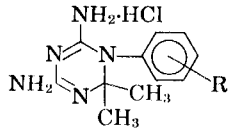
hyde, as described for the preparation of XXXIX, was obtained 1.43 Gm. (51%) of recrystallized product, m.p. 152–154°. For analysis a sample was recrystallized once more from ethanol to give crystals, m.p. 156–159°. $\lambda_{max.}$ (EtOH): 333 mμ; $\nu_{max.}$ 1640 (C=O); 1570 (C=C); 1500, 1335 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{17}H_{13}NO_3$: C, 73.1; H, 4.69; N, 5.01. Found: C, 72.9; H, 4.65; N, 5.05.

Pfeiffer *et al.* (17) have recorded m.p. 157–158° for this compound.

1 - (p - Aminophenyl) - 4 - (2,4 - dichlorophenyl)-butane Hydrochloride (XXXVc)—*Method D*—A mixture of 2.2 Gm. (6.8 mmoles) of XXXIIIc, 200 ml. of absolute ethanol, and 100 mg. of platinum oxide catalyst was shaken with hydrogn at 2–3 Atm. until reduction was complete (3.5 hr.). The filtered solution was evaporated *in vacuo*. The oily residue was dissolved in 25 ml. of reagent ether, then the solution was treated with hydrogen chloride gas. The product was collected on a filter and washed

TABLE V—PHYSICAL CONSTANTS OF



Compd.[a]	R	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
XII	3-C_4H_9-*n*	39	179–180[b]	58.1	7.81	22.6	57.9	7.81	22.4
XIII	3-$(CH_2)_3C_6H_5$	85	181–182[c]	64.6	7.05	18.8	64.3	7.07	18.6
XIV	3-$(CH_2)_4C_6H_5$	67	175–176[c]	65.4	7.31	18.2	65.1	7.46	18.0
XV	3-$(CH_2)_5C_6H_5$	47	180–181[b]	66.1	7.56	17.5	66.0	7.50	17.3
XVI[d]	3-$CH_2NHC_6H_5 \cdot HCl$	32	181–183*d*[e,f]	53.4[f]	6.60	19.7	53.1	6.60	19.7
XVIII[d]	3-OC_3H_7-*n*	77	184–185[c]	53.9	7.11	22.5	53.7	7.12	22.5
XIX	3-$(CH_2)_4C_6H_3Cl_2$-3,4	18	173–174[g]	55.5	5.76	15.4	55.6	5.99	15.1
XX[h]	3-$(CH_2)_4C_6H_3Cl_2$-2,4	73	174–175[g]	55.5	5.76	15.4	55.3	5.93	15.1
XXI[i]	3-$(CH_2)_4C_6H_3Cl_2$-2,6	39	165–169[c]	52.3[i]	5.91	13.3	52.4	5.83	13.3
XXII	3-$(CH_2)_4C_6H_4Cl$-2	65	176–177[c]	60.0	6.48	16.7	60.3	6.66	16.8
XXIII	4-$(CH_2)_2C_6H_5$	78	200–202[c]	63.8	6.76	19.6	63.8	6.91	19.7
XXIV	4-$(CH_2)_4C_6H_3Cl_2$-2,4	68	181–182[c]	55.5	5.76	15.4	55.6	5.95	15.6
XXV	3-Cl-4-$(CH_2)_4C_6H_5$	85	191–192[c]	60.0	6.48	16.7	60.0	6.50	16.6
XXVI	4-$(CH_2)_4C_6H_5$	58	199–200[j]	65.4	7.31	18.2	65.7	7.51	17.9

[a] All compounds had ultraviolet and infrared spectra in agreement with their assigned structures. [b] Recrystallized from isopropyl alcohol–ether. [c] Recrystallized from absolute ethanol–ether. [d] The starting amine was prepared by catalytic reduction of the corresponding nitro compound. [f] Mono-methanolate. [g] Recrystallized from 0.01 *N* aqueous hydrochloric acid. [h] The ethanesulfonate salt was prepared by condensation of the appropriate aniline, cyanoguanidine with ethanesulfonic acid in 50% yield; white crystals from ethanol–ether, m.p. 174–175°. *Anal.*—Calcd. for $C_{21}H_{25}Cl_2N_5 \cdot C_2H_6SO_3H$: C, 52.3; H, 5.91; N, 13.3. Found: C, 52.0; H, 6.01; N, 13.4. [i] Ethanesulfonic acid salt. [j] Recrystallized from ethanol–petroleum ether (b.p. 30–60°).

with ether; yield, 1.97 Gm. (88%), m.p. 151–153°, that was suitable for further transformation. Recrystallization from absolute ethanol–ether gave nearly white crystals, m.p. 155–156°. $\nu_{max.}$ 2950, 2850, 2610, 1950 (NH, NH^+), 1610, 1575, 1505 $cm.^{-1}$ (NH, C=C). (See Table IV for additional data.)

m-Amino-1,3-diphenylpropane Hydrochloride (XLI)—*Method E*—A mixture of 2.53 Gm. (10 mmoles) of XXXIX, 200 ml. of glacial acetic acid, 2.20 Gm. (20 mmoles) of ethanesulfonic acid, and 0.2 Gm. of 5% palladium-charcoal catalyst was shaken with hydrogen at 2–3 Atm. for 17 hr., during which time reduction ceased. The filtered solution was evaporated *in vacuo*. The residual, oily, ethanesulfonate was dissolved in 40 ml. of water, then the solution was adjusted to pH 8–9 with 10% aqueous sodium hydroxide. The mixture was extracted with four 15-ml. portions of benzene. The combined extracts, dried with magnesium sulfate, were evaporated *in vacuo*. The residue was dissolved in 40 ml. of reagent ether, then the solution was treated with hydrogen chloride gas. The solvent was decanted from the separated gum which was crystallized by trituration with fresh ether; yield, 2.2 Gm. (89%), m.p. 117–118°, that was suitable for further transformation. Recrystallization of a sample from isopropyl alcohol–ether with the aid of decolorizing carbon gave white crystals, m.p. 118–119°; $\nu_{max.}$ 2950, 2850, 2600, 1960 (NH, NH^+); 1620, 1595, 1520 $cm.^{-1}$ (NH, C=C). (See Table IV for additional data.)

4,6-Diamino-1-{m-[4-(2,4-dichlorophenyl)-butyl]phenyl}-1,2-dihydro-2,2-dimethyl-s-triazine Hydrochloride (XX)—*Method F*—A solution of 727 mg. (2.2 mmoles) of XXXIV*c*, 198 mg (2.35 mmoles) of cyanoguanidine, and 3.5 ml. of acetone was refluxed with magnetic stirring for 18 hr.; after about 3 hr., the product began to separate. The product was collected and washed with acetone; yield, 730 mg. (73%), m.p. 180–182°. Recrystallization from 0.01 *N* aqueous hydrochloric acid gave white crystals, m.p. 178–189°. $\lambda_{max.}$ (H_2O): 243 mμ; $\nu_{max.}$ 3450, 3320, 3150 (NH); 1660, 1645, 1600, 1560, 1545, 1520 $cm.^{-1}$ (NH, C=NH^+, C=C, C=N). (See Table V for additional data on this and other compounds prepared by this method.)

REFERENCES

(1) Delmonte, L., and Jukes, T. H., *Pharmacol. Rev.*, **14**, 91(1962).
(2) Wood, R. C., and Hitchings, G. H., *J. Biol. Chem.*, **234**, 2381(1959).
(3) Pine, M. J., *J. Bacteriol.*, **79**, 827, 835(1960).
(4) Werkheiser, W. C., *Proc. Am. Assoc. Cancer Res.*, **3**, 371(1962).
(5) Werkheiser, W. C., Law, L. W., Roosa, R. A., and Nichol, C. A., *ibid.*, **4**, 71(1963).
(6) Fischer, G. A., *Biochem. Pharmacol.*, **11**, 1233(1962).
(7) Frenkel, J. K., and Hitchings, G. H., *Antibiot. Chemotherapy*, **7**, 630(1957).
(8) Hakala, M., *Biochim. Biophys. Acta*, **102**, 210(1965).
(9) Bertino, J. R., private communication.
(10) Wood, R. C., and Hitchings, G. H., *J. Biol. Chem.*, **234**, 2377(1959).
(11) Baker, B. R., Santi, D. V., Almaula, P. I., and Werkheiser, W. C., *J. Med. Chem.*, **7**, 24(1964).
(12) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
(13) Bertino, J. R., Booth, B. A., Bieber, A. L., Cashmore, A., and Sartorelli, A. C., *J. Biol. Chem.*, **239**, 479(1964).
(14) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 72(1965).
(15) *Ibid.*, **2**, 335(1965).
(16) Modest, E. J., *J. Org. Chem.*, **21**, 1(1956).
(17) Pfeiffer, P., Prahl, E., Fitz, W., and Stoll, W., *J. Prakt. Chem.*, **109**, 41(1925).
(18) McDonald, R. N., and Campbell, T. W., *J. Org. Chem.*, **24**, 1969(1959).
(19) Bergmann, E., and Shapiro, D., *ibid.*, **12**, 57(1947).
(20) L'Ecuyer, P., and Turcotte, F., *Can. J. Res.*, **25B**, 575(1947).
(21) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(22) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **53**, 1137 (1964).
(23) Baker, B. T., and Ho, B.-T., *J. Heterocyclic Chem.*, **2** 162(1965).

Interaction of Sodium and Calcium Salicylates with Cationic Surfactants

By LUCY S. C. WAN

Sodium and calcium salicylates have been found to interact with surfactants of the quaternary ammonium type in much the same way as salicylic acid, as stated in a previous report. The interaction produces a marked change in viscosity which increases with salicylate concentration until the system becomes a gel. This increase in viscosity is not related to pH and is reduced with a rise in temperature. This complex interaction is probably due to attraction between the salicylate ion and the cation of the surfactant molecule leading to an increase in size of the surfactant molecule resulting in a rise in viscosity. The change in viscosity is further enhanced by an increase in the electrical charges in the system brought about by the addition of sodium salts, the order of effectiveness of the anion being as follows: citrate > phosphate > sulfate > carbonate > bromide > (except at low concentration) chloride, and by the addition of various sulfates, the order of effectiveness of the cation being as follows: sodium > potassium > ammonium > magnesium > zinc. Phenyl, ethyl, and methyl salicylates fail to show this complex interaction due to the absence of salicylate ion in aqueous solution. In addition, sodium lauryl sulfate and cetomacrogol 1000 do not demonstrate any change in viscosity in the presence of both salicylates.

COMPLEX INTERACTIONS play an increasingly important role in pharmaceutical formulations today. Interaction of quaternary ammonium bactericides with various compounds including anionic and nonionic surfactants, dyes, and polymeric compounds have been studied by various workers (1–4). Cationic drugs have been shown to form complexes with sodium carboxymethylcellulose (5), and sodium salicylate has been reported to interact with polyvinylpyrrolidone (6) and with oxytetracycline dihydrate and tetracycline trihydrate (7) forming complexes. The factors involved in complexation and the postulations offered for the mechanisms of complexation are many and varied (8–12). As salicylic acid is the only one of the substituted benzoic acids which interact with the quaternary compounds (13), it will be interesting to discover if the salicylates behave in like manner and it is hoped to learn more of the possible mechanism of this highly specific interaction.

EXPERIMENTAL

Materials—Sodium salicylate (British Drug Houses Ltd.) and calcium salicylate (E. Merck, Darmstadt) were used as supplied by the manufacturers. Phenyl salicylate, 42–43°; ethyl salicylate, density 1.140; methyl salicylate, density 1.184 (Hopkin and Williams, Ltd.); the chloride and bromide of sodium and potassium (May and Baker, Ltd.); sodium citrate, carbonate, and phosphate, and the sulfates of sodium, potassium, magnesium, zinc, and ammonium (British Drug Houses Ltd.) were used without purification. The cationic surfactants, dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide (Glovers Chemicals Ltd.), cetyltrimethylammonium bromide, (Imperial Chemical Industries), sodium lauryl sulfate (Sipon Products Ltd.), and cetomacrogol 1000 B.P.C,[1] were the same as those described in a previous paper (13). The approximate molecular weights of dodecyltrimethylammonium, tetradecyltrimethylammonium, and cetyltrimethylammonium bromides are 314, 342, and 370, respectively.

Apparatus—Portable Ferranti viscometer, Pye tropical universal pH meter.

Measurement of Viscosity—The required amounts of salicylate were weighed into a series of 100-ml. volumetric flasks containing the required concentration of surfactant. The flasks were allowed to rotate in a thermostatically controlled water bath at 25 ± 0.1° for 24 hr. For the study of the effect of addition of salts, the required volume of a concentrated salt solution was incorporated and then equilibrated in like manner. The viscosities of the solutions were measured using the Ferranti portable viscometer placed in a thermostatically controlled water bath at 25 ± 0.5°. The shear rates employed ranged from 78.56 to 483.0 sec.$^{-1}$. An interval of 30 sec. was allowed between readings.

RESULTS AND DISCUSSION

Viscosity Measurements—Figures 1 and 2 show the effect of sodium salicylate on the viscosity of cetyltrimethylammonium bromide and tetradecyltrimethylammonium bromide solutions at 25°. A marked change in viscosity is observed with increased concentration of sodium salicylate; this change is seen with all five of the different shear rates used. This viscosity effect occurs in all surfactant concentrations studied, ranging from 0.05 to 0.18 moles/L., but is much less prominent in dodecyltrimethylammonium bromide systems. Similarly, calcium salicylate exerts this viscosity effect when dispersed in the same quaternary surfactants (Figs. 3 and 4).

Salicylic acid has been shown to demonstrate this viscosity effect (13) but unlike the salicylates it does not gel the system even after it has become saturated with the acid. However, the salicylates produce a

Received December 13, 1966, from the School of Pharmacy, University of Singapore, Singapore.
Accepted for publication January 30, 1967.

[1] Texofor A1P, Glovers Chemicals Ltd.

gel of the system at a particular concentration, probably due to the presence of the sodium or calcium ion. Gelling is taken as the state in which the viscosity cannot be determined by the Ferranti viscometer because the dial readings continually fluctuate. The molar concentration at which gelling occurs is greater for sodium salicylate than for calcium salicylate, and in addition the amount required for

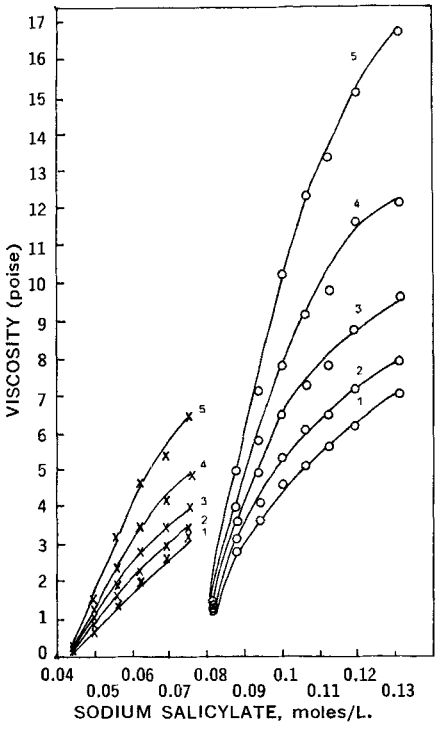


Fig. 1—Effect of sodium salicylate on the viscosity of cetyltrimethylammonium bromide solution at 25°. Key: ×, 0.081 M; ○, 0.162 M. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

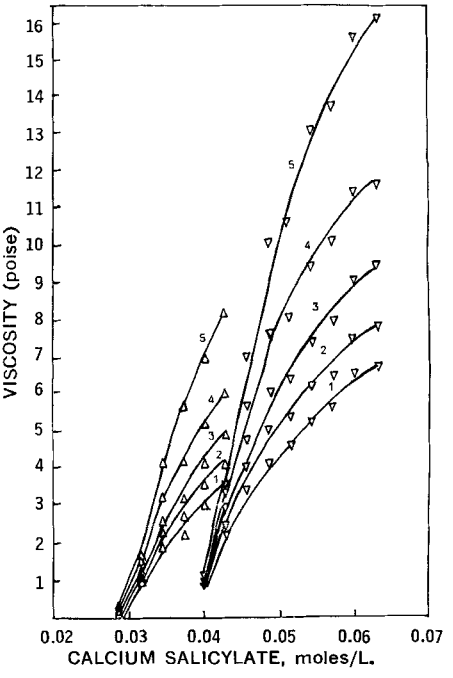


Fig. 3—Effect of calcium salicylate on the viscosity of cetyltrimethylammonium bromide solution at 25°. Key: △, 0.108 M; ▽, 0.162 M. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

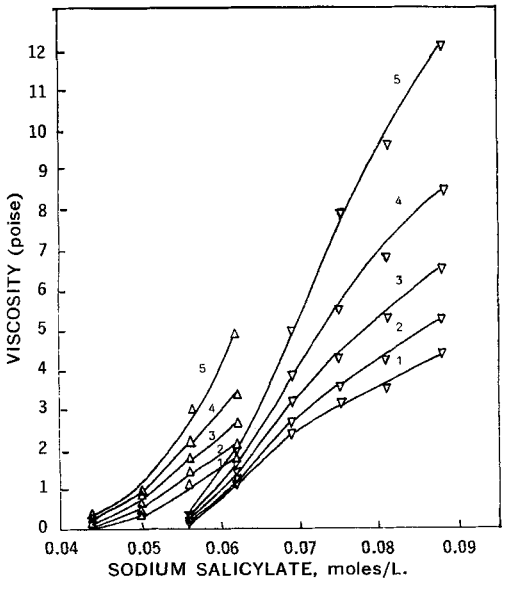


Fig. 2—Effect of sodium salicylate on the viscosity of tetradecyltrimethylammonium bromide solution at 25°. Key: △, 0.117 M; ▽, 0.1754 M. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

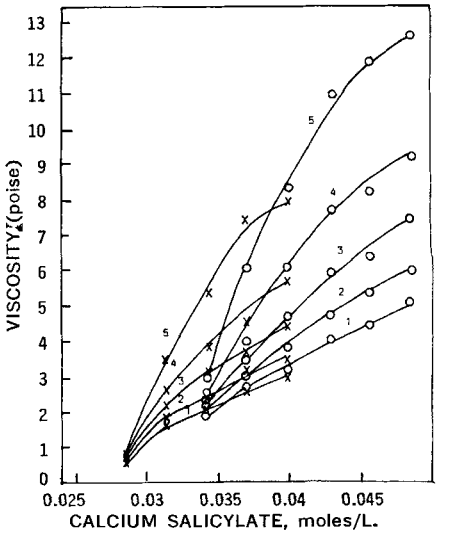


Fig. 4—Effect of calcium salicylate on the viscosity of tetradecyltrimethylammonium bromide solution at 25°. Key: ×, 0.1462 M; ○, 0.1754 M. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

gelling by sodium salicylate is approximately twice that required by calcium salicylate (Table I). This could be due to the fact that the calcium salicylate molecule produces twice as many salicylate ions as the sodium salicylate molecule. The ratios of salicylate to surfactant concentration at which the system becomes a gel decrease with increasing concentration of the quaternary compound, indicating that as the number of surfactant molecules increases following increased concentration of surfactant less salicylate molecules are required to produce a gel, and that probably at high surfactant concentration, the molecules interact less with the salicylates because of the extremely viscous nature of the system in which the surfactant molecules may find their movement restricted or are less mobile. Hence, they do not interact to the same degree as at low concentration of surfactant. From a graphical representation of the data presented in Table I, the amount of salicylate that has to be added to a given concentration of the quaternary compound to produce a gel or maximal measurable viscosity can be calculated.

Table I—Relationship Between Gelling of Surfactant Solutions by Salicylates and Surfactant Concentration

CTAB,[a] moles/L. *A*	Na Salicylate, moles/L. Required to Gel *B*	*B/A*	Ca Salicylate, moles/L. Required to Gel *C*	*C/A*
0.027	0.031	1.148	0.014	0.519
0.054	0.050	0.926	0.026	0.481
0.081	0.075	0.926	0.037	0.456
0.108	0.094	0.870	0.046	0.426
0.135	0.113	0.837	0.054	0.401
0.162	0.131	0.810	0.063	0.388
TTAB,[b] moles/L. *D*	*E*	*E/D*	*F*	*F/D*
0.059	0.044	0.746	0.020	0.341
0.088	0.056	0.636	0.029	0.329
0.117	0.069	0.590	0.037	0.316
0.146	0.081	0.555	0.043	0.294
0.175	0.094	0.536	0.049	0.280

[a] CTAB, cetyltrimethylammonium bromide. [b] TTAB, tetradecyltrimethylammonium bromide.

Effect of Temperature—Figure 5 shows the effect of increasing temperature on the viscosity of sodium salicylate dispersed in cetyltrimethylammonium bromide solution. There is a sharp decrease in viscosity followed by a gradual fall. The viscosity decreases with increasing shear rates but approaches the same value at about 42°, after which there is practically no change with further rise of temperature, suggesting the conversion to a Newtonian liquid. At about 49° and above the viscosity is about the same as that of a solution of cetyltrimethylammonium bromide without the salicylate at 25°. With increased Brownian motion arising from high temperatures, the system offers less resistance to shear.

Effect of pH—Table II shows the pH of sodium salicylate and calcium salicylate dissolved in cetyltrimethylammonium bromide solutions. There is a small difference in pH for both systems and a gradual increase in pH with increasing concentration of salicylate. The effect of additives on the pH of solutions of cetyltrimethylammonium bromide containing sodium salicylate is seen in Table III. Generally a slight change in pH is observed with increased concentration of the additive. pH seems to have no effect on the viscosity change.

Table II—pH of CTAB[a] Solutions Containing Sodium Salicylate and Calcium Salicylate

CTAB (0.135 *M*) + Na Salicylate (*M*)	pH	CTAB (0.135 *M*) + Ca Salicylate (*M*)	pH
0.075	5.5	0.034	5.7
0.081	5.6	0.371	5.9
0.088	5.7	0.040	5.95
0.094	5.8	0.043	5.95
0.100	5.85	0.046	6.15
0.106	6.0	0.049	6.3
0.113	6.0	0.052	6.4

[a] CTAB, cetyltrimethylammonium bromide.

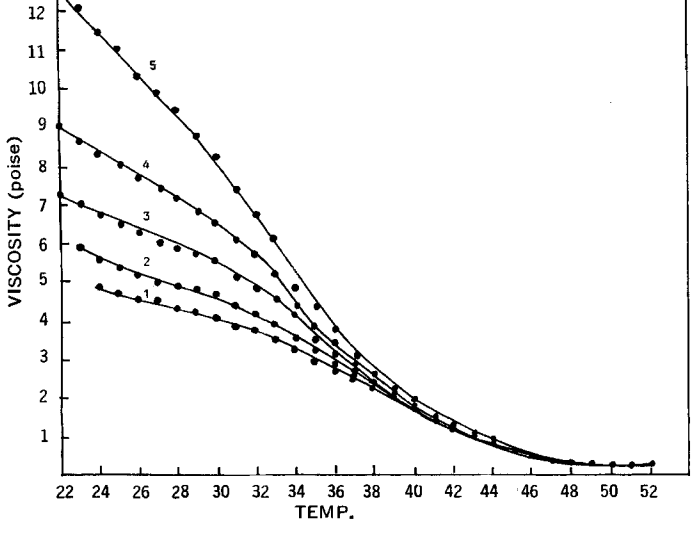


Fig. 5—Effect of temperature on the viscosity of sodium salicylate (0.1 M) dispersed in cetyltrimethylammonium bromide solution (0.135 M). Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

TABLE III—EFFECT OF ADDITIVES ON pH OF CETYLTRIMETHYLAMMONIUM BROMIDE SOLUTIONS (0.108 *M*) CONTAINING SODIUM SALICYLATE (0.0625 *M*)

Additives Concn., moles/L.	pH									
	Na_2SO_4	K_2SO_4	$(NH)_2SO_4$	$MgSO_4$	$ZnSO_4$ (moles/L.)	$Na_3C_6H_5O_7$	Na_2HPO_4	Na_2CO_3	NaBr	NaCl
0.1	6.35	5.8	5.7	5.7	5.55	7.8	8.7	9.9	5.9	...
0.2	6.3	5.9	5.65	5.8	5.4	7.8	8.75	9.95	6.0	6.05
0.3	6.25	5.95	5.55	5.8	5.3	7.85	8.8	10.1	...	...
0.4	6.25	6.0	5.5	5.85	5.25	7.85	8.85	10.1	...	...
0.5	6.2	6.0	5.5	5.85	5.25	7.9	8.85	10.15	6.2	6.1
0.6	6.2	...	5.45	5.9	5.2	7.9	8.9	10.3	...	...
0.7	6.1	...	5.45	5.9	5.1	8.0	8.9	10.3	...	...
0.75	6.0	...	5.45	5.95	5.1	8.1	8.95	10.35	6.25	6.45

Effect of Anion—The addition of sodium chloride, bromide, citrate, phosphate, sulfate, and carbonate to a cetyltrimethylammonium bromide solution containing sodium salicylate further enhances the viscosity effect as seen in Fig. 6. This is true for different shear rates used. The order of effectiveness of the anion is: citrate > phosphate > sulfate > carbonate > bromide > (except at low concentration) > chloride. The valency of the charge on the anion appears to determine the effectiveness on the enhancement as the higher the valency the greater is the additional increase in viscosity. For the sodium halides the effect is different in that a much higher concentration of the corresponding salt is required to effect an additional increase in viscosity in which case the increase is also gradual. In addition, up to a concentration of 1.8 moles/L. of the salt the system does not become a gel, in contrast to the other sodium compounds, all of which form a gel at about 0.7 to 0.75 moles/L. of the salt concentration. Sodium iodide has been included in the investigation but it forms a white precipitate and the viscosity of the system decreases because the surfactant has been removed by precipitation and thus is not available for interaction with the salicylate. Potassium chloride and bromide also show an enhanced viscosity effect similar to that produced by the corresponding sodium compounds, the potassium salts exerting a relatively larger effect on the viscosity. Since the above-mentioned salts form insoluble compounds with calcium salicylate, only sodium citrate, chloride, bromide, and potassium chloride and bromide were added to aqueous solutions of cetyltrimethylammonium bromide containing calcium salicylate. The additives also increase the viscosity of the system similar to that in the systems containing sodium salicylate (Fig. 7). Here again the concentration of sodium citrate required to gel the system is the same as that containing sodium salicylate. The viscosity of cetyltrimethylammonium bromide solutions containing the sodium or potassium compound in the absence of the salicylate remains unaffected.

Effect of Cation—Figure 8 shows the effect of adding the sulfates of ammonium, sodium, potassium, magnesium, and zinc to a solution of cetyltrimethylammonium bromide containing sodium salicylate. The order of enhanced viscosity effect is sodium > potassium > ammonium > magnesium > zinc. This order seems to be related to the valency of the charge on the cation and is the reverse of that observed with the effect of anions in that the smaller the valency of the charge of the cation, the greater the enhancement. A higher concentration of potassium sulfate could not be used since it became insoluble in the system at above 0.5 moles/L.

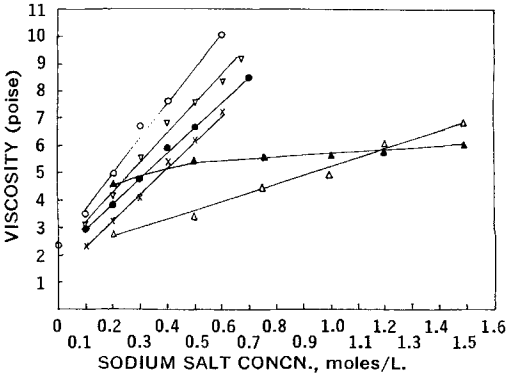


Fig. 6—Effect of various anions of a series of sodium salts on the viscosity of cetyltrimethylammonium bromide solution (0.108 M) containing sodium salicylate (0.0561 M) at 25°. Key: ◌, without salt; ○, $C_6H_5O_7Na_3$; ▽, Na_2HPO_4; ●, Na_2SO_4; ×, Na_2CO_3; ▲, NaBr; △, NaCl; shear rate, 78.56 sec.$^{-1}$.

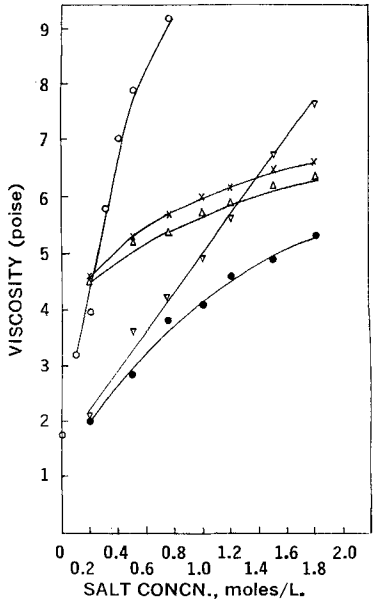


Fig. 7—Effect of sodium and potassium salts on the viscosity of cetyltrimethylammonium bromide solution (0.108 M) containing calcium salicylate (0.0314 M) at 25°. Key: ◌, without salt; ○, $C_6H_5O_7Na_3$; ×, KBr; △, NaBr; ▽, KCl; ●, NaCl; shear rate, 78.56 sec.$^{-1}$.

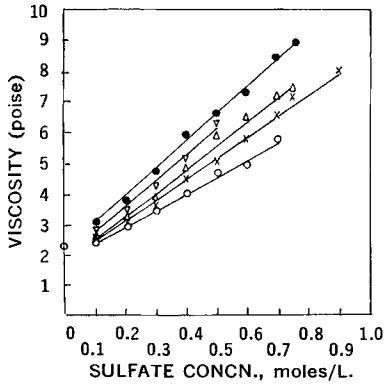


Fig. 8—Effect of various cations of a series of sulfates on the viscosity of cetyltrimethylammonium bromide solution (0.108 M) containing sodium salicylate (0.0561 M) at 25°. Key: ①, without sulfate; ●, Na_2SO_4; ▽, K_2SO_4; △, $(NH_4)_2SO_4$; ×, $MgSO_4$; ○, $ZnSO_4$; shear rate, 78.56 sec.$^{-1}$.

The results of this investigation indicate that sodium and calcium salicylates interact with the quaternary surfactants in much the same way as salicylic acid itself. They further confirm that the salicylate ion is of prime importance in the interaction, since phenyl, ethyl, and methyl salicylates do not demonstrate any viscosity effect. The complex interaction of salicylates with the quaternary surfactants may be due to a strong attraction between the salicylate ion and the bulky cation of the surfactant, thus enlarging its size and leading to an increase in viscosity. The attraction is enhanced by the addition of anions such as chloride, bromide, citrate, phosphate, sulfate, and carbonate and also by the addition of cations such as sodium, potassium, magnesium, ammonium, and zinc since they reinforce the electrical charges in the system. No change in viscosity is observed either with dispersions containing any of the salts investigated and a salicylate in the absence of the surfactant or with dispersions containing any of the salts studied and the surfactant in the absence of the salicylate. Hence the anions and cations only affect the viscosity in the presence of the complex formed by the interaction of salicylate and the surfactant.

REFERENCES

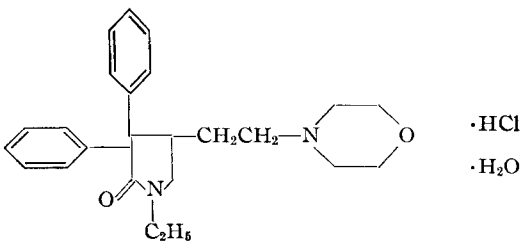
(1) Walter, G. R., and Gump, W. S., *J. Pharm. Sci.*, **51**, 770(1962).
(2) DeLuca, P. P., and Kostenbauder, H. B., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 430(1960).
(3) Lachman, L., Kuramoto, R., and Cooper, J., *ibid.*, **47**, 87(1958).
(4) Lawrence, C. A., "Surface Active Quaternary Ammonium Germicides," Academic Press Inc., New York, N.Y., 1950.
(5) Kennon, L., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 157(1956).
(6) Higuchi, T., and Kuramoto, R., *ibid.*, **43**, 393(1954).
(7) Gans, E. H., and Higuchi, T., *ibid.*, **46**, 458(1957).
(8) Cohen, J., and Lach, J. L., *J. Pharm. Sci.*, **52**, 132 (1963).
(9) Schlenk, H., and Sand, D., *J. Am. Chem. Soc.*, **83**, 2312(1961).
(10) Lach, J. L., and Chin, T. F., *J. Pharm. Sci.*, **53**, 69 (1964).
(11) Higuchi, T., and Drubulis, A., *ibid.*, **50**, 905(1961).
(12) Goudah, M. W., and Guth, E. P., *ibid.*, **54**, 298(1965).
(13) Wan, L. S. C., *ibid.*, **55**, 1395(1966).

Qualitative and Quantitative Tests for Doxapram Hydrochloride

By EDWARD F. SALIM* and A. EDWIN MARTIN†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1-ETHYL-4-(2-MORPHOLINOETHYL)-3,3-DIPHENYL-2-PYRROLIDINONE HYDROCHLORIDE HYDRATE; $C_{24}H_{30}N_2O_2 \cdot HCl \cdot H_2O$; mol. wt. 433.00. The structural formula of doxapram hydrochloride may be represented as



Physical Properties—Doxapram hydrochloride occurs as a white to off-white, odorless, crystalline powder and melts within a range of 2° between 217° and 221° (U.S.P., class I). It is sparingly soluble in water and in alcohol, soluble in chloroform, and practically insoluble in ether. The pH of a solution of doxapram hydrochloride in carbon dioxide-free water (1 in 100) is between 3.5 and 5.0.

Identity Tests—A 1 in 2500 solution of doxapram hydrochloride in water exhibits ultraviolet absorbance maxima at about 252 mμ, 258 mμ [absorptivity (a) about 1.1], and 264 mμ and absorbance minima at about 244 mμ, 254 mμ, and 262 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of doxapram hydrochloride in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of doxapram hydrochloride, accurately weighed, at 105° for 2 hr.: it loses not more than 4.5% of its weight.

Char about 1 Gm. of doxapram hydrochloride, accurately weighed, in a tared platinum crucible, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.3%.

Dissolve 1.0 Gm. of doxapram hydrochloride in 25 ml. of water by gentle warming, and cool: the heavy metals limit determined by U.S.P. method I is 20 p.p.m.

Assay—*Chloride*—Transfer about 300 mg. of doxapram hydrochloride, accurately weighed, to a 250-ml. iodine flask, and dissolve in 50 ml. of water. Add 25.0 ml. of 0.1 N silver nitrate, 3 ml. of nitric acid, 5 ml. of nitrobenzene, and 2 ml. of ferric ammonium sulfate T.S. Shake well, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate. Each milliliter of 0.1 N silver nitrate is equivalent to 3.545 mg. of chloride (Cl). The amount of chloride found is not less than 7.90% and not more than 8.30% of the weight of the sample taken.

Doxapram Hydrochloride—Transfer about 800 mg. of doxapram hydrochloride, accurately weighed, to a 200-ml. tall form beaker, add 50 ml. of glacial acetic acid, and stir until the sample is completely dissolved. Add 10 ml. of mercuric acetate T.S., 1 drop of crystal violet T.S., and titrate with 0.1 N perchloric acid to a blue-green end point. Perform a blank titration and make any necessary correction. Each milliliter of 0.1 N perchloric acid is equivalent to 43.30 mg. of $C_{24}H_{30}N_2O_2 \cdot HCl \cdot H_2O$. The amount of doxapram hydrochloride found is not less than 98.0% and not more than 100.5% of the weight of the sample taken.

DOSAGE FORMS OF DOXAPRAM HYDROCHLORIDE

Doxapram Hydrochloride Injection

Physical Properties—Doxapram hydrochloride injection is a sterile aqueous solution with chlorobutanol present as a preservative. The pH of the solution is between 3.5 and 5.0.

Received December 20, 1966, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication February 10, 1967.

† A. H. Robins Co., Inc., Richmond, VA 23220. A. H. Robins Co., Inc., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The Drug Standards Laboratory gratefully acknowledges the assistance of Miss Carolyn Damon and Miss Hannah Klein.

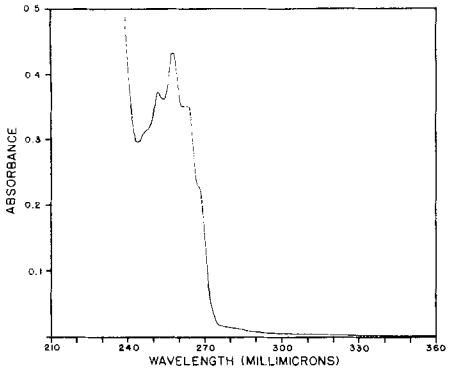


Fig. 1—Ultraviolet absorption spectrum of doxapram hydrochloride in water (400 mcg./ml.); Beckman model DK-2A spectrophotometer.

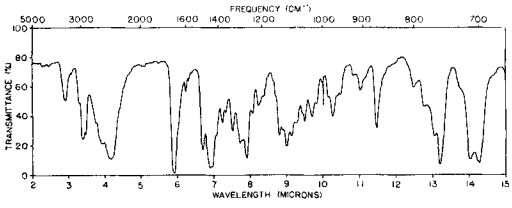


Fig. 2—Infrared spectrum of doxapram hydrochloride in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Identity Test—The solution prepared by dilution of the injection in the *Assay* exhibits absorbance maxima and minima at the same wavelengths as the doxapram hydrochloride standard solution.

Assay—Pipet a volume of doxapram hydrochloride injection, equivalent to 100 mg. of doxapram hydrochloride, into a 250-ml. volumetric flask, dilute to volume with water, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of doxapram hydrochloride, in water, at a concentration of about 400 mcg./ml., in 1-cm. cells, at the maximum at about 258 mμ, with a suitable spectrophotometer, using water as the blank. Calculate the quantity, in milligrams, of $C_{24}H_{30}N_2O_2 \cdot HCl \cdot H_2O$ in each milliliter of injection, by the formula $0.25 \times (C/V) \times (A_u/A_s)$, in which C is the exact concentration of the standard solution, in micrograms per milliliter, V is the volume, in milliliters, of the injection taken, A_u is the absorbance of the sample solution, and A_s is the absorbance of the doxapram hydrochloride standard solution. The amount of doxapram hydrochloride found is not less than 95.0% and not more than 105.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Doxapram hydrochloride[1] is a respiratory stimulant which acts primarily to increase tidal volume and secondarily to increase respiratory rate. It is particularly effective following general anesthesia to prevent postanesthetic respiratory depression or hypoventilation.

Quantitative Methods—The assay for chloride determines the nonactive portion of the molecule but serves as a control on the purity of the bulk material. Analysis of doxapram hydrochloride by the indicator titration method gave an average value of 8.18 ± 0.09%[2] chloride (Cl). The calculated value for chloride content is 8.19%.

The nonaqueous titrimetric procedure for doxapram hydrochloride gave an average value of 99.4 ± 0.3%.[2] The indicator end point was determined by simultaneous indicator and potentiometric observation during the titration. The electrode system employed was glass *versus* calomel modified by replacement of the aqueous salt bridge with 0.02 *N* lithium chloride in glacial acetic acid.

Analysis of commercial doxapram hydrochloride injection by the spectrophotometric method gave an average value of 99.6 ± 0.5%[2] of the labeled amount of doxapram hydrochloride. This approach can be used for analysis of bulk doxapram hydrochloride provided suitable reference standard material is available for absorbance measurement.

[1] Marketed as Dopram by A. H. Robins Co., Inc., Richmond, Va.

[2] Maximum deviation from the mean value.

Lubricity of Dilute Solutions of Hydrocolloids

By T. WERNER SCHWARZ

To determine relative lubricity of hydrocolloids at low viscosities, the friction values of dilute solutions of seven hydrocolloids were measured with the lubrimeter. At very low viscosities, hydrocolloids impart no lubricity to water. A certain consistency appears to be a prerequisite for hydrocolloid dispersions to be effective as lubricants, except for a high molecular weight polymer of ethylene oxide.

IN A PREVIOUS paper on lubricity (1) it was shown that, in the case of hydroxypropyl methylcellulose gels, lubricity was not a function of viscosity. Rather, lubricity was directly related to the solid content of the dispersion regardless of the viscosity grade of the methylcellulose. Thus, dispersions of equal concentrations of two hydroxypropyl methylcelluloses[1] had identical lubricity values (but quite different viscosities).

The lubrimeter, with which the decrease in friction was measured (2), consists basically of two cylinders separated by a film of lubricant. The lower cylinder is rotated at a constant speed; the upper one, to which a spring is attached, rests on the lower cylinder and its motion is activated by the turning of the lower one. The extent of the rotation of the upper cylinder depends on the friction between the surfaces of the two cylinders. If friction is high, the upper cylinder is pulled along up to half of its circumference thereby stretching the spring until the restoring force of the extended spring equals the pull of the upper cylinder. The extension of the spring is a measure of the friction value, which is expressed in grams. The greater the stretch, the greater the friction, and *vice versa*.

The movements of the two cylinders in the lubrimeter facilitate the formation of a converging fluid wedge along the line of nearest approach of the cylinders. This fluid wedge, which is an essential feature of hydrodynamic lubrication, increases the flow velocity of the lubricant and subjects it to great shear, which, in turn, decreases the apparent viscosity of hydrocolloids. Thus the lubricant effect of hydrocolloid gels, all of which have non-Newtonian flow characteristics, may not parallel empirical impressions of lubricity. By rotating the driving cylinder at a slow speed, however, this effect on the viscosity was decreased. Although it was established that lubricity was not simply related to viscosity, at least in the case of methylcellulose, viscosity is, nevertheless, a contributing factor to lubricity. A comparison of different hydrocolloid gels with regard to their lubricity might well be invalidated by the distortion of viscosities. Since no quantitative expression exists for lubricity of aqueous systems, such as Petroff's equation for friction and lubricity in metal bearings (3), the contribution of viscosity to the lowering of friction remains shrouded.

Very dilute solutions of hydrocolloids have low viscosities and they approach, furthermore, Newtonian flow characteristics as the concentration of hydrocolloid decreases. The effect of viscosity on the measurement of lubricity in such dilute solutions would be minimal. In addition to the fundamental aspects of such a study, practical applications are also conceivable. Although medicinal lubricants are usually of gel consistency, mobile solutions, as used for irrigations, enemas, and lotions, can be improved by the addition of a water-soluble lubricant.

Among the several hydrocolloids which were studied were representatives of the natural plant gums, semisynthetic cellulose ethers, and totally synthetic products. Where several viscosity grades of a product existed, a low viscosity type was selected. Except for one hydrocolloid, only solutions with viscosities of less than 1000 cps. were incorporated in the study.

EXPERIMENTAL

Materials—Commercially available hydrocolloids were used except for a polyethylene glycol resin of very high molecular weight[2] and hydroxyethyl cellulose,[3] samples of which had been furnished by their manufacturers. Naturally occurring hydrocolloids were represented by nonionic guar gum[4] and anionic carrageenin.[5] Three cellulose derivatives were used: nonionic hydroxypropyl methylcellulose,[6] anionic sodium carboxymethylcellulose,[7] and nonionic hydroxyethylcellulose.[3] Representatives of the synthetic hydrocolloids were anionic neutralized carboxy vinyl polymer[8] and nonionic polyethylene glycol resin.[2]

Received October 6, 1966, from the School of Pharmacy, San Francisco Medical Center, University of California, San Francisco, CA 94122

Accepted for publication April 5, 1967.

This investigation was supported in part from research funds of the Academic Senate, University of California.

The author wishes to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

[1] Methocel 90 HG 400 and Methocel 90 HG 4000. Dow Chemical Co., Midland, Mich.

[2] Polyox WSR 301. Union Carbide Corp., New York, N. Y.

[3] Natrosol, low viscosity type. Hercules Powder Co., Wilmington, Del.

[4] JaGuar A-20-D. Stein Hall and Co., New York, N. Y.

[5] Viscarin, standard grade. Marine Colloids, Springfield, N. J.

[6] Methocel 90 HG 400. Dow Chemical Co., Midland, Mich.

[7] Cellulose Gum 7 L. Hercules Powder Co., Wilmington, Del.

[8] Carbopol 934. B. F. Goodrich Chemical Co., Cleveland, Ohio.

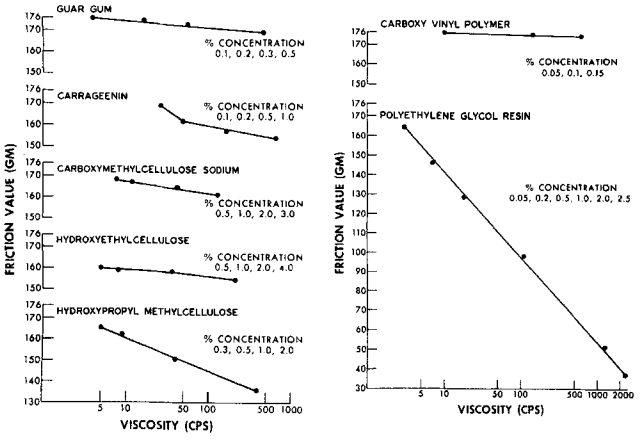


Fig. 1—The effect of hydrocolloid concentration on lubricity and viscosity.

Apparatus and Procedure—The lubrimeter and its use were described previously (2). A single spring was used which covered the whole range of lubricities examined. Its total range was somewhat less than the range of lubricities covered with several springs and, therefore, the difference between extremes of high and low friction was less than in previous experiments. Thus water had a friction value of 176 Gm. as compared with previous readings of 203 and 210 Gm., respectively. Glycerin gave a reading of 29 Gm. (previously 22.7 Gm.). All measurements were carried out at room temperature, which fluctuated between 21° and 24°. Otherwise, the same procedure was followed as described previously.

Viscosity determinations were made with the Brookfield viscometer, model LVF, at temperatures of 22° ± 1° and spindles No. 1 and No. 3. Each hydrocolloid solution was measured at speeds of 6, 12, and 30 r.p.m. and the scale readings were converted to cps. Up to about 500 cps. differences in apparent viscosities, if any, were small. The viscosities used for the purpose of comparison were based on the scale readings at 12 r.p.m. At very low viscosities (up to about 8 cps.) the instrument's readings were erratic; such low viscosity values may, therefore, be inaccurate.

RESULTS AND DISCUSSION

Figure 1 illustrates the results for each of the hydrocolloids examined. All but one exhibit little or no lubricity. This was not unexpected since in aqueous systems generally only full fluid or hydrodynamic lubrication is effective. This type of lubrication requires a substantial layer of lubricant between the opposing surfaces which remains in place by virtue of its viscosity. It could not be taken for granted that the lubrimeter, which was designed to measure hydrodynamic lubrication in aqueous systems as applied in a physiological environment was capable of measuring also lubricity when viscosity, a basic condition of hydrodynamic lubrication, was practically absent or very low.

The friction value of water, 176 Gm., was used as the upper limit. Actually, water itself has a lubricating effect, but the addition to it of any hydrocolloid could only lower its friction value. Very low concentrations of a hydrocolloid, as in the case of the carboxy vinyl polymer, have no effect whatever on the friction value. Even at a viscosity of more than 2000 cps. for a 0.2% solution of this neutralized hydrocolloid—not included in Fig. 1—the friction value was only slightly less than that for the 0.05% solution. A higher concentration of solute, in general, produced a reduction in friction, which was in line with the conclusion of a previous report (1) that the solid content of the solution has a direct bearing on the friction reducing effect. Yet when compared with a known lubricant, such as glycerin, even 3% of one of the hydrocolloids in solution, carboxymethylcellulose, produced no significant lubrication. Its 3% solution had only slightly less friction value than a 0.5% solution. Guar gum, which forms much more viscous systems than carboxymethylcellulose at equal concentrations, had about the same friction value at 0.5% concentration as carboxymethylcellulose. Some of the hydrocolloids had, at low concentrations, a slight effect on the lubricity of their aqueous solutions, others practically none. Only hydroxypropyl methylcellulose, aside from polyethylene glycol resin, improved the lubricity of water at low concentrations and low viscosities.

It was, therefore, surprising to observe a very marked lubricity in polyethylene glycol resin in concentrations comparable to those of other hydrocolloids. At a concentration of 0.5%, the friction value of this resin was already lower than that of any other hydrocolloid tested regardless of concentration. And the slope representing the decrease in friction value with increasing viscosity was considerably steeper than that for methylcellulose, which showed somewhat better lubricity than any of the other five hydrocolloids. Extending the measurements of polyethylene glycol resin to still higher concentrations resulted in a continuing increase of lubricity. Viscosity increased proportionally with the increase of lubricity. At the highest concentration tested, the consistency approached that of a thin gel which was non-Newtonian. Nevertheless, the slope remained the same. However, there was no corresponding further change in lubricity when the concentration was increased beyond 2.5%, only a marked change in viscosity (not indicated on the graph.) The friction value of a 2.5% solution of polyethylene glycol resin was almost as low as that of 95% glycerin (29 Gm.).

The extraordinary reduction of friction by low concentrations of polyethylene glycol resin in water begs an explanation. Aqueous solutions of this resin are non-Newtonian; their apparent viscosities decrease with increasing shear except at very low concentrations. Other liquids having consistencies similar to those of polyethylene glycol resin solutions such as glycerin, or polyethylene glycol of low molecular weight, have also very low friction values. It is worth noting that of three polyols previously measured (1), polyethylene glycol had the lowest friction value and even at a dilution of one part with four parts of water, it raised the lubricity of water significantly.

Hydrodynamic lubrication depends on the inherent viscosity of the lubricant. With decreasing viscosity, hydrodynamic lubrication changes to boundary lubrication, in which a very thin film of only a few layers of the lubricant molecules may separate the surfaces. Solutions and gels of polyethylene glycol resin are apparently effective lubricants at any concentration. It is suggestive to attribute the striking lubricating effect to the linearity and the size of the resin macromolecules; among the most effective lubricants in engineering are linear hydrocarbons (4). At low viscosities, partial hydrodynamic and boundary lubrication coexist because no liquid wedge can be sustained under the pressure of opposing surfaces. If the lubricant film is only a few molecules thick, it must strongly adhere to one or both surfaces or it will be worn away. Solutions of polyethylene glycol resin are very slippery to the touch and stretch fiber-like when being removed from the main body of the liquid. This resistance to being pulled apart is defined as tack (5). The cohesiveness together with adhesion to opposing surfaces distinguish polyethylene glycol resin from the other hydrocolloids and may be partly responsible for its lubricity even in low concentrations. Measurements of adhesiveness of hydrocolloids, with a tackmeter (5, 6) may well contribute to further elucidation of the nature of lubrication with hydrocolloids.

The relationship between friction value and viscosity appears to be linear in the semilog graph. However, a mathematical analysis was not attempted because of too many variables inherent in the lubrimeter. Although the elasticity of various springs could readily be calibrated, friction values of the same lubricant obtained with different springs but identical plastic sheets and by the same operator were different. The plastic sheets, with which one cylinder of the lubrimeter was covered, gave different results, which necessitated averaging a minimum of 6 measurements. The deviations from the average were always less than 5% and were largest at low friction values. Sheets cut from a different piece of the same type of ribbed polyethylene did not match previous results. Nevertheless, the instrument is capable of comparing quantitatively friction values under carefully controlled conditions.

It is of historical interest that the first instrument to measure the "sliding ability" of pharmaceutical preparations was described more than 30 years ago in the *Journal of the American Pharmaceutical Association* (7); an improved version was described more recently (8).

SUMMARY

The lubricity of several dilute solutions of hydrocolloids was measured with the lubrimeter.

Only a high molecular weight polyethylene glycol resin reduced the friction values of water markedly.

The experimental results were discussed and evaluated.

REFERENCES

(1) Levy, G., and Schwarz, T. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 504(1960).

(2) *Ibid.*, **46**, 558(1957).

(3) Fuller, D. D., "Theory and Practice of Lubrication for Engineers," John Wiley & Sons, Inc., New York, N. Y., 1956, p. 12.

(4) Bowden, F. P., and Tabor, D., "Friction and Lubrication," Methuen & Co., London, England, 1956, pp. 97, 107–108.

(5) Green, H., "Industrial Rheology and Rheological Structures," John Wiley & Sons, Inc., New York, N. Y., 1949, pp. 113 *ff.*

(6) Wood, J. H., and Lapham, E. A., *J. Pharm. Sci.*, **53**, 825(1964).

(7) Lloyd, J. U., Ostwald, W., and Erbring, H., *J. Am. Pharm. Assoc.*, **25**, 386(1936).

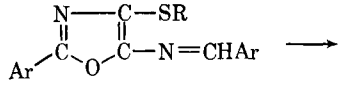
(8) Füller, W., and Münzel, K., *Pharm. Acta Helv.*, **34**, 246(1959).

Somnifacient Action of α-Methylmercaptohippuramide

By ARNOLD R. MARTIN*, F. H. MEYERS, and ROGER KETCHAM

α-Methylmercaptohippuramide has an LD_{50} in mice of 670 ± 20 mg./Kg. Sublethal doses produce sleep but not anesthesia. In preliminary tests on a dog, the drug showed no effect on the autonomic system, nor did it alter the normal responses to the autonomic drugs or histamine. Its pharmacology appears to be similar to that of trimeglamide and thalidomide to which it is structurally related.

DURING THE COURSE of chemical studies on the oxazole condensation products (I) from *S,S'*-dialkyl or diaryl dithioöxaldiimidates and aromatic aldehydes (1), it was found that these oxazole derivatives are rapidly hydrolyzed by acids to open chain nitriles (II), or, under only slightly more vigorous conditions, to the corresponding amides (III).



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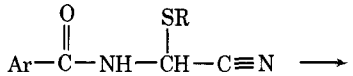

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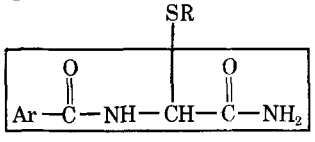
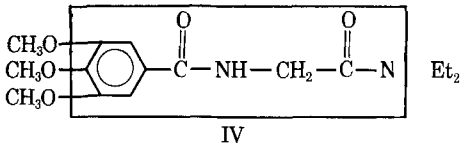


IIIa, R = CH_3, Ar = C_6H_5



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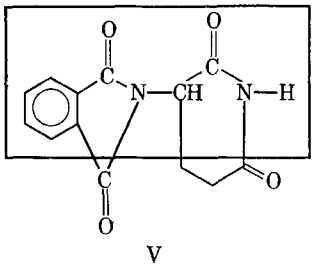

V

Scheme I

The structural similarity of these amides (III) to trimeglamide (IV) and thalidomide (V), which is pointed out in Scheme I, suggested that these sulfur-containing hippuramides might possess similar biological properties. Compound IIIa was therefore subjected to preliminary pharmacological testing.

EXPERIMENTAL

Experiments with Mice—Male Swiss albino mice, weighing 19–23 Gm., were injected (i.p.) with a suspension containing 25 mg./ml. of IIIa in 20% glycerin in water with 0.1% polysorbate 80[1] as a suspending agent. Ten mice at each dose level were treated in a group and observed periodically in a well-lighted room. The data (Table I) give an LD_{50} of 670 ± 20 mg./Kg. (2).

At doses below the LD_{50}, the compound caused the mice to show increased spontaneous activity, to become sluggish and depressed, and to fall asleep if left alone. The mice were easily aroused by external stimuli and behaved normally during the period of stimulation. Neither ataxia nor excitement was observed. The induction period was 15 to 45 min.; the duration of action was 2 to 6 hr.

The gross effects of thalidomide, compound IIIa, trimeglamide, and the thiazolothiazole mentioned below were compared following intraperitoneal injection into dogs, rats, mice, and guinea pigs. The effect, induction of normal sleep in the undisturbed animal, was the same for each drug in each species, including the guinea pig.

Mice given doses near and above the LD_{50} exhibited the same general symptoms mentioned above. However, all of the mice which died passed from a depressed to a convulsive state. Marked ataxia and disorientation were observed at these doses. The convulsions did not appear asphyxial, although some depression of respiration was noted. Several mice survived the convulsions which lasted for about 10–20 min.

Three mice, each given 750 mg./Kg. of IIIa and kept in separate cages in a semidarkened environment, exhibited qualitatively the same symptoms as mice given subtoxic doses, *i.e.*, they moved to a darkened corner of the cage and slept. All of these mice survived and none exhibited convulsions. Thus an LD_{50} determined by keeping the treated mice in separate cages would probably be higher than the group LD_{50}.

Anesthetized Dog Experiment—A dog was anesthetized with sodium pentobarbital, 30 mg./Kg. i.v. Carotid blood pressure was measured with a pressure transducer and respiration with a pneumotachygraph in the airway. These functions and the electrocardiogram were recorded on a polygraph. The effects of intravenous injections into the femoral vein of 10 mcg./Kg. of epinephrine, methacholine, and histamine, and the response to vagal stimulation

Received November 4, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122

Accepted for publication February 28, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This paper was given a Lederle Pharmacy Faculty Award.

Abstracted in part from the Ph.D. thesis of A. R. Martin.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

This work was supported in part by training grant 5 TI GM 728 and mental health grant MH 08787 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE I—ACUTE TOXICITY DATA ON MICE

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500	0/10
600	1/10
650	5/10
700	6/10
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DISCUSSION

The pattern of effects suggests that this compound is not a sedative-hypnotic since it does not produce anesthesia even in large doses. Nor does it have behavioral or autonomic effects suggestive of a tranquilizer of the phenothiazine type. It appears to be similar in its action to thalidomide and trimeglamide to which it is chemically related and to the chemically unrelated 2,5-bis(1,1-dimethyl-3-cyanopropyl)thiazolothiazole (3).

The specific and tragic toxic effect of thalidomide need not obscure the fact that thalidomide defines a purely somnifacient class of drugs potentially safer than the barbiturates. The experience referred to in this paper suggests that drugs of this new class can be recognized during a routine screening and that the action may reside in structures chemically distant from thalidomide.

REFERENCES

(1) Martin, A. R., and Ketcham, R., *J. Org. Chem.*, **31**, 3612(1966).
(2) Litchfield, J. T., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
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Opium Alkaloids IV. Isolation of Isoboldine

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The aporphine alkaloid isoboldine has been isolated from opium. It has been identified by NMR and mass spectrometry and by comparison of its infrared spectrum with that of synthetic (±)-isoboldine. ORD measurements have shown that the isolated alkaloid has the absolute configuration corresponding to the (*S*)-series. Possible biosynthetic pathways for isoboldine are discussed briefly.

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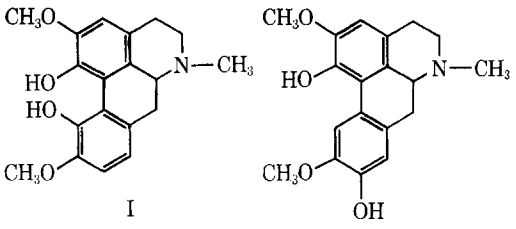
All naturally occuring aporphine alkaloids which are known so far are substituted in positions 1 and 2 (III). The amine nitrogen may be secondary, tertiary, or quaternary, and ring D is either unsubstituted or has one or more oxygen functions in positions 8, 9, 10, and 11.



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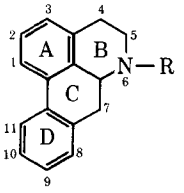

III

EXPERIMENTAL

Isolation—Dried and powdered opium of Indian origin (1,755 Gm.) was first extracted with petroleum ether, b.p. 40–60°, in a Soxhlet extractor. After drying in air, the opium powder was moistened with an aqueous solution of sodium carbonate (185 Gm. anhydrous Na_2CO_3 in 750 ml. H_2O) and dried

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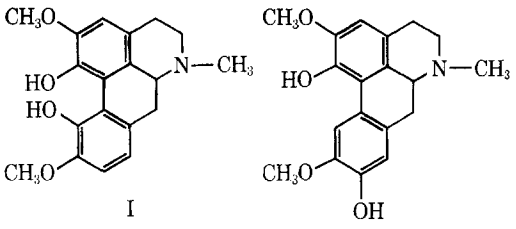

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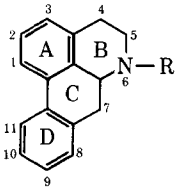

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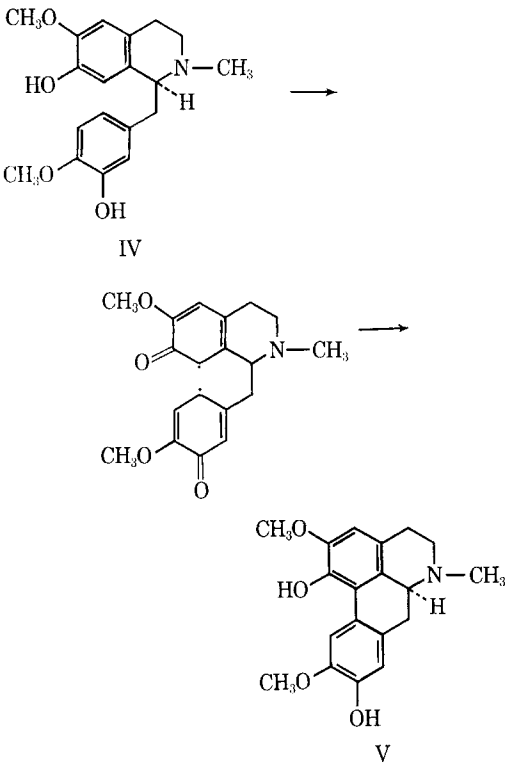
in a vacuum oven at about 30°. The alkalinized material was powdered and extracted with ether in a Soxhlet extractor. The solution in the extraction flask was replaced with fresh ether several times and evaporated to dryness in a rotating vacuum evaporator. The total dry extract obtained in this way weighed 365 Gm. It was dissolved in chloroform and extracted in a separator with McIlvain buffer of pH 6.2. This removed most of the codeine. The chloroform phase was shaken repeatedly with tartaric acid solution (2%), leaving most of the very weak bases (mainly papaverine and narcotine) in the chloroform. When the acid extract was kept in a refrigerator overnight, crystals of thebaine tartrate were formed. These were filtered off and the alkaloids in the filtrate separated into phenolic and nonphenolic bases by extraction with chloroform at pH 13–14. The aqueous layer was adjusted to pH 9–10 with sodium bicarbonate and again extracted with chloroform. After evaporation of the chloroform in a rotating vacuum evaporator, the residue, consisting mainly of diphenolic alkaloids (6.5 Gm.), was subjected to preparative thin-layer chromatography on silica gel (4). Several alkaloid bands could be observed. These were scraped off, the silica gel extracted with warm methanol, and the solutions evaporated under reduced pressure. Two alkaloids obtained in this way were identified as reticuline (2.8 Gm.) and scoulerine (0.5 Gm.) which had previously been isolated from the mother liquors produced during the purification of morphine (5–8). A third alkaloid fraction was crystallized repeatedly from methanol and gave almost colorless crystals (160 mg.) which rapidly became dark when exposed to air, m.p. 125° (micro m.p. K.).

Identification of Isoboldine—The NMR spectrum[1] in deuterochloroform with internal TMS standard revealed three aromatic protons at 2.05, 3.28, and 3.54 τ, two methoxyl groups (singlet, 6.15 τ), an *N*-methyl group at 7.46 τ, and a broad phenolic proton band at 4.3–4.6 τ. Several investigators have studied the nuclear magnetic resonance spectra of aporphine alkaloids and have demonstrated that the NMR spectrum can yield valuable information regarding their substitution pattern (9–11). The low field absorption band (2.05 τ) is characteristic of the aromatic proton at C-11, whereas the bands at 3.28 τ and 3.54 τ are due to the C-8 and C-3 protons, respectively (10). The chemical shift of the methoxyl groups (6.15 τ) indicates that these substituents are located in positions 2, 9, or 10. A methoxyl group at C-1 appears at higher field (6.37–6.58 τ), and a methoxyl group at C-11 has an intermediate chemical shift (6.28–6.35 τ) (9).

When the NMR spectrum of the isolated substance was compared with that of authentic synthetic (±)-isoboldine, the two were found to be identical.

The mass spectrum[2] gave a molecular ion with mass 327. Major fragments appeared in the mass spectrum at *m/e* 326, 312, 296, 284, 269, 253, 165, and 152, corresponding to the favored cleavage of aporphines having an *N*-methyl function (12). The M-31 peak (*m/e* 296) is small, indicating that the alkaloid does not have a methoxyl group in position 1 (12, 13). The M-1 peak (*m/e* 326) is of greater intensity than the peak for the parent ion. This is characteristic of aporphines which are substituted in positions 1,2,9, and 10, while aporphines having a 1,2,10, and 11 substitution pattern exhibit the oppositive relative intensities for these two peaks (14).



Scheme I

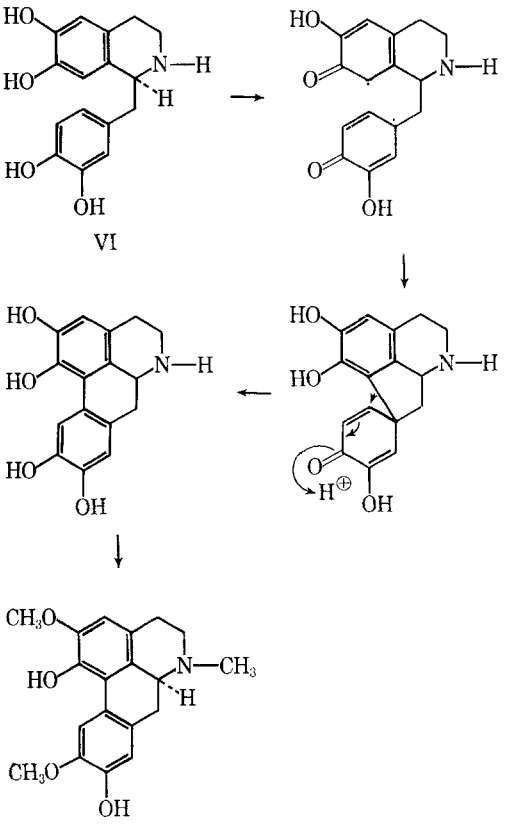
The U.V. spectrum[3] in 95% ethanol gave maxima at 303, 280, and 220 mμ typical of 1,2,9,10 substituted aporphines (15–18). The I.R. spectrum[4] in chloroform was identical with that of authentic (±)-isoboldine.

Stereochemistry—Isoboldine isolated from opium gave a specific rotation of $[\alpha]_D^{25}$ + 66° (c = 0.48 in chloroform). [Lit. + 83.2° (2); + 65.3° (3).] ORD measurements[5] gave a large positive Cotton effect at 251 mμ and a small negative Cotton effect at 320 mμ. From the sign of the Cotton effect in the neighborhood of 250 mμ, it is possible to conclude that the absolute configuration of isoboldine corresponds to the *S*-series according to the Cahn-Ingold-Prelog convention or the L-series if based on the amino acids (19). A sample of isoboldine obtained through the courtesy of Professor Tomita exhibited the same ORD curve as the alkaloid isolated from opium.

DISCUSSION

Robinson (20, 21) was the first to propose that aporphine alkaloids might arise in nature by oxidative coupling of a suitably substituted benzyltetrahydroisoquinoline. Barton and Cohen (22) carried this idea one step further when they suggested that

[1] The instrument used was a Varian HA-100 spectrometer.
[2] Instrument: Associated Electrical Industries, MS9.
[3] Instrument: Unicam SP-800 ultraviolet spectrophotometer.
[4] Unicam SP-200 infrared spectrophotometer.
[5] ORD measurements were made in 95% ethanol at 20° on a Jasco ORD/CD-5 instrument.



VI

Scheme II

the oxidative coupling reaction was the result of a phenol oxidation involving *ortho* or *para* coupling *via* an intermediate quinoid biradical. Two possible mechanisms were envisaged, either a direct coupling to give the aporphine structure, or formation of an intermediate dienone which in turn might rearrange to give rise to a great variety of aporphines (22, 23). The first mechanism is illustrated for isoboldine in Scheme I. The logical precursor is (+)-reticuline (IV) which has the same absolute configuration as (+)-isoboldine (V) (7). It occurs in the opium poppy (7, 24) and has been shown to play an important role in the biosynthesis of a large number of opium alkaloids (25, 26).

An *in vitro* synthesis has been carried out in accordance with the oxidative coupling reaction of Scheme I (27, 28).

If the second mechanism (Scheme II) is used to explain the biosynthesis of isoboldine, a reasonable precursor might be (−)-norlaudanosoline (VI) which also has the correct configuration. Although it has not as yet been isolated from opium or the opium poppy, feeding experiments with radioactively labeled (−)-norlaudanosoline have indicated that it is involved in the biosynthesis of opium alkaloids, presumably as a precursor for (+)-reticuline (25). A closely related mechanism has been demonstrated for the biosynthesis of the aporphine alkaloid isothebaine in *P. orientale* (29).

REFERENCES

(1) Nijland, M. M., *Pharm. Weekblad.* **100,** 88(1965).
(2) Chikamatsu, H., Tomita, M., and Kotake, M., *J. Chem. Soc., Japan,* **82,** 1708(1961).
(3) Tomita, M., and Fujie, M., *J. Pharm. Soc. Japan,* **82,** 1457(1962).
(4) Brochmann-Hanssen, E., Nielsen, B., and Utzinger, G. E., *J. Pharm. Sci.,* **54,** 1531(1965).
(5) Brochmann-Hanssen, E., and Furuya, T., *ibid.,* **53,** 575(1964).
(6) Brochmann-Hanssen, E., and Furuya, T., *Planta Med.,* **12,** 328(1964).
(7) Brochmann-Hanssen, E., and Nielsen, B., *Tetrahedron Letters,* **1965,** 1271.
(8) *Ibid.,* **1966,** 2261.
(9) Bick, J. R. C., Harley-Mason, J., Sheppard, N., and Vernengo, M. J., *J. Chem. Soc.,* **1961,** 1896.
(10) Baarscher, W. H., Arndt, R. R., Pachler, K., Weisbach, J. A., and Douglas, B., *ibid.,* **1964,** 4778.
(11) Bhacca, N. S., Johnson, L. F., and Shoolery, J. N. NMR Spectra Catalog, Varian Associates, Palo Alto, Calif., 1962.
(12) Budzikiewicz, H., Djerassi, C., and Williams, D. H., "Structure Elucidation of Natural Products by Mass Spectrometry, Volume I: Alkaloids," Holden-Day, Inc., San Francisco, Calif., 1964, p. 175.
(13) Ohashi, M., Wilson, J. M., Budzikiewicz, H., Shamma, M., Slusarchyk, W. A., and Djerassi, C., *J. Am. Chem. Soc.,* **85,** 2807(1963).
(14) Jackson, A. H., and Martin, J. A., personal communication.
(15) Shamma, M., and Slusarchyk, W. A., *Chem. Rev.,* **64,** 59(1964).
(16) Djerassi, C., Mislow, K., and Shamma, M., *Experientia,* **18,** 53(1962).
(17) Shamma, M., *ibid.,* **16,** 484(1960).
(18) Sangster, A. W., and Stuart, K. L., *Chem. Rev.,* **65,** 69(1965).
(19) Craig, J. C., and Roy, S. K., *Tetrahedron,* **21,** 395 (1965).
(20) Gulland, J. M., and Robinson, R., *Mem. Proc Manchester Lit. Phil. Soc.,* **69,** 69(1925).
(21) Robinson, R., and Sugasawa, S., *J. Chem. Soc.,* **1931,** 3163.
(22) Barton, D. H. R., and Cohen, T., in "Festschrift A. Stoll," Birkhäuser Verlag, Basel, Switzerland, 1957, p. 117.
(23) Boit, H.-G., "Ergebnisse der Alkaloid-Chemie bis 1960," Akademie-Verlag, Berlin, Germany, 1961, p. 263.
(24) Battersby, A. R., Evans, G. W., Martin, R. O., Warren, M. E., Jr., and Rapoport, H., *Tetrahedron Letters,* **1965,** 1275.
(25) Battersby, A. R., Foulkes, D. M., and Binks, R., *J. Chem. Soc.,* **1965,** 3323.
(26) Brochmann-Hanssen, E., *Medd. Norsk Farm. Selskap,* **28,** 186(1966).
(27) Jackson, A. H., and Martin, J. A., *Chem. Commun.,* **1965,** 420.
(28) Chan, W., and Maitland, P., *J. Chem. Soc., Part C,* **1966,** 753.
(29) Battersby, A. R., Brown, R. T., Clemments, J. H., and Iverach, G. G., *Chem. Commun.,* **1965,** 230.

Molecular Weight Determination in Aqueous and Nonaqueous Solvents II. Thermodynamic Properties of Various Solvents

By RICHARD D. JOHNSON* and FRANK M. GOYAN

The change in the resistance of a thermistor from its value when surrounded by a pure solvent is plotted against the concentration of solutions substituted for the solvent. The solutes are chosen for stability and freedom from tendency to associate or dissociate in order to establish a calibration curve for the determination of molecular weights. Within the limit of experimental error, the curves appear to be straight lines in dilute solutions. The slopes of these lines may be used to estimate the approximate thermodynamic properties of solvents by methods analogous to those used in boiling point elevation studies.

THE THERMOELECTRIC vapor-phase osmometer formerly used to study colligative properties of various solutes (1) was improved upon by its developers (2) in a later study of molecular weight determination. This improved instrument has been used to measure changes in the resistance of a thermistor in contact successively with pure solvent and dilute solutions. A matching thermistor in contact with pure solvent during each measurement was used as a reference. The solutes were carefully selected for stability and freedom from tendency to associate or dissociate, and solutions measured ranged in concentration from 0.01 to 0.2 molal. These resistance changes plotted against their respective concentrations give, for each different solvent, a calibration curve that is used for the determination of the molecular weights of solutes. Within the limit of experimental error, these curves appear to be straight lines. The slopes of these lines are shown to be related to thermodynamic properties of certain solvents. The use of equations derived on the assumption of equilibrium show to a very good approximation that these results may be treated like boiling point elevation data at room temperature.

The aforementioned instrument (2) consists of a Wheatstone bridge circuit containing a pair of 100,000 ohm thermistors, a recording potentiometer, and a temperature control system. The measuring thermistor is in series with a decade resistance box which is used to balance the bridge. These thermistors have a high negative temperature coefficient of resistance of about 4% or 4000 ohms/1°C. Instrument sensitivity is such that a change in resistance of less than 1 ohm may be observed. Therefore, a temperature change of approximately 2×10^{-4}° can be observed. Each thermistor is housed in a separate glass cell which is lined with platinum gauze. This gauze, when wet with solvent, provides an atmosphere of solvent vapor inside the cell. Using a ground-glass tipped medicine dropper, drops of solution are placed on the thermistor tip *via* a capillary tube. Following each measurement, the drop of solution is rinsed from the thermistor tip with several successive solvent washings. A lower reservoir and ejection capillary allow for the removal of this waste material.

Both cells are immersed in a constant-temperature bath maintained at 25.00° ± 0.005° by the temperature control system, involving a water bath in a Dewar flask controlled by an Electron-o-therm Sr., model 148, Technical Equipment Co., Walnut Creek, Calif. The entire apparatus is maintained in a constant-temperature controlled atmosphere of a closed cabinet.

One thermistor, wet with a drop of solvent, is always used as a reference resistor. The second holds the various solutions under study including the solvent as the initial calibrating drop. The thermistors measure a slight increase in temperature caused by condensation of the solvent vapor upon the drop of solution, which has a lower vapor pressure. This process in turn changes the resistance of the thermistor in question causing an off-balance condition across the bridge. The recording potentiometer measurement of the off-balance condition is directly related to the change in resistance necessary to return the bridge to balance. The resistance so indicated is added to the decade box in series with the sample thermistor. The difference between this reading and the initial reading of the same box when pure solvent was on the thermistor is the "ΔR" value that is required for the calculations.

EXPERIMENTAL

Five solvents selected for study were water, 95% ethanol, benzene, ethyl ether, and carbon tetrachloride, while sucrose and benzil were the solutes

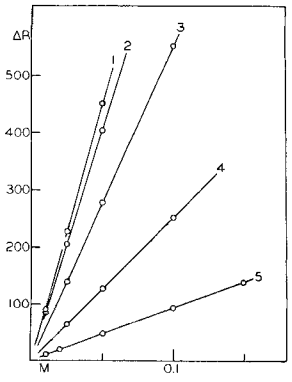


Fig. 1—Calibration curves. Key: 1, *carbon tetrachloride;* 2, *ethyl ether;* 3, *benzene;* 4, *95% ethanol;* 5, *water.* Δ R (*ohms*) vs. *molal concentrations.*

Received August 24, 1965, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication October 6, 1966.

Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.

The authors gratefully acknowledge the support from the Academic Research Funds, University of California, San Francisco, and grant B-TI-GM-728-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

* American Foundation for Pharmaceutical Education Fellow. Holder of the Sir Henry S. Wellcome Memorial Fellowship, 1963–1965.

Previous paper: Goyan, F. M., and Johnson, R. D., *J. Pharm. Sci.*, **53**, 328(1964).

TABLE I—AVERAGE RESISTANCES AND CONCENTRATIONS FOR SOLUTIONS STUDIED

Substance	No.	Concn., molal	Av. ΔR, ohms
Sucrose (in water)	5	0.01	9
		0.02	18
		0.05	46
		0.10	92
		0.15	138
		0.20	184
Benzil (in 95% ethanol)	4	0.025	63
		0.05	126
		0.10	252
Benzil (in benzene)	3	0.025	138
		0.05	277
		0.10	554
Benzil (in ether)	2	0.01	85
		0.025	205
		0.05	404
Benzil (in carbon tetrachloride)	1	0.01	89
		0.025	227
		0.05	452

employed. The organic solvents were all of reagent quality, the benzene being toluene-free. Ordinary distilled water was used. Sucrose and benzil were also of reagent quality, and purification procedures included recrystallization of the benzil from absolute alcohol and sucrose from water.

Several concentrations of solutions were prepared for each solvent between the range of 0.01–0.2 molal. The solutes were weighed out accurately using an analytical balance, placed in a tared flask, and proper solvent added by weight to give the desired w/w solutions. ΔR readings were obtained as previously described, and a weighted average value was computed for each concentration. These values were plotted against their respective molal concentrations, and the slopes of the curves computed.

RESULTS

Figure 1 and Table I show the results obtained for the various solvents under study. The data for benzene appear in an earlier paper (2), and most of the other data were presented in the doctoral thesis of one of the authors (3). However, the values for 95% ethanol represent later work. The slopes of the curves in Fig. 1 and their reciprocal values have been tabulated in Table II.

DISCUSSION AND RESULTS

From classical thermodynamics the well-known relationship of physical chemistry (4) may be written:

$$\Delta T_b = \frac{RT^2}{l_v \ 1000}\, m \qquad \text{(Eq. 1)}$$

where ΔT_b is the boiling point elevation, l_v is the latent heat of vaporization in calories per gram, m is the molal concentration, R represents the gas constant, and T is the absolute temperature. The linearity of the results shown in Fig. 1 indicate that ΔR is a colligative property proportional to ΔT_b. Therefore,

$$\Delta R = \frac{K}{l_v} \cdot m \qquad \text{(Eq. 2)}$$

TABLE II—SLOPES, RECIPROCAL SLOPES, AND LATENT HEATS OF VAPORIZATION CORRESPONDING TO CURVES IN FIG. 1

Curve No.	Solvent	Slope $\frac{(dR)}{(dm)} = S$	Reciprocal Slope $1/S$	l_v (cal./Gm. at 25°)
1	CCl_4	9060	0.110×10^{-3}	50.2
2	Ether	8050	0.124×10^{-3}	86.1
3	Benzene	5540	0.181×10^{-3}	103
4	95% Ethanol	2520	0.397×10^{-3}	224
5	Water	920	1.087×10^{-3}	584

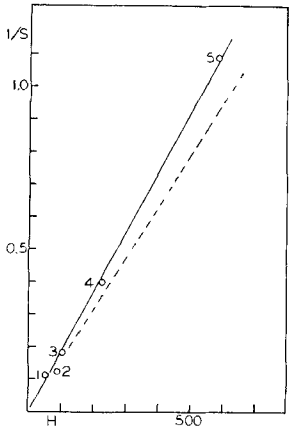


Fig. 2—Reciprocal slopes (× 10³) of curves of Fig. 1 vs. latent heats of vaporization (cal./Gm.). Numbers correspond to those listed for Fig. 1.

where K is a proportionality constant from whence it follows that:

$$l_v = K \cdot \frac{dm}{d\Delta R} \qquad \text{(Eq. 3)}$$

$\frac{dm}{d\Delta R}$ represents the reciprocal slopes listed in Table II. These values plotted against the corresponding latent heats of vaporization should give a straight line. Figure 2 shows this graphically. Latent heats of vaporization displayed in Table II were obtained for 25° by graphical interpolation of established values at various temperatures (5). The value for 95% ethanol was calculated for 25° from the value at the boiling point given by Licht and Denzler (6) by assuming that pure ethanol and the azeotrope required the same correction in going from the boiling point to 25°.

It will be noted from Fig. 2 that all of the points are close to the solid straight line. Ether, which seemed to be the most difficult to work with experimentally, comes closer to the theoretical value shown by the dotted line. This line was established by assuming the relationship:

$$\Delta T_b = \Delta R \cdot f \qquad \text{(Eq. 4)}$$

where ΔT_b and ΔR are taken from Eq. 1, and f represents a conversion factor found to be 2.8×10^{-4}°/ohm by direct measurement with a thermometer. Combining Eqs. 1 and 4 and evaluating all terms gives the slope of the dotted line. By the same reasoning, the solid line gives a value for f of 3.26×10^{-4}°/ohm.

SUMMARY AND CONCLUSION

Calibration curves described above for determining colligative properties in various solvents may be combined in such a way as to reveal expected thermodynamic properties of the solvents. Measurements made on drops of solutions held on the tips of glass rods containing a thermistor show that the vapor exchange is sufficient to minimize the effect of the heat loss which must occur. This situation is no different in principle from the use of a thermometer in a typical boiling point experiment. Although undoubtedly refinements should include the use of instrument constants accounting for heat conduction and other factors, the apparent approximate validity of a classical thermodynamic treatment is a good indication of the effectiveness of this particular instrument.

REFERENCES

(1) Goyan, F. M., and Johnson, R. D., *J. Pharm. Sci.*, **52**, 390(1963).
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(3) Johnson, R. D., "A Study of Physical and Chemical Properties of Solutions by Thermal Electric Methods," Thesis, University of California, San Francisco, Calif., 1965.
(4) Moore, W. J., "Physical Chemistry," 3rd ed., Prentice-Hall Inc., Englewood Cliffs, N. J., 1964, p. 132.
(5) "International Critical Tables," vol. V, McGraw-Hill Book Co., New York, N. Y., 1928, p. 138.
(6) Licht, W., Jr., and Denzler, C. G., *Chem. Eng. Progr.*, **44**, 627(1948).

Absorption and Permeability of a Flavor in Plastic Containers as Determined by Head-Space Gas Chromatography

By SAM J. DONATO

When guarana flavor was used as the flavoring agent in an oral preparation and placed in plastic containers, and when head-space gas–liquid chromatography was applied to the investigation of the stability of this product, the method was found to be rapid and specific. The flavoring constituents of guarana were detected as having been absorbed by the wall of the container and having permeated to the atmosphere surrounding the outside surface of polyethylene bottles. Polyvinyl chloride bottles showed definite absorption of guarana and some change in the appearance of the containers but gave no evidence of permeability.

THE INCREASED importance of plastic containers in the packaging industry has emphasized the need for determining their compatability as a substitute for glass. The object of this work was to develop a rapid method for the study of one phase of this compatability. Much has been accomplished to improve plastics (1) and this achievement has progressed to the point that plastics almost look like glass. Because of its nonfragile nature, lighter weight, and low production cost there is much interest in the pharmaceutical industry for its use as a substitute for glass. A major problem, that of permeability, is associated with plastics and this has led to some reservation as to their total acceptance. In studies thus far reported in the literature, the observations have been arrived at by noting loss in weight and change in appearance, *i.e.*, swelling or collapse of container (2), these being methods which required long standing, careful weighing and handling, and are useful only on a macro scale. Where very small amounts of flavoring are utilized, the aforementioned approach is ineffective and necessitates another analytical technique. In this laboratory such a technique was devised. The author is now able to detect minute traces of flavors as they become absorbed or permeate into new zones within the plastics by using head-space GLC. The procedure is simple, and the graphs obtained are a permanent record of the observations. The use of head-space GLC has been reported in several publications, especially those pertaining to studies in wine and beer flavor (3, 4). These efforts are merely a continuation of these studies. Since glass is very inert and impermeable,

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Accepted for publication March 9, 1967.

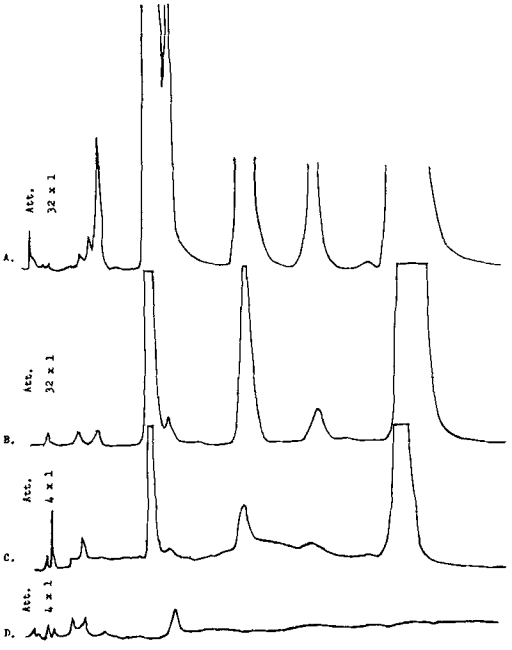

Fig. 1—Head-space gas chromatography of 0.0002% guarana flavor in water. Key: A, *air sample above solution of 0.0002% guarana;* B, *air sample withdrawn from the interior of a washed plastic container that had contained a solution of 0.0002% guarana;* C, *air sample from a closed glass jar containing a polyethylene bottle filled with the guarana flavor;* D, *outside surface air adjacent to glass jar containing the guarana flavor (completely comparable to laboratory air).*

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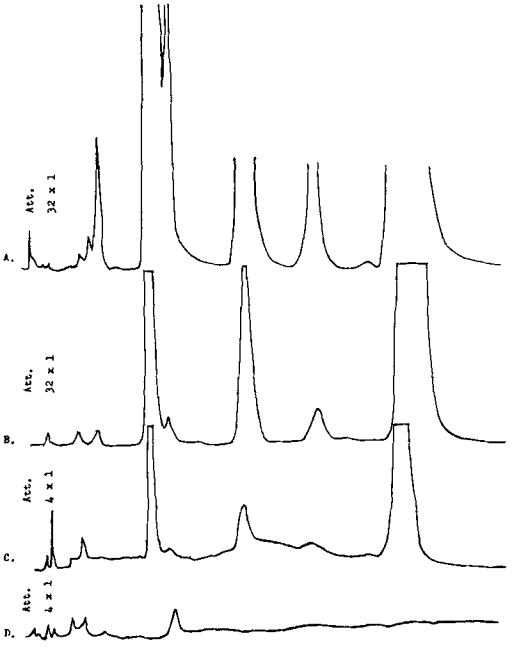

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the plastic sample container was allowed to equilibrate in a tightly closed glass receptacle and the enclosed atmosphere was analyzed.

EXPERIMENTAL

Polyethylene, polyvinyl chloride, and glass bottles were each filled with a 0.0002% v/v guarana–water solution, were separately placed in glass jars with aluminum screw caps, and stored at room temperature for at least 24 hr. A 2.0-ml. aliquot of the air space in each case was withdrawn with a Hamilton gas syringe after piercing through the metal cap with a small nail. This air sample was injected into the F & M model 609 flame ionization gas chromatograph. The small opening would always be sealed with silicone grease immediately after withdrawal of the sample. This sealing technique proved very satisfactory. The results are shown in Fig. 1. The analysis was carried out using a column of copper 10 ft. in length, 1/4 in. o.d., packed with 10% Carbowax 20M on 60–80 mesh Diatoport W. The column temperature was kept isothermally at 90° after an overnight equilibration. The injection port was 200° while the detector block temperature was 130°.

Nitrogen was used as the carrier gas with a flow rate of 45 ml./min. Hydrogen and air flow were kept at 45 and 400 ml./min., respectively. The attenuation was as indicated in Fig. 1; the range was ×1 in each case. The recorder speed was set at 15 in./hr. Each determination required approximately 30 min.

DISCUSSION

This method was found to be effective and simple. Some precautions, however, had to be employed. New septums had to be used on each day's run of the GC since the punctures made by the No. 22 gauge needle would, after a day's use, severely damage the septum and create loss in sensitivity. The cap in each sample bottle had to be kept well sealed and care had to be taken when filling the sample bottles so as not to spill any of the solution on the neck or outside surface of the bottles. The bottles were always thoroughly washed.

The column had to be conditioned overnight to give linear base at the range of ×1.

CONCLUSION

At the conclusion of the experiment the plastic containers were removed from the jars, and the containers thoroughly washed. After this washing there was still a strong odor of the guarana flavor in the air inside the plastic containers. This odor was not observed in the glass jars used in the experiment. Samples of air were withdrawn from the interior of the washed plastic containers and injected into the gas chromatograph. At an attenuation of ×32 the characteristic peaks of guarana flavor were present. Figure 1, B, is an indication that there is a considerable amount of guarana constituents still present in the plastic.

The results suggest that the method would be useful with even more dilute solutions, and quantitation is possible.

REFERENCES

(1) Goldstein, G. F., *Package Engineering*, **1966**, 108–113.
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Alkaloids of the *Papaveraceae* V. Muramine and Berberine from *Argemone squarrosa*

By FRANK R. STERMITZ*

***Argemone squarrosa* Greene (subsp. *squarrosa*) from several locations in central New Mexico was found to contain mainly muramine and berberine, but no allocryptopine. This is in contrast to an earlier literature finding that the same species from southern Colorado contained allocryptopine as the major, if not only, alkaloid. Muramine is now available for the first time in quantities sufficient for pharmacological testing.**

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the only chemical investigation (7) reported that β-allocryptopine (I*a*) was essentially the only alkaloid present in *A. squarrosa* Greene (subsp. *squarrosa*) collected in southern Colorado. Thus, this species seemed to be one which could not be placed in any of the above alliances with certainty. In the course of a collecting trip in New Mexico it was noted that *A. squarrosa* in most locations had a bright yellow sap, which has been found indicative of considerable concentrations of berberine. It was therefore deemed advisable to chemically investigate the species from this area.

Collections were made at two locations in Lincoln County, where *A. squarrosa* (subsp. *squarrosa*) had previously been collected (4), and one location in Eddy County, where this subspecies had been reported (4) to grow mixed with *A. squarrosa* (subsp. *glabrata*). Each of the three collections was found to contain approximately 0.5% alkaloids, with the Lincoln County plants containing about a 4:1 ratio of muramine (I*b*) to berberine. This ratio was approximately reversed in the Eddy County plants. Only traces of other alkaloids could be detected by thin-layer chromatography of the last mother liquors remaining after removal of muramine and berberine. Because of the similarity of structure and melting point of muramine and β-allocryptopine, an authentic sample of the latter, which had been isolated (7) from the same plant species, was obtained[1] and compared with I*b*. The two were indeed found to be different and the original assignment of structure (7) of the β-allocryptopine was confirmed by additional spectral methods.

On the basis of these results and considering plant morphology, *A. squarrosa* is therefore tentatively assigned to the alliance composed of *A. polyanthemos*, *A. corymbosa*, *etc.* It still remains somewhat distinctive, however, in that all other members of the alliance contain protopine, allocryptopine, and berberine and apparently do not contain muramine.

Muramine has, up to now, only been isolated in trace amounts from *Argemone* (8) and a few *Papaver* species (9) and its synthesis (10) has only recently been accomplished in small yield. The discovery of a good source has now provided sufficient material for pharmacological testing, the results of which will be reported at a later date.

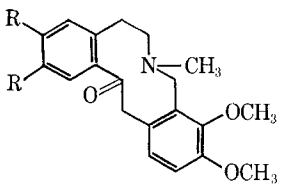
EXPERIMENTAL[2]

Material—The three collections of *A. squarrosa* (subsp. *squarrosa*) used in the present study were made in June 1966 as follows:[3]

(*a*) Lincoln County, N. Mex., 2 mi. east of Capitan on U.S. highway 380; in blossom, but capsules rare. Voucher sample: No. 111162.

(*b*) Lincoln County, N. Mex., east city limits of Lincoln; in blossom, but past peak blossom time. Many capsules. Voucher sample: No. 111156.

(*c*) Eddy County, N. Mex., 4.9 mi. west of Hope on U.S. highway 82; similar growth stage to



I*a*, R, R = —O—CH_2—O—
I*b*, R′ = R = OCH_3

collection (*b*). The presence of *A. squarrosa* (subsp. *glabrata*) as well as intermediates between the two subspecies was noted at this location, but the attempt was made to collect homogeneous subsp. *squarrosa*. Voucher sample: No. 111155.

Isolation and Identification of Alkaloids—The following description [for that of collection (*a*) above] is typical. A total of 521 Gm. of dried, ground whole plant material was extracted for 24 hr. with methanol in a Soxhlet extractor. The methanol was evaporated to a viscous liquid and this was partitioned between 1 *M* sulfuric acid and a 1:1 *n*-butyl alcohol–benzene solution. Some insoluble tar was discarded. After the layers were separated, the organic layer was extracted with further portions of acid. These were combined and washed once with chloroform. The combined aqueous acid solution was made basic with sodium carbonate and extracted three times with equal volumes of chloroform. The chloroform solution was dried with anhydrous sodium sulfate and evaporated *in vacuo* to leave 2.4 Gm. of brown-yellow gum. This was dissolved in 30 ml. of hot methanol and cooled. Brownish transparent prisms (0.38 Gm., m.p. 168–172°) were formed and these were recrystallized once from methanol to yield pure transparent prisms of muramine, m.p. 174–176°. [Lit. (11) m.p. 176–177°.] This was spectroscopically identical to muramine isolated (8) from *A. munita* (subsp. *rotundata*). The methanolic solution from the first muramine crystallization was evaporated to dryness and dissolved in 30 ml. of warm, dilute hydrochloric acid. Cooling the solution yielded yellow-brown microcrystals (0.09 Gm.) of berberine chloride which were spectroscopically identical to a commercial sample. From these results, further work-up of the mother liquors and thin-layer chromatography of residues, the ratio of muramine to berberine in the total alkaloid fraction was estimated at 4:1. Only by heavily loading thin-layer plates with final residues could traces of two other alkaloids be observed. These were not further isolated. Since it was found (7) that ethanol Soxhlet extraction removed only a portion of the allocryptopine from Colorado *A. squarrosa*, the plant residue from the present methanol extraction was submitted to the standard (8) direct butanol–benzene method, but no further alkaloids were obtained.

Collection (*b*) yielded results not differing significantly from those just described. However, when the same procedure was applied to collection (*c*) the first crystallization of the total crude alkaloid fraction yielded berberine, not muramine. Only after redissolving residues in acid and repeating the cycle of extractions and pH adjustment could muramine be isolated. The final results from collection (*c*), again based on isolated amounts plus thin-layer chromatographic estimation of residue

[1] The author is indebted to Professor Soine for this sample.

[2] Melting points were determined on a Mel-Temp apparatus and are uncorrected. Spectral comparisons were made using an Hitachi Perkin-Elmer RMU6-E mass spectrometer and Varian A-60 (NMR), Beckman IR-8 (IR), and Cary 15 (UV) spectrometers. The purchase of the mass spectrometer was made possible through a grant from the National Science Foundation to the Department of Chemistry.

[3] The author is indebted to Professor G. B. Ownbey for confirming the identity of each of the above voucher samples. The numbers given are accession numbers in the Intermountain Herbarium, Utah State University.

contents, showed an approximate ratio of berberine to muramine of about 4:1. This reversal of concentrations might be related to environmental factors or to a genetic nonhomogeneity of the subspecies. (See under *Materials*.)

REFERENCES

(1) Stermitz, F. R., symposium on "Recent Advances in Phytochemistry," Austin, Tex., 1966, proceedings to be published.
(2) Stermitz, F. R., and Seiber, J. N., unpublished data.
(3) Fedde, F., "Das Pflanzenreich," Wilhelm Engelmann, Verlag, Leipzig, Germany, 1909, vol. 40, pp. 271–287.
(4) Ownbey, G. B., *Mem. Torrey Botanical Club*, **21**, No. 1 (1958).
(5) Stermitz, F. R., McMurtrey, K., and Coomes, R. M., unpublished data.
(6) Slavik, J., and Slavikova, L., *Coll. Czech. Chem. Commun.*, **28**, 1728(1963).
(7) Soine, T. O., and Willette, R. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 368(1960).
(8) Stermitz, F. R., and Seiber, J. N., *J. Org. Chem.*, **31**, 2925(1966).
(9) Kühn, L., and Pfeifer, S., *Pharmazie*, **18**, 819(1963).
(10) Giacopello, D., and Deulofeu, V., *Tetrahedron Letters*, **1966**, 2859.
(11) Boit, H. G., and Flentje, H., *Naturwissenschaften*, **47**, 180(1960).

Alkaloids of the *Papaveraceae* VI. Protopine and Allocryptopine from *Arctomecon californica*

By FRANK R. STERMITZ* and V. P. MURALIDHARAN

Protopine and allocryptopine (in approximately equal amounts) make up over 95 per cent of the alkaloidal content of *A. californica*.

THE GENUS *Arctomecon* consists of three species (*A. californica* Torr. et Fremont, *A. humilis* Coville, and *A. merriami* Coville) whose range is restricted to the extreme southwestern corner of Utah, southern Nevada, and the adjoining California desert (1). As part of the continuing investigation of the *Papaveraceae*, it was deemed advisable to investigate this relatively rare genus. Although extensive searches were conducted in 1962–1964 at locations where previous collections of each species had been made, only *A. californica* could be found.[1]

From 700 Gm. of powdered stems and leaves, a total of 2.3 Gm. of crude alkaloid mixture was obtained after treating the powder with 1:1 butanol–benzene, extracting this solution with 1 *M* sulfuric acid, adjusting to pH 9, extracting with chloroform, and evaporating the chloroform layer to dryness. Thin-layer chromatography showed the presence of two alkaloids. A small portion of root material treated in the same fashion showed similar results except with a slightly higher total alkaloid content.

The alkaloid mixture was treated with hot methanol, and protopine was precipitated from the cooled solution. Identification was made by NMR, U.V., and I.R. comparison with a known sample (2). Evaporation of the mother liquor, followed by crystallization of the residue from methanol, was accomplished three times with each crystallization yielding protopine. The fourth crystallization yielded a different alkaloid (m.p. 150°) which, after seven further recrystallizations, had m.p. 156° and was identical in all respects (NMR, I.R., U.V.) to α-allocryptopine, m.p. 160°.[2]

Thin-layer chromatographic analysis of the mother liquors again showed mainly protopine and allocryptopine. An additional two spots could be faintly seen, but sufficient material for further work was not available. Based on the isolated pure alkaloids and thin-layer chromatographic analysis of the mother liquors, protopine and allocryptopine occur in approximately equal amounts in *A. californica* and together represent probably 95% of the total alkaloid content.

General experimental procedures were as previously reported (3).

REFERENCES

(1) Fedde, F., "Das Pflanzenreich," Wilhelm Engelmann, Verlag, Leipzig, Germany, 1909, vol. 40, pp. 134–135.
(2) Stermitz, F. R., Chen, L., and White, J. L., *Tetrahedron*, **22**, 1095(1966).
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Received November 18, 1966, from the Department of Chemistry, Utah State University, Logan, UT 84321
Accepted for publication February 10, 1967.
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(6) Slavik, J., and Slavikova, L., *Coll. Czech. Chem. Commun.*, **28**, 1728(1963).
(7) Soine, T. O., and Willette, R. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 368(1960).
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(10) Giacopello, D., and Deulofeu, V., *Tetrahedron Letters*, **1966**, 2859.
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(2) Stermitz, F. R., Chen, L., and White, J. L., *Tetrahedron*, **22**, 1095(1966).
(3) Stermitz, F. R., *J. Pharm. Sci.*, **56**, 760(1967).

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Effect of the Component Parts of Nicotinamide Adenine Dinucleotide ($NAD^{\oplus}$) as Inhibitors of Lactic Dehydrogenase

By ALBERT J. FORLANO

Kinetic experiments were used to determine the K_m value for $NAD^{\oplus}$ and the K_i values for the component parts of $NAD^{\oplus}$ functioning as inhibitors of the coenzyme. The inhibitory powers of these substances progressively increased as the structure of the inhibitors more closely resembled that of the complete coenzyme. The increasing degree of inhibition appears to be due to more complete binding at the active site of LDH as the structure of the inhibitor approaches the structure of $NAD^{\oplus}$ and effectively prevents the normal binding of the coenzyme.

LACTIC ACID DEHYDROGENASE (LDH), an enzyme found in animal cells, catalyzes the reaction:

$$\text{L(+) lactate} + NAD^{\oplus} \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{pyruvate} + NADH + H^{\oplus} \quad \text{Reaction } 1$$

LDH from rat muscle is specific for L(+)-lactate; however, D(−)-lactate is neither a substrate for LDH nor is it a competitive inhibitor of the normal substrate (1). With reference to the coenzyme portion of LDH one finds that $NAD^{\oplus}$ is approximately 170 times more effective than $NADP^{\oplus}$ for oxidizing lactate (2). Winer and Schwert (3) determined the Michaelis constant (K_m) for $NAD^{\oplus}$ in the reversible reaction *1* over the pH range 5.58 to 10.20. The values ranged from 45.0×10^{-5} moles/L. (pH 5.58) to 5.4×10^{-5} moles/L. (pH 8.59) to 61.0×10^{-5} moles/L. (pH 10.20). The K_m for $NAD^{\oplus}$ in reaction *1* exhibited a minimum at pH 8.7 (3).

The mechanism of LDH inhibition is not well understood. Green and Brosteaux (4) first observed that anions, presumably as competitive inhibitors of lactate binding, decreased the activity of LDH. Kubowitz and Ott (1) showed that high concentrations of pyruvate acted as an inhibitor of LDH. Since pyruvate can act as a competitive inhibitor of reaction *1*, it was decided that the forward reaction would be facilitated if the pyruvate was tied up as soon as it was formed. In the present study semicarbazide hydrochloride was added to the reaction mixture to form the semicarbazone of pyruvic acid and thus ensure maximal forward reaction.

A study of the competitive inhibitors resembling the component parts of $NAD^{\oplus}$ showed that ADP ribose and diadenylic acid are potent enzymatic inhibitors, whereas AMP, ADP, IMP, nicotinamide mononucleotide, nicotinamide, and adenosine were rated as weak inhibitors (5).

The literature appears to have a number of studies involving the inhibitory properties of the component parts of $NAD^{\oplus}$; however, many of these studies were performed by different workers under different experimental conditions. Since there appears to be a correlation between the chemical similarity of $NAD^{\oplus}$ inhibitors and degree of inhibition, it was decided to make a systematic study of the major single components of $NAD^{\oplus}$, followed by combinations of the single components joined together by covalent bonds. The degree of inhibition by these substances in terms of K_i values was determined under the same experimental conditions as the K_m value for $NAD^{\oplus}$.

EXPERIMENTAL

Materials—Lithium lactate (51.1% L-isomer), β-diphosphopyridine nucleotide ($NAD^{\oplus}$), lactic dehydrogenase type I, 70,000 units/mg. protein, all from Sigma Chemical Co.; niacinamide U.S.P.; D(−)-ribose, Eastman Red Label; adenine hemisulfate; 1 H_2O grade B, adenosine-5′-monophosphate morpholidate, grade B; adenosine CFP, the latter three from California Biochemicals; K_2HPO_4, Baker reagent; adenosine-5′-diphosphate sodium salt, ribose-5′-phosphate barium salt, and adenosine-3′-phosphate, the last three from Schwartz Laboratories; Tris, Mann Biochemicals.

KINETIC PROCEDURES

$NAD^{\oplus}$ and the inhibitors were assayed for their effect on LDH in the following manner. The solutions of reagents were added to the spectrophotometer cells in the specified order: 1.0 ml. lithium lactate solution, 0.10 ml. semicarbazide HCl solution, varying amounts of the $NAD^{\oplus}$ solution (as indicated in Table I) to give different concentrations of the $NAD^{\oplus}$ and enough 0.1 *M* Tris buffer (pH 8.8) to make a total volume of 3.10 ml. In the case of the inhibited solutions the specified amount of inhibitor (Table I) was also added and the volume was brought up to 3.10 ml. with 0.1 *M* Tris buffer (pH 8.8). Then 0.03 ml. of the enzyme solution (absorbance 280 mμ = 0.050), which was prepared fresh daily, was added last from a microsyringe. The tubes were rapidly shaken and placed into a Gilford model 2000 multiple sample absorbance recorder equipped with a Beckman DU monochromater. The increases in absorbance at 340 mμ were determined every 20 sec. at 25°. Since the increase in absorbance at 340 mμ is a function of the conversion of $NAD^{\oplus} \rightarrow NADH$ which occurs when the lactate is oxidized, the absorbance increase was used as a means of determining the reaction rate. Inhibitors and combinations of inhibitors were handled in the same fashion. The K_m for $NAD^{\oplus}$ was determined at the different concentrations specified in Table I in the absence of inhibitors and all other conditions remained constant. In the K_i determinations the amount of inhibitor or combinations of inhibitors remained constant and the $NAD^{\oplus}$ concentration was varied as specified in Table I. The final concentrations of the inhibitors in the reaction mixture are given in Table II.

RESULTS

The change in absorbance at 340 mμ *versus* time was followed over a period of 15 min. There was an initial reaction rate that appeared to be maximal which subsequently tapered off. The slope of the initial plot of absorbance *versus* time was utilized in the calculation of the Michaelis constant (K_m) and the inhibitor constants (K_i). The calculation for K_m was based on Eq. 1 from White *et al.* (6) utilizing the

Received February 9, 1967, from the College of Pharmacy, Ferris State College, Big Rapids, MI 49307
Accepted for publication March 15, 1967.

TABLE I—REAGENT CONCENTRATION AND AMOUNTS USED IN REACTION VOLUME OF 3.1 ml.[a]

Reagent	Vol., ml.
0.240 *M* Lithium lactate	1.00
0.384 m*M* $NAD^{\oplus}$	0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.00
0.050 *M* Semicarbazide HCl	0.10
3.840 *M* Niacinamide	0.10
3.840 *M* D(−)-Ribose	0.75
0.019 *M* Adenine	1.00
3.840 *M* K_2HPO_4	1.00
0.041 *M* Adenosine-5′-phosphate	0.20
0.100 *M* Adenosine-5′-diphosphate	0.10
0.050 *M* Ribose-5′-phosphate	1.00
0.033 *M* Adenosine	1.00
0.100 *M* Adenosine-3′-phosphate	0.20
0.100 *M* Tris buffer pH 8.8	

[a] All the solutions were prepared in 0.1 *M* Tris buffer. The differences in concentrations were due to differences in solubilities of these reagents. Maximal solubility was desired wherever possible.

Lineweaver-Burk modification of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{K_m}{V_{max.}} \cdot \frac{1}{[S]} \qquad \text{(Eq. 1)}$$

where $[S]$ = substrate concentration $[NAD^{\oplus}]$, v = reaction velocity at $[S]$, $V_{max.}$ = maximal velocity, and K_m = Michaelis constant for $NAD^{\oplus}$. If a plot of $1/v$ *versus* $1/[S]$ is made, the slope of the line is equal to $K_m/V_{max.}$ and the y intercept equals $1/V_{max.}$; consequently K_m can easily be calculated. The K_m value for $NAD^{\oplus}$ determined in the absence of inhibitors is found in Table II. The calculation of the K_i value utilized Eq. 2 from White *et al.* (6):

$$\frac{1}{v} = \frac{K_m}{V_{max.}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max.}} \qquad \text{(Eq. 2)}$$

where $[I]$ represents the concentration of inhibitor, and K_i is the dissociation constant for the enzyme inhibitor complex EI. The K_i is analogous to the K_m value which is equal to the concentration of substrate present at $^1/_2$ the numerical maximal velocity. Consequently, the smaller the value of K_i, the more effective the inhibitor. The reaction velocities obtained in the presence of the inhibitors were used for the calculation of the K_i values. A plot of $1/v$ *versus* $1/[S]$ (S = $[NAD^{\oplus}]$) in the presence of a constant amount of inhibitor was made and the slope of the line was equal to $(K_m/V_{max.})(1 + [I]/K_i)$. Recall that the value of $(K_m/V_{max.})$ is the same as for the uninhibited reaction. The inhibited plots also have the same ordinate value $(1/V_{max.})$ as the uninhibited reaction because these agents function as competitive inhibitors of $NAD^{\oplus}$. The K_i values are presented in Table II.

DISCUSSION

The K_m value of 8.52×10^{-5} *M* determined at pH 8.8 in this study compares favorably with the literature value of Winer and Schwert (3) who found a value of 5.4×10^{-5} *M* at pH 8.59 and 12.0×10^{-5} *M* at pH 8.96. Since the K_i values were determined with the same enzyme preparation, reagents, *etc.*, as the K_m value, there should be a good degree of correlation with each other.

The data in Table II indicate that none of the inhibitors or combination of inhibitors was more firmly bound to LDH than $NAD^{\oplus}$. The high K_i values for ribose and phosphate indicate that these portions individually were weakly bound; however, when they were joined by a covalent bond as in the case of ribose-5′-phosphate, the K_i value dropped sharply indicating much stronger binding than either of the component portions alone. Adenine itself had a moderately high K_i value, however, when it was joined covalently to ribose-5′-phosphate to yield adenosine-5′-phosphate, the K_i of the nucleotide was about $^1/_2$ that of the ribose-5′-phosphate. This is a good indication that adenine is a necessary binding portion of the coenzyme. When an additional phosphate was joined covalently to AMP to yield ADP, the K_i of ADP dropped to $^1/_3$ the value for AMP, indicating the additional attraction afforded by the second covalently bonded phosphate. The increased binding ability contributed by the extra phosphate is understandable because phosphate groups are ionized at this pH and could easily form ionic bonds with some positive group at the active site of LDH. Niacinamide, which is not a potent

TABLE II—K_m FOR $NAD^{\oplus}$ AND K_i VALUES IN THE LDH CATALYZED CONVERSION OF LACTATE TO PYRUVATE IN 0.1 *M* TRIS BUFFER AT pH 8.8 at 25° C.

Substance	K_m or K_i (moles/L)	Concn. of Inhibitor in Reaction Mixture, moles/L.	Ratio $K_i \times 10^{-2}/K_m$
$NAD^{\oplus}$	8.52×10^{-5}	...	...
D(−)-Ribose	23.68	0.93	2780.00
Niacinamide	7.08×10^{-2}	0.12	8.31
Adenine	4.51×10^{-2}	6.19×10^{-3}	5.30
Niacinamide + adenine[a]	6.36×10^{-2}	$0.12 + 6.19 \times 10^{-3}$	7.47
Phosphate	1.80	1.24	212.00
Adenosine-5′-PO_4	4.57×10^{-3}	2.60×10^{-3}	0.54
Adenosine-5′-$DiPO_4$	1.48×10^{-3}	3.22×10^{-3}	0.17
Adenosine-5′-$DiPO_4$ + niacinamide[a]	5.21×10^{-4}	$3.22 \times 10^{-3} + 0.123$	0.06
Adenosine-5′-$DiPO_4$ + niacinamide + ribose[a]	4.67×10^{-4}	$3.22 \times 10^{-3} + 0.123 + 0.929$	0.06
D(−)-Ribose-5′-PO_4	1.07×10^{-2}	1.61×10^{-2}	1.26
Adenosine	1.56×10^{-2}	1.07×10^{-2}	1.83
Adenosine-3′-PO_4	7.65×10^{-3}	6.45×10^{-3}	0.90

[a] The calculations of these K_i values are based solely upon the molar concentration of the first inhibitor even though the other inhibitors were present.

inhibitor of NAD⊕, potentiated the inhibitory powers of ADP when used in physical combination indicating that another one of the points that NAD⊕ uses for attachment to the LDH had been partially blocked. The same was true for the mixture of ADP with niacinamide and D(−)-ribose.

The K_i for adenosine was fairly high; however, if a phosphate group was joined to the 5′ position covalently to yield the adenosine-5′-PO_4 the K_i decreased to $^1/_3$ of its value. The placement of a covalently bonded phosphate in the 3′ position of adenosine reduced the K_i of adenosine to only $^1/_2$ of its original value. This indicates that the phosphate of a nucleotide is a required portion for bonding to the enzyme; in addition there are steric requirements for maximum binding since both isomers do not have equal K_i values.

Ringold (7) recently theorized that there is an interaction between the quaternary nitrogen of the NAD⊕ and the basic nitrogen of the imidazole ring from histidine at the active site of LDH. With this idea in mind we can see why niacinamide, without a quaternary nitrogen, is not a catalytic species, nor is it very strongly bound to the active site of the enzyme.

One can conclude that the individual components of NAD⊕ are not very strongly bound to LDH, especially in the case of ribose or phosphate alone. However, the amount of inhibition increases as one systematically joins these portions together covalently and the products more closely resemble NAD⊕. This is expected since they become more effective in preventing the complete binding of NAD⊕ to LDH which apparently is a necessary prerequisite for enzymatic activity.

SUMMARY AND CONCLUSIONS

The K_m value for NAD⊕ and the K_i values for the component parts of NAD⊕ as inhibitors of the LDH catalyzed conversion of lactate to pyruvate were determined in 0.1 *M* Tris buffer at pH 8.8.

One finds that the individual component parts of NAD⊕, especially the phosphate and D(−)-ribose, were very poor inhibitors of the coenzyme; however, as the component parts were joined together covalently to make the nucleoside, nucleotide, nucleotide phosphate, *etc.*, their inhibitory powers increased because of greater binding at the active site thus preventing the normal binding of NAD⊕ to LDH.

REFERENCES

(1) Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94(1943).
(2) Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961(1948).
(3) Winer, A. D., and Schwert, G. W., *ibid.*, **231**, 1065 (1958).
(4) Green, D. E., and Brosteaux, J., *Biochem. J.*, **30**, 1489 (1936).
(5) Baker, B. R., and Sachdev, A. S., *J. Pharm. Sci.*, **52**, 933(1963).
(6) White, A., Chandler, P., and Smith, E., "Principles of Biochemistry," 3rd ed., McGraw-Hill Book Co., Inc., New York, N. Y., pp. 223–236.
(7) Ringold, H. J., *Nature*, **210**, 535(1966).

Alterations in Hyaluronidase Activity and Serum Protein Electrophoretic Patterns After Chronic Heat Stress

By M. M. CLAY* and M. W. ADLER

The effects of a hot environment upon plasma proteins and dermal hyaluronidase spreading activity was studied in rats. Hyaluronidase activity decreased after 1 day of heat, increased after 4 and 16 days, and again decreased after 48 days. Swimming for 1.5 hr. after each period of heat stress resulted in inhibition of hyaluronidase spreading activity with the exception of the 48-day period when activity increased. Swimming caused adrenal gland hypertrophy in unstressed but not in heat stressed rats. The relative concentration of plasma albumin increased during heat stress.

Wittersfors *et al.* (1, 2) have reported hypoalbuminemia with increased extravascular albumin distribution in some patients with gastric carcinoma. The authors suggested increased vascular permeability as a mechanism for loss of circulating albumin. Enhanced as well as diminished dermal connective tissue permeability to colloidal particles has been reported after stress and cortisone administration by this laboratory (3–5). The above work and that of Menkin (6) show a role for glucocorticoid hormones in regulation of vascular as well as connective tissue permeability.

The authors studied the effects of stress upon circulating proteins of rats. They also attempted to determine whether changes in serum proteins accompanied changes in dermal connective tissue permeability. For the first study environmental heat was selected as the stress and intradermally injected India ink and hyaluronidase–India ink were selected as indicators of connective tissue permeability to colloidal particles.

Received April 25, 1966, from the College of Pharmacy, Columbia University, New York, NY 10032
Accepted for publication February 20, 1967.
Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
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EXPERIMENTAL

Male albino Wistar strain rats initially weighing 90–120 Gm. were used. Food and tap water were supplied *ad libitum* at all times. Unless otherwise indicated, the rats were maintained at 24 ± 2°

Animal Treatment—Five groups of 24 rats each were maintained at 32 ± 2° for 1, 4, 16, 32, or 48 days in a ventilated semidark chamber. Each group was then divided into three equal subgroups. Dermal permeability was assayed 2 hr. and 24 hr. after removal from the heat in groups *A* and *B*, respectively. Twenty-four hours after heat stress, the remaining rats of group *C* were forced to swim for 1.5 hr. in water at 25 ± 1°. After swimming,

inhibitor of NAD⊕, potentiated the inhibitory powers of ADP when used in physical combination indicating that another one of the points that NAD⊕ uses for attachment to the LDH had been partially blocked. The same was true for the mixture of ADP with niacinamide and D(−)-ribose.

The K_i for adenosine was fairly high; however, if a phosphate group was joined to the 5′ position covalently to yield the adenosine-5′-PO_4 the K_i decreased to $^1/_3$ of its value. The placement of a covalently bonded phosphate in the 3′ position of adenosine reduced the K_i of adenosine to only $^1/_2$ of its original value. This indicates that the phosphate of a nucleotide is a required portion for bonding to the enzyme; in addition there are steric requirements for maximum binding since both isomers do not have equal K_i values.

Ringold (7) recently theorized that there is an interaction between the quaternary nitrogen of the NAD⊕ and the basic nitrogen of the imidazole ring from histidine at the active site of LDH. With this idea in mind we can see why niacinamide, without a quaternary nitrogen, is not a catalytic species, nor is it very strongly bound to the active site of the enzyme.

One can conclude that the individual components of NAD⊕ are not very strongly bound to LDH, especially in the case of ribose or phosphate alone. However, the amount of inhibition increases as one systematically joins these portions together covalently and the products more closely resemble NAD⊕. This is expected since they become more effective in preventing the complete binding of NAD⊕ to LDH which apparently is a necessary prerequisite for enzymatic activity.

SUMMARY AND CONCLUSIONS

The K_m value for NAD⊕ and the K_i values for the component parts of NAD⊕ as inhibitors of the LDH catalyzed conversion of lactate to pyruvate were determined in 0.1 *M* Tris buffer at pH 8.8.

One finds that the individual component parts of NAD⊕, especially the phosphate and D(−)-ribose, were very poor inhibitors of the coenzyme; however, as the component parts were joined together covalently to make the nucleoside, nucleotide, nucleotide phosphate, *etc.*, their inhibitory powers increased because of greater binding at the active site thus preventing the normal binding of NAD⊕ to LDH.

REFERENCES

(1) Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94(1943).
(2) Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961(1948).
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(6) White, A., Chandler, P., and Smith, E., "Principles of Biochemistry," 3rd ed., McGraw-Hill Book Co., Inc., New York, N. Y., pp. 223–236.
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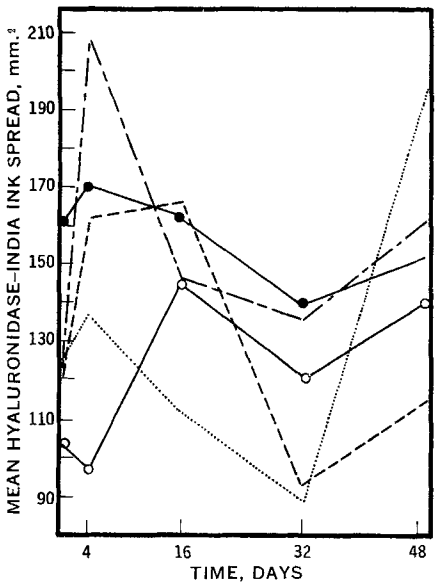

Fig. 1—Effect of stress on the spread of hyaluronidase–India ink indicator solution. Group A, *heat stress; group* B, *heat stress, followed by 24 hr. recovery; group* C, *heat stress, recovery and 1.5 hr. of swimming; group* A′, *nonstressed controls; group* C′, *nonstressed controls, 1.5 hr. of swimming. Key:* – – –, *group* A; — · —, *group* B; ····, *group* C; ●, *group* A′; O, *group* C′.

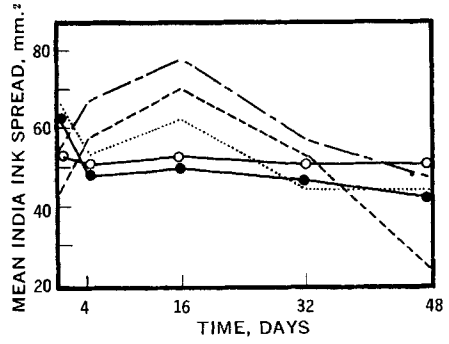

Fig. 2—Effect of stress on the spread of India ink. Group A, *heat stress; group* B, *heat stress, followed by 24 hr. recovery; group* C, *heat stress, recovery, and 1.5 hr. of swimming; group* A′, *nonstressed controls; group* C′, *nonstressed controls, 1.5 hr. of swimming. Key:* – – –, *group* A; — · —, *group* B; ····, *group* C; ●, *group* A′; O, *group* C′.

the rats were gently wiped and kept for 2 hr. in a draft-free cage maintained at 27°. Dermal permeability was then assayed. For each group of 24 treated rats there were 16 control rats of equal initial age and body weight. After 1, 4, 16, 32, or 48 days, dermal permeability was assayed in control rats, group *A′*. The remaining eight control rats, group *C′*, were treated in the same manner as group *C* above.

Assay of Dermal Permeability—Dermal permeability was assayed by the method of Clay and Nelson (7). A volume of 0.05 ml. of each indicator

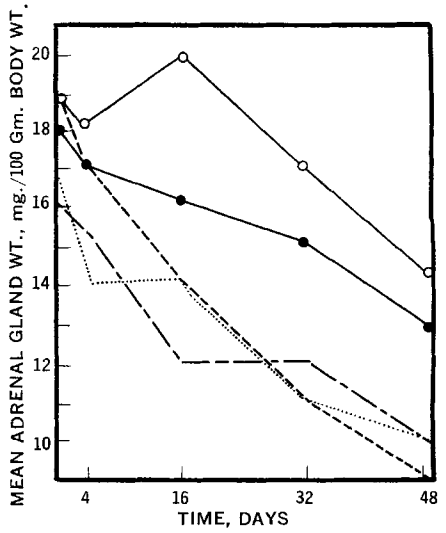

Fig. 3—Effect of stress on adrenal gland weight. Group A, *heat stress; group* B, *heat stress, followed by 24 hr. recovery; group* C, *heat stress, recovery, and 1.5 hr. of swimming; group* A′, *nonstressed controls; group* C′, *nonstressed controls, 1.5 hr. of swimming. Key:* – – –, *group* A; — · —, *group* B; ····, *group* C; ●, *group* A′; O, *group* C′.

solution was injected into the skin of the flank of rats lightly anesthetized with ether. The indicator solution contained 17% by volume of India ink and 0.9% NaCl or the same solution with 150 N.F. units of hyaluronidase[1] added. The area of spread of the India ink in the skin was calculated by substituting the largest and widest dimensions of the ink spot in the formula for an ellipse. Measurements were made 1 hr. after injection under light ether anesthesia. Immediately after measurement of ink spread, the rats were weighed, decapitated, and blood collected. Adrenal glands were removed, dissected free of fat, and weighed on a Christian Becker analytical balance.

Serum Protein Electrophoresis—The blood was permitted to stand for 20 min. The coagulated blood was centrifuged for 10 min. The sera of selected groups were pooled for electrophoretic analysis by means of the Perkin-Elmer model 38 Tiselius electrophoresis apparatus. Pooled sera were diluted 1:3 with barbital buffer of pH 8.6 which was 0.1 *M* with respect to sodium diethyl barbiturate and 0.015 *M* with respect to diethyl barbituric acid, with an ionic strength of 0.1. Each sample was dialyzed in a bag of Visking tubing against the barbiturate buffer for 24 hr. at 1.5°. A volume of 2 ml. of the dialyzed diluted serum was subjected to electrophoretic analysis. The electrophoretic patterns obtained by the scanning method of Longsworth (8) were projected, magnified, traced, and the areas under the peaks measured by means of a planimeter.

RESULTS AND DISCUSSION

Connective Tissue Permeability—Spread of the hyaluronidase–India ink indicator solution in the control rats of the *A′* groups did not vary signifi-

[1] The authors thank G. D. Searle and Co. for the supply of hyaluronidase, trademarked Alidase.

TABLE I—SERUM ELECTROPHORESIS DATA

Treatment	% Total Protein				
	Albumin	α1-Globulin	α2-Globulin	β-Globulin	γ-Globulin
4 Days heat stress	40.7	22.7	7.8	18.0	8.7
24 hr. recovery	50.9	16.2	7.2	16.4	6.7
Swimming	53.6	11.7	7.3	21.6	4.4
32 days heat stress	58.3	9.7	6.1	12.0	5.5
24 hr. recovery	51.0	16.2	10.3	16.4	5.2
Swimming	45.0	25.2	5.7	12.9	7.5
48 days heat stress	69.8	[a]	8.5	15.0	4.8
24 hr. recovery	58.6	14.2	7.6	14.0	5.3
Swimming	66.3	[a]	9.8	16.5	7.2
Control	51.2	13.1	6.0	20.5	9.0
Swimming	53.6	11.7	7.3	21.6	4.4

[a] Values included with α2-globulin, as peaks were indistinct. *Note:* totals do not equal 100% due to presence of unidentified components.

cantly during the 48 days of the study (Fig. 1). The acute stress of swimming caused spread to decrease at each time interval ($P = <0.05$, in each case), accompanied by increased adrenal gland weights in the control rats of the C' groups.

Heat stress (group A) resulted in decreased hyaluronidase spreads compared to control levels (group A') at each time interval except 16 days when the spreads were approximately equal. The spreads were decreased as follows: 1 day, (−) 47.6 mm.^{2}; 4 days, (−) 10.7 mm.^{2}; 32 days, (−) 48.1 mm.^{2}; and 48 days, (−) 38.4 mm.^{2}. The differences were significant only at 32 and 48 days ($P = <0.01$). At 32 and 48 days adrenal gland weights decreased from control levels ($P = <0.01$). (Fig. 3.) Clay and Sinai (4) reported that rats maintained at 12° also showed decreased spreads accompanied, however, by increased adrenal gland weights. Hellman *et al.* (9) reported no change in urinary output of 17-hydroxycorticosteroids in human subjects exposed to heat stress. There was, however, an increase in aldosterone secretion.

Figure 1 shows that hyaluronidase–India ink spreads generally increased 24 hr. after removal from the heat (group B) compared to heat stress levels (group A). The differences were, however, not significant. The acute stress of swimming (Group C) caused decreased spreads at the 4, 16, and 32-day intervals compared to the 24-hr. recovery levels ($P = <0.05$ in each case). After 48 days of heat stress, however, swimming resulted in an increase in spread of 40% ($P = <0.05$) and 70% ($P = <0.01$) compared to 24 hr. recovery and heat stress levels, respectively. Swimming caused a similar rebound in spread after only 4, 8, or 16 days of cold stress (4). In this laboratory a single subcutaneous injection of cortisone acetate (5 mg./Kg. body weight) has also caused a rebound in spread in heat and cold stressed rats. In the present study, the changes in spread produced by swimming after 32 and 48 days of heat stress were not accompanied by significant alterations in adrenal gland weight. Fediay and Clay (5) have reported that the combined stress of cold and restriction of movement for 14 days resulted in increased spread, adrenal gland weight, and plasma corticosterone levels.

Spread of the India ink solution not accompanied by hyaluronidase (Fig. 2) failed to show significant differences among the various treatments.

Serum Electrophoresis—In the data presented in Table I, only a single pattern in serum protein composition can be related to dermal permeability changes. Serum albumin rose from 41% after 4 days of stress to 58% and 70% after 32 and 48 days of stress, respectively. Figure 1 shows that hyaluronidase–India ink spread in the skin fell after these time intervals. Decreased connective tissue permeability to colloidal particles might act to restrict movement of albumin out of the vascular system.

The remaining changes in serum protein composition cannot be related to alterations in connective tissue permeability.

REFERENCES

(1) Wittersfors, S., Liljedahl, O., Plantin, L. O., and Birke, G., *Acta Med. Scand.*, **168,** 347(1960).
(2) *Ibid.*, **172,** 163(1962).
(3) Clay, M. M., and Nelson, J. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43,** 230(1954).
(4) Clay, M. M., and Sinai, C. R., *J. Pharm. Sci.*, **54,** 469(1965).
(5) Fediay, Z., and Clay, M. M., *Nature*, **202,** 907(1964).
(6) Menkin, V., *Am. J. Physiol.*, **166,** 518(1951).
(7) Clay, M. M., and Nelson, J. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43,** 222(1954).
(8) Longsworth, L. G., *J. Am. Chem. Soc.*, **61,** 529(1939).
(9) Hellman, K., Collins, K. J., Gray, C. H., Jones, R. M., Lunnon, J. B., and Weiner, J. S., *J. Endocrinol.*, **14,** 207 (1956).

Use of a Technique for Measuring Pulmonary Resistance to Airflow for the Pharmacological Evaluation of Bronchodilator Agents

By ROBERT G. FAMILIAR, JOE R. WARDELL, JR., and LEON C. GREENE

A technique for measuring pulmonary resistance to airflow without the interference of compliance changes was used to obtain data on the bronchodilator activity of ephedrine sulfate and methoxyphenamine hydrochloride. The resting pulmonary resistance of cats was increased by injecting histamine (100 mcg., base, i.v.) or 5-hydroxytryptophan creatinine sulfate (25 mcg., base, i.v.) or by electrical stimulation (5 v., 1.5 msec., 15 c.p.s.) of the peripheral ends of both vagus nerves. The average maximum per cent inhibition of the increase in pulmonary resistance induced by the constrictor agents was used to calculate ED_{50}'s of the test drugs. Drugs were administered either intraduodenally or intravenously. The ability to obtain valid quantitative data for the evaluation of potential bronchodilator agents offers a distinct advantage over the methods presently employed.

MEASUREMENTS of lung volume changes with constant pressure inflations or tracheal pressure changes with constant volume inflations have been used to evaluate bronchodilator agents in the intact animal (1–3). At the ventilation frequencies used, these methods are sensitive to changes in lung stiffness and relatively less sensitive to changes in pulmonary resistance to airflow (4). Such measurements are only indirectly related to airway smooth muscle tone and can be influenced by other factors such as pulmonary vascular congestion, edema, or alveolar collapse. It must be appreciated also that no valid quantitative deductions can be made from these methods about airway smooth muscle.

Obviously, a method which measures changes in airway size separate from changes in lung stiffness or lung compliance would be advantageous in the evaluation of bronchodilator agents. With a technique previously described (5), it is possible to separate these parameters and measure pulmonary resistance without the interference of compliance changes. Pulmonary resistance includes both airway resistance and tissue resistance. Tissue resistance represents only a small part of the pulmonary resistance and does not vary markedly even in pathological states (6). Therefore changes in pulmonary resistance are a valid index of changes in airway resistance. This technique has been used in the past to study the action of bronchoconstrictor agents on the airways (7–9), but has not been used for comparative pharmacological evaluation of bronchodilator agents. To demonstrate the application of this technique, two bronchodilators, ephedrine sulfate and methoxyphenamine hydrochloride, have been studied for their ability to inhibit the increase in pulmonary resistance induced by histamine, serotonin, and vagal stimulation.

EXPERIMENTAL

Cats of either sex weighing 1.6–4.0 Kg. are anesthetized with sodium pentobarbital (30 mg./Kg. i.p.), paralyzed with gallamine triethiodide (40 mg. i.v.), and respired through a tracheal cannula at a constant tidal volume (50 ml.) and frequency of 28 breaths per minute. Transpulmonary pressure (P_{tp}) is measured by introducing a No. 12 Malecot catheter through the 5th or 6th intercostal space into the pleural space, and connecting it to one side of a Statham differential pressure transducer (type PM 131 TC). The other side of this transducer is connected to a hole in the side of the tracheal cannula. Airflow is measured with a Fleisch pneumotachograph (type 0.157) in conjunction with a Statham differential pressure transducer (type PM 5 TC). A second Statham differential pressure transducer (type PM 15 TC) is also connected to the pneumotachograph, and a volume signal is obtained by electrical integration of the airflow signal from this transducer. A schematic illustration of the experimental set-up is shown in Fig. 1.

Individual parameters of transpulmonary pressure, airflow, and volume are monitored *via* their respective amplifiers on the upper screen of an Electronics for Medicine recorder (model DR 8). The lower screen of the recorder is used to monitor pulmonary resistance utilizing the subtraction technique of Mead and Whittenberger (5).

Once the resting pulmonary resistance has been established, an increase in resistance is induced either by injecting histamine (100 mcg., base) or serotonin (25 mcg. base) i.v. or by electrically stimulating the peripheral ends of cut vagus nerves. In experiments where vagal stimulation is used, the animal is pretreated 1 hr. before stimulation with 3–5 mg. of neostigmine i.v. to improve the consistency of the response. Control experiments have shown that the responses obtained with any of the constrictor agents are quite consistent over at least a 2-hr. period.

Intraduodenal Administration of Test Drug—To administer a test drug intraduodenally, a midline incision, 4 cm. long, is made in the abdomen and the duodenum is exposed. Two control responses to the constric or agent of choice are made 3 min. apart. The drug is then injected directly into the duodenum, either in suspension (0.5% tragacanth), or solution (distilled water) in a volume of 4 ml. Postdrug responses to the constrictor agent are made at 10, 20, 40, 60, 90, and 120 min.

Intravenous Administration of Test Drug—Two control responses to the constrictor agent of choice are taken 3 min. apart. The test drug is then administered i.v., in saline. Postdrug responses to the constrictor agent are made at 30 sec., 5 min., 15

Received February 24, 1967, from the Pharmacology Section, General Therapeutics Department, Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication March 22, 1967.

The authors are indebted to Miss Marilyn Lebbing, Mr. Joseph Loux, and Miss Rosemarie Leandri for their technical assistance and thank Mr. Roy Eby for statistical analyses of the data.

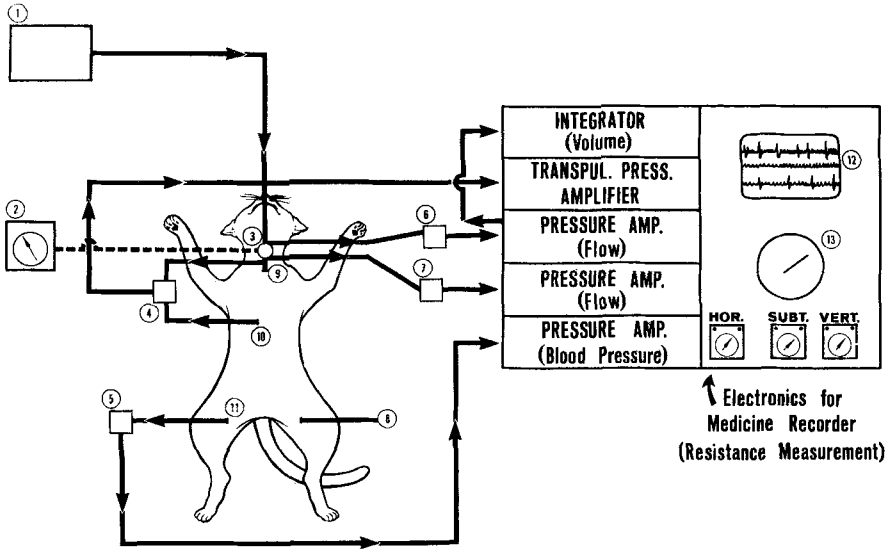

Fig. 1—Apparatus for measurement of pulmonary resistance. Key: 1, Harvard pump; 2, heater with rheostat; 3, pneumotachograph (A. Fleisch); 4, differential strain gauge (PM 131 TC 2.5); 5, pressure transducer; 6, differential pressure transducer (PM 15); 7, differential pressure transducer (PM 5); 8, intravenous catheter (injections); 9, tracheal cannula; 10, intrapleural catheter; 11, arterial cannula; 12, multitrace screen; 13, vector scope-resistance.

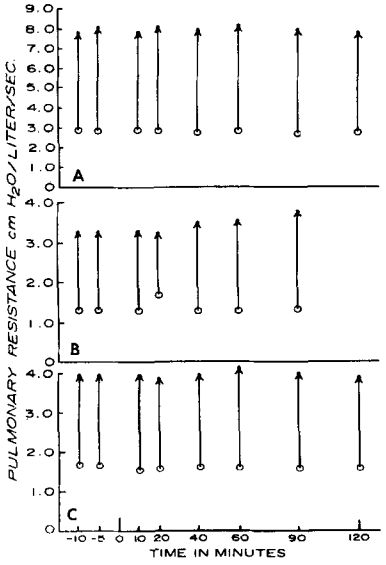

Fig. 2—Average control responses to: A, vagal stimulation (5 v., 1.5 msec., 15 c.p.s.) (two cats); B, histamine (100 mcg., i.v.) (three cats); and C, serotonin (25 mcg., i.v.) (three cats). Open circles represent pulmonary resistance preceding each administration of constrictor stimuli, while the dots on the top of the arrows represent pulmonary resistance 30 sec. after each administration. Distilled water was administered intraduodenally, 4 ml. vol. at zero time.

min., and at various intervals until the response returns to control values.

Evaluation of Data—The per cent inhibition of the increase in pulmonary resistance is calculated by the following formula. The test response is the increase in pulmonary resistance produced by the constrictor stimulus:

$$\% \text{ inhibition} = \frac{\text{av. predrug test response} - \text{postdrug test response}}{\text{av. predrug test response}} \times 100$$

The resting pulmonary resistance during the predrug period serves as the baseline for both the predrug and postdrug test responses. Postdrug test responses which are greater than the predrug value are considered zero per cent inhibition.

A dose-response curve is plotted for any test drug by obtaining a minimum of 3 to 5 individual responses for each dose, and calculating an average maximum per cent inhibition of pulmonary resistance increase. For intraduodenal administration each cat received only one dose of test drug. Several doses of a test drug, the number depending on the duration of individual responses, could be administered for intravenous evaluation. An ED_{50} and 95% Fieller limits are calculated by a method described by Finney (10).

RESULTS

Control Responses to Individual Constrictor Stimuli—The pulmonary resistance increase induced by histamine, serotonin, or vagal stimulation was consistent over time. The average results of the control experiments, in which distilled water (4 ml.) was injected intraduodenally, are shown in Fig. 2.

Intraduodenal Administration of Test Drugs—Ephedrine was shown to inhibit the pulmonary resistance increase induced by histamine, serotonin, and vagal stimulation. ED_{50}'s have been obtained for ephedrine against each of the constrictor agents. The onset of activity was between 20 and 60 min.; the duration was usually greater than 90 min. The dose-response curves are shown in Fig. 3.

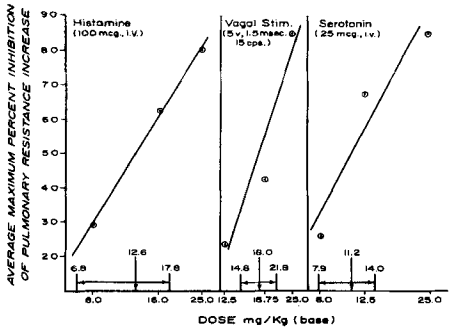

Fig. 3—Effect of intraduodenal ephedrine on the pulmonary resistance increase induced by histamine, serotonin, and vagal stimulation. Each point on the curves represents a minimum of three individual cats. ED_{50} (95% Fieller limits) in each case.

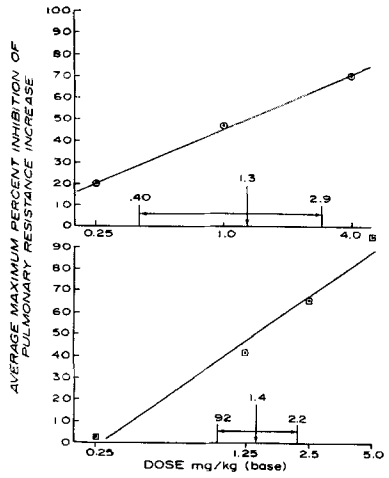

Fig. 4—Effect of intravenous ephedrine (top) and methoxyphenamine (bottom) on the pulmonary resistance increase induced by serotonin (25 mcg. i.v.). Each point on the curves represents a minimum of three responses. ED_{50} (95% Fieller limits) in each case.

Intravenous Administration of Test Drugs—To demonstrate the feasibility of using i.v. administration for drug evaluation, dose-response curves were obtained for ephedrine and methoxyphenamine against serotonin injection (25 mcg., i.v.). Both compounds were shown to inhibit the pulmonary resistance increase induced by this constrictor agent. The results are shown in Fig. 4.

DISCUSSION

The control of airway muscle has been frequently investigated. In the main, the studies fall into two categories. The first includes *in vitro* studies of the action of drugs and chemicals on excised airways or on denervated lung. Such methods are obviously unphysiological, the studies being limited to certain portions of the airways, and correlation with clinical conditions impossible. The second category includes *in vivo* studies involving the assessment of the relationship between inflation pressure and tidal volume during cyclical changes in pressure or volume. These methods measure all three pressures of breathing: elastic, resistive, and accelerative; therefore, changes in either compliance or resistance will affect the measurement. In fact, at the usual frequencies of ventilation, such measurements are relatively insensitive to changes in airway diameter (*i.e.*, resistive and accelerative forces) but are greatly influenced by alterations in lung stiffness (*i.e.*, elastic forces). Although lung compliance may change in the present method, this change is quite obvious (opening of pressure–flow trace) and correction can be made. In other words, the changes measured are due to changes in the size of the airways.

All of the agents used to induce the increase in pulmonary resistance have been implicated in hypersensitivity reactions (11–13). A more meaningful evaluation of potential bronchodilators can be made if they are compared to standard agents for their effect against all three of these constrictor stimuli, rather than any one.

The feasibility of using this method for intraduodenal drug evaluation is demonstrated in Fig. 2. The stable resting pulmonary resistance along with consistent responses to each constrictor stimulus over time makes the method ideal for this purpose. Some drugs may decrease an animal's resting pulmonary resistance in addition to inhibiting the response to a constrictor stimulus. Both effects are reflected in the calculations of drug activity since the predrug resting resistance is used as the baseline for postdrug test responses.

This method of drug evaluation offers a distinct advantage over the other methods since the measurements are not influenced to any great extent by factors such as pulmonary vascular congestion, edema, or alveolar collapse. In addition, as shown, the data can be quantitatively analyzed for a more valid comparison of test agents.

REFERENCES

(1) Hanna, C., *Arch. Intern. Pharmacodyn.*, **134**, 426 (1962).
(2) Cambridge, G. W., Janses, A. B. A., and Jarman, D. A., *Nature*, **196**, 1217(1962).
(3) Amin, A. H., and Mehta, D. R., *ibid.*, **184**, 317 (1959).
(4) Widdicombe, J. G., *Phys. Rev.*, **43**, 1(1963).
(5) Mead, J., and Whittenberger, J. L., *J. Appl. Physiol.*, **5**, 779(1953).
(6) Nadel, J. A., and Widdicombe, J. G., *J. Physiol.*, **163**, 13(1962).
(7) Widdicombe, J. G., Kent, D. C., and Nadel, J. A., *J. Appl. Physiol.*, **17**, 613(1962).
(8) Nadel, J. A., Salem, H., Tamplin, B., and Tokiwa, Y., *ibid.*, **20**, 164(1965).
(9) Colebatch, H. J. H., Olsen, C. R., and Nadel, J. A., *ibid.*, **21**, 217(1966).
(10) Finney, D. J., "Statistical Methods and Biological Assay," Hafner Publishing Co., New York, N. Y., 1952.
(11) Frank, D. E., *Ann. Allergy*, **17**, 200(1959).
(12) Curry, J. J., *J. Clin. Invest.*, **25**, 785(1946).
(13) West, G. B., *Intern. Arch. Allergy Appl. Immunol.*, **18**, 56(1961).

Phytopharmacologic Studies of *Ipomoea muricata* Seeds

By M. L. GUPTA, J. P. TEWARI, S. N. KHANNA*, P. C. GUPTA*, M. C. SRIVASTAVA, and S. S. MISHRA

A glycoside muricatin ($C_{28}H_{52}O_{11}$), isolated from the seeds of *I. muricata*, appears to consist of 4-*O*-L-rhamnopyranocyl-L-rhamnopyranose moiety attached glycosidically to (+)-11 hydroxyhexadecanoic acid. It is cardiac depressant, spasmolytic to the smooth musculature of gut, lowers the blood pressure of the anesthetized dog, and has no action on the respiration, uterus, skeletal muscle, and blood vessels of the frog.

UNDER the research program on systematic phytochemical and pharmacological investigations of the plants belonging to genus *Ipomoea* (5, 6, 9–11), studies on the seeds of *Ipomoea muricata* were undertaken in this department. *I. muricata* (family, Convolvulacae), commonly known as kaladana, is found in the Himalyan region from Kangra to Sikkim and on Dacan hills. Seeds of the plant are bitter, purgative, and used as a substitute for *I. hyderacea* and in powder form as febrifuge (2, 4). Leaves are used in apthous (8). In this communication, characterization and preliminary pharmacodynamic actions of the glycoside muricatin isolated from the seeds of *I. muricata* are reported.

MATERIALS AND METHODS

Chemical—The glycoside muricatin has been isolated from the petroleum ether extract of the authenticated seeds of *I. muricata* by the method of Mishra and Tewari (7).

A chemical examination of the seeds of this plant was also made by Mishra and Tewari (7). They reported an enzyme, a lipoid, an organic acid, a glucoside, $C_{30}H_{54}O_{13}.1^1/_2H_2O$, and a dark brown resinous mass. However, the glycoside muricatin (m.p. 108–109°, $[\alpha]_D^{25} - 44.5°$) isolated in the present study is a white crystalline solid. It was found to be soluble in water, methanol, ethanol, sparingly soluble in acetone, and insoluble in ether, benzene, and chloroform. The elemental analysis of muricatin indicated the empirical formula $C_{28}H_{52}O_{11}$. This water-soluble glycoside was a weak acid with neutralization equivalent 543. Acid hydrolysis yielded 2 moles of L-rhamnose and 1 mole of fatty acid. On the basis of physical and chemical studies, muricatin appears to consist of 4-*O*-L-rhamnopyranocyl-L-rhamnopyranose moiety attached glycosidically to (+)-11-hydroxyhexadecanoic acid.

Pharmacodynamic Studies of Muricatin—The pharmacodynamic actions of this water-soluble glycoside muricatin have been studied on various organ systems. In each case 10 experiments were performed to draw the inference.

To study the effect of muricatin on the frog's heart, perfusion of the frog heart *in situ* and isolated frog's heart was carried out according to the method of Bulbring, as quoted by Burn (1). The ventricular contractions were recorded on a slowly moving drum through a starling heart lever.

The effect of drug was studied on the isolated frog rectus abdominis muscle preparation (set up in a 5-ml. bath), according to the method described by Burn (1). Acetylcholine responses were obtained by offering it in a strength of 1:2,000,000 for 90 sec.

The effect of drug on carotid blood pressure, respiration, carotid occlusion response, and intestine *in situ* was studied on anesthetized mongrel dogs of either sex, weighing between 6 and 10 Kg. Sodium pentobarbital (35 mg./Kg. i.p.) was used as an anesthetic agent. Blood pressure was recorded by introducing an arterial cannula in the left carotid artery and connecting the cannula to a mercury manometer. Respiration was recorded by connecting the trachea with Mary's tambur. All the injections were made through the cannulated femoral vein. Auricular and ventricular contractions were registered in anesthetized dogs by the suspension method of Jackson (3). Intestinal movements in dogs were recorded through Jackson's enterograph.

In the dog the common carotid arteries of both sides were exposed. Gentle pressure by putting bull dog clips on both the carotid arteries for 10 sec., at a point just below the bifurcation of carotid arteries into external and internal carotid arteries, brought about occlusion and rise in blood pressure. In the case of carotid occlusion studies in dogs, the blood pressure was recorded from the femoral artery.

The action of muricatin was also observed on electrically induced contractions (preganglionic stimulation) of the cat's nictitating membrane. Experiments were also performed to study the effect of the drug on blood pressure of the cat, following spinal transection at the C_2 level, using the method of Burn (1).

The effect of the drug was observed on isolated preparations of rabbit's and rat's ileum. The freshly removed segments were suspended in a 10-ml. isolated organ bath, containing Tyrode solution (NaCl, 8.0 Gm.; KCl, 0.2 Gm.; $MgCl_2$, 0.5 Gm.; $NaHCO_3$, 1.0 Gm.; Na_2HPO_4, 0.5 Gm.; distilled water, 1 L.) maintained at 37 ± 1° and freely oxygenated.

The action was also observed on isolated uterus of the gravid and nongravid albino rat, guinea pig, and rabbit. One of the uterine horns was suspended in a 10-ml. bath containing oxygenated modified Locke's solution (NaCl, 9.0 Gm.; KCl, 0.42 Gm.; $CaCl_2$, 0.06 Gm.; glucose, 0.5 Gm.; $NaHCO_3$, 0.5 Gm.; distilled water, 1 L.) at 32°. The effect of a single dose was recorded for 90 sec. and an interval of 5 min. was allowed between the successive doses.

Blood vessels of the frog were perfused with Ringer's solution (NaCl, 0.65%; KCl, 0.014%; $CaCl_2$, 0.012%; $NaHCO_3$, 0.02%; Na_2HPO_4, 0.001%; distilled water, 100 ml.) by introducing a cannula in the innominate artery and counting the drops of perfusate. Injections of the drug were made in the ventral lymph sac.

OBSERVATIONS

Muricatin in graded doses causes a decrease in rate, amplitude, and tone of the frog's heart *in situ* and isolated frog's heart. The repetition of higher doses produced tachyphylaxis. The cardiac depressant

Received December 13, 1966, from the Department of Pharmacology, G.S.V.M. Medical College, Kanpur, India.
Accepted for publication March 9, 1967.
* Address inquiries to Department of Chemistry, University of Allahabad, India.
This work was generously supported by grant from I.C.M.R., New Delhi, India.

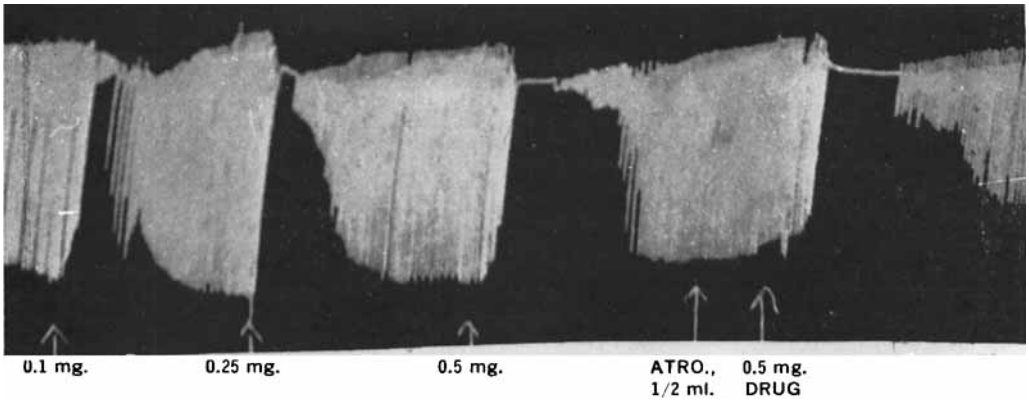

Fig. 1—Effect of muricatin on isolated frog's heart.

effect of the drug could not be blocked by pretreatment with atropine, which, however, blocked the activity of equidepressant doses of acetylcholine (Fig. 1).

Muricatin (1 mg./Kg.) caused a sharp fall in blood pressure (Fig. 2) without having any significant action on respiration of anesthetized dogs. This effect was also noticed in cats. Atropine sulfate (2 mg./Kg.) partially blocked this hypotensive effect of muricatin. But unlike acetylcholine, the drug did not give any pressure response when given after administration of atropine and eserine sulfate (1 mg./Kg.). This hypotensive effect in cats persists to the same degree even after spinal transection at the C_2 level. The hypotensive effect was more marked in experimentally induced hypertension in dogs with norepinephrine infused at the rate of 20 drops/min. at a concentration of 1:1000. Muricatin inhibited both auricles and ventricles *in situ* in dogs. The drug had no significant effect on the dog's carotid occlusion reflex and ileum *in situ* nor on electrically induced contractions of the cat's nictitating membrane.

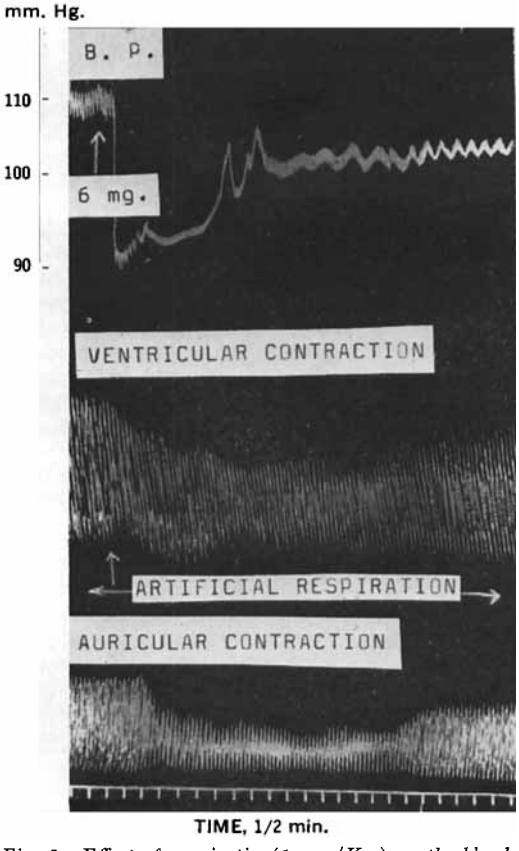

Fig. 2—Effect of muricatin (1 mg./Kg.) on the blood pressure and auriculo ventricular contractions of a female dog (6 Kg.) anesthetized with pentobarbital.

On isolated rabbit and rat ileum, muricatin in all the doses tried exhibited a spasmolytic activity. In these doses this was quite marked in magnitude. The antispasmodic activity of muricatin against spasms induced by posterior pituitary, acetylcholine chloride (0.2 mcg./ml.), histamine diphosphate (0.2 mcg./ml.), barium chloride (0.1 mg./ml.), and 5 HT (0.4 mcg./ml.) in isolated ileum of rabbit and rat has been studied. A 0.5-mg. quantity (in a bath of 10 ml.) of the drug antagonized these spasms (Fig. 3). The spasmolytic activity of papaverine was also of similar pattern, but it was about 4 to 5 times stronger in activity than muricatin in this respect.

The drug failed to have any effect on uterine strips of the various species tried and did not antagonize the spasm of the uterus induced by posterior pituitary. On the frog rectus muscle the drug had no action at any concentration. It did not affect the acetylcholine induced contractions. Muricatin had no effect on the perfused blood vessels of the frog at any concentration.

CONCLUSIONS

Ipomoea provides many plants which are employed in the Ayurvedic system of medicine. A glycoside has been isolated from *I. carnea* (12), but its pharmacological properties have not been reported.

The glycoside muricatin isolated from *I. muricata* in the present communication is water-soluble and thermostable. It shows a characteristic depressant action on the frog's heart. In older work on glycosides this was the principal effect on which attention was focused. In the initial stages fuller contractions in systole occur; various arrhythmias develop later (13).

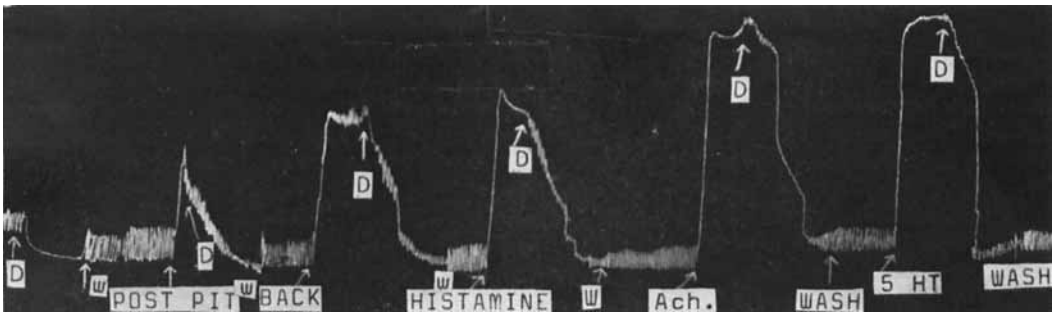

Fig. 3—Effect of muricatin on isolated rabbit ileum. Key: D, *drug (0.5 mg.).*

The blood pressure lowering property of muricatin is a characteristic response, which is partially blocked by atropinization. But this lowering of blood pressure persists even after spinal transection at the C_2 level. Therefore, it can be inferred that the lowering of blood pressure may be due to a myocardial depressant property of muricatin as evidenced by the observations on auricular and ventricular contractions.

The drug shows its benignity toward other organ systems of the body except a direct potent spasmolytic effect on gastrointestinal smooth musculature. In general, the pattern of relaxant and spasmolytic activity of muricatin was similar to that of papaverine. The difference was that the former was a weaker relaxant and spasmolytic compared to the latter. It therefore appears that the mechanism of action of muricatin on ileum is a papaverine-like, direct, nonspecific musculotropic action.

REFERENCES

(1) Burn, J. H., "Practical Pharmacology," J & A Churchill Ltd., London, England, 1952.
(2) Chopra, R. N., Nayer, S. L., and Chopra, I. C., "Glossary of Indian Medicinal Plants," C.S.I.R. Publication, New Delhi, India, 1958.
(3) Jackson, D. E., "Experimental Pharmacology and Materia Medica," 2nd ed., C. V. Mosby & Co., London, England, 1939, p. 285.
(4) Kirtikar, K. R., and Basu, B. D., "Indian Medicinal Plants," Part II, L. M. Basu, Allahabad, India, 1933, p. 873.
(5) Mishra, S. S., Tewari, J. P., and Matin, M. A., *J. Pharm. Sci.*, **54**, 471(1965).
(6) Mishra, S. S., and Dutta, K. C., *Ind. J. Med. Res.*, **50**, 43(1962).
(7) Mishra, A. L., and Tewari, J. D., *Indian J. Chem. Soc.*, **29**, 430(1952).
(8) Nadkarni, A. K., and Nadkarni, K. M., "Indian Materia Medica," Dhootpapeswar Pakashan, Bombay, India, 1954.
(9) Tewari, J. P., Matin, M. A., and Mishra, S. S., *Ind. J. Appl. Chem.*, **27**, 155(1964).
(10) Tewari, J. P., and Dutta, K. C., *Labdev. J. Sci. Tech.*, **2**, 1(1964).
(11) Tewari, J. P., and Mishra, S. S., *ibid.*, **3**, 72(1965).
(12) Ventura, S. S., through *Chem. Abstr.*, **42**, 7838(1948).
(13) Valpian, E., *Compt. Rend.*, **88**, 1293(1879).

Testing of Tablets with Prolonged Action. Enzyme Activity During the Modified Half-Change Method

By W. A. RITSCHEL and H. ORTH

The enzyme activities of pepsin, pancreatic lipase, trypsin, and amylase were studied. It was found that the activity of the enzymes in freshly prepared artificial gastric fluid and in artificial intestinal fluid decreases during heating from room temperature to 37° and by keeping at 37°. To maintain proper enzyme levels during an 8-hr. interval when testing tablets with prolonged action, a modified half-change method is suggested. A survey of enzyme levels when using this modified method is given.

MANY SUBSTANCES used in tableting as excipients and fillers, such as zein, starch, stearates, and others, are digestible by gastrointestinal enzymes. Therefore, the possible effect of enzymes on disintegration and/or drug dissolution of oral tablets in *in vitro* tests must be taken into consideration, particularly in the case of procedures of several hours' duration applicable to sustained-release medication. In prolonged-release medication forms the drug is often embedded in synthetic fats such as glyceryl monostearate (1), glyceryl myristate (1), glyceryl palmitostearate (2), *etc.* Such glyceryl fatty acid esters are hydrolyzed by pancreatic lipase (3). The enzymatic hydrolysis depends on the length of the carbon chain of the fatty acids (4). The liberation of the active principle embedded in the tablet follows a characteristic inverted curve, because beside diffusion of the drug, digestion of the tablet base material takes place after 3 hr. when optimal pH conditions are reached for the enzyme activity (5). Gelatin, used as a binder in wet granulation as well as in direct compression, is liquified by trypsin (6), while casein (7) and peptides used for coatings (8) are digested. Amylose, suggested as a dry binder for direct compression (9), is digested by amylase. Gelatin, casein, and other peptides are also digested by pepsin (10).

Studying the stability of gastrointestinal enzymes in artificial gastric and intestinal fluid, it was found that the activity of the enzymes was partly destroyed during the time needed to heat the test solutions from room temperature to 37°, and while

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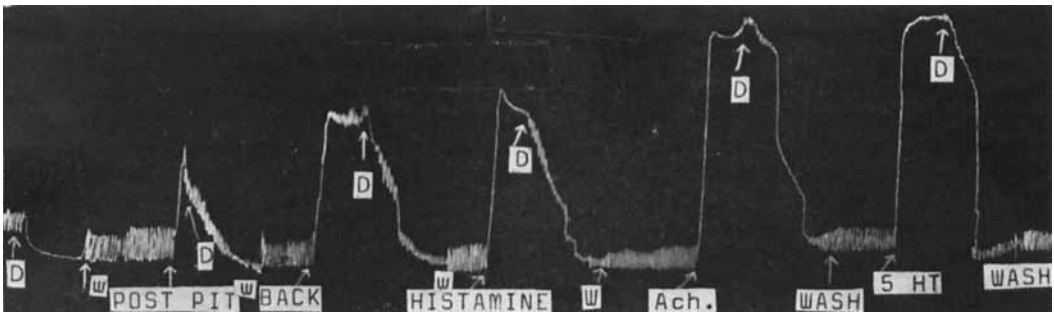
Accepted for publication March 6, 1967.



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REFERENCES

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(2) Chopra, R. N., Nayer, S. L., and Chopra, I. C., "Glossary of Indian Medicinal Plants," C.S.I.R. Publication, New Delhi, India, 1958.
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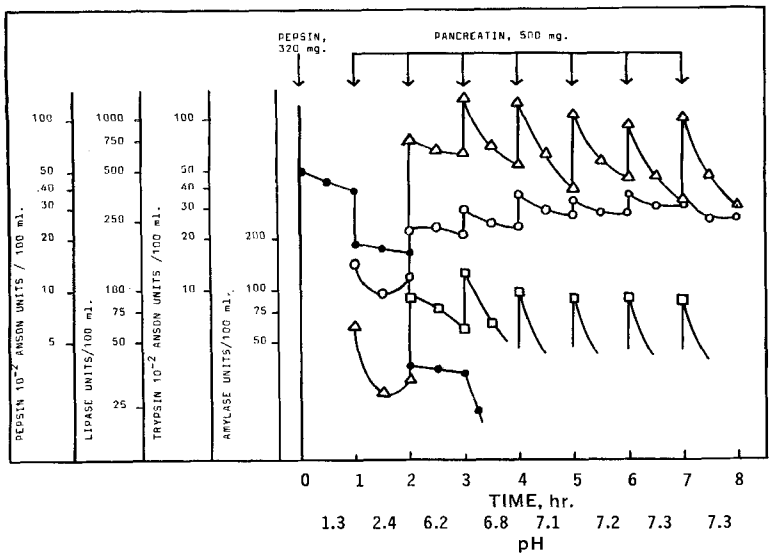


Fig. 1—Semilog plots for the ferment activity of gastrointestinal enzymes during testing with the modified half - change method at 37°. Key: ●, pepsin; △, lipase; ○, trypsin; □, amylase.

keeping artificial gastrointestinal test solutions at 37°. Loss of pepsin activity was 20% within 1 hr.; loss of lipase activity was 68% within 30 min. and 84% within 1 hr.; loss of trypsin activity was 50% within 1 hr.; and loss of amylase activity was more than 50% within 10 min. Based on the results obtained with lipase, it was felt that utilization of the principle of the so-called "half-change method" (11), in order to bring about uniformity of enzymatic activity during the course of the test period, was desirable.

EXPERIMENTAL

Materials—*Artificial Enzyme-Free Gastric Fluid*—Sodium chloride, 0.2%, and 0.7% hydrochloric acid in water. One hundred milliliters of the artificial enzyme-free gastric fluid at 37° was used in each test; 320 mg. of pepsin was added; pepsin activity was determined 30 and 60 min. thereafter.

Artificial Enzyme-Free Intestinal Fluid—Predried sodium phosphate, ($Na_2HPO_4 \cdot 2H_2O$), 0.805%, 0.165% sodium biphosphate ($NaH_2PO_4 \cdot 2H_2O$) in water. Fifty milliliters of the artificial enzyme-free intestinal fluid at 37° was used in each test.

Modified Half-Change Method—After a 60-min. interval, half of the volume of the artificial gastric fluid was withdrawn and substituted with 50 ml. of artificial enzyme-free intestinal fluid at 37°. At hourly intervals thereafter, again half of the fluid was withdrawn and substituted with fresh, prewarmed 50-ml. portions of artificial intestinal fluid. Immediately after each addition, 500 mg. of pancreatin powder was added. The test period was extended for 8 hr.

The activities of enzyme lipase, trypsin, and amylase were tested immediately after addition of each portion of pancreatin to the test solution, 30 min. thereafter, and just before the next addition.

Pepsin and trypsin were analyzed according to Anson (12); lipase activity was determined by the method described by Lazo-Wasem (13). For the estimation of amylase, a method described in the documentation of a manufacturer of pancreatin (14) was used; the method was based on the dextrinication point.

RESULTS AND DISCUSSION

The enzyme levels of the gastrointestinal enzymes during an 8-hr. run of the modified half-change method are shown in Fig. 1. Additions of pepsin and pancreatin are indicated by arrows; the different pH values of the test solution are also indicated in the figure. The change from pH 2.4 to pH 6.2 in the third hour is due to the effectiveness of the buffer substances in the artificial intestinal fluid.

It may be noted from the illustration that the pepsin level falls approximately 20% during the first hour at 37°. On substitution of half of the volume of the artificial gastric fluid by enzyme-free artificial intestinal fluid and addition of 500 mg. pancreatin, the decrease in pepsin activity corresponds to the level of dilution, but after the second substitution of the test solution by enzyme-free artificial intestinal fluid and subsequent addition of pancreatin in the third hour, remaining pepsin activity is negligible. The trypsin levels remain relatively constant during the whole test period, while lipase activity decreases to about 50% of initial between pancreatin additions. The expected amylase level could be found only after the second addition of pancreatin. The hourly additions of pancreatin result in short-lived measurable amylase levels.

Testing with the modified half-change method at a lower temperature to improve enzyme stability is not feasible since optimum enzyme activity depends on temperature and is correspondingly decreased at room or cooler temperatures.

The results obtained indicate that, when testing oral depot tablets, the dissolution or disintegration of which is dependent on gastrointestinal enzymes, it is necessary to use a method such as the "modified half-change method" in order to obtain uniformly effective enzyme levels.

REFERENCES

(1) Martin, E. W., and Cook, E. F., "Remington's Practice of Pharmacy," 12th ed., Mack Publishing Co., Easton, Pa., 1961, p. 500.

(2) Jaminet, Fr., and Hazee, A., *Pharm. Acta Helv.*, **41**, 530(1966).

(3) Baur, H., *Schweiz. Apoth. Ztg.*, **96**, 497(1958).

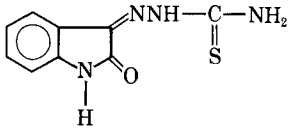
(4) Speiser, P., *A.P.V.-Informationsdienst*, **3**, 53(1964).
(5) Ritschel, W. A., "*Die Tablette*," Aulendorf, 1966, p. 306.
(6) Willstätter, R., *Z. Physiol. Chem.*, **161**, 194, 206 (1926).
(7) Kunitz, M., *J. Gen. Physiol.*, **30**, 291(1947).
(8) Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643(1939).
(9) Kwan, K. C., and Milosovich, G., *J. Pharm. Sci.*, **55**, 340(1966).
(10) "Die Substitutionstherapie mit Verdauungsfermenten," *Pharm. Ztg.*, **106**, 594(1961).
(11) Ritschel, W. A., and Orth, H., *Pharm. Ind.*, **28**, 454 (1966).
(12) Anson, M. L., *J. Gen. Physiol.*, **22**, 79(1939).
(13) Lazo-Wasem, E. A., *J. Pharm. Sci.*, **50**, 999(1961).
(14) "Bestimmung der Dextrinierungs-Einheiten," Schweizerische Ferment AG., Basle, Switzerland.

Thiosemicarbazones Derived from Indanedione-1,3

By RAJENDRA S. VARMA and W. LEWIS NOBLES

A series of thiosemicarbazones derived from indanedione-1,3 has been synthesized. Preliminary biological screening data are provided.

ANTIVIRAL (1–7) and tuberculostatic (8–14) activity has been reported for thiosemicarbazones of different carbonyl compounds. Further work on the thiosemicarbazones led to the discovery of the antiviral activity of isatin-3-thiosemicarbazone (I) against the pox group of viruses in human and type 2 polio in ERK cells (15). A number of monocyclic thiosemicarbazones derived from nicotinaldehyde, isonicotinaldehyde, and 2- and 3-thenaldehydes have also exhibited high antiviral activity (5). 4-Bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone (II), when given orally, was found to protect mice infected intracerebrally with neurovaccinia (15). Thiosemicarbazones derived from substituted pyrrolidine-2,3-diones demonstrated protection against experimental influenza infection in mice (15).



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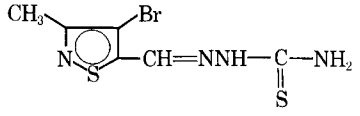
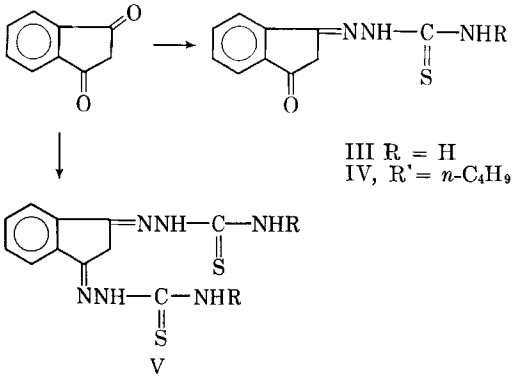


II

In view of the antiviral, antituberculous, and antitumor (16, 17) activity demonstrated by certain thiosemicarbazones, the synthesis and biological evaluation of compounds III, IV, and V appeared to be of interest (Scheme I).

Biological Evaluation—Several of the compounds described in this report have been subjected to preliminary antiviral and antibacterial screening procedures. The method with respect to the antiviral screening involved the use of the plastic panel and agar diffusion techniques and, in general, the



III R = H
IV, R' = n-C_4H_9

Scheme I

compounds were screened for possible activity against poliomyelitis type II, *Herpes simplex*, measles, and parainfluenza 3(HA-1) viruses.

In connection with the antibacterial screening, filter paper disks (6.35 mm.) saturated with 2 drops of a suspension of the test compounds (20 mg./ml. in alcohol or water) were tested with various organisms by the use of the agar diffusion method.

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EXPERIMENTAL[1]

Indanedione-1,3-thiosemicarbazone (III)—Indanedione-1,3 (0.05 mole) was dissolved in 50 ml. of hot ethanol. To this solution a slurry of thiosemicarbazide (0.05 mole) in 50 ml. of ethanol was added. Instantaneous condensation occurred resulting in the separation of a yellow solid. The reaction mixture was heated for 10 min. and allowed to cool to room temperature. The product was removed by filtration and crystallized from dimethyl-

Received January 5, 1967, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication January 30, 1967.
This investigation was supported in part by grant A104701 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors are grateful to Dr. Lyman Magee and Mr. William Bing, Department of Biology, University of Mississippi, for the antiviral and antibacterial screening data.

[1] All melting points were taken in capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. The infrared spectra were determined using a Perkin-Elmer 137 model spectrophotometer.

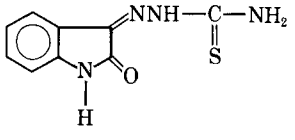
(4) Speiser, P., *A.P.V.-Informationsdienst*, **3**, 53(1964).
(5) Ritschel, W. A., "*Die Tablette*," Aulendorf, 1966, p. 306.
(6) Willstätter, R., *Z. Physiol. Chem.*, **161**, 194, 206 (1926).
(7) Kunitz, M., *J. Gen. Physiol.*, **30**, 291(1947).
(8) Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643(1939).
(9) Kwan, K. C., and Milosovich, G., *J. Pharm. Sci.*, **55**, 340(1966).
(10) "Die Substitutionstherapie mit Verdauungsfermenten," *Pharm. Ztg.*, **106**, 594(1961).
(11) Ritschel, W. A., and Orth, H., *Pharm. Ind.*, **28**, 454 (1966).
(12) Anson, M. L., *J. Gen. Physiol.*, **22**, 79(1939).
(13) Lazo-Wasem, E. A., *J. Pharm. Sci.*, **50**, 999(1961).
(14) "Bestimmung der Dextrinierungs-Einheiten," Schweizerische Ferment AG., Basle, Switzerland.

Thiosemicarbazones Derived from Indanedione-1,3

By RAJENDRA S. VARMA and W. LEWIS NOBLES

A series of thiosemicarbazones derived from indanedione-1,3 has been synthesized. Preliminary biological screening data are provided.

ANTIVIRAL (1–7) and tuberculostatic (8–14) activity has been reported for thiosemicarbazones of different carbonyl compounds. Further work on the thiosemicarbazones led to the discovery of the antiviral activity of isatin-3-thiosemicarbazone (I) against the pox group of viruses in human and type 2 polio in ERK cells (15). A number of monocyclic thiosemicarbazones derived from nicotinaldehyde, isonicotinaldehyde, and 2- and 3-thenaldehydes have also exhibited high antiviral activity (5). 4-Bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone (II), when given orally, was found to protect mice infected intracerebrally with neurovaccinia (15). Thiosemicarbazones derived from substituted pyrrolidine-2,3-diones demonstrated protection against experimental influenza infection in mice (15).



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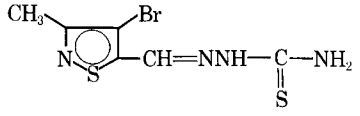
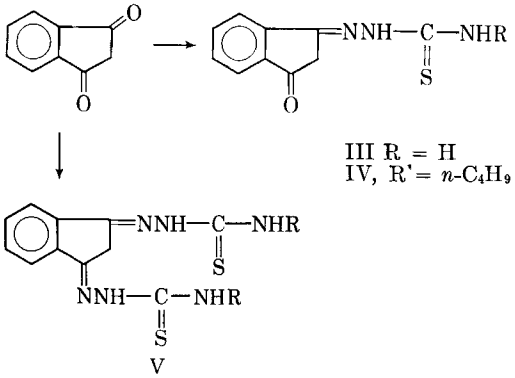


II

In view of the antiviral, antituberculous, and antitumor (16, 17) activity demonstrated by certain thiosemicarbazones, the synthesis and biological evaluation of compounds III, IV, and V appeared to be of interest (Scheme I).

Biological Evaluation—Several of the compounds described in this report have been subjected to preliminary antiviral and antibacterial screening procedures. The method with respect to the antiviral screening involved the use of the plastic panel and agar diffusion techniques and, in general, the



III R = H
IV, R' = n-C_4H_9

Scheme I

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EXPERIMENTAL[1]

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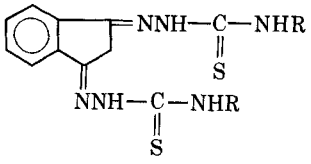
TABLE I—INDANEDIONE-1,3-DITHIOSEMICARBAZONES (V)



No.	R	M.p., °C.	Yield, %	Mol Formula	Anal., % Calcd.	Found
1	Methyl	263	78	$C_{13}H_{16}N_6S_2$	C, 48.73	49.15
					H, 5.03	5.17
					S, 20.02	19.79
2	*n*-Butyl	244–245	89	$C_{19}H_{28}N_6S_2$	C, 56.40	56.52
					H, 6.97	6.94
					N, 20.77	19.94
3	Ethyl	245	51	$C_{15}H_{20}N_6S_2$	C, 51.68	52.00
					H, 5.78	5.93
					N, 24.12	23.98
4	Allyl	237	94	$C_{17}H_{20}N_6S_2$	C, 54.80	54.80
					H, 5.41	5.44
					N, 22.56	22.76
					S, 17.22	17.00

formamide–water. Yield 10.5 Gm. (96%), m.p. 230–232° dec. $\nu_{max.}^{KBr}$ 5.85 μ (carbonyl).

Anal.—Calcd. for: $C_{10}H_9N_3OS$: C, 54.79; H, 4.13; N, 19.17. Found: C, 54.82; H, 4.19; N, 19.03.

Indanedione-1,3-n-butylthiosemicarbazone (IV)—To a boiling solution of 4-*n*-butylthiosemicarbazide (0.02 mole) in 25 ml. of ethanol was added indanedione (0.02 mole) dissolved in 25 ml. of ethanol. The resulting reaction mixture was refluxed for 20 min. A solid product separated on cooling; this was suspended in 100 ml. of ethanol–acetone mixture (1:1). The suspension was stirred and filtered. The filtrate, upon refrigeration, yielded a crystalline solid melting in the range of 173–174°. Yield, 5.0 Gm. (91%). $\nu_{max.}^{Nujol}$ 5.88 μ (carbonyl).

Anal.—Calcd. for: $C_{14}H_{17}N_3OS$: C, 61.07; H, 6.22; N, 15.26. Found: C, 61.11; H, 6.14; N, 15.10.

Indanedione-1,3-dithiosemicarbazones—The appropriate thiosemicarbazide (0.15 mole) was dissolved in 50 ml. of ethanol; to this there was added indanedione (0.05 mole). The resulting reaction mixture was refluxed for 4 hr. At the end of this period, the contents were cooled and the product removed by filtration and washed with ethanol. The thiosemicarbazones were recrystallized either from ethyl-acetate or ethanol–dimethylformamide–water. The analyses, melting points, and other pertinent data are recorded in Table I.

REFERENCES

(1) Sadler, P. W., O'Sullivan, D. G., and Bauer, D. J., *Antibiot. Chemotherapy*, **2**, 403(1963).
(2) Bauer, D. J., and Sadler, P. W., *Brit. J. Pharmacol.*, **15**, 101(1960).
(3) Buttimore, D., Jones, D. H., Slack, R., and Wooldridge, K. R. H., *J. Chem. Soc.*, **1963**, 2032.
(4) Slack, R., Wooldridge, K. R. H., McFadzean, J. A., and Squires, S., *Nature*, **204**, 587(1964).
(5) Thompson, R. L., Minton, S. A., Officer, J. E., and Hitchings, G. H., *J. Immunol.*, **70**, 229(1953).
(6) Campaigne, E., Thompson, R. L., and Van Werth, J. E., *J. Med. Pharm. Chem.*, **1**, 577(1959).
(7) Hamre, D., Bernstein, J., and Donovick, R., *Proc. Soc. Expll. Biol. Med.*, **73**, 275(1950).
(8) Domagk, G., *Naturwissenschaften*, **33**, 315(1946).
(9) Behnisch, R., Mietzsch, F., and Schmidt, H., *Angew. Chem.*, **60**, 113(1948).
(10) Buu-Hoi, N. P., Welsch, M., Dechamps, G., Bihan, H. L., Binon, F., and Xuong, N. D., *J. Org. Chem.*, **18**, 121 (1953).
(11) Varma, R. S., *J. Indian Chem. Soc.*, **43**, 558(1966).
(12) Nobles, W. L., *J. Am. Chem. Soc.*, **77**, 6675(1955).
(13) Caldwell, H. C., and Nobles, W. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 729(1956).
(14) Dodgen, D. F., Jr., and Nobles, W. L., *ibid.*, **46**, 437 (1957).
(15) Whipple, H. E., *Ann. N.Y. Acad. Sci.*, **130**, 71(1965).
(16) Petering, H. G., Buskirk, H. H., and Underwood, G. E., *Cancer Res.*, **24**, 367(1964).
(17) Petering, H. G., VanGiessen, G. J., Crim, J. A., and Buskirk, H. H., *J. Med. Chem.*, **9**, 420(1966).

Dualistic Thiourea Moiety Taste Response of Methimazole

By GLENN H. HAMOR and AIDA LAFDJIAN

Determination of the taste responses to phenylthiourea, to propylthiouracil, and to 1-methyl-2-mercaptoimidazole (methimazole) using impregnated filter paper strips on a group of 191 individuals showed that methimazole, contrary to its literature description of "almost no taste," is quite bitter to 30 per cent of the population studied, and does possess a dualistic taste similar to the other two compounds tested.

INDIVIDUALS differ in their ability to taste substances containing the thioureylene, —NHCS-NH—, or thiocarbamoyl, —NHC(S)— groups. Phenylthiourea and the cyclic thioureide, propylthiouracil, taste bitter to a considerable portion of the population, and tasteless to the rest; the inability to taste is apparently a recessive, inherited Mendelian characteristic (1–3). Structures of representative compounds, with their taste responses, are given in Figs. 1–3.

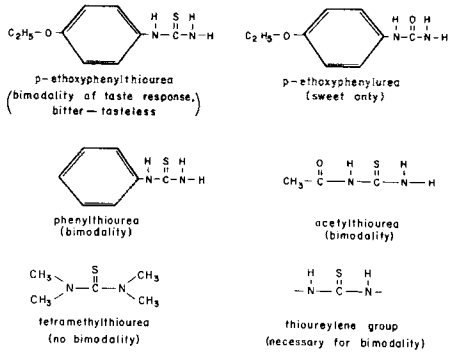


Fig. 1—Thiourea derivatives with taste responses.

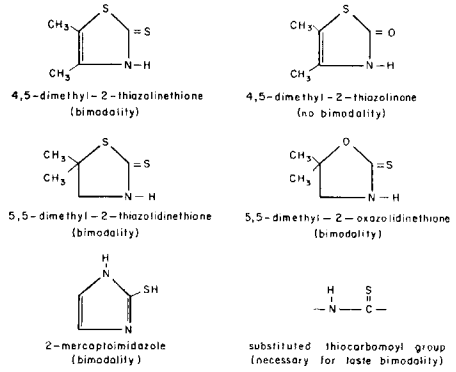


Fig. 2—Thiourea derivatives with taste responses.

The antithyroid substances, L-5-vinyl-2-oxazolidinethione (goitrin) (4, 5), 2-mercaptoimidazole (2), and propylthiouracil (2) possess the dualistic taste response. Harris, Kalmus, and Trotter found that nontasters of phenylthiourea were slightly more prone to develop adenomatous goiter than tasters (6). This finding has been confirmed by Kitchin *et al.*, who also found that toxic diffuse goiter is more prevalent among tasters (7). Shepard and Gartler

Received May 16, 1963, from the School of Pharmacy, University of Southern California, Los Angeles, CA 90007
Accepted for publication February 16, 1967.

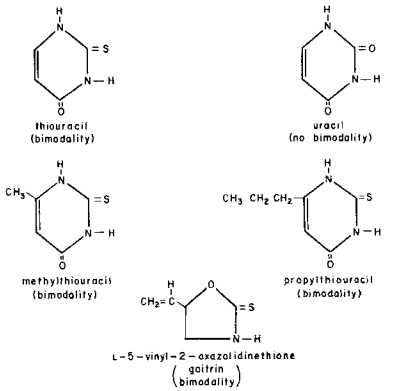


Fig. 3—Thiourea derivatives with taste responses.

reported an increased incidence of nontasters of phenylthiourea among congenital athyreotic cretins (8).

This study extends the determination of the taste response to the antithyroid drug, 1-methyl-2-mercaptoimidazole (methimazole). The propylthiouracil taste response and the phenylthiourea taste threshold were also determined. In order to contribute data to help in possible correlation of taste and thyroid disease, the phenylthiourea taste threshold was also ascertained on a group of thyroid patients. The three compounds in this study are shown in Fig. 4.

EXPERIMENTAL

Serial Dilutions Method—The phenylthiourea taste threshold was determined by the serial dilutions method of Harris and Kalmus (2). A control

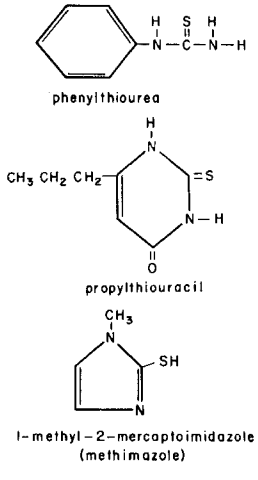


Fig. 4—Structures of compounds studied.

TABLE I—SUMMARY OF TASTE RESPONSES TO MEDICATED PAPER STRIPS

Impregnating Soln. Concn. Gm./100 ml.	Drug	Total	Tasters	Nontasters	Percentage of Nontasters
		Weak			
0.040	Phenylthiourea	191	97	94	49.2
0.040	Propylthiouracil	191	92	99	51.8
0.040	Methimazole	191	56	135	70.6
		Strong			
1.0	Phenylthiourea	279	246	33	11.8
1.0	Propylthiouracil	279	240	39	13.9

group of 171 individuals[1] were tested with 14 concentrations of phenylthiourea. Dilution No. 1 contains 1300 mg./L., and each subsequent number dilution is one-half the strength of the preceding concentration. The threshold is the weakest concentration at which the individual is able to differentiate four cups of distilled water from four cups of the test concentration. The people tested were then divided into two groups; tasters are persons who can distinguish phenylthiourea solutions numbered 5 (81.25 mg./L.) or higher, and nontasters are those who can taste only solutions numbered 4 (162.5 mg./L.) or lower. The phenylthiourea taste threshold was also determined on a group of 148 thyroid patients.[2]

Filter Paper Strip Method—The paper strip method of Parr (9) was used to obtain the relative taste response of persons to phenylthiourea, propylthiouracil, and methimazole,[3] and to establish a correlation between this and the serial dilutions method. Two types of filter paper strips were used for phenylthiourea and propylthiouracil. One type was prepared by soaking the paper strips in a 1% solution of the drug, so that each strip contained approximately 2.5 mg. of compound, which is nearly equal to the 2.6 mg. found in 8 ml. of solution No. 3 in the serial dilutions method. A weaker concentration paper strip was prepared by immersing the strips in a 0.04% solution of the substance in acetone, so that each strip contained about 0.1 mg. of drug, corresponding to the 0.08 mg. present in 8 ml. of the serial dilutions No. 8. Only the more weakly medicated strips of methimazole were prepared.

In the procedure of tasting, the persons were asked to put the weaker of the two strips on the back of the tongue, where sensitivity to bitter taste is pronounced. When a taste sensation was observed, a blank strip was given in order to check the result. If the strip with weak medication did not produce a sensation, the stronger was administered (except in the case of methimazole).

Using the serial dilutions method, the percentage of nontasters found in the white control group of 134 students was 21.6; while that found in 37 Mongolian students was 8.1. Preliminary data obtained by similar performance of the phenylthiourea threshold on a group of 148 thyroid patients indicates a relatively high percentage of nontasters in euthyroid patients and in male thyroid patients, with a low percentage of nontasters in hyperthyroid individuals.

Table I shows that methimazole, like phenylthiourea and propylthiouracil, exhibits the dualistic taste response; and contrary to its literature description of "almost no taste" (10), it is quite bitter to almost 30% of the population. Nontaster percentages found for filter paper strips weakly medicated with phenylthiourea, propylthiouracil, and methimazole were 49.2, 51.8, and 70.6, respectively. Therefore, taste sensitivity to phenylthiourea was the strongest, followed closely by propylthiouracil, whereas sensitivity to methimazole was lower. A similar correlation was observed with strongly medicated phenylthiourea and propylthiouracil paper strips, the percentage of nontasters being, respectively, 11.8 and 13.9.

[1] Consisting mainly of first year students at the School of Pharmacy, University of Southern California, Los Angeles.

[2] The thyroid patients were those of Dr. Boris Catz, Thyroid Clinic, Los Angeles County Hospital, Los Angeles, to whom the authors extend thanks for generous advice. Certain of the patients were those of Dr. Donald E. Ross, Ross-Loos Medical Group, Los Angeles.

[3] The authors are grateful to Dr. Glenn W. Irwin, Eli Lilly and Co., Indianapolis, Ind., for supplying the methimazole.

REFERENCES

(1) Fox, A. L., *Proc. Natl. Acad. Sci. U.S.*, **18**, 115(1932).
(2) Harris, H., and Kalmus, H., *Ann. Eugen. Lond.*, **15**, 24(1950).
(3) Snyder, L. J., Muller, H. K., and Little, C. C., "Genetics, Medicine, and Man," Cornell University Press, Ithaca, N. Y., 1947, p. 148.
(4) Astwood, E. B., Greer, M. A., and Ettlinger, M. G., *J. Biol. Chem.*, **181**, 121(1949).
(5) Boyd, W. C., *Science*, **112**, 153(1950).
(6) Harris, H., Kalmus, H., and Trotter, W. R., *Lancet*, **2**, 1038(1949).
(7) Kitchin, F. D., Howel-Evans, W., Clarke, C. A., McConnell, R. B., and Sheppard, P. M., *Brit. Med. J.*, **1**, 1069(1959).
(8) Shepard, T. H., and Gartler, S. M., *Science*, **131**, 929 (1960).
(9) Parr, L. W., *J. Heredity*, **25**, 187(1934).
(10) "New and Nonofficial Drugs," Council on Drugs of the American Medical Association, J. B. Lippincott Co., Philadelphia, Pa., 1964, p. 721.

Oxidation of Benzyl Alcohol and Benzaldehyde with Xenon Trioxide

By HAROLD J. RHODES, RONALD KLUZA, and MARTIN I. BLAKE

Benzyl alcohol is oxidized by xenon trioxide in acidic or neutral solution to benzaldehyde and benzoic acid. The course of the reaction was followed by gas chromatography. Thin-layer chromatography was used to identify the products of oxidation.

THE OXIDIZING properties of xenon trioxide in aqueous solution have been reported by Williamson and Koch (1) and by Appelman and Malm (2). In acid solution xenon trioxide was shown to be a very powerful oxidizing agent with the advantage that in the reduced form (xenon gas) it is relatively insoluble and does not contaminate the reaction mixture. However, only a few reports have appeared in the literature dealing with the application to the oxidation of organic compounds. Jaselskis and Vas (3) performed a series of qualitative studies on the oxidation of certain *vic*-diols with xenon trioxide. It was presumed that the reactivity of this oxidizing agent was similar to that of periodic acid. Xenon trioxide was found to react readily with ethylene glycol, 2,3-butanediol, and certain primary alcohols in neutral and basic solution. No reaction was noted in acidic solution. In a subsequent paper Jaselskis and Warriner (4) reported a quantitative method for the determination of primary and secondary aliphatic alcohols in aqueous solution. This study was limited to methanol, ethanol, and 2-propanol. It was applicable for both macro and semimicro quantities of alcohol. Jaselskis and Krueger (5) extended the procedure to include a series of mono- and dicarboxylic acids.

In the present paper the xenon trioxide oxidation of benzyl alcohol and benzaldehyde to benzoic acid is reported.

EXPERIMENTAL

Apparatus—A WCLID 1670 gas chromatograph[1] was employed for detection of the products of oxidation and for following the course of the reaction. It was equipped with dual hydrogen flame ionization detectors and stainless steel columns. The 6 ft. × 1/8 in. o.d. columns[2] were packed with 60–80 mesh flux calcined diatomaceous earth coated with 15% diethylene glycol succinate and 13% phosphoric acid.[3] The columns were operated at the following temperatures: 140° for the determination of benzyl alcohol, 120° for benzaldehyde, and 195° for benzoic acid. The injection port was maintained at 200°. A Hamilton syringe (701-N) was employed for injection of samples. Hydrogen gas flow rate was maintained at 5 ml./min., air at 300 ml./min., and nitrogen gas at 50 ml./min.

For the thin-layer chromatography studies, 20 × 5 cm. glass plates, a Desaga variable-gate spreader, and cylindrical development tank were used. A Mineralight ultraviolet lamp,[4] short wave UVS-12, was employed for visualization of colorless zones.

Solutions and Reagents—Benzyl alcohol solution was 0.05 *M* in concentration. For benzoic acid and benzaldehyde, saturated solutions in water were used. Xenon trioxide solution was 0.02 or 0.06 *M* and was prepared by diluting a concentrate (0.2 *M*) with water. The preparation of concentrated xenon oxide solutions is described by Appelman and Malm (2). Since solid xenon trioxide has a tendency to explode, solutions were stored in tightly stoppered flasks at all times. A saturated aqueous solution of potassium iodide was kept at hand to inactivate any spilled or unspent xenon trioxide solution.

Ammoniacal fuchsin reagent (6), 2,4-dinitrophenylhydrazine T.S. (7), and ferric chloride T.S. (6) were used for identification of benzaldehyde and benzoic acid.

Silica Gel HF_{254} (Merck) and Silica Gel G according to Stahl were used for the preparation of the thin-layer chromatographic plates. All chemicals and solvents were reagent grade.

Procedure—*Gas Chromatography*—Exactly 250 μl. of 0.05 *M* benzyl alcohol and 250 μl. of 0.06 *M* xenon trioxide solution were transferred by micropipet to a 2 dr. clear glass screw-top vial. The vial was tightly capped and permitted to stand at room temperature. Corresponding reaction mixtures of benzaldehyde and benzoic acid were prepared in a similar manner. Four controls were prepared containing 250 μl. of the following solutions: benzyl alcohol, benzaldehyde, benzoic acid, and xenon trioxide.

One microliter of each reaction mixture was injected into the gas chromatograph at zero time, after 5 min., and then every 15 min. for 9 hr. The retention times for the oxidation products were determined. The control solutions were injected into the chromatograph and retention times were calculated and used for identification of the oxidation products. The concentrations of the components were calculated by integration of the peak areas and comparison with those obtained from standard solutions of varying concentrations.

Thin-Layer Chromatography—Exactly 250 μl. of 0.05 *M* benzyl alcohol and 500 μl. of 0.02 *M* xenon trioxide solution were transferred by micropipet to a 2 dr. vial. A second reaction mixture was prepared using exactly 250 μl. of 0.025 *M* benzaldehyde solution in place of the benzyl alcohol. The vials were tightly stoppered and permitted to stand at room temperature for 48 hr.

Adsorbent layers, 500 μ in thickness, were prepared by applying aqueous slurries (40 Gm. with 100 ml. water) of Silica Gel HF_{254} or Silica Gel G onto glass plates. Air-drying overnight at room temperature provided adequate activation of the adsorbents. Sample solutions of the reaction mixtures and controls were each spotted portionwise in 5-μl. quantities. Development of the plates was conducted in an ascending manner in tanks pre-

Received October 3, 1966, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Accepted for publication March 15, 1967.

The authors gratefully acknowledge E. H. Appelman and J. G. Malm, Argonne National Laboratory, Argonne, Ill., for supplying the xenon trioxide used in this study.

[1] Purchased from Warner-Chilcott Laboratories, Instruments Division, Richmond, Calif.

[2] Prepared by Chemical Research Services, Inc., Addison, Ill.

[3] Gas-Pack W.

[4] Ultraviolet Products, Inc., San Gabriel, Calif.

TABLE I—RATE OF OXIDATION OF BENZYL ALCOHOL AND BENZALDEHYDE AND THE RATE OF FORMATION OF BENZOIC ACID BY VAPOR PHASE CHROMATOGRAPHY

Benzyl Alcohol[a]		Benzaldehyde[b]		Benzoic Acid[c]	
Time, min.	Concn., mm./ml.	Time, min.	Concn., mm./ml.	Time, min.	Concn., mm./ml.
0	0.0500	0	0.0250	180	0.0010
60	0.0400	90	0.0200	300	0.0015
165	0.0250	225	0.0150	480	0.0050
240	0.0200	285	0.0125	540	0.0050
360	0.0125	390	0.0100		

[a] Column temperature, 140°; retention time, 65 sec. [b] Column temperature, 120°; retention time, 54 sec. [c] Column temperature, 195°; retention time, 40 sec.

viously equilibrated for 20 min. with the appropriate solvent mixture. Visualization of the plates was carried out either directly or by scanning with an ultraviolet lamp.

For the detection of benzoic acid, a plate layered with Silica Gel HF_{254} was spotted with the reaction mixtures and a control solution of benzoic acid, developed 70 min. to a solvent front migration of 14.7 cm. with chloroform–cyclohexane–acetic acid (80:20:10), and visualized by ultraviolet light scanning.

For the detection of benzaldehyde, aliquots of the benzyl alcohol reaction mixture and a control solution of benzaldehyde were first treated with an excess of 2,4-dinitrophenylhydrazine T.S., before spotting on an adsorbent layer of Silica Gel G. A sample of the 2,4-dinitrophenylhydrazine T.S., as a control, was also spotted on the plate. The chromatogram was developed for 30 min. to a solvent front migration of 17.0 cm. with benzene–ethyl acetate (95:5), air-dried, and examined visually.

RESULTS AND DISCUSSION

In acidic or neutral solution benzyl alcohol is oxidized at room temperature, first to benzaldehyde, and then to benzoic acid. The course of the reaction was followed by vapor phase chromatography. The data are shown in Table I. The retention times for benzyl alcohol, benzaldehyde, and benzoic acid were 65, 54, and 40 sec. The half-time for the oxidation of benzyl alcohol to benzaldehyde was 165 min., and for benzaldehyde it was 285 min. The presence of benzaldehyde and benzoic acid in the reaction mixture was confirmed by comparison of the retention times with those of the control solutions. In addition, the reaction mixture was augmented with samples of known benzaldehyde and benzoic acid. Single peaks were produced for each component and the area under the curve increased by the amount of the component added to the reaction mixture. The presence of benzaldehyde and benzoic acid in the reaction mixture was further confirmed by testing qualitatively with ammoniacal fuchsin and ferric chloride test solutions, respectively.

The reaction mixture was also studied by thin-layer chromatography of Silica Gel HF_{254}, a fluorescent adsorbent. Prior to development, the spotted zones at the origin appeared as dark areas against a brightly fluorescent background when viewed under ultraviolet light. After development of the chromatogram, dark zones were detected at an R_f of 0.65 for the reaction mixtures as well as for the known benzoic acid sample. This test confirmed the presence of benzoic acid in the reaction mixtures. The test proved unsatisfactory for the detection of benzaldehyde because of its loss by volatilization during the drying treatment of the chromatogram. Detection of benzaldehyde, however, was successful through the formation of the 2,4-dinitrophenylhydrazone derivative. The benzaldehyde in the reaction mixture and a known sample of benzaldehyde were converted to the hydrazone derivative which was detected as bright yellow zones on Silica Gel G with an R_f value of 0.75. A corresponding zone was absent for the reagent sample which served as a control. A yellow zone corresponding to 2,4-dinitrophenylhydrazine and having an R_f of 0.50 was apparent for the reaction mixture, the known benzaldehyde solution, and the reagent solution.

Under the conditions of this study the reaction appears to be quantitative. Further studies are now in progress involving the oxidation of other aromatic alcohols, certain organic sulfides, and a variety of amines.

REFERENCES

(1) Williamson, S. M., and Koch, C. W., "Noble Gas Compounds," Hyman, H. H., ed., University of Chicago Press, Chicago, Ill., 1963, pp. 158–166.
(2) Appelman, E. H., and Malm, J. G., *J. Am. Chem. Soc.*, **86**, 2141(1964).
(3) Jaselskis, B., and Vas, S., *ibid.*, **86**, 2078(1964).
(4) Jaselskis, B., and Warriner, J. P., *Anal. Chem.*, **38**, 563 (1966).
(5) Jaselskis, B., and Krueger, R. H., *Talanta*, **13**, 954 (1966).
(6) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 985, 1075.
(7) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 543.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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VOLUME 56

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JANUARY TO DECEMBER, 1967

JOURNAL OF **Pharmaceutical Sciences**

July 1967 volume 56, number 7

Review Article

Pharmaceutical Sciences—1966

A Literature Review

By GERALD P. POLLI* and LOUIS J. RAVIN†

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Received from the * Pharmaceutical Research and Development Department, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, and the † Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101

THIS SURVEY of the literature is the fifth compilation to be published in *J. Pharm. Sci.* (1–4). It represents a comprehensive cross-section of the research and development efforts in the various disciplines of the pharmaceutical sciences. Numerous periodicals and selected sections of *Chemical Abstracts* were selectively abstracted. Two changes have been made from the 1965 literature review. The *Pharmacognosy* section has been discontinued and sections on *Sustained Release* and *Cosmetics* have been created. In order to maintain continuity with the previous pharmaceutical sciences reviews of *J. Pharm. Sci.*, their general format was retained.

GENERAL PHARMACY

A series of papers presented an exhaustive review of the problems associated with the production and marketing of new drugs (5, 6). Another survey outlined the application of a broad knowledge of physical and chemical properties of materials in the formulation of new drugs into suitable dosage forms (7). A systematic

approach to the development of a product formula, using the development of a gelled alcoholic formulation as an example, was described (8). The problems involved in the construction, design, and equipment of a new drug plant were reviewed (9). Meyers described in detail the information which might be supplied to obtain approval for marketing a new drug (10). He also commented on good record keeping as essential to maintaining high standards of quality control (11). Another review article, containing 54 references, was concerned with the application of statistical methodology in quality control functions of the pharmaceutical industry (12). The need for good quality control and how it can be achieved was stressed (13). A discussion of the need for good record keeping during the manufacturing and prepackaging of drugs was also published (14).

Excellent review articles on over-the-counter (O-T-C) antacids, O-T-C antiacne aids, O-T-C laxatives, cough depressants, nasal decongestants, antihistamines and other allergy aids, sleep aids and other O-T-C sedatives, and dry skin and chapping aids appeared in *J. Am. Pharm. Assoc.* (15–22). A survey on dimethylsulfoxide also appeared in the literature (23).

Preservatives—Garrett suggested a basic model for the quantification of preservative action which considered the thermodynamic activity of the biologically effective concentration of the preservative in the aqueous phase (24). Instructions and tabulated data, with general rules and exceptions for testing and preservation of medicinals, were published (25). Methods for the evaluation of preservative systems in proprietary products were discussed (26). The factors affecting the binding of phenol derivatives to *Micrococcus lysodeikticus* cells were evaluated (27). Another study was concerned with the stability of chlorobutanol in aqueous solutions (28). Another paper assessed the importance of the lack of moisture to prevent the growth of microorganisms in soap (29). The results of tests on 41 different preservatives in polypeptide containing cosmetic formulations were described (30). In a study designed to examine the effects of preservatives on the aging characteristics of acacia solutions, the unpreserved control solution showed the most pronounced reduction in pH and viscosity (31).

Paruta and Sheth determined the solubilities of methyl, ethyl, propyl, butyl, and benzylparabens in syrup vehicles of varying concentration (32). The effect of surfactants on activity of phenolic-type germicides and preservatives was reviewed (33). The addition of surfactants to 1,3-dichloro-5,5-dimethylhydantoin increased its solubility but did not impair its bactericidal activity as tested on staphylococcus (34). The interaction between methyl *p*-hydroxybenzoate and polysorbate 80[1] has been determined using a molecular sieve technique (35). The use of the Coulter counter to detect the inactivation of preservatives by polysorbate 80 was demonstrated (36). In another study benzoic acid was shown to interact with glycol ethers in water and in aqueous polysorbate 80 solutions (37).

Flavor, Aroma, and Color—Wesley discussed the possibility of using flavor combinations called nondescript flavors instead of individual flavors to get the desired taste for products (38). Several useful tips were presented toward the achievement of good taste and mouthfeel in a vehicle as well as high resistance to cap-locking (39). The solubilization of essential oils by surfactants was the subject of two papers (40, 41). A complete review of sweetening agents was presented (42). One paper showed a direct dependence of relative sweetness on hydrophobic bonding (43). In another paper the use of malic acid in place of citric acid in hard candy was discussed (44). The mechanism of chemical excitation of taste and olfactory receptors was shown to depend upon adsorption of the stimulus molecules to the receptor surfaces (45). The present state of the stereochemical theory of odor was described (46). Some 400 essential oils and pure perfumes were examined for suitability to scent aerosol products (47).

Bhatia, Sokoloski, and Bhatia studied dye–polyethylene glycol 6000 interactions in film-coating solutions and their effect on color uniformity (48). Carotenoids were successfully incorporated into sugar-coated tablets as color agents (49). In another paper the fading of colors was attributed to reduction or oxidation of the dyes; the actual mechanism was not determined (50). In two publications concerned with the influence of ultraviolet absorbers on the color stability of certified dyes, one paper showed the ultraviolet absorbers have little value as color stabilizers for tablet coating, whereas the other publication showed the ultraviolet absorbers can be used to protect certified colors against fading by incorporating the absorbers into the product or into the coating of the package (51, 52). A modified Godlove's equation was adapted to the color changes of various pharmaceutical preparations (53).

Adjuvants—The effect of humidity and temperature on the cohesion of wheat starch,

[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

cornstarch, potato starch, acacia, tragacanth, alginic acid, lactose, dextrose, and sucrose powders was determined by Shotton and Harb (54). Anhydrous lactose was found to possess excellent tableting properties and could be run directly on a high-speed tablet machine (55). The montmorillonites were studied for their use as disintegrants, binders, and lubricants in the manufacture of compressed tablets (56). Experimental data indicated that inositol, due to its nonhygroscopic nature, chemical inertness, nontoxicity, physical stability, superior mouthfeel, and texture, could be beneficially employed as the base for the formulation of chewable tablets (57). Other researchers reported on the use of mannitol as the preferred excipient for chewable tablets (58). Microcrystalline cellulose has been recommended as an extremely valuable tableting agent (59). Based on the results of compression effects, physical properties, stability, and drug availability, amylose was shown to have the characteristics desired of the ideal direct compression tablet binder (60). The utilization of sodium carboxymethylcellulose in the preparation of tablets has been proposed (61). Other investigators announced the use of haluasite, a clay mineral of the kaolinite group, as a useful tablet ingredient (62).

The application of silicones to cosmetic and pharmaceutical preparations was the object of publications by Leberl and Dias (63, 64). The relative effects of tortuosity, electrostatic attraction, and increased viscosity of water on the self-diffusion rates of cations in bentonite–water systems were described (65). Karaya gum and ghatti gum were found to be good emulsifying agents at all pH's (66). Corn oil was found to be less stable to oxidation than olive oil in the formulation of topical preparations (67). Malucidin, a protein obtained from brewer's yeast, has been recommended for cosmetic use in the formulation of skin and hair products (68).

Stability—A paper by Parrott discussed the stability of pharmaceuticals, covering parameters such as biological contamination, container, light, age, temperature, moisture, and oxidation (69). Those properties of emulsions, suspensions, and solids which are amenable to measurement and which can be used as parameters for establishing and predicting deterioration of finished consumer products were considered (70, 71). Another report examined seven of the most important chemical reactions taking place during the storage of drugs. Examples of each kind of reaction were given; the kinetics and parameters of these reactions were discussed and counteracting agents were suggested (72). An apparatus for accelerated temperature cycling was disclosed (73). A comparison of short-time storage tests at higher temperatures with the usual commercial storage time period has been made (74). An outline of recommendations regarding methods for accelerated testing of the stability of pharmaceutical preparations has been presented (75). Refractometry was used as a tool to measure the stability of solutions of pharmaceutical preparations (76).

Several investigators studied the hydrolysis of acetylsalicylic acid in the presence of accompanying substances in powder mixtures. Decomposition was shown to be dependent on the pH of the powder mixture (77–79). Brown *et al.* enhanced the stability of pilocarpine hydrochloride solutions by adding methylcellulose (80). The degradation of sodium bisulfite in dextrose solutions was followed (81). Ergotamine injections were stabilized by the addition of ethylenediaminetetraacetic acid and thiourea (82). Heat sterilization (100° for 15 min.) did not affect solutions of lobeline which were acidified to pH 2.8–4.0 by the addition of hydrochloric acid or sodium bisulfite (83).

Doppert and Staverman investigated the kinetics of the retrogradation of amylose in aqueous solutions of pH 4.0, 9.2, and 11.9 (84). In another study the degradation of pectin by γ-radiation under various moisture conditions was reported (85). The autooxidation of fats and oils was shown to be a chain reaction catalyzed by traces of metals or enzymes from bacteria or fungi (86). The results of stability tests of several medium-chain and liquid long-chain triglycerides have been correlated with the results of peroxide number determinations after storage (87). The effect of antioxidants on the stability of hydrated and anhydrous ointment bases was tested (88). Phenylbutazone stability in suppositories was shown to be dependent on the vehicle employed (89). The stability of chloramine-T in acidified aqueous solutions was recorded (90). Matsuoka *et al.* reported that α-chymotrypsin in aqueous solution is stabilized by the addition of glyceryl acetate (91). Standards have been suggested for shipping thermolabile drugs and biologicals (92). The use of tartaric acid as an efficient synergist for antioxidant action in cosmetic products was reported (93).

Stability Kinetics—A detailed analysis was presented on the use of the first-order rate equation with data which only approximates first-order kinetics (94). Pincock and Kiovsky published on the kinetics of reactions in frozen solutions (95). Some of the methods currently

available for studying chemical reactions in the time range 1 to 10^{-10} sec. were considered in another publication (96). A review, with 134 references, covered the subject of acid-catalyzed hydrolysis of glycosides (97).

The rates of hydrolysis of chlorobutanol were found to be reduced by surface-active agents but not by polyethylene glycols (98). Other workers investigated the rates of alkaline hydrolysis of ethyl benzoates in water and in aqueous dimethylsulfoxides (99, 100). The optimum pH for the stability of injection solutions of tetracaine HCl (dicaine), bencaine, and procaine was reported as 3.8, 2.7, 4.4, respectively (101). A difference in rate of degradation of phenobarbital and pentobarbital in alkaline solution was shown (102). The hydroxyl ion-catalyzed hydrolysis of glyceryl esters in aqueous medium in pharmaceutical systems was investigated (103).

Wadke and Guttman showed that the unionized species were much more susceptible to hydrolysis than the ionized form while investigating the hydrolytic behavior of 9-methylisoalloxazine (104, 105). The heats of activation for the reaction of nonionized isoniazid and isoniazid-anion were found to be approximately 11 and 5.6 Kcal./mole, respectively (106). The order of reactions, rate constants, and half-life periods for the hydrolysis of acetylsalicylic acid occurring in aqueous solution in the presence of the salts of phenobarbital,[2] codeine, caffeine, sodium benzoate, magnesium oxide, calcium oxide, calcium carbonate, and calcium gluconate were studied (107). The kinetics of hydrolysis of colchicine and some related tropolone methyl ethers were announced (108). The decomposition of idoxuridine was shown to be first order and maximum stability in solution was obtained at pH's below 7.0 (109). A series of publications on phenothiazine derivatives appeared in the literature (110–114): the rate of photooxidation of propericiazine and its sulfoxide increased with increasing amounts of oxygen and lowering pH; acetylpromazine and its sulfoxide showed less photodegradation than thioproperazine and its sulfoxide; the mechanism of the photodegradation of 10-(2-dialkylaminopropyl)phenothiazines was found to be dependent on the amount of air present; the 10-alkylaminoalkyl side chain was observed to influence photodegradation.

Herd, Eberson, and Higuchi investigated the mechanisms and the relative rates of alkaline hydrolysis of succinanil and methyl-substituted succinanils (115). The anionic forms of polycarboxylic acids, such as succinic and citric acids, were shown to interact reversibly and rapidly with glutaric anhydride in aqueous solution at room temperature to produce species which undergo subsequent hydrolysis (116). The same researchers also studied the irreversible interaction of acetic anhydride with di- and tricarboxylic acids in aqueous solution (117). The kinetics of the reaction of reserpine with nitrous acid were published (118). The acid demineralization kinetics of enamel were reported in an attempt to explore further a recently developed mathematical model in which it was assumed that hydroxyapatite is the thermodynamically governing phase in the mineralization of enamel (119). One paper considered the kinetics and transformations of cycloheximide as a function of pH and temperature (120). The decomposition of meperidine hydrochloride was found to be first order with respect to hydronium-ion concentration and ester concentration (121). Optimum stability for *N,N*-dimethyleneoxide-bis-(pyridinium-4-aldoxime)-dichloride in aqueous solution was reported at pH 3 (122). General equations were derived which related the half-life of tr methylene-bis-(4-formylpyridinium bromide)-dioxime in aqueous solutions to pH and temperature (123).

Antibiotic Stability—Belen'kii *et al.* announced that water, acids, increased temperature, and ultraviolet irradiation inactivate sodium nystatin. They reported that both the photo- and thermoinactivations of nystatin were prevented by the addition of the antioxidant, propyl gallate (124). The kinetics of the stability of oxacillin in aqueous solution were researched over the pH range of 1.44–10.93 (125). Data were collected showing aqueous solutions of methicillin to be most stable at pH 7.44 (126). Streptomycin sulfate, neomycin sulfate, and tetracycline hydrochloride were suspended in a dry mixture of tragacanth and sucrose; after addition of water the suspensions lost 7, 3, and 36%, respectively, of their antibiotic activities when stored for 10 days at room temperature in daylight (127). The stabilization of penicillin G solutions by sodium edetate was demonstrated (128).

Garrett *et al.* quantified and predicted the biological activities of chloramphenicol analogs through the use of microbial kinetics (129). The best viomycin stability in aqueous solution was disclosed to be in the range of pH 3.0 to 4.0 (130). Gramicidin was shown to be hydrolyzed by a *Bacillus subtilis* proteinase at the peptide linkage between L-valine and L-ornithine; the resulting peptide had no antibacterial activity (131). Stability studies of neomycin and penicillin in ointments were presented (132).

[2] Marketed as Luminal by Winthrop Laboratories, New York, N. Y.

Vitamin Stability—The stability of vitamins in pharmaceutical preparations was the subject of several publications. The stability of vitamins A, B, B_2, B_6, ascorbic acid, vitamin D_2, and niacinamide in oral liquid formulations was followed as a function of sucrose, glycerol, and water concentrations (133). The changes of vitamin contents and pH values of liquid medicines during storage in ampuls were also announced (134). Gurevich *et al.* employed Na_2SO_3 to stabilize ascorbic acid in a compound injection solution (135). The effect of liver extract on the stability of vitamins A, B_1, and ascorbic acid when present alone or in multivitamin formulations was determined in a syrup–glycerol–water vehicle (136). Over 100 samples of vitamin preparations were checked for vitamin content after storage for 1–16 years under various climatic conditions (137).

Carstensen *et al.* illustrated moisture stress studies, using vitamin A to exemplify this point (138). The effect of various suppository bases on the stability of a synthetic vitamin A palmitate and of a natural vitamin concentrate was investigated with and without the addition of antioxidants (139). Disodium edetate was found to be the most suitable autooxidation inhibitor for ascorbic acid (140). In comparison with KCNS, edetic acid (Idranal III) was shown to be a more effective inhibitor of oxidation of ascorbic acid in aqueous solutions, at room and at elevated temperatures, as well as in solutions containing copper, iron, or zinc ion (141). All salts studied, except sodium chloride, were shown to have a deleterious effect on the stability of ascorbic acid (142). The stability of dehydroascorbate was observed to be best at a pH of 2.4, the half-life being greater than 1000 min. at 20° (143). Second-order reaction kinetics were shown for the oxidation of ascorbic acid by various quinones using a polarographic technique (144). Stability tests on 12 ascorbic acid formulations at 80°, 60°, and 55° showed the failure of Arrhenius' law in a heterogeneous solid system (145).

Attention continues to be focused on thiamine stability. Two different groups of researchers studied the stability of thiamine solutions in the presence of dipyrone (Sulpyrin) (146–148). Data were presented which showed thiamine nitrate to be more stable than thiamine hydrochloride in a mixture containing vitamin B complex (149). The effects of γ-radiation on vitamin B_6 stability were probed (150). The effects of initial riboflavin concentration, hydrogen-ion concentration, and ionic strength on the light fading of riboflavin solutions were recorded (151).

PHARMACEUTICAL TECHNOLOGY

A discussion of controls on tablets (hardness, disintegration, and drug accuracy), injections (foreign particles and closure), and ointments appeared in the literature (152). Fluidization and its applications in pharmaceutical technology were covered in a two-part review. The first part covered the medical aspects, whereas the second part covered practical applications (153, 154). Ridgway and Segovia elaborated on fluidization and gas suspension techniques in pharmaceutical manufacturing, tablet coating, and granulating (155). Tablet coating by the air suspension technique was commented on by other researchers (156). Punch and die control was the subject of a publication which outlined a program to ensure the protection from defects of punches and dies (157).

Sterility—The use of a biological-chemical indicator for ethylene oxide sterilization of disposable medical and pharmaceutical materials was reported by Brewer and Arnsberger. The indicator system affords an immediate visible indication of gas penetration into the materials during the sterilization cycle and also serves as a biological control (158). Oils, such as liquid petrolatum and castor oil, were safely sterilized by using 0.1% ethylene oxide (159). A review of the properties of ethylene oxide and results of sterilizing tests with filter cartons, membrane filters, and vegetable parchment were summarized (160). Gelatin, when exposed to ionizing radiation, was shown to undergo molecular weight changes through free radical mechanisms involving scission, crosslinking, and weak bond formation (161). A review, with 66 references, was tabulated on the effects of radioactive irradiation on pharmaceutical preparations (162). Sterilization by electrohydraulic treatment was demonstrated by applying this technique to suspensions of *Escherichia coli*, spores of *Bacillus subtilis* var. niger, *Saccharomyces cerevisiae*, and bacteriophage T-2 (163). Methods for the preparation of sterile ophthalmic solutions were discussed in detail (164). Experimental data, collected from various laboratories, were published on the sterility tests of penicillin (165). Membrane filtration procedures for antibiotic sterility testing were offered as an improved approach for determining the sterility of antibiotics (166).

Parenterals—A review of the solubilizing agents used in parenterals appeared in the literature (167). Zoellner and Vastagh demonstrated that *m*-xylidine is a contaminant which causes discoloration of injectable solutions of lidocaine (168). Information on sodium chloride equiv-

alents, cryoscopic properties, and hemolytic effects of certain medicinals in aqueous solution was compiled (169). A discussion on the extemporaneous compounding of intravenous additives was presented (170). A survey of the literature pertaining to problems of incompatibilities and instabilities of parenteral solutions with admixtures was tabulated (171). Mixtures of potassium penicillin G–tetracycline hydrochloride, and potassium penicillin G–chlortetracycline hydrochloride in 5% dextrose were investigated for physicochemical incompatibilities (172). An incompatibility chart of 61 frequently used intravenous additive combinations was published (173).

Tablets and Capsules—Tanaka, Matsushita, and Utsumi ascertained that the disintegration time of calcium *p*-aminosalicylate increased with time due to formation of the hexahydrate which was detected by calorimetric measurements (174). Many manuscripts appeared on the subject of tablet disintegration. In a study on the mechanism of action of starch as a tablet disintegrant, pH was shown to have little effect on swelling, whereas salts were shown to affect swelling (175). In another publication the concentration of water-soluble medicament did not significantly change the disintegration time of tablets (176). Temperature of liquid and tablet compressional force was reported to directly affect the wetting of powder and disintegration time (177). In a study on the mechanism of the action of starch as a disintegrating agent in aspirin tablets, the authors concluded that the primary mechanism appeared to be a swelling action; capillarity *per se* did not appear to have a disintegrating effect (178). Tablets disintegrated 2 to 10 times faster when polysorbate 80 was added to the tablet formulation (179). A comparison was made of the influence of various chemicals on the disintegration time and on the crumbling rate of compressed tablets of acetylsalicylic acid at various stages of storage (180). The disintegration of tablets was studied by changing the ratio of aluminum hydroxide gel and diluent, such as potato starch, lactose, and cornstarch (181). Two methods were developed for testing the swelling capacity of tablet disintegrants (182).

A preliminary report on experiments carried out to find optimum tableting pressures, based on crystallographic consideration for potato starch, lactose, ascorbic acid, phenacetin, phenobarbital, and aminopyrine (amidazophen) appeared in the literature (183). The compression of mixtures of lactose and starch with different binding agents was studied using the dry granulation technique (184). A review of the physical phenomena which occur during the compression of tablets was published (185). An Instron physical testing instrument was adapted to permit its utilization in the evaluation of the compressing characteristics of drug particles and granules (186). In a two-part series, the physical properties of particles prepared by five different granulation methods and factors involved in the sieving of pharmaceutical granules were analyzed (187, 188). A method of measuring the fluidity of semifluid powders was described and used to investigate the role of various silica-type glidants in the direct compression of microcrystalline cellulose and a spray-dried lactose–microcrystalline cellulose blend on two different tablet presses (189). The influence of the addition of lubricants upon the flow rate of granulates was studied by measuring the flow velocity in an apparatus based on the device of Paksy and Sabjan. All lubricants delayed flow, especially polyethylene glycol 4000 and the stearates (190). The rate of flow of varying ratios of four sieve size fractions of rock salt through the hopper of a rotary press was represented by a second degree polynomial equation. No correlation was found between the tangent of the angle of repose and flow rate (191). Gold *et al.* studied the effect of glidants on flow rate and angle of repose (192). An article concerned with the influence of particle size of a glyceryl tri-ester lubricating agent on the physical properties of compressed tablets was presented (193). Tablet binders were evaluated by means of adhesion tension determinations (194). Spray-dried flour was evaluated against spray-dried lactose as a suitable carrying agent for direct compression formulas in tableting (195). Two new physical forms of lactose, spray dried and agglomerated, were used in the direct compression of tableted formulations (196).

Daragan suggested that the rate of dissolution of ethylcellulose films could be increased by including polysorbate 80 or citric acid in the composition of the film (197). A new method of coating tablets, using the revolving coating pan, was published. The coating materials were applied in suspensions or solutions as a spray from a centrifugal disk atomizer (198). A new dosage form, tablets of coated aspirin microspherules, was developed (199). The influence of particle size distribution, particle shape, apparent bulk density, moisture content, additives, and punch shape on the directly compressible characteristics of potassium chloride was investigated (200). Gastric-soluble films were applied to tablets by the dry-pressing method and by the spraying method (201). A three-part series on

medicated candies described the method of manufacture, the problems peculiar to medicated candies with hard sugar bases, stability, and the control aspects (202–204).

Sustained Release—A review dealing with the development and biokinetics of oral sustained-release medications was published (205). A two-part publication discussed the various patents obtained for prolonged-action dosage forms (206, 207). The quantitation of procedures and quantitative studies involving the release of sulfanilamide, caffeine, and potassium acid phthalate from polyethylene matrix disks into aqueous media were presented (208, 209). The mechanism of drug release from tablets made from mixtures of drug and polyvinyl chloride particles was investigated (210). One author pointed out that the development of oral sustained-release dosage forms is predicated on sound physical, chemical, and pharmacological properties of drugs and their bases (211). Another investigation dealt with the result of a mathematical and an analog computer analysis of the kinetic relationships governing the rate of release of drugs from sustained-release dosage forms (212).

A new method of preparing a sustained-action dosage form was developed. This entailed the tableting of film coated granules with subsequent recovery of the intact granules on disintegration of the tablet (213). The formulation and evaluation of a sustained-action compressed tablet with a plastic matrix composed of a vinyl acetate–vinyl chloride copolymer, zein, and gypsum were described (214). A sustained-release tablet preparation containing microcrystalline potassium chloride in a fatty base was developed which was free from any local irritant effect (215). Sucrose distearate, sucrose monostearate, and cellulose acetate phthalate were used for the preparation of prolonged-release granules and pills of sulfisoxazole (216). Draper and Becker observed a direct relationship between *in vitro* drug release rate and average theoretical surface area of sulfaethylthiadiazole–wax[3] particles, but did not observe this relationship for sulfaethylthiadiazole–beeswax particles (217). Spray-dried formulations of sulfaethylthiadiazole for prolonged-release medication were prepared by using shellac, cellulose acetate phthalate, a synthetic wax-like ester,[3] hydrogenated castor oil,[4] aluminum monostearate, and glyceryl monostearate as the dissolution-retarding materials (218). An article appeared which discussed the rationale for sustained-release aspirin (219). Factors affecting the release of a water-soluble drug from a compressed hydrophilic matrix were investigated (220). The influence of the concentration and particle size of the active substance and of polyethylene matrix on the speed of release of the active substance from a delayed-release tablet was published (221).

Suspensions—Nakagaki and Kitamura used an integrating sphere turbidimeter to study particle size distribution of silica suspensions (222). A review on pharmaceutical suspensions was presented which covered types of suspensions, particle size determination, and dependence of stability on factors such as viscosity and agglomeration (223). The effect of ultrasonic vibration on the deformation processes and stability of water dispersions of montmorillonites was investigated (224). The stabilization of clay suspensions during ultrasonic treatment and the effect of electrolytes were presented (225). Ultrasonic waves, at a frequency of 100 Kc., were found to cause a significant reduction in the particle size of polyethylene spheres only if surfactants were present (226). Using an aqueous sulfamerazine suspension, experiments showed good correlation which tested the validity of an accelerated procedure for determining settling volumes, following both centrifugation and storage under ambient conditions (227). A novel method was suggested for accelerating the physical changes in suspensions (228).

Nakanishi published three articles on the viscosity and sedimentation of the dispersed systems of barium sulfate in methylcellulose and carboxymethylcellulose (229–231). The use of high molecular weight compounds such as methylcellulose, sodium carboxymethylcellulose, and polysorbate 60[5] in the production of sulfamethazine (sulfadimezine) and sulfanilamide (streptocide) suspensions was suggested (232). Formulas were given which modified the unpleasant taste of calcium and phosphorus salts (233, 234). The influence of the disperse medium on the stability of an aqueous suspension of propyliodone was studied (235).

Emulsions—A review, with 18 references, on the theory of emulsion stability appeared in the literature (236). Another review covered tests for determining emulsion types, emulsion viscosity, and emulsion stability as influenced by emulsifying agents, particle size, temperature, viscosity, density, and types of emulsion (237). It was pointed out that the key to "leveling" in aqueous emulsion systems is that the product must wet both the substrate and its own drying

[3] Glyco Wax, Glyco Chemicals, Inc., New York, N. Y.
[4] Castorwax, Baker Castor Oil Co., Bayonne, N. J.
[5] Marketed as Tween 60 by Atlas Chemical Industries, Inc., Wilmington, Del.

film (238). Emulsion stability was considered with regard to inversion and HLB balance (239). The electrical conductivity of emulsions was used as a tool for testing and production control of emulsions (240). The effect of the shape of dispersed particles on the dielectric constant of emulsions with a low content of the dispersed phase was studied (241). One manuscript indicated that inversion of emulsions from water-in-oil to oil-in-water is a characteristic of the oil and appears to be most directly related to the viscosity of the oil (242). The relation between the solvent power and the hydrophile–lipophile balance of the emulsifier was determined (243).

In a study dealing with phase interfaces, Ford and Furmidge concluded that a strong interfacial film should be formed which prevents the coalescence of water droplets while permitting the coalescence of oil droplets (244). Flocculation of oil droplets in dilute emulsions was shown to depend upon concentration of oil droplets, the size distribution of the primary droplets, and the amount of salt present (245). The nature and influence of the attractive and repulsive forces which occur during emulsion film formation were discussed (246). Aggregation behavior was observed when an emulsion was diluted in saline containing cetrimide below the micelle concentration (247). The mechanism of emulsion stabilization with reference to yield value and surfactant chain length was outlined (248). New calculations showed that larger amounts of energy are required to overcome the viscous forces and secure emulsification than have been assumed hitherto (249). The effects of aging and surfactant concentration on the rheology and droplet size distribution of a nonaqueous emulsion were presented (250). Interfacial viscosities were studied by means of a viscous traction interfacial viscometer (251). The flocculation and coalescence of emulsion droplets of the normal and inverse types, studied simultaneously, confirmed that the attainment of a stable coalesced emulsion always requires the same amount of any given surfactant, the optimal concentration being independent of the size of the electrostatic energy barrier and the oil medium (252). The factors affecting stability in nonaqueous emulsions were presented (253).

Fox and Shangraw described an automated reciprocating capillary emulsator which duplicates the low-pressure homogenization principle of the interconnected glass syringe method used to prepare repository water/oil emulsions (254). These same authors showed that inorganic electrolytes at concentrations as low as 0.01 *M* increased apparent viscosity, retarded sedimentation, and had a marked stabilizing influence (255). Emulsions made with an ultrasonic emulsifier were reported to be superior to those made with the standard homogenizer (256). New emulsions were developed in which filter and screen light-protective substances were incorporated (257). The relative viscosities of water-in-oil type emulsions were shown to be a function of volume fractions of the dispersoid and also of the sorbitan monostearate[6] concentration (258). Systems having low interfacial viscosities were shown to give relatively stable emulsions (259). A comparison was made of results obtained on a typical pharmaceutical emulsion using optical and electron microscopes, and the Coulter counter for determining emulsion particle size (260). The emulsifying properties of sodium β-boswellate were investigated and shown to be quite good (261).

Ointments and Creams—Whitworth and Becker published on the effects of solubility factors on the diffusion of sulfacetamide and sulfathiazole from ointment bases (262). A "dialysis cell" method was developed for comparatively measuring the release of salicylic acid from various ointment bases (263). The rate of diffusion of procaine from an ointment base and several water-in-oil emulsions was shown to be inversely proportional to the amount of sodium lauryl sulfate present (264). Adsorption was shown to be responsible for a decrease of drug release from ointments (265). The diffusion of sulfanilamide from ointments was less when sulfanilamide was solubilized in the surfactants as compared to when the surfactant was incorporated into the ointment base (266). The diffusion of both salicylic acid and sodium salicylate from hydrophobic bases was shown to be very slow (267). A new apparatus, a rake bladed stirrer which keeps the ointment in constant circulation during an experiment, has been described and used in *in vitro* experiments (268). The rate of chlortetracycline and chloral hydrate release from ointment bases containing bentonic gel was studied (269).

Kutscher *et al.* applied sheets of polyvinyl alcohol coated with gentian violet to oral mucosal tissue and noted dye absorption in 5–55 min. indicating the usefulness of the vehicle as a base for drugs (270). Other investigators used solutions of ethylcellulose and sodium carboxymethylcellulose containing antibiotics as bactericidal film-forming liquids in treating cuts and small skin wounds (271). A rheological investigation of various ointments was described to

[6] Marketed as Span 60 by Atlas Chemical Industries, Inc., Wilmington, Del.

facilitate the selection of the most convenient base for medicinal ointments (272). An easy procedure was developed for the sterilization of ointments (273). Anhydrous ointment vehicles containing palm oil were best stabilized by using butylhydroxytoluene and butylhydroxyanisole, whereas hydrated ointment vehicles containing palm oil were best stabilized by using gallates (274). Polymethylphenylsiloxane liquid has been investigated as a component for ointment bases (275). The hydrophilic capacity of official polyethylene glycol ointment was increased 48% by the addition of 0.5% of dry unneutralized carboxypolymethylene compound (276). Various topical preparations for application to lips to reduce chapping were evaluated (277).

Suppositories—The absorption of streptomycin and chloramphenicol was increased slightly when sodium lauryl sulfate was incorporated in the fatty suppository base (278). Rectal absorption studies of various sulfonamides from aqueous polyethylene solutions in rabbits showed the ionized drugs to be better absorbed than the nonionized drugs, and the sodium salts of sulfonamides to be more effective than their free form (279). The adsorption of a brand of purified siliceous earth[7] in a cocoa butter suppository base was the same as that in a brand name glycol ester suppository base (280). The influence of various commonly used viscosity-inducing modifiers of suppository bases was determined *in vitro* using antipyrine (phenazone) and aminopyrine (aminophenazone) (281). An apparatus was described which can be employed for the control of the physical properties of suppositories made with fatty bases or with water-soluble or water-dispersible bases (282). Upon partial hydrogenation, some liquid vegetable oils assumed consistencies which made it possible to use these reacted oils as vehicles for rectal suppositories (283).

Aerosols—All types of pharmaceutical aerosols were discussed in detail in a review article by Marshall (284). New developments in aqueous foams, and their many interesting applications in cosmetics, were considered (285). In a two-part series, the properties of aqueous alcohol aerosol foams and how they apply to products were discussed, along with the effects of additives and how they may affect the foaming properties of these aerosols (286, 287). The changes in size distribution of an initially homogeneous aerosol were studied as the aerosol coagulated and deposited on the walls of a closed chamber (288). One author demonstrated that molecular complexes formed in aerosol emulsion systems increased emulsion and foam stability and decreased foam drainage (289). The use of the Rotovisco rotational viscometer was described in evaluating the rheological properties of pressurized foams (290). The microbiological testing of aerosol preparations was conducted using *Chaetomium globosum* under conditions that were applicable to the natural growth habits of the organism (291).

The development of water washable spray-on bandages was discussed (292). A new aerosol antiperspirant was considered along with safety data and formulation suggestions (293). A review on germicidal preparations in the form of aerosols along with the qualitative compositions of various air fresheners and surgical antiseptic aerosols was published (294). Chemical specialties foamed with water-dispersed colloidal alumina were studied (295). Hardness, clarity, and gloss retention of leading commercial hair sprays were evaluated to determine formulating goals in preparing aerosol hair sprays (296). Formulation and activity of water-based aerosols containing pyrethrum was the subject of still another manuscript (297).

Cosmetics—deNavarre predicted the development of more treatment cosmetics, some only for prescription sale (298). The physical and chemical properties of branch-chain hydrocarbons, fatty acids, alcohols and esters, both of synthetic and natural origin, which have found increasingly growing application in bases of creams and other cosmetics, were reviewed (299). "Some New Keys to Cosmetic Chemistry 1965" was the title of a review article which contained 278 references (300). One author's experience led to the publication of a manuscript entitled "Cosmetics for Use Under Tropical Conditions" (301). Another review article on cosmetics and toilet preparations contained 47 references (302). A discussion of lipids and their derivatives and other fats and waxes commonly used in cosmetology and dermatology was reported (303).

Rees analyzed the various properties of a good shampoo and also described the numerous types of modern shampoo formulations, including liquid, lotion, cream, powder, and jelly shampoos (304). Appearance, performance during use, and effect on hair after use were the three major criteria for evaluation of shampoos in the opinion of a team of investigators (305). The type of surface-active agents used in modern shampoos was commented on in detail (306). Quaternary ammonium salts were used in hair treatment preparations (307). Another publication presented the various hair dressing formulas for

[7] Trademarked as Aerosil by Degussa, Frankfurt on Main, Germany.

men and the requirements for each type (308). The use of oxidation dyes in hair coloring preparations was surveyed (309). Progress has been made with the formulation of stable, clear, emulsion systems, particularly hairdressing gels (310). The emulsifying, wetting, and foam properties of quaternary ammonium compounds, as applies to their use in hair treatment preparations, were reviewed (311). Protein based detergents were suggested for application in shampoos (312).

Goldrick discussed the need of vitamins for topical application and also gave several typical formulas (313). Water standards were suggested for use in the manufacture of cosmetic preparations (314). Aspects of microbiology, with reference to antimicrobial agents, in cosmetic products were presented (315). The problems relating to the proof of efficacy of antiperspirants and deodorants were examined (316). The significance in the choice of an appropriate vehicle for antiperspirants and deodorants was explored in detail (317). New techniques contributing to the laboratory evaluation of chemical, physical, and chemical/physical combination sunscreen products were presented (318). The nature and type of dermatological testing were discussed with special reference to different categories of cosmetics (319).

Schlossman categorized the types of skin cleaning preparations and gave sample formulations of each (320). The properties of cationic surface-active agents were reviewed and their suitability for use as emusifiers in cosmetics was discussed (321). The various surface-active agents used in bubble bath formulas were disclosed (322). The clear gel system was examined and typical formulas were outlined (323, 324).

Packaging—Lees discussed the use of plastics for packaging (325). A package development checklist was presented along with an explanation on how it is to be used (326). Reports of the committee developing test procedures for the determination of the safety of plastics in pharmaceutical and medinical uses were given (327). A very searching test for the suitability of plastics for cosmetic packaging was suggested (328). Published data were used to establish the superiority of foamed plastics over other pharmaceutical packaging materials. Among the foamed plastics, polystyrene foam was rated best on the basis of its mechanical, heat insulating, water-repellent, and aging properties (329).

The feasibility of using ultrahigh frequency power to accelerate freeze-drying was demonstrated and the facilities were described (330). The engineering aspects of ultrahigh frequency dielectric heating to accelerate freeze-drying was outlined (331). A method for determining the bacterial permeability of plastic films was announced (332). The permeability of plastics was shown to cause problems in the packaging of pharmaceuticals (333). The permeability of various solvents through polyethylene was expressed by an equation (334). The designing of machines for the packaging of sterile pharmaceuticals was described (335).

EQUIPMENT

A recording powder flowmeter consisting of a hopper, a strain gauge, and a recorder presented a new approach to the measurement of powder flow (336). Cohn *et al.* employed an IBM 1620 computer to evaluate data obtained from a compactor optimized to prepare a basic granulation to which other drugs could be added and directly compressed into tablets (337). In another paper the surface areas of powders were determined by flow microcalorimetry (338). A laboratory glass spray drier made from borosilicate glass was described (339). The design, critical components, method, and costs of spray driers were presented (340).

One mixer was described which utilized compressed air to mix solids rapidly, while another was described to blend or agglomerate solids by atomizing liquid into air-suspended solids (341). Nash and Haeger stressed the importance of zeta potential in the development of stable pharmaceutical suspensions (342). Some basic theories about the use of instrumentation in cosmetic color control with emphasis on practical application were reviewed by one author, while another elaborated on an instrument that measured color objectively (343, 344). A new machine for unit dose packaging of tablets and capsules was also described (345). In addition, the automatic packaging of individual ointment-impregnated gauze bandages was a subject for discussion (346).

PHYSICAL PHARMACY

Biles studied the distribution of quaternary ammonium salts between chloroform and water (347). It was also shown that pH affected the distribution of certain medicinal preparations between chloroform and water (348). The kinetics of reactions involved in penicillin allergy revealed that the aminolysis of penicillins by glycine is general base catalyzed (349). A number of antacids were granulated by dry, wet, and spray methods and compared for specific surface area, angle of contact, porosity, and acid-neutralizing capacity (350). An interesting paper concerned with the kinetics of swelling of dextran

gels in water was published (351). The diffusion of water vapor through a hydrophilic polymer film was determined using a multifilm technique (352).

Mark *et al.* studied the oxygen permeability of amylomaize starch films over a wide range of relative humidity (353). The permeation, sorption, and diffusion of water in ethylcellulose were also investigated (354). Freezing point curves were published for the system dimethylsulfoxide–water (355). Phase relationships were presented for a system containing isoelectric gelatin, ethanol, and water (356). A study of membrane characterization by measurement of transient osmotic pressures was reported (357). A review article concerned with the physical aspects of drug action appeared in the literature (358).

Solubility—Bates *et al.* published several papers concerned with the solubilizing properties of bile salt solutions on glutethimide, griseofulvin, and hexestrol as a function of temperature, bile salt concentration, and inorganic electrolyte (359–361). The solubility of 21 steroids in water at 25° was determined and compared to previously reported data (362). The dissolution rates of powdered preparations of sulfonamides and benzoic acid derivatives were studied, and the data were found to be in good agreement with the Noyes-Nernst equation concerning transport controlled dissolutions (363–365). The solubility of sulfonamides in phosphate–citrate buffer systems appeared in the literature (366). Sulfanilamide solubility increased with an increase in concentration and chain length of nonionic solubilizers and temperature (367). Another study presented data giving methods of solubilizing difficultly soluble materials by way of intermediates (368).

Paruta *et al.* investigated the solubility of xanthines, antipyrine, and succinic acid as a function of dielectric constant in several solvent systems (369–372). The dissolution rate and solubility behavior of 3-(1-methyl-2-pyrrolidinyl)-indole as a function of pH have been evaluated (373). In another study data were presented showing the effect of complex formation on dissolution kinetics (374). Benzoic acid was solubilized by polyoxyethylene compounds (375). Another article explored the mechanism of solubilization of water-insoluble substances with sodium benzoate derivatives (376). In addition, spectroscopic examination revealed that benzoic acid was solubilized by nonionic surfactants (377). The rate of crystal growth and dissolution rate of cholesterol crystals in aqueous media were probed (378).

The hydrophilic–lipophilic balance concept was utilized effectively to solubilize a wide variety of perfumery materials (379). Nonionic surfactants have been widely used to effect solution of a number of pharmaceuticals (380, 381). Antibiotics such as erythromycin and chlortetracycline have been the subject of papers in which their solubility and stability have been investigated (382, 383). Goldberg *et al.* presented a series of papers which demonstrated that dissolution rates and gastrointestinal absorption of drugs could be increased by preparing solid solutions and eutectic mixtures (384–386). A new method of solid state dispersion for increasing dissolution rates was described (387). Dissolution rate data appeared for drugs in powder form, granules, and tablets (388).

Slightly soluble acidic drugs were solubilized by using mixtures of alcohol and water (389). It was reported that the rate of rehydration of lyophilized serum globulin can be influenced by temperature, dilution, and the addition of sodium chloride, glucose, or sucrose (390). The solubility of acetanilide and several derivatives was determined in aqueous sucrose solutions (391). Anderson and Morgan showed that the solubilization of hexachlorophene had some effect on its antibacterial activity (392). Another study pointed out that supersaturated micelles form during solubilization (393). Poly-*n*-vinyl-5-methyl-2-oxazolidinone was found to solubilize several barbituric acid derivatives (394).

Complexation—In a study of the interaction of weak organic acids and phenols it was indicated that the drug–plastic interactions were due to hydrogen bonding of the agents to the polyamide, but that secondary valence forces of the Van der Waal's type play a predominate role in the binding mechanism for the more hydrophobic molecules (395). Several papers were concerned with the complexation tendencies of various antibiotics in the presence of metal ions and bovine serum albumin (396–399). Lach and Bornstein reported on solid–solid interactions by diffuse reflectance studies (400, 401). A potential mechanism for aspirin allergy due to an interaction with amines has been mentioned (402). Metal complexes of *d*-glucosamine and its derivatives with copper were found to be stable (403). The interaction of phenothiazine drugs with riboflavine and xanthine dyes was the subject of two papers (404, 405). It was pointed out that pK values, molecular weight, and the number of hydrophilic groups of organic ions are important in dye–drug salt interactions (406). The binding tendencies of cationic dyes to nucleic acids and other biological polyanions were investigated (407); also the nature of bonding in dye aggregates was reported (408).

Connors and Mollica analyzed comparative

studies of complex formation and found that different experimental approaches may not always yield the same numerical result and that comparative studies with several techniques may yield valuable information concerning the natures of the complexes (409). Viscosity measurements were used to study the interaction of benzoic acid derivatives and cationic surfactants (410). It was also reported that imidazole derivatives are useful as chelating agents (411, 412). The general nature of the interaction of phenol and certain macromolecules was investigated utilizing equilibrium dialysis techniques (413, 414).

The solubility product principle and phase rule diagrams were used to demonstrate interactions involving sulfanilamide and sulfathiazole (415). Data were also published which pointed out that β-cyclodextrin and sodium deoxycholate interact with many pharmaceutical agents in aqueous solution (416). Static dialysis techniques were utilized in studying the transfer of drug molecules from the micellar phase (417–419). The mechanism of solubilization of water-insoluble substances in aqueous sodium benzoate solutions has been determined (420, 421). The degree of binding of 8-hydroxyquinoline sulfate to polysorbate 80 was shown to be a function of the concentration of the nonionic surface-active agent (422). The interaction of aqueous polyethylene glycol solutions with iodine was investigated (423). The interaction of several local anesthetic drugs and sodium polyethylenesulfonate was evaluated by membrane dialysis measurements (424). The binding tendencies of methyl orange with proteins were also observed in a similar manner (425). Metal ions were shown to form complexes with ascorbic acid (426); molybdenum-thiol complexes were used as models for molybdenum bound in enzymes (427).

Beckett and Choulis investigated molecular interactions of stereoisomers in the solid state (428). Differential thermal analysis, infrared, and X-ray analysis were employed to evaluate the interactions between ionized surfactants and long chain polar compounds (429). The chelation of sugars and polyols with iron was exhibited most strongly by those compounds that contained a dihydroxyacetone structure in their molecule (430). Hydrophilic gel beads were utilized to form insoluble biologically active compounds (431). The metal chelating tendencies of glyoxylic acid have been investigated (432). The interaction of cationic drugs with polyacrylic acid was studied by phase separation as a function of temperature, polymer, and drug concentration. Separation temperature varied with polymer added, drug concentration, basic structure of drug, and valence of drug cation (433).

Surface Phenomena—A review article, containing 146 references, was concerned with particle–particle interactions (434). Another review commented on the study of the texture of porous solids (435). Micelle formation and other typical phenomena of surface-active substances were the subject of still another review (436). The scope of methods for estimating surface areas of solids from results of adsorption from solution has been described (437). A computer program was set up to reduce the calculations in surface area determinations and to provide an estimate of accuracy and reliability of a set of experimental data (438). The difference between flocculation and coagulation was discussed in detail by Ecanow *et al.* (439). The various methods of determining the surface tension of solids and their shortcomings have been described (440).

The drop volume method was used to study the surface activity of several phenothiazines at the air–solution interface (441). It was also pointed out that the presence of organic cations inhibited surface activity at this interface (442). Solid adsorbents such as kaolin, talc, and activated charcoal were shown to adsorb phenothiazine derivatives (443). Horikoshi and Hata published several papers concerned with a physicochemical study on water contained in solid medicaments (444–446). The neutralization rate, crystal structure, and crystal growth rate of dried aluminum hydroxide gel were shown to be affected by aqueous environment (447, 448). The sedimentation and aggregation of aluminum hydroxide and aluminum oxide particles were explored (449). It was also pointed out that the volume of aluminum hydroxide gels changes when it crystallizes (450).

A new method, comparing the flux magnitudes corresponding to different conditions of gas flow through porous media, has been proposed for the determination of surface areas of powders (451). Adsorption studies were conducted on a number of steroid powders (452). An investigation of the flow rate of loosely packed magnesium hydroxide has shown that the general equation developed by Jones and Pilpel can be applied to single as well as multicomponent mixtures in a wide size range (453). The sorption and desorption data for methylene blue on kaolinite fit the Langmuir equation (454). Brunauer published a review article which discussed the surface behavior of solids (455). Spray-dried clays were evaluated *versus* oven-dried clays and found to vary somewhat in their physical characteristics (456).

The influence of lanolin derivatives on the physical aspects of dispersions was discussed by Conrad *et al.* with particular reference to pigment wetting (457–459). By using the Levich model for the effect of surfactants, a quantitative conclusion was drawn concerning the hydrodynamics of a laminar liquid film in the presence of surfactant, when their distribution on the surface depends on the rate of the adsorption and desorption processes (460). Surface-active agents were shown to have a marked influence on the electrokinetic potential of some sulfonamides in aqueous syrup suspensions (461). In another study the general criteria for the stability of lyophobic sols was established and found valid for any potential of the colloid particles in the critical state (462). From the general criteria of the stability of disperse systems, complete curves were calculated which characterized the coagulating effect of mixtures of various electrolytes for potentials of colloid particles (463). Using a hydrosol of titanium dioxide stabilized by sodium oleate, it was shown that, when the stabilizer content in the dispersion medium is close to the micelle forming concentration, the disperse particles aggregate (464). A quantitative theory was presented which describes the kinetics of coagulation of colloidal systems containing more than one dispersed species (465).

The adsorption of three quaternary ammonium salts has been measured at the oil–water interface (466). In another investigation the electrophoretic mobilities, the rates of agglomeration, and the rates of flow through sedimented particles were studied for a model suspension consisting of spherical particles (467). Nash elaborated on structural vehicles for stabilized suspensions along with physical stability test methods and special factors in producing good parenteral suspensions (468). A detailed study has been made on the destabilization of dilute clay suspensions with a cationic polymer under controlled conditions of pH, ionic strength, initial clay concentration, and intensity and duration of agitation (469). A simple and inexpensive *in vitro* test was utilized to evaluate the defoaming activity of a silicone defoamer in tablet combination with a number of commonly used antacid materials (470). The equilibrium adsorption of kanamycin A by cationic exchange resins was explored (471). The adsorptive mechanism of a palm oil–water emulsion stabilized by a mixture of casein and gelatin was elucidated by combining surface tension studies of the oil–water interface with electrophoresis studies (472). The kinetics of particle adhesion for glass beads in liquid media containing surfactants were investigated (473).

Crystallization—The crystallization of petrolatum waxes was the subject of a review article (474). Morawetz observed that the reactivity of organic crystals is sometimes different from reactions that take place in the liquid state (475). The water solubility of chlortetracycline hydrochloride varied with the change in crystal structure of the compound (476). It was also observed that the biological properties of erythromycin polymorphs were similar, but the melting points and the water of hydration were different (477). The effect of polymorphism on the solubility of pharmaceuticals was shown (478).

Rheology—A visco-coagulograph was developed which records continuously the structure formation in liquids (479). Neuwald and Scheel did a rheological study on new cream bases as a function of temperature, while Boylan presented a rheological study on 13 pharmaceutical semisolids (480–482). A method was described for the continuous monitoring of solutions of stirred reaction media (483). The problems encountered in determining the viscosity of shampoos were discussed (484). The parallel plate plastometer was modified for the measurement of Newtonian and non-Newtonian fluids (485). The rheological behavior of granular material was investigated by another investigator (486). Another paper described the various methods of characterizing the thixotropic properties of an oil-in-water lotion (487). Extrusion type cells have been developed for measuring and recording rheological properties of gels (488).

In another study a modified Brookfield viscometer was used to evaluate dispersions of lipophilic suspending agents (489). Powell *et al.* studied the effects of high shear processing and thermal exposure on the molecular weight and the solution viscosity of selected polymers and found that the viscosity degradation followed a first-order reaction rate with the viscosity half-life for the higher molecular weight grade polymer being considerably less than that of the lower molecular weight grade polymer (490). It was also shown that the limiting shear stress and torque under which a gelatin solution can retain its structure are a function of concentration, time, and temperature (491). The various techniques used for assessing the rheological properties of toiletry and cosmetic products were considered (492). A rheological evaluation of pressurized pharmaceutical foams was presented (493).

PHARMACOCHEMICAL ASPECTS

This section of the review considers many of the papers on polymers, antibiotics, and radio-

isotopes which might be of interest to the pharmaceutical scientist. It is not intended to encompass the vast area of pharmaceutical chemistry concerned with synthesis, structure activity studies, reaction mechanisms, analysis, *etc.* These related disciplines are reviewed annually in other publications and are, therefore, omitted from this report.

Polymers—Carpenter presented a review article, containing 135 references, in which irreversible, reversible, macromolecular, and association colloids were reviewed (494). Recent theory and developments relating to the formation and modification of synthetic polymeric films in relation to the pharmaceutical uses of such films in dosage form development have been discussed (495). Several polymeric materials, currently used in the plastics industry, were evaluated for their use in pharmaceutical film coatings (496). Five commercially available polymers were considered for the preparation of enteric compression coatings utilizing a unique polymer incorporation method which was part of the granulating process (497). In another investigation cellulose acetophthalate was examined as an enteric tablet coating (498). Surfactant polymeric materials have been used to prepare prolonged action insulin preparations (499). Film forming macromolecules have been utilized as protective coatings for skin wounds and for antiseptic sprays and balms (500, 501). The water vapor transmission properties of free polymer films were explored (502). In addition, the water vapor transmission of applied polymer films on tablets was found to be a function of polymer coat formulation, film coat thickness, and nature of the tablet matrix (503).

Antibiotics—Schumacher presented a review, containing 27 references, on the chemistry, microbiology, pharmacology, and the therapeutic application of cephalosporin antibiotics (504). Another paper reviewed both broad spectrum and narrow range antibiotics for activity against Gram-positive or Gram-negative organisms (505). A complete discussion of the newer semisynthetic penicillin derivatives was given (506). The properties of some of the new antibiotics, spinamycin, leucomycin A_1, ericamycin, xanthocidin, and cineromycins A and B were disclosed (507–511). The kinetics and mechanisms of action of chloramphenicol and tetracycline on the generation rates of *E. coli* were investigated (512). Another paper discussed the compatibility of some pharmaceutical preparations with antibiotics (513).

The binding tendencies of sulfonamide drugs to serum protein have been the subject of several papers (514–517). A number of papers appeared in the literature concerning the protein binding of antibiotics, especially the penicillins (518–525). Several papers contained data on blood serum levels of penicillin and some semisynthetic penicillins (526–529). A comparison of tetracycline concentrations, following oral and intravenous administration, indicated that intravenous administration gave superior therapeutic response (530).

Radioisotopes—Wagner and Emmons reviewed the characteristics of an ideal radiopharmaceutical (531). The problems characteristic of radioactive pharmaceuticals have been outlined (532). A biochemical view of radiopharmaceutical developments indicated that to date more attention has been paid to the physical characteristics of the isotopes incorporated into radiopharmaceuticals than to the biochemical aspects of their use (533). Another author pointed out the value of computer analysis of kinetic biological parameters (534).

BIOPHARMACEUTICS

The area of biopharmaceutics considers research efforts directed toward studying the influence of pharmaceutical formulations on the biological activity of drugs. Numerous review articles appear in the literature. An article, containing 67 references, was published on the noncompetitive mechanism governing the action of medicinal drugs (535). Another review discussed pharmaceuticals which modified the activities of gastric enzymes (536). Beckett reviewed the problem of drug distribution and metabolism (537). In addition, another review article discussed the aspects of clinical trials of new drugs and the difficulties of evaluating them for one species from the results obtained in another species (538). Pharmacokinetics were reviewed and discussed (539). The induction of drug-metabolizing enzymes in liver microsomes was the subject of another review article containing 83 references (540). The application of statistics and biometry in the comprehension of drug action was noted (541).

Methods were described which utilize the analog computer as a laboratory aid in the preparation of drug formulations with improved therapeutic efficacy (542). A mathematical and theoretical interpretation of drug distribution and dosage, involving complex pharmacokinetic models and the analog computer, was presented (543). The analog computer was used to help clarify the distribution processes of *d*-tubocurarine in experimental animals (544). Protein binding tendencies of some azo dyes, thyroxine, diphenylhydantoin, and pentaerythritol have

been the subjects of several papers (545–547). The biphasic elimination of noscapine was published (548). Human bioassay techniques have been used for determining the availability of vitamins from preparations resistant to *in vitro* disintegration (549). Data concerned with the modification of drug responses by hydrolytic enzymes were recorded (550).

Effects of Physicochemical Properties—The urinary excretion kinetics of methylamphetamine in man were studied by Beckett and Rowland (551). In a similar study, it was shown that the rate of excretion of methylephedrine and its metabolite, ephedrine, fluctuated with a change in urinary pH (552). Several papers reported data on some basic *in vitro* and *in vivo* studies with six different sulfa drugs (553, 554). The transport of volatile fatty acids across rumen epithelium was altered by concentration gradient, pH, and metabolism (555). It was demonstrated that cholestryamine resin interacted with commonly used, orally administered drugs in both *in vivo* and *in vitro* systems (556). The relation between the physicochemical and pharmacological properties of iron and chromdoitin sulfate colloid for intravenous injection was studied (557).

Effects of Formulation—Lammers reviewed the ways in which a drug can interact with an inactive substance to produce a potentiation or a lessening of the effect of the drug (558). Another review article commented on the effect of drug formulations on the absorption rate (559). Percutaneous absorption of lipid substances and their effect on skin surface was the subject of another review (560). The relationship between the *in vitro* dissolution kinetics and the *in vitro* intestinal absorption characteristics of tablet preparations of coumarin revealed that the composition of the dissolution medium had a significant quantitative and qualitative effect on dissolution kinetics (561). It was also pointed out that the absorption of a number of alcohols and barbiturates was affected by the concentration of nonionic surfactant (562). Static dialysis studies indicated that it was possible to anticipate the amount of drug absorbed in the small intestinal tract containing polysorbate 80 solution (563). Drug release from water-in-oil emulsion systems, containing tensides of a sorbitan[8] series, was investigated (564, 565). Another study further substantiated that drugs are absorbed from micellar solutions (566). It was also shown that a relationship existed between the concentration of polysorbate 80 and the rate of absorption of salicylamide from the small intestine of the rat (567).

A comparative investigation on the rectal absorption of aminophylline and theophylline from various medicated preparations was carried out in man by Neuwald and Ackad (568). The rectal absorption of sulfonamides from oily solutions seemed to take different paths: that of absorption through the rectal membrane after being liberated into the excretion fluid and that of absorption directly from the fatty oil through the rectal membrane (569). Surfactants were found to enhance the release and absorption of barbiturates from suppository bases in most cases; however, binding of the drug was suspected in some bases (570). The rectal absorption of aminopyrine and isopropylantipyrine was carried out in rabbits from an improved hydrophilic ointment base (571). Inert suppository ingredients sometimes have an adverse effect on the rectal absorption coefficient of drugs; this was apparent when antipyrine (phenazone) was evaluated (572).

Several papers were published on the action of proteolytic enzymes on the serum concentrations of the tetracyclines (573–575). Other investigators evaluated the feasibility of using an enzyme–substrate system as a mechanism for controlling the rate of drug release from a dosage form (576, 577). When ethinylestradiol was introduced directly into the small intestine of the rat as an oily solution, it was absorbed more slowly than when administered as an aqueous suspension (578). An antidiarrheal preparation containing attapulgite and citrus pectin was studied for its potential effect on the absorption of promazine from the human gastrointestinal tract (579). The inverted sac technique was used by Aguiar and Fifelski to investigate the effect of pH on the *in vitro* absorption of flufenamic acid. The study revealed that the passage of this drug through the gut membrane was by passive diffusion (580). An evaluation of the blood level duration properties of procaine benzylpenicillin in oil with aluminum monostearate was carried out (581).

Micronized medroxyprogesterone acetate displayed more metabolite in 8 hr. than a nonmicronized preparation, indicating improved absorption with smaller particles (582). Rosen *et al.* reported on the absorption and excretion of radioactively tagged dextroamphetamine sulfate from a sustained-release form. The data suggested that the sustained-release dosage form is a reliable way to administer the drug (583). The absorption, distribution, and excretion of iron following oral administration in the presence

[8] Marketed as the various Spans by Atlas Chemical Industries, Inc., Wilmington, Del.

of various ligands was the subject of another communication (584). Plasma and urinary excretion levels of 5-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyridine were measured after application of various ointments and solutions to the skin of rats or rabbits (585). Flame photometry was used to determine the activity of ointments (586). The topical absorption of various polypeptides in the presence of dimethylsulfoxide has been evaluated (587). Wagner *et al.* pointed out that the rates of absorption of indoxole after oral administration varied with the nature of the dosage form (588). A study evaluating the effect of polyethylene glycol 4000 on the absorption of several barbiturates has also been reported (589).

Absorption Control—A review on the absorption and biochemistry of vitamin B_{12} was published (590). Holt demonstrated that competitive inhibition of intestinal bile salts in the rat was apparent (591). It was also further substantiated that iron was transported across membranes, using a loop of gut with an artificial circulation (592). The release rate characteristics of sulfaethylthiadiazole from various wax formulations were revealed (593).

Absorption Mechanism—Palva discussed the drug-induced malabsorption of vitamin B_{12}, pointing out that *p*-aminosalicylic acid interfered with certain metabolic processes mediated by folic acid (594, 595). An investigation of the prolongation of the therapeutic effect of penicillin by polymeric preparations indicated that the mobility and the rate of elimination of the polymer molecules from the body were responsible for the delayed effect (596). In a similar study polyvinylpyrrolidone was shown to prolong the activity of tetracycline (597). Other authors studied the effect of cations on the transport of sulfonamides through the small intestine of rats and found that absorption of sulfisoxazole was reduced when potassium ion was substituted for sodium ion (598). Ascorbic acid was shown to increase the absorption of iron (599), while allopurinol had no effect on the absorption of iron (600). It was reported that the topical application of dimethylsulfoxide enhanced the topical absorption of phenylbutazone; however, when the dimethylsulfoxide was administered subcutaneously there was no effect (601).

Kinetic Studies—The kinetics of the metabolism, absorption, and decomposition of drugs were reviewed in a paper citing 14 references (602). Another article discussed the pharmacokinetics of various tetracyclines (603). Nelson presented data showing the application of results of pharmacokinetic estimations and their significance (604). A theoretical study of drug action was outlined and the concept was tested with hormones of the neurohypophysis and their analogs (605). Kinetic interpretation was used as a tool to explain pharmacological response (606). A pharmacokinetic model involving first-order processes for drug release, absorption and elimination, for sustained-release preparations, has been described (607). Several papers surveyed the distribution, absorption, and excretion of sulfonamides (608–611).

Gibaldi and Kanig studied the effect of body position and pH on the gastrointestinal absorption of salicylate and creatinine in man (612). The pharmacodynamic behavior of the salicylates has been the subject of several papers (613–617). Tritiated digoxin was used to determine the distribution and excretion of this material in experimental animals (618). Using plasma salicylate levels, the rates of drug absorption and elimination from the blood were determined for salicylic acid and sodium salicylate administered in four different suppository bases (619). In studying protein binding tendencies of sulfonamides it was observed that there was a deviation from first-order kinetics, characterized by a diminishing steepness of the slope of the time–concentration curve (620). The analysis of expired air was shown to offer several advantages over the analysis of blood and urine samples (621).

A kinetic model to explain the elimination of bromsulfophthalein using the Michaelis-Menten expressions has been proposed by Winkler (622). In another study, urinary excretion data were utilized to evaluate some oral prolonged-release forms of dextroamphetamine in man (623). The first apparent demonstration of competitive inhibition of drug metabolism in man was reported (624). The elimination constants and biological half-lives of thiamine were determined for a series of thiamine derivatives (625). Radioactive tracer techniques were used to follow the absorption and excretion of hydroxycyanocobalamin, benzoylthiamine disulfide, and a hypoglycemic metabolite of glycodiazine (626–628). A complete pharmacodynamic evaluation of a sustained-release aspirin product was published (629). The absorption, excretion, and metabolism of nalidixic acid and hydroxynalidixic acid was the subject of two papers (630, 631). The use of kinetic models to study antibiotic absorption, metabolism, and excretion has been proposed (632).

Drug Absorption—Munies described a method for investigating *in vivo* percutaneous absorption based on the determination of methyl ethyl ketone in expired air following topical

application (633). In another paper the absorption, excretion, and elimination of chlorphenesin carbamate in the dog were discussed (634). A large volume liquid scintillation detector was used to determine the rates of excretion, absorption, and intercompartmental clearance in ambulatory dogs (635). A similar study was carried out with cyanocobalamin (636). Radiotracer techniques were used to follow the absorption of furosemide and thimerosal (637, 638). The problems associated with sensitivity and cross sensitivity to drugs were pointed out (639). The absorption, duration of circulation, and elimination of sodium sulfapyridazine by different routes of administration have been evaluated (640). It was reported that the absorption of a vitamin A–casein complex was more efficient than absorption of vitamin A (641).

Levy and Jusko pointed out that the enhanced absorption of riboflavin in the presence of food appeared to be due to the decrease in intestinal transit rate causing the vitamin to remain at absorption sites longer (642). The absorption, distribution, and elimination of a long-acting vitamin B_{12} preparation have been studied (643); and the absorption and excretion of 5-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyridine were determined when it was administered by different routes (644, 645). It has been shown that sulfaphenazole appears in the saliva after intravenous administration (646). Data were presented for the absorption of betamethasone from the gastrointestinal tract in dogs (647). Another publication presented data concerned with the effect of physical activity on the absorption rates of procaine penicillin G implants (648). Another investigator carried out research on the absorption and activity of griseofulvin and some derivatives in rabbits (649). Sweetening agents were also the subject of absorption and excretion studies in humans, rats, and rabbits (650).

REFERENCES[9]

(1) McKeehan, W. J., *J. Pharm. Sci.*, **52**, 613, 717 (1963).
(2) McKeehan, W. J., and Lappas, L. C., *ibid.*, **53**, 695, 847(1964).
(3) Lappas, L. C., and Dempski, R. E., *ibid.*, **54**, 931 (1965).
(4) Dempski, R. E., and Polli, G. P., *ibid.*, **55**, 645(1966).
(5) Buechi, J., *Pharm. Acta Helv.*, **41** (1), 1(1966); through *Chem. Abstr.*, **64**, 9505(1966).
(6) Buechi, J., *Pharm. Acta Helv.*, **41** (2), 65(1966); through *Chem. Abstr.*, **64**, 14769(1966).
(7) Macek, T. J., *Am. J. Pharm.*, **138**, 22(1966).
(8) Phares, R. E., Jr., and Hammond, E. K., *Am. J. Hosp. Pharm.*, **23**, 145(1966).
(9) Blazey, L. W., *Drug Cosmetic Ind.*, **99**, 52(1966).
(10) Meyers, E. L., *J. Chem. Doc.*, **5** (4), 251(1965); through *Chem. Abstr.*, **64**, 9506(1966).
(11) Meyer, E. L., *Drug Cosmetic Ind.*, **99**, 70(1966).
(12) Olson, T. N. T., and Lee, I., *J. Pharm. Sci.*, **55**, 1(1966).
(13) Johnson, J. L., *Drug Cosmetic Ind.*, **99**, 50(1966).
(14) Benya, T. J., *Am. J. Hosp. Pharm.*, **23**, 384(1966).
(15) Penna, R. P., *J. Am. Pharm. Assoc.*, **NS6**, 463(1966).
(16) Hopponen, R. E., *ibid.*, **NS6**, 466(1966).
(17) Darlington, R. C., *ibid.*, **NS6**, 470(1966).
(18) Chalmers, R. K., *ibid.*, **NS6**, 538(1966).
(19) Zupko, A. G., and Stempel, E., *ibid.*, **NS6**, 543(1966).
(20) Weinswig, M. H., *ibid.*, **NS6**, 550(1966).
(21) Erickson, C. K., *ibid.*, **NS6**, 547(1966).
(22) Day, R. L., *ibid.*, **NS6**, 650(1966).
(23) Engelund, A., *Arch. Pharm. Chemi*, **72** (23), 840 (1965); through *Chem. Abstr.*, **64**, 4862(1966).
(24) Garrett, E. R., *J. Pharm. Pharmacol.*, **18**, 589(1966).
(25) Horsch, W., *Pharm. Praxis, Beilage Pharmazie*, **1965** (10), 220; through *Chem. Abstr.*, **64**, 11025(1966).
(26) Marinaro, A., *Am. Perfumer Cosmet.*, **81** (7), 29 (1966).
(27) Judis, J., *J. Pharm. Sci.*, **55**, 803(1966).
(28) Patwa, N. V., and Huyck, C. L., *J. Am. Pharm. Assoc.*, **NS6**, 372(1966).
(29) Jakubowska, J., Nowakowska-Waszczuk, A., and Losata-Dulkowska, E., *Tluszcze i Srodki Piorace*, **9** (6), 354 (1965); through *Chem. Abstr.*, **64**, 17351(1966).
(30) Riepl, G., Modde, H., and Schuster, G., *Am. Perfumer Cosmet.*, **81** (6), 39(1966).
(31) Araujo, O. E., *J. Pharm. Sci.*, **55**, 636(1966).
(32) Paruta, A. N., and Sheth, B. B., *ibid.*, **55**, 1208 (1966).
(33) Evans, W. P., and Dunbar, S. F., *Soc. Chem. Ind. (London), Monograph No. 19*, **1965**, 169; through *Chem. Abstr.*, **64**, 17873(1966).
(34) Balykova, L. A., *et al.*, *Zh. Mikrobiol., Epidemiol. i Immunobiol.*, **43** (6), 121(1966); through *Chem. Abstr.*, **65**, 8680(1966).
(35) Ashworth, R. W., and Heard, D. D., *J. Pharm. Pharmacol.*, **18**, 98S(1966).
(36) Parker, M. S., Barnes, M., and Bradley, T. J., *ibid.*, **18**, 103S(1966).
(37) Donbrow, M., and Jacobs, J., *ibid.*, **18**, 92S(1966).
(38) Wesley, F., *Drug Cosmetic Ind.*, **99**, 37(1966).
(39) Ward, D. R., Lathrop, L. B., and Lynch, M. J., *ibid.*, **99**, 48(1966).
(40) Kanjo, K., *Yukagaku*, **15** (6), 267(1966); through *Chem. Abstr.*, **65**, 6993(1966).
(41) Angla, B., *Soap, Perfumery Cosmetics*, **39**, 375(1966).
(42) Wicker, R. J., *Chem. Ind. (London)*, **1966**, 1708.
(43) Deutsch, E. W., and Hansch, C., *Nature*, **211**, 75 (1966).
(44) Berger, S. E., and Carr, D. R., *Food Technol.*, **20** (11), 97(1966).
(45) Beidler, L. M., *Advan. Chem. Ser.*, **56**, 1(1966); through *Chem. Abstr.*, **65**, 19151(1966).
(46) Amoore, J. E., *Parfum. Cosmet. Savons*, **9** (5), 195 (1966); through *Chem. Abstr.*, **65**, 8663(1966).
(47) Feustel, G., *Riechstoffe, Aromen, Koerperpflegemittel*, **16** (1), 4(1966); through *Chem. Abstr.*, **64**, 12457(1966).
(48) Bhatia, D. G., Sokoloski, T. D., and Bhatia, V. N., *J. Pharm. Sci.*, **55**, 1116(1966).
(49) Magid, L., *Drug Cosmetic Ind.*, **99**, 64(1966).
(50) McLaren, K., *Am. Perfumer Cosmet.*, **81** (11), 43 (1966).
(51) Vaidya, B. R., *Dissertation Abstr.*, **26**, 6655(1966).
(52) Thomas, W. G., Jr., *J. Soc. Cosmetic Chemists*, **17** (9), 553(1966).
(53) Otani, S., *Yakuzaigaku*, **25** (4), 45(1965); through *Chem. Abstr.*, **65**, 18431(1966).
(54) Shotton, E., and Harb, N., *J. Pharm. Pharmacol.*, **18**, 175(1966).
(55) Batuyios, N. H., *J. Pharm. Sci.*, **55**, 727(1966).
(56) Wai, K., DeKay, H. G., and Banker, G. S., *ibid.*, **55**, 1244(1966).
(57) Nasir, S. S., and Wilken, L. O., Jr., *ibid.*, **55**, 794 (1966).
(58) Daoust, R. G., and Lynch, M. J., *Texas J. Pharm.*, **4** (3), 314(1963); through *Chem. Abstr.*, **64**, 12466(1966).
(59) Reier, G. E., and Shangraw, R. F., *J. Pharm. Sci.*, **55**, 510(1966).
(60) Kwan, K. C., and Milosovich, G., *ibid.*, **55**, 340 (1966).
(61) Zabaria, N., *et al.*, *Ann. Pharm. Franc.*, **24** (4), 275 (1966); through *Chem. Abstr.*, **65**, 8681(1966).
(62) Salo, D. P., Panasevich, O. O., and Slin'ko, N. D. *Farmatsevt. Zh. (Kiev)*, **20** (6), 5(1965); through *Chem. Abstr.*, **64**, 14034(1966).
(63) Leberl, O., *Kosmetik-Prafum-Drogen-Rundschau*, **13** (7), 99(1966); through *Chem. Abstr.*, **65**, 18423(1966).
(64) Dias, M., *Rev. Port. Farm.*, **16** (1), 12(1966); through *Chem. Abstr.*, **65**, 2063(1966).
(65) Gast, R. G., *Intern. Clay Conf., Proc. Conf. Stockholm*, **1963** (1), 251; through *Chem. Abstr.*, **64**, 8965(1966).
(66) Mukerjee, L. N., and Shukla, S. D., *J. Indian Chem. Soc.*, **42** (11), 805(1965); through *Chem. Abstr.*, **64**, 9516 (1966).
(67) Bajohr, F. C., *Deut. Apotheker-Ztg.*, **104**, 1103(1964); through *Chem. Abstr.*, **65**, 15166(1966).
(68) Goldschmidt, H., *Am. Perfumer Cosmet.*, **81** (1), 23 (1966).
(69) Parrott, E. L., *J. Am. Pharm. Assoc.*, **NS6**, 73 (1966).
(70) Kennon, L., *J. Soc. Cosmetic Chemists*, **17** (3), 135 (1966).
(71) *Ibid.*, **17** (6), 313(1966).
(72) Krowczynski, L., *Farm. Polska*, **21** (23), 893(1965); through *Chem. Abstr.*, **65**, 2063(1966).

[9] *Index Medicus* abbreviations were used for journals not listed in *Chemical Abstracts*.

(73) Carless, J. E., Foster, A. A., and Jolliffe, G. O., *J. Pharm. Pharmacol.*, **18,** 815(1966).
(74) Lukesch, H., *Arch. Biochim. Cosmetol.*, **9** (87), 158 (1966); through *Chem. Abstr.*, **65,** 15148(1966).
(75) Castagnola, V., and Rigamonti, S., *Boll. Chim. Farm.*, **104** (7), 444(1965); through *Chem. Abstr.*, **64,** 6412 (1966).
(76) Deak, F., and Tokes, B., *Rev. Med.*, **12** (2), 192 (1966); through *Chem. Abstr.*, **65,** 19935(1966).
(77) Tanaka, N., Fujimoto, K., and Utsumi, I., *Yakugaku Zasshi*, **86,** 282(1966).
(78) Grabowska, I., *Gdanskie Towarzyst Nauk. Wydzial Nauk Mat. Przyrodniczych, Rozprawy Wydzialu III, No. 1,* 193(1964); through *Chem. Abstr.*, **64,** 14029(1966).
(79) Grabowska, I., *Gdanskie Towarzyst. Nauk. Wydzial Nauk Mat. Przyrodniczych Rozprawy No. III, 1,* 207(1964); through *Chem. Abstr.*, **64,** 15678(1966).
(80) Brown, I. R., *et al., Can. J. Pharm. Sci.*, **1** (1), 22 (1966); through *Chem. Abstr.*, **65,** 18432(1966).
(81) Schumacher, G. E., and Hull, R. L., *Am. J. Hosp. Pharm.*, **23,** 245(1966).
(82) Kurchenko, T. N., and Konev, F. A., *Farmatsevt. Zh. (Kiev)*, **20** (5), 13(1965); through *Chem. Abstr.*, **64,** 7973 (1966).
(83) Yalcindag, O. N., *Turk Hijyen Tecrubi Biyol. Dergisi*, **26** (1), 93(1966); through *Chem. Abstr.*, **65,** 8678 (1966).
(84) Doppert, H. L., and Staverman, A. J., *J. Polymer Sci.*, **1966A-1,** 2353.
(85) Wahba, I. J., and Massey, L. M., Jr., *ibid.*, **1966A-1,** 1759.
(86) Galczynska, M., Baranowska, B., and Jurkowska, E., *Ann. Acad. Med. Lodz*, **6,** 98(1965); through *Chem. Abstr.*, **65,** 574(1966).
(87) Neuwald, F., and Winkler, A., *J. Soc. Cosmetic Chemists*, **16** (12), 679(1965).
(88) Wisniewski, W., and Golucki, Z., *Acta Polon. Pharm.*, **23** (3), 235(1966); through *Chem. Abstr.*, **65,** 16796(1966).
(89) Pasich, J., and Szczesniewska, B., *Acta Polon. Pharm.*, **23** (3), 239(1966); through *Chem. Abstr.*, **65,** 16796(1966).
(90) Nair, C. G. R., *Talanta*, **12** (11), 1057(1965); through *Chem. Abstr.*, **64,** 530(1966).
(91) Matsuoka, Y., *et al., Yakuzaigaku*, **25** (4), 59(1965); through *Chem. Abstr.*, **65,** 18432(1966).
(92) Beretta, J. J., *Am. J. Hosp. Pharm.*, **23,** 27(1966).
(93) Pellati, S., *Riv. Ital. Essenze-Profumi, Piante Offic., Aromi-Saponi, Cosmet.-Aerosol*, **48** (1), 31(1966); through *Chem. Abstr.*, **65,** 6994(1966).
(94) DeLos, D. F., and Day, V. M., *J. Phys. Chem.*, **70,** 495(1966)
(95) Pincock, R. E., and Kiovsky, T. E., *J. Chem. Educ.*, **43,** 358(1966).
(96) Hammes, G. G., *Science*, **151,** 1507(1966).
(97) Nuhn, P., and Wagner, G., *Pharmazie*, **21** (5), 261 (1966); through *Chem. Abstr.*, **65,** 8665(1966).
(98) Anderson, R. A., and Slade, A. H., *J. Pharm. Pharmacol.*, **18,** 640(1966).
(99) Hojo, M., Utaka, M., and Yoshida, Z., *Tetrahedron Letters*, **1966,** 19.
(100) *Ibid.*, **1966,** 25.
(101) Denisov, M. D., *Farmatsevt. Zh.*, **20** (1), 46(1965); through *Chem. Abstr.*, **64,** 6412(1966).
(102) Chafetz, L., Robinson, J. R., and Petrick, R. J., *J. Assoc. Offic. Agr. Chemists*, **48** (5), 1068(1965); through *Chem. Abstr.*, **64,** 3287(1966).
(103) Patel, M. B., *Dissertation Abstr.*, **26,** 3880(1966).
(104) Wadke, D. A., and Guttman, D. E., *J. Pharm. Sci.*, **55,** 1088(1966).
(105) *Ibid.*, **55,** 1363(1966).
(106) Kakemi, K., Sezaki, H., and Inoue, S., *Yakugaku Zasshi*, **86,** 163(1966).
(107) Grabowska, I., *Gdanskie Towarzyst. Nauk., Wydzial Nauk Mat., Przyrodniczych, Rozprawy Wydzialu III, No. 1,* 223(1964); through *Chem. Abstr.*, **64,** 17362(1966).
(108) Fitzgerald, T. J., *Dissertation Abstr.*, **27,** 6251(1966).
(109) Backer, E. T., and van de Langerijt, J. J. A. M., *Pharm. Weekblad*, **101** (22), 489(1966); through *Chem. Abstr.*, **65,** 18431(1966).
(110) Fujisawa, S., and Kawabata, S., *Yakugaku Zasshi*, **86,** 504(1966).
(111) *Ibid.*, **86,** 510(1966).
(112) *Ibid.*, **86,** 514(1966).
(113) Fujisawa, S., Kawamura, M., and Kawabata, S., *ibid.*, **86,** 708(1966).
(114) *Ibid.*, **86,** 714(1966).
(115) Herd, A. K., III, Eberson, L., and Higuchi, T., *J. Pharm. Sci.*, **55,** 162(1966).
(116) Robinson, J. R., Repta, A. J., and Higuchi, T., *ibid.*, **55,** 1196(1966).
(117) Repta, A. J., Robinson, J. R., and Higuchi, T., *ibid.*, **55,** 1200(1966).
(118) Haycock, R. P., Sheth, P. B., Higuchi, T., Mader, W. J., and Paperiello, G. J., *ibid.*, **55,** 826(1966).
(119) Patel, P. R., *Dissertation Abstr.*, **26,** 7055(1966)
(120) Notari, R. E., *ibid.*, **26,** 6654(1966).
(121) Patel, R., *ibid.*, **26,** 5735(1966).
(122) Bernasconi, R., *Pharm. Acta Helv.*, **40** (10), 564 (1965); through *Chem. Abstr.*, **64,** 4874(1966).
(123) Ellin, R. I., Easterday, D. E., Zvirblis, P., and Kondritzer, A. A., *J. Pharm. Sci.*, **55,** 1263(1966).
(124) Belen'kii, B. G., *et al., Antibiotiki*, **11** (2), 112(1966); through *Chem. Abstr.*, **64,** 17356(1966).
(125) Chi'i, N., Chao, F., and Feng, Y., *Yao Hsueh Hseuh Pao*, **12** (9), 615(1965); through *Chem. Abstr.*, **64,** 4873(1966).
(126) Hsi, N., Liu, T., and Wang, W., *Yao Hsueh Hsueh Pao*, **13** (1), 62(1966); through *Chem. Abstr.*, **65,** 8678(1966).
(127) Gecgil, S., *Istanbul Univ. Eczacilik Fak. Mecmuasi*, **2** (1), 56(1966); through *Chem. Abstr.*, **65,** 19927(1966).
(128) Tank, K., and Li, C., *Yao Hsueh Hsueh Pao*, **13** (1), 64 (1966); through *Chem. Abstr.*, **65,** 8678(1966).
(129) Garrett, E. R., *et al., J. Med. Chem.*, **9,** 203(1966).
(130) Libinson, G. S., *Antibiotiki*, **10** (11), 997(1965); through *Chem. Abstr.*, **64,** 7973(1966).
(131) Yukioka, M., Saito, Y., and Otani, S., *J. Biochem.*, **60** (3), 295(1966); through *Chem. Abstr.*, **65,** 19145(1966).
(132) Yousef, R. T., *Arzneimittel-Forsch.*, **16** (3), 446(1966); through *Chem. Abstr.*, **65,** 2069(1966).
(133) Sabri, M. I., and Mohan Rao, V. K., *Indian J. Technol.*, **3** (9), 287(1965); through *Chem. Abstr.*, **64,** 531 (1966).
(134) Misawa, T., *et al., Hokkaido-ritsu Eisei Kenkyu-shoho, No. 16,* 97(1966); through *Chem. Abstr.*, **65,** 19934 (1966).
(135) Gurevich, I. Ya., *et al., Aptechn. Delo*, **15** (1), 29 (1966); through *Chem. Abstr.*, **64,** 17362(1966).
(136) Sabri, M. I., and Mohan Rao, V. K., *Indian J. Technol.*, **4** (6), 180(1966); through *Chem. Abstr.*, **65,** 8678 (1966).
(137) Katerburgskii, A. M., Konstantinovskaya, L. A., and Zemlyanoi, S. V., *Voenno-Med. Zh.*, **1965** (3), 55; through *Chem. Abstr.*, **64,** 19326(1966).
(138) Carstensen, J. T., Aron, S. E., Spera, D. C., and Vance, J. J., *J. Pharm. Sci.*, **55,** 561(1966).
(139) Rigamonti, S., *Boll. Chim. Farm.*, **104** (11), 744 (1965); through *Chem. Abstr.*, **64,** 9516(1966).
(140) Dezelic, M., Grujic-Vesic, J., and Popovic, R., *Tehnika (Belgrade)*, **19,** 2087*a*(1964); through *Chem. Abstr.*, **65,** 15163(1966).
(141) Grujic-Vasic, J., and Popovic, R., *Glasnik Hemicara Tehnol. Bosne Hercegovine*, **13–14,** 113(1965); through *Chem. Abstr.*, **65,** 12062(1966).
(142) Sabri, M. I., and Mohan Rao, V. K., *Indian J. Technol.*, **4** (6), 191(1966); through *Chem. Abstr.*, **65,** 8679 (1966).
(143) Terada, K., and Ohmura, T., *Nippon Nogei Kagaku Kaishi*, **40** (4), 196(1966); through *Chem. Abstr.*, **65,** 2069 (1966).
(144) Seelert, K., and Schenck, G., *Arch. Pharm.*, **299** (9), 757(1966); through *Chem. Abstr.*, **65,** 18425(1966).
(145) Macian, R. S., and del Pozo, A., *Galenica Acta*, **17** (3–4), 165(1964); through *Chem. Abstr.*, **64,** 3288(1966).
(146) Inazu, K., and Yamamoto, R., *Bitamin*, **34** (3), 328 (1966); through *Chem. Abstr.*, **65,** 18432(1966).
(147) Samejima, M., Sugimoto, I., and Utsumi, I., *Yakugaku Zasshi*, **86,** 900(1966).
(148) Inazu, K., and Yamamoto, R., *Bitamin*, **34** (3), 334 (1966); through *Chem. Abstr.*, **65,** 19432(1966).
(149) Bojarski, A., Blitek, D., and Borkowski, B., *Dissertationes Pharm.*, **17** (3), 345(1965); through *Chem. Abstr.*, **64,** 4874(1966).
(150) Galatzeanu, I., and Antoni, F., *Intern. J. Appl. Radiation Isotopes*, **17,** 369(1966).
(151) Otani, S., *Yakugaku Zasshi*, **86,** 263(1966).
(152) Wahlgren, S., *Svensk Farm. Tidskr.*, **69** (34), 965 (1965); through *Chem. Abstr.*, **64,** 7964(1966).
(153) Kovacs, B., *Gyogyszereszet*, **9** (12), 441(1965); through *Chem. Abstr.*, **64,** 14033(1966).
(154) Keseru, P., *Gyogyszereszet*, **9** (12), 445(1965); through *Chem. Abstr.*, **64,** 14033(1966).
(155) Ridgway, K., and Segovia, E., *Mfg. Chemist Aerosol News*, **37** (12), 39(1966).
(156) Singiser, R. E., Heiser, A. L., and Prillig, E. B., *Chem. Eng. Progr.*, **62** (6), 107(1966); through *Chem. Abstr.*, **65,** 8681(1966).
(157) Mathison, H. R., *Drug Cosmetic Ind.*, **99,** 52(1966).
(158) Brewer, J. H., and Arnsberger, R. J., *J. Pharm. Sci.*, **55,** 57(1966).
(159) Menczel, E., Rabinovitz, M., and Megued-Madjar, A., *Harokeach Haivri*, **11,** 83/241–94/230(1965); through *Chem. Abstr.*, **65,** 8680(1966).
(160) Thon, D., *Verpackungs Rundschau*, **17** (1), 1(1966); through *Chem. Abstr.*, **65,** 2063(1966).
(161) Prusak, L. P., and Sciarrone, B. J., *J. Pharm. Sci.*, **55,** 407(1966).
(162) Kotsyurba, V. A., *Aptechn. Delo*, **15** (3), 67(1966); through *Chem. Abstr.*, **65,** 5299(1966).
(163) Allen, M., and Soike, K., *Science*, **154,** 155(1966).
(164) Parker, M. S., Bradley, T. J., and Barnes, M., *Soap, Perfumery Cosmetics*, **39,** 371(1966).
(165) Maniar, A. C., *Mfg. Chemist Aerosol News*, **37** (11), 71(1966).
(166) Bowman, F. W., *J. Pharm. Sci.*, **55,** 818(1966).
(167) Anschel, J., *Drug Cosmetic Ind.*, **99,** 70(1966).
(168) Zoellner, E., and Vastagh, G., *Pharm. Zentralhalle*, **105,** 369(1966).
(169) Hammarlund, E. R., and Van Pevenage, G. L., *J. Pharm. Sci.*, **55,** 1448(1966).
(170) Meisler, J. M., and Skolaut, M. W., *Am. J. Hosp. Pharm.*, **23,** 557(1966).
(171) Bair, J. N., *ibid.*, **23,** 344(1966).
(172) Im, S., and Latiolais, C. J., *ibid.*, **23,** 332(1966).
(173) Patel, J. A., and Phillips, G. L., *ibid.*, **23,** 409 (1966).
(174) Tanaka, N., Matsushita, H., and Utsumi, I., *Yakugaku Zasshi*, **86,** 554(1966).

(175) Ingram, J. T., and Lowenthal, W., *J. Pharm. Sci.*, **55**, 614(1966).
(176) Feinstein, W., and Bartilucci, A. J., *ibid.*, **55**, 332 (1966).
(177) Nogami, H., Nagai, T., and Uchida, H., *Chem. Pharm. Bull. (Tokyo)*, **14**, 152(1966).
(178) Patel, N. R., and Hopponen, R. E., *J. Pharm. Sci.*, **55**, 1065(1966).
(179) Borzunov, E. E., Shevchenko, S. M., and Nosovitskaya, S. A., *Med. Prom. SSSR*, **19** (11), 31(1965); through *Chem. Abstr.*, **64**, 4874(1966).
(180) Delonca, H., *et al.*, *J. Pharm. Belg.*, **21** (1–2), 67 (1966); through *Chem. Abstr.*, **65**, 8681(1966).
(181) Niwa, H., and Hikichi, N., *Tohoku Yakka Daigaku Kenkyu Nempo, No. 11*, 37(1964); through *Chem. Abstr.*, **64**, 19327(1966).
(182) Modrzejewski, F., and Wochna, L., *Acta Polon. Pharm.*, **22** (4), 305(1965); through *Chem. Abstr.*, **64**, 6411 (1966).
(183) Bidlo, G., Takacs, G., and Somogyi, I., *Magy. Kem. Lapja*, **21** (3), 122(1966); through *Chem. Abstr.*, **65**, 3674 (1966).
(184) Jaminet, F., and Hess, H., *Pharm. Acta Helv.*, **41** (1), 39(1966); through *Chem. Abstr.*, **64**, 9515(1966).
(185) Seth, P. L., and Speiser, P., *Pharm. Acta Helv.*, **41**, (7), 385(1966); through *Chem. Abstr.*, **65**, 16797(1966).
(186) Varsano, J., and Lachman, L., *J. Pharm. Sci.*, **55**, 1128(1966).
(187) Fonner, D. E., Jr., Banker, G. S., and Swarbrick, J., *ibid.*, **55**, 181(1966).
(188) *Ibid.*, **55**, 576(1966).
(189) Augsburger, L. L., and Shangraw, R. F., *ibid.*, **55**, 418(1966).
(190) Leoveanu, O., Zaharia, N., and Pilea, V., *Rev. Chim. (Bucharest)*, **17** (2), 112(1966); through *Chem. Abstr.*, **65**, 2070(1966).
(191) Sumner, E. D., Thompson, H. O., Poole, W. K., and Grizzle, J. E., *J. Pharm. Sci.*, **55**, 1441(1966).
(192) Gold, G., Duvall, R. N., Palermo, B. T., and Slater, J. G., *ibid.*, **55**, 1291(1966).
(193) Jaminet, Fr., and Hazee, A., *Pharm. Acta Helv.*, **41** (10), 530(1966); through *Chem. Abstr.*, **65**, 18433(1966).
(194) Lim, J. K., *Dissertation Abstr.*, **27**, 1204-B(1966).
(195) Morris, R. M., *ibid.*, **26**, 3880(1966).
(196) Bishop, R. E., *Am. Perfumer Cosmet.*, **81** (5), 31 (1966).
(197) Daragan, A. G., *Aptechn. Delo*, **15** (4), 10(1966); through *Chem. Abstr.*, **65**, 16796(1966).
(198) Anderson, W., and Sakr, A. M., *J. Pharm. Pharmacol.*, **18**, 783(1966).
(199) Green, D. M., *J. New Drugs*, **6**, 294(1966).
(200) Lazarus, J., and Lachman, L., *J. Pharm. Sci.*, **55**, 1121(1966).
(201) Efimova, L. S., Minina, S. A., and Rosentsveig, P. E., *Med. Prom. SSSR*, **20** (8), 35(1966); through *Chem. Abstr.*, **65**, 18433(1966).
(202) Lieberman, H. A., and Richardson, G., *Drug Cosmetic Ind.*, **99**, 45(1966).
(203) Richardson, G., Lieberman, H. A., and Lachman, L., *ibid.*, **99**, 50(1966).
(204) Lachman, L., Lieberman, H. A., and Richardson, G., *ibid.*, **99**, 60(1966).
(205) Soliva, M., and Speiser, P., *Pharm. Acta Helv.*, **41** (3), 176(1966); through *Chem. Abstr.*, **64**, 15678(1966).
(206) Stempel, E., *Drug. Cosmetic Ind.*, **98**, 44(1966).
(207) *Ibid.*, **98**, 36(1966).
(208) Desai, S. J., Singh, P., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **55**, 1224(1966).
(209) *Ibid.*, **55**, 1230(1966).
(210) *Ibid.*, **55**, 1235(1966).
(211) Krause, G. M., *Am. J. Pharm. Educ.*, **30**, 261(1966).
(212) Robinson, J. R., and Eriksen, S. P., *J. Pharm. Sci.*, **55**, 1254(1966).
(213) Nessel, R. J., *Dissertation Abstr.*, **27**, 529-B(1966).
(214) Rodriguez, J. B., *Anales Fac. Quim. Farm., Univ. Chile*, **16**, 9(1964); through *Chem. Abstr.*, **64**, 14034(1966).
(215) Bervenmark, H., *et al.*, *Acta Pharm. Suecica*, **3** (1), 45(1966); through *Chem. Abstr.*, **64**, 18242(1966).
(216) Kakemi, K., *et al.*, *Yakuzaigaku*, **25** (1), 19(1965); through *Chem. Abstr.*, **64**, 532(1966).
(217) Draper, E. B., and Becker, C. H., *J. Pharm. Sci.*, **55**, 376(1966).
(218) Asker, A. F., and Becker, C. H., *ibid.*, **55**, 90(1966).
(219) O'Reagen, T., *Drug Cosmetic Ind.*, **98**, 35(1966).
(220) Lapidus, J., and Lordi, N. G., *J. Pharm. Sci.*, **55**, 840 (1966).
(221) Ionescu-Stoian, P., *et al.*, *Ann. Pharm. Franc.*, **24** (5), 367(1966); through *Chem. Abstr.*, **65**, 12062(1966).
(222) Nakagaki, M., and Kitamura, S., *Yakugaku Zasshi*, **86**, 894(1966).
(223) Horsch, W., *Pharm. Praxis, Beilage Pharmazie*, **1966**, 109; through *Chem. Abstr.*, **65**, 8665(1966).
(224) Kruglitskii, N. N., *et al.*, *Fiz.-Khim. Mekhan. Dispernykh Struktur, Akad. Nauk SSSR, Sb. Statei*, **1966**, 151; through *Chem. Abstr.*, **65**, 4693(1966).
(225) Kruglitskii, N. N., *et al.*, *Dopovidi Akad. Nauk Ukr. RSR*, **1965** (10), 1336; through *Chem. Abstr.*, **64**, 4308 (1966).
(226) Sheikh, M. A., Price, J. C., and Gerraughty, R. J., *J. Pharm. Sci.*, **55**, 1048(1966).
(227) Dakkuri, A., and Ecanow, B., *Am. Perfumer Cosmet.*, **81** (5), 33(1966).
(228) Carless, J. E., and Foster, A. A., *J. Pharm. Pharmacol.*, **18**, 697(1966).
(229) Nakanishi, Y., *Yakugaku Zasshi*, **86**, 993(1966).
(230) *Ibid.*, **86**, 997(1966).
(231) *Ibid.*, **86**, 1001(1966).
(232) Tentsova, A. I., *Aptechn. Delo*, **11** (4), 15(1962); through *Chem. Abstr.*, **64**, 14034(1966).
(233) Bogs, U., and Bormann, G., *Pharm. Praxis, Beilage Pharmazie*, **1965** (12), 265; through *Chem. Abstr.*, **64**, 15673 (1966).
(234) Bugnariu, O., *et al.*, *Farmacia (Bucharest)*, **8** (11), 653(1965); through *Chem. Abstr.*, **64**, 7975(1966).
(235) Varlamova, L. S., and Pozharskaya, A. M., *Med. Prom. SSSR*, **20** (8), 39(1966); through *Chem. Abstr.*, **65**, 18432(1966).
(236) Lange, H., *J. Soc. Cosmetic Chemists*, **16** (12), 697 (1965); through *Chem. Abstr.*, **64**, 4859(1966).
(237) Mueller, R., *Melliand Textilber*, **47** (8), 897(1966); through *Chem. Abstr.*, **65**, 11386(1966).
(238) Fichtner, G. G., and Branton, P. L., *Chem. Specialties Mfrs., Assoc., Proc. Ann. Meeting*, **52**, 206(1965); through *Chem. Abstr.*, **65**, 9780(1966).
(239) Griffin, W. C., *et al.*, *Am. Perfumer Cosmet.*, **81** (9), 31(1966).
(240) Holzner, G., *Seifen-Oele-Fette-Wachse*, **92** (12), 399(1966); through *Chem. Abstr.*, **65**, 15147(1966).
(241) Klugman, I. Y., *Kolloidn. Zh.*, **28** (1), 51(1966); through *Chem. Abstr.*, **64**, 11897(1966).
(242) Griffin, W. C., Ranauto, H. J., and Adams, A. D., *Arch. Biochim. Cosmetol.*, **9** (87), 93(1966); through *Chem. Abstr.*, **65**, 18422(1966).
(243) Rimlinger, G., *Parfum. Cosmet. Savons*, **9** (4), 158 (1966); through *Chem. Abstr.*, **65**, 10422(1966).
(244) Ford, R. E., and Furmidge, C. G. L., *J. Colloid Interface Sci.*, **22**, 331(1966).
(245) Mima, H., and Kitamori, N., *J. Pharm. Sci.*, **55**, 44 (1966).
(246) Brown, G. L., *J. Macromol. Chem.*, **1** (1), 151(1966); through *Chem. Abstr.*, **64**, 7406(1966).
(247) Groves, M. J., *J. Pharm. Pharmacol.*, **18**, 305(1966).
(248) Taubman, A. B., Nikitina, S. A., and Mochalova, O. S., *Kolloidn. Zh.*, **28** (5), 769(1966); through *Chem. Abstr.*, **65**, 19343(1966).
(249) Subramanyam, S. V., and Gopal, E. S. R., *Kolloid-Z. Z. Polymere*, **210** (1), 80(1966); through *Chem. Abstr.*, **65**, 9773(1966).
(250) Hamill, R. D., and Petersen, R. V., *J. Pharm. Sci.*, **55**, 1268(1966).
(251) *Ibid.*, **55**, 1274(1966).
(252) Sonntag, H., Netzel, J., and Klare, H., Jr., *Kolloid-Z. Z. Polymere*, **211** (1), 121(1966); through *Chem. Abstr.*, **65**, 16100(1966).
(253) Hamill, R. D., *Dissertation Abstr.*, **26**, 5732(1966).
(254) Fox, C. D., and Shangraw, R. F., *J. Pharm. Sci.*, **55**, 318(1966).
(255) *Ibid.*, **55**, 323(1966).
(256) Haavisto, M. L., and Hagner, R., *Farm. Aikakauslehti*, **75** (2), 75(1966); through *Chem. Abstr.*, **64**, 15677 (1966).
(257) Longhin, S., *et al.*, *Dermato-Venerol.*, **10** (5), 385 (1965); through *Chem. Abstr.*, **64**, 9505(1966).
(258) Matsumoto, S., *Nippon Kagaku Zasshi*, **86** (3), 324 (1965); through *Chem. Abstr.*, **64**, 1389(1966).
(259) Carless, J. E., and Hallworth, G., *Chem. Ind. (London)*, **1966**, 30.
(260) Freshwater, D. C., Scarlett, B., and Groves, M. J., *Am. Perfumer Cosmet.*, **81** (5), 43(1966).
(261) Motawi, M. M., and El-Shibini, H. A. M., *ibid.*, **81** (5), 47(1966).
(262) Whitworth, C. W., and Becker, C. H., *Am. J. Hosp. Pharm.*, **23**, 574(1966).
(263) Howze, J. M., and Billups, N. F., *Am. J. Pharm.*, **138**, 193(1966).
(264) Vila, L., and Cadorniga, R., *Gattefosse-SFPA, Bull. Tech., No. 61*, 59(1964); through *Chem. Abstr.*, **64**, 1899 (1966).
(265) Motawi, M. M., Czetsch-Lindenwald, H. V., and Khalil, S. A., *Egypt. Pharm. Bull.*, **44** (4), 311(1962); through *Chem. Abstr.*, **64**, 4874(1966).
(266) Yousef, R. T., and Khawam, M. N., *Arch. Mikrobiol.*, **53** (2), 159(1966); through *Chem. Abstr.*, **64**, 17363 (1966).
(267) Kucera, J., and Veber, V., *Cesk. Dermatol.*, **41** (4), 229(1966); through *Chem. Abstr.*, **65**, 18431(1966).
(268) Krogerus, V. E., *Farm. Aikakauslehti*, **74** (12), 298 (1965); through *Chem. Abstr.*, **64**, 6414(1966).
(269) Trandafilov, T., and Tomasini, L., *Farmatsiya*, **16** (3), 32(1966); through *Chem. Abstr.*, **65**, 16796(1966).
(270) Kutscher, A. H., *et al.*, *Oral Surg., Oral Med., Oral Pathol.*, **21** (1), 32(1966); through *Chem. Abstr.*, **64**, 9515 (1966).
(271) Istomina, I. A., Botvinik, S. A., and Rozentsveig, P. E., *Sb. Nauchn. Tr. Vitebskogo Med. Inst.*, **1964** (11), 171; through *Chem. Abstr.*, **64**, 6402(1966).
(272) Fenyi-Demeny, M., *et al.*, *Gyogyszereszet*, **9** (11), 423 (1965); through *Chem. Abstr.*, **64**, 9518(1966).
(273) Eperjessy, E. T., and Fodor, G., *Pharmazie*, **21** (7), 430(1966); through *Chem. Abstr.*, **65**, 19936(1966).
(274) Wisniewski, W., and Golucki, Z., *Acta Polon. Pharm.*, **23** (1), 63(1966); through *Chem. Abstr.*, **65**, 3673(1966).
(275) Alyushin, M. T., *Sb. Nauchn. Tr., Tsentr. Aptechn. Nauchn-Issled. Inst.*, **6**, 60(1964); through *Chem. Abstr.*, **64**, 14035(1966).

(276) Chandranondnaiwinit, W., and Sommers, E. B., *J. Pharm. Sci.*, **55**, 1221(1966).
(277) Silverman, H. I., *Am. J. Pharm.*, **138**, 66(1966).
(278) Ciocanelea, V., *et al., Farmacia*, **13** (12), 713(1965); through *Chem. Abstr.*, **64**, 15673(1966).
(279) Kakemi, K., Arita, T., and Muranish, S., *Yakuzaigaku*, **25** (3), 41(1965); through *Chem. Abstr.*, **65**, 6147(1966).
(280) Katona, K., *Acta Pharm. Hung.*, **36** (3), 126(1966); through *Chem. Abstr.*, **65**, 2071(1966).
(281) Falk, G., and Voigt, R., *Pharm. Zentralhalle*, **105** (9), 573(1966).
(282) Braidotti, L., *Boll. Chim. Farm.*, **105** (1), 82(1966); through *Chem. Abstr.*, **64**, 17363(1966).
(283) Aktulga, A., and Gueven, K. C., *Eczacilik Bulteni*, **8** (2), 19; (3) 50; (4) 82(1966); through *Chem. Abstr.*, **65**, 16797(1966).
(284) Marshall, J. R., *Drug. Cosmetic Ind.*, **98**, 41(1966).
(285) Sanders, P. A., *Am. Perfumer Cosmet.*, **81** (2), 31 (1966).
(286) Sanders, P. A., *Drug Cosmetic Ind.*, **99**, 56(1966).
(287) *Ibid.*, **99**, 57(1966).
(288) Devir, S. E., *J. Colloid Interface Sci.*, **21**, 9(1966).
(289) Sanders, P. A., *J. Soc. Cosmetic Chemists*, **17** (13), 801(1966).
(290) Richman, M. D., and Shangraw, R. F., *Aerosol Age*, **11** (8), 39(1966); through *Chem. Abstr.*, **65**, 17730(1966).
(291) Blasecki, J. W., Jr., and Iannarone, M., *J. Pharm. Sci.*, **55**, 1471(1966).
(292) Lange, W. E., and Fang, V. S., *J. Soc. Cosmetic Chemists*, **17** (2), 115(1966).
(293) Martin, J. J., Jr., *et al., Drug. Cosmetic Ind.*, **99**, 54 (1966).
(294) Leberl, O., *Kosmetik-Parfum-Drogen Rundschau*, 13 (1–2), 11(1966); through *Chem. Abstr.*, **64**, 19315(1966).
(295) More, E. P., *Soap Chem. Specialties*, **41** (11), 113 (1965); through *Chem. Abstr.*, **64**, 18454(1966).
(296) McFarland, J. H., *Soap Chem. Specialties*, **42** (1), 97(1966).
(297) Jones, G. D., and Weaning, A. J. S., *Aerosol Age*, **11** (3), 21(1966); through *Chem. Abstr.*, **65**, 17736(1966).
(298) deNavarre, M. G., *Am. Perfumer Cosmet.*, **81** (1), 25(1966).
(299) Rybczynska, B., *Tluszcze i Srodki Piorace*, **9** (4), 231(1965); through *Chem. Abstr.*, **64**, 17351(1966).
(300) Lauffer, P. G. T., *Proc. Sci. Sect. Toilet Goods Assoc.*, **44**, 7(1965); through *Chem. Abstr.*, **64**, 11023(1966).
(301) Tio, T. H., *Am. Perfumer Cosmet.*, **81** (4), 45(1966).
(302) Green, E., *Rept. Progr. Appl. Chem.*, **49**, 297(1964); through *Chem. Abstr.*, **64**, 523(1966).
(303) Francois, M. Th., *Journees Inform. Corps Gras Ind. Chim., Huiles Ind., Acides Gras, Lipochim., Paris*, **1965**, 49; through *Chem. Abstr.*, **64**, 19315(1966).
(304) Rees, T., *Am. Perfumer Cosmet.*, **81** (4), 37(1966).
(305) Sorkin, M., Shapiro, B., and Kass, G. S., *J. Soc. Cosmetic Chemists*, **17** (9), 539(1966).
(306) Cook, M. K., *Drug Cosmetic Ind.*, **99**, 52(1966).
(307) Kluge, A., *Am. Perfumer Cosmet.*, **81** (3), 35(1966).
(308) Kalish, J., *Drug Cosmetic Ind.*, **98**, 40(1966).
(309) Cook, M. K., *ibid.*, **99**, 52(1966).
(310) Harding, W. H., *Arch. Biochim. Cosmetol.*, **9** (87), 130 (1966); through *Chem. Abstr.*, **65**, 18423(1966).
(311) Kluge, A., *Am. Perfumer Cosmet.*, **81** (3), 35(1966).
(312) Anon., *Soap, Perfumery Cosmetics*, **39**, 309(1966)
(313) Goldrick, E. A., *Am. Perfumer Cosmet.*, **81** (12), 43(1966).
(314) Thistlewayte, D. K. B., *ibid.*, **81** (12), 29(1966).
(315) Bell, K. W., *ibid.*, **81** (5), 25(1966).
(316) Van Abbe, N. J., *Perfumery Essent. Oil Record*, **57** (8), 501(1966); through *Chem. Abstr.*, **65**, 15149(1966).
(317) Mannheim, P., *Soap, Perfumery Cosmetics*, **39**, 807 (1966).
(318) Master, K. J., Sayre, R. M., and Everett, M. A., *J. Soc. Cosmetic Chemists*, **17** (10), 581(1966).
(319) Tronnier, H., *Soap, Perfumery Cosmetics*, **39**, 123 (1966).
(320) Schlossman, M. L., *Drug Cosmetic Ind.*, **98**, 50(1966).
(321) Godfrey, K. M., *J. Soc. Cosmetic Chemists*, **17** (1), 17 (1966).
(322) Argiriadi, A., *Drug Cosmetic Ind.*, **99**, 49(1966).
(323) Martin, J. R. L., *Soap, Perfumery Cosmetics*, **39**, 895 (1966).
(324) Schlossman, M. L., *Drug Cosmetic Ind.*, **98**, 32(1966).
(325) Lees, J. R., *Food Engineering*, **38** (3), 80(1966).
(326) Anon., *ibid.*, **38** (3), 89(1966).
(327) Krueger, E. O., *Am. Chem. Soc., Div. Org. Coatings, Plastics Chem., Preprints*, **24** (2), 195(1964); through *Chem. Abstr.*, **64**, 12459(1966).
(328) Greber, W., *Kosmetik-Parfum-Drogen Rundschau*, **13** (5–6), 72(1966); through *Chem. Abstr.*, **65**, 15150(1966).
(329) Lewalski, S., and Korzeniowski, A., *Farm. Polska*, **22** (1–2), 42(1966); through *Chem. Abstr.*, **65**, 8681(1966).
(330) Hoover, M. W., Markantonatos, A., and Parker, W. N., *Food Technol.*, **20**, 103(1966).
(331) *Ibid.*, **20**, 107(1966).
(332) Ronsivalli, L. R., Bernsteins, J. B., and Tinker, B. L., *ibid.*, **20**, 98(1966).
(333) Jaminet, F., *Tribune CEBEDEAU (Centre Belge Etude Doc. Eaux)*, **18** (265), 580(1965); through *Chem. Abstr.*, **64**, 17363(1966).
(334) Davis, D. S., *Am. Perfumer Cosmet.*, **81** (9), 54 (1966).
(335) Hetherington, S., *Harokeach Haivri*, **11**, 4(1965); through *Chem. Abstr.*, **64**, 19328(1966).
(336) Gold, G., Duvall, R. N., and Palermo, B. T., *J. Pharm. Sci.*, **55**, 1133(1966).
(337) Cohn, R., Heilig, H., and deLorimier, A., *ibid.*, **55**, 328(1966).
(338) Groszek, A. J., *Chem. Ind. (London)*, **1966**, 1754.
(339) Topham, J. D., *J. Pharm. Sci.*, **55**, 203(1966).
(340) Entwistle, R. E., *Food Engineering*, **38** (2), 82(1966).
(341) Anon., *ibid.*, **38** (1), 45(1966).
(342) Nash, R. A., and Haeger, B. E., *J. Pharm. Sci.*, **55**, 829(1966).
(343) Davidson, H. R., *J. Soc. Cosmetic Chemists*, **17** (6), 329(1966).
(344) Simmons, P. M., *Food Engineering*, **38** (1), 52(1966).
(345) Samuels, T. M., and Guthrie, D. L., *Am. J. Hosp. Pharm.*, **23**, 4(1966).
(346) Buechner, C., *Verpack. Mag.*, **1966**, 48; through *Chem. Abstr.*, **65**, 19937(1966).
(347) Biles, J. A., Plakogiannis, F. M., Wong, B. J., and Biles, P. M., *J. Pharm. Sci.*, **55**, 909(1966).
(348) Konyushko, V. S., Gurevich, T.Sh., and Kartasheva, L.Kh., *Sb. Nauchn. Tr. Vitebskogo Med. Inst.*, **1964** (11), 189; through *Chem. Abstr.*, **64**, 7974(1966).
(349) Schwartz, M. A., and Wu, G. M., *J. Pharm. Sci.*, **55**, 550(1966).
(350) Yasui, C., and Kosasa, S., *Yakuzaigaku*, **25** (4), 29 (1965); through *Chem. Abstr.*, **65**, 18430(1966).
(351) Vavruch, I., *Kolloid-Z. Z. Polymere*, **105** (1), 32 (1965); through *Chem. Abstr.*, **64**, 2778(1966).
(352) Gillespie, T., and Williams, B. W., *J. Polymer Sci.*, **1966A-1**, 933.
(353) Mark, A. M., *et al., Food Technol.*, **20**, 75(1966).
(354) Wellons, J., and Stannett, V., *J. Polymer Sci.*, **1966A-1**, 593.
(355) Havemeyer, R. N., *J. Pharm. Sci.*, **55**, 851(1966).
(356) Nixon, J. R., Khalil, A. H., and Carless, J. E., *J. Pharm. Pharmacol.*, **18**, 409(1966).
(357) Coll, H., *J. Polymer Sci.*, **1966A-2**, 659.
(358) Perlia, X., *Pharm. Acta Helv.*, **41** (4), 193(1966); through *Chem. Abstr.*, **64**, 18218(1966).
(359) Bates, T. R., Gibaldi, M., and Kanig, J. L., *J. Pharm. Sci.*, **55**, 901(1966).
(360) *Ibid.*, **55**, 191(1966).
(361) Bates, T. R., Gibaldi, M., and Kanig, J. L., *Nature*, **210**, 1331(1966).
(362) Kabasakalian, P., Britt, E., and Yudis, M. D., *J. Pharm. Sci.*, **55**, 642(1966).
(363) Nogami, H., Nagai, T., and Suzuki, A., *Chem. Pharm. Bull. (Tokyo)*, **14**, 329(1966).
(364) *Ibid.*, **14**, 339(1966).
(365) Nogami, H., Nagai, T., and Ito, K., *ibid.*, **14**, 351 (1966).
(366) Likholet, N. M., *Farmatsevt. Zh. (Kiev)*, **20** (5), 44 (1965); through *Chem. Abstr.*, **64**, 7974(1966).
(367) Kawam, M. N., Tawashi, R., and v. Czetsch-Lindenwald, H., *Sci. Pharm.*, **33** (3), 153(1965); through *Chem. Abstr.*, **64**, 532(1966).
(368) Savopol, E., *Farmacia (Bucharest)*, **14** (8), 479 (1966); through *Chem. Abstr.*, **65**, 19936(1966).
(369) Paruta, A. N., and Irani, S. A., *J. Pharm. Sci.* **55**, 1060(1966).
(370) *Ibid.*, **55**, 1055(1966).
(371) Paruta, A. N., *Am. J. Pharm.*, **138**, 137(1966).
(372) Paruta, A. N., and Sheth, B. B., *J. Pharm. Sci.*, **55**, 896(1966).
(373) Hamlin, W. E., and Higuchi, W. I., *ibid.*, **55**, 205 (1966).
(374) Kildsig, D. O., *Dissertation Abstr.*, **26**, 3879(1966).
(375) Anderson, R. A., and Slade, A. H., *Australasian J. Pharm.*, **46**, S53(1965); through *Chem. Abstr.*, **64**, 9517 (1966).
(376) Ueda, S., *Chem. Pharm. Bull. (Tokyo)*, **14**, 39(1966).
(377) Donbrow, M., and Rhodes, C. T., *J. Pharm. Pharmacol.*, **18**, 424(1966).
(378) Saad, H. Y., *Dissertation Abstr.*, **26** 5114(1966)
(379) Angla, B., *France Parfums*, **8** (45), 323(1965); through *Chem. Abstr.*, **64**, 4857(1966).
(380) Henderson, G., and Newton, J. M., *Pharm. Acta Helv.*, **41** (4), 228(1966); through *Chem. Abstr.*, **64**, 17363 (1966).
(381) Regdon-Kiss, E., and Mester, L., *Pharmazie*, **21** (2), 109(1966); through *Chem. Abstr.*, **64**, 17363(1966).
(382) Wang, H., and Wang, P., *Yao Hsueh Hsueh Pao*, **13** (1), 63(1966); through *Chem. Abstr.*, **65**, 5307(1966).
(383) Monciu, D., *et al., Farmacia (Bucharest)*, **8** (12), 727 (1965); through *Chem. Abstr.*, **64**, 7973(1966).
(384) Goldberg, A. H., Gibaldi, M., Kanig, J. L., and Mayersohn, M., *J. Pharm. Sci.*, **55**, 581(1966).
(385) Goldberg, A. H., Gibaldi, M., and Kanig, J. L., *ibid.*, **55**, 487(1966).
(386) *Ibid.*, **55**, 482(1966).
(387) Mayersohn, M., and Gibaldi, M., *ibid.*, **55**, 1323 (1966).
(388) Finholt, P., *et al., Medd. Norsk Farm. Selskap*, **28** (2), 17; (3) (3) 31(1966); through *Chem. Abstr.*, **65**, 8679 (1966).
(389) Gusyakov, V. P., and Mis'kiv, G. M., *Farmatsevt. Zh. (Kiev)*, **21** (1), 28(1966); through *Chem. Abstr.*, **65**, 5306 (1966).
(390) Kittnar, E., *et al., Cesk. Farm.*, **15** (1), 5(1966); through *Chem. Abstr.*, **64**, 19326(1966).
(391) Sheth, B. B., Paruta, A. N., and Ninger, F. C., *J. Pharm. Sci.*, **55**, 1144(1966).

(392) Anderson, R. A., and Morgan, K. J., *J. Pharm. Pharmacol.*, **18**, 449(1966).
(393) Kuettel, D., *Pharm. Zentralhalle*, **104** (8), 570(1965); through *Chem. Abstr.*, **64**, 532(1966).
(394) Wolfson, B. B., *Dissertation Abstr.*, **27**, 530-B(1966).
(395) Rodell, M. B., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **55**, 1429(1966).
(396) Benet, L. Z., and Goyan, J. E., *ibid.*, **55**, 1184(1966).
(397) Niebergall, P. J., *et al.*, *J. Pharm. Pharmacol.*, **18**, 729(1966).
(398) Cressman, W. A., *et al.*, *ibid.*, **18**, 801(1966).
(399) Keen, P. M., *Biochem. Pharmacol.*, **15**, 447(1966).
(400) Lach, J. L., and Bornstein, M., *J. Pharm. Sci.*, **55**, 1040(1966).
(401) Bornstein, M., and Lach, J. L., *ibid.*, **55**, 1033(1966).
(402) Schwartz, M. A., and Amidon, G. L., *ibid.*, **55**, 1464 (1966).
(403) Miyazaki, M., Senshu, T., and Tamura, Z., *Chem. Pharm. Bull. (Tokyo)*, **14**, 114(1966).
(404) Labos, E., *Nature*, **209**, 201(1966).
(405) Maruchin, J. E., *Farm. Polska*, **22** (3), 177(1966); through *Chem. Abstr.*, **65**, 10424(1966).
(406) Shih, I., and Teare, F., *Can. J. Pharm. Sci.*, **1** (1), 20(1966); through *Chem. Abstr.*, **65**, 18429(1966).
(407) Scott, J. E., and Willett, I., *Nature*, **209**, 985(1966).
(408) Rohatgi, K. K., and Singhal, G. S., *J. Phys. Chem.*, **70**, 1695(1966).
(409) Connors, K. A., and Mollica, J. A., Jr., *J. Pharm. Sci.*, **55**, 772(1966).
(410) Wan, L. S., *ibid.*, **55**, 1395(1966).
(411) Yamauchi, O., Tanaka, H., and Uno, T., *Chem. Pharm. Bull. (Tokyo)*, **14**, 952(1966).
(412) *Ibid.*, **14**, 948(1966).
(413) Kabadi, B. N., and Hammarlund, E. R., *J. Pharm. Sci.*, **55**, 1072(1966).
(414) *Ibid.*, **55**, 1069(1966).
(415) Ito, K., and Sekiguchi, K., *Chem. Pharm. Bull. (Tokyo)*, **14**, 255(1966).
(416) Lach, J. L., and Pauli, W. A., *J. Pharm. Sci.*, **55**, 32 (1966).
(417) Matsumoto, H., Matsumura, H., and Iguchi, S., *Chem. Pharm. Bull. (Tokyo)*, **14**, 385(1966).
(418) *Ibid.*, **14**, 391(1966).
(419) Matsumoto, H., *ibid.*, **14**, 398(1966).
(420) Ueda, S., *ibid.*, **14**, 29(1966).
(421) *Ibid.*, **14**, 22(1966).
(422) Landi, S., and Held, H. R., *J. Pharm. Sci.*, **55**, 18 (1966).
(423) Hiskey, C. F., and Cantwell, F. F., *ibid.*, **55**, 166 (1966).
(424) Leonardi, G., Solinas, M., and Botré, C., *ibid.*, **55**, 526(1966).
(425) Nakagaki, M., Koga, N., and Terada, H., *Yakugaku Zasshi*, **86**, 447(1966).
(426) Finholt, P., Kristiansen, H., Krowczynski, L., and Higuchi, T., *J. Pharm. Sci.*, **55**, 1435(1966).
(427) Meriwether, L. S., Marzluff, W. F., and Hodgson, W. G., *Nature*, **212**, 465(1966).
(428) Beckett, A. H., and Choulis, N. H., *J. Pharm. Sci.*, **55**, 1155(1966).
(429) Goddard, E. D., and Kung, H. C., *Chem. Specialties Mfrs. Assoc., Proc. Ann. Meeting*, **52**, 124(1965); through *Chem. Abstr.*, **65**, 10812(1966).
(430) Davis, P. S., and Deller, D. J., *Nature*, **212**, 404 (1966).
(431) Johnson, S. L., *Dissertation Abstr.*, **26**, 5636(1966).
(432) Sakaguchi, T., and Taguchi, K., *Yakugaku Zasshi*, **86**, 26(1966).
(433) Tanaka, N., Hirata, G., and Utsumi, I., *Chem. Pharm. Bull. (Tokyo)*, **14**, 414(1966).
(434) Hiestand, E. N., *J. Pharm. Sci.*, **55**, 1325(1966).
(435) Combarnous, M., and Marle, C., *Genie Chim.*, **94** (2), 29(1965); through *Chem. Abstr.*, **64**, 13426(1966).
(436) Schwen, G., *Melliand Textilber*, **47** (9), 1009(1966); through *Chem. Abstr.*, **65**, 16093(1966).
(437) Wright, E. H. M., *Chem. Ind. (London)*, **1966**, 245.
(438) Beaven, C. J. H., and Eadington, P., *ibid.*, **1966**, 1484.
(439) Ecanow, B., Grundman, R., and Wilson, R., *Am. J. Hosp. Pharm.*, **23**, 404(1966).
(440) Sell, P. J., and Neumann, A. W., *Angew. Chem.*, **78** (6), 321(1966); *Angew. Chem., Intern. Ed., Engl.*, **5** (3), 299(1966); through *Chem. Abstr.*, **64**, 18455(1966).
(441) Zografi, G., and Zarenda, I., *Biochem. Pharmacol.*, **15**, 591(1966).
(442) Patel, R. M., and Zografi, G., *J. Pharm. Sci.*, **55**, 1345(1966).
(443) Sorby, D. L., Plein, E. M., and Benmaman, J. D., *ibid.*, **55**, 785(1966).
(444) Horikoshi, I., and Himuro, I., *Yakugaku Zasshi*, **86**, 324(1966).
(445) Horikoshi, I., and Hata, S., *ibid.*, **86**, 356(1966).
(446) Horikoshi, I., and Himuro, I., *ibid.*, **86**, 353(1966).
(447) Nogami, H., *et al.*, *Chem. Pharm. Bull. (Tokyo)*, **14**, 159(1966).
(448) *Ibid.*, **14**, 225(1966).
(449) Nicolescu, A., and Gruia, M., *Rev. Roumaine Chim.*, **11** (7), 847(1966); through *Chem. Abstr.*, **65**, 17742(1966).
(450) Aldcroft, D., and Bye, G. C., *Chem. Ind. (London)*, **1966**, 1846.
(451) Izrailevich, I. S., and Novikov, S. N., *Dokl. Akad. Nauk SSSR*, **165** (1), 77(1965); through *Chem. Abstr.*, **64**, 4296(1966).
(452) Chapman, J. H., and Neustadter, E. L., *J. Pharm. Pharmacol.*, **17(S)**, 138S(1965); through *Chem. Abstr.*, **64**, 7975(1966).
(453) Jones, T. M., and Pilpel, N., *J. Pharm. Pharmacol.*, **18(S)**, 429S(1966).
(454) De Chakrvarti, D. K., and Mukherjee, S. K., *Sci. Cult. (Calcutta)*, **32** (4), 182(1966); through *Chem. Abstr.*, **65**, 11378(1966).
(455) Brunauer, S., *Pure Appl. Chem.*, **10** (4), 293(1965); through *Chem. Abstr.*, **65**, 17741(1966).
(456) Palmer, H. A., *Dissertation Abstr.*, **26**, 6654(1966).
(457) Conrad, L. I., Maso, H. F., and DeRagon, S. A., *J. Soc. Cosmetic Chemists*, **17** (3), 157(1966).
(458) Conrad, L. I., Maso, H. F., and DeRagon, S. A., *Arch. Biochim. Cosmetol.*, **9** (87), 122(1966); through *Chem. Abstr.*, **65**, 18422(1966).
(459) Conrad, L. I., Maso, H. F., and DeRagon, S. A., *Am. Perfumer Cosmet.*, **81** (3), 47(1966).
(460) Boyadzhiev, K., *Izv. Inst. Obshta Neorg. Khim., Bulgar. Akad. Nauk.*, **3**, 137(1965); through *Chem. Abstr.*, **64**, 16148(1966).
(461) Nakagaki, M., and Kawamura, S., *Yakugaku Zasshi*, **86**, 287(1966).
(462) Barboi, V. M., *Kolloidn. Zh.*, **25** (4), 385(1963); through *Chem. Abstr.*, **64**, 56(1966).
(463) Barboi, V. M., *Kolloidn. Zh.*, **27** (5), 643(1965); through *Chem. Abstr.*, **64**, 2778(1966).
(464) Shits, L. A., *Kolloidn. Zh.*, **26** (3), 397(1964); through *Chem. Abstr.*, **65**, 7471(1966).
(465) Hogg, R., Healy, T. W., and Fuerstenau, D. W., *Trans. Faraday Soc.*, **62** (6), 1638(1966); through *Chem. Abstr.*, **65**, 1429(1966).
(466) Weiner, N. D., Parreira, H. C., and Zografi, G., *J. Pharm. Sci.*, **55**, 187(1966).
(467) Bruh, R. R., *Anales Fac. Quim. Farm., Univ., Chile*, **16**, 23(1964–1965); through *Chem. Abstr.*, **64**, 19327(1966).
(468) Nash, R. A., *Drug Cosmetic Ind.*, **98**, 39(1966).
(469) Black, A. P., Birkner, F. B., and Morgan, J. J., *J. Colloid Sci.*, **21**, 626(1966).
(470) Rezek, M., *J. Pharm. Sci.*, **55**, 538(1966).
(471) Libinson, G. S., and Slugina, M. D., *Zh. Fiz., Khim.*, **39** (11), 2813(1965); through *Chem. Abstr.*, **64**, 4864(1966).
(472) Mussellwhite, P. R., *J. Colloid Interface Sci.*, **21** (1), 99(1966); through *Chem. Abstr.*, **64**, 11898(1966).
(473) Zimon, A. D., and Klishin, N. G., *Kolloidn. Zh.*, **27** (5), 685(1965); through *Chem. Abstr.*, **64**, 2774(1966).
(474) Birdwell, B. F., and Jessen, F. W., *Nature*, **209**, 366 (1966).
(475) Morawetz, H., *Science*, **152**, 705(1966).
(476) Karmarkar, S. S., and Thirumalachar, M. J., *Hindustan Antibiot. Bull.*, **6** (1), 9(1963); through *Chem. Abstr.*, **64**, 9507(1966).
(477) Shtol'ts, M. G., *et al.*, *Antibiotiki*, **11** (4), 291(1966); through *Chem. Abstr.*, **65**, 18426(1966).
(478) Brandstaetter-Kuhnert, M., and Martinek, A., *Mikrochim. Ichnoanal. Acta*, **1965** (5–6), 909; through *Chem. Abstr.*, **64**, 14034(1966).
(479) Zesche, G., *Kolloid-Z. Z. Polymere*, **209** (1), 43 (1966); through *Chem. Abstr.*, **65**, 4700(1966).
(480) Neuwald, F., and Scheel, D., *Arch. Biochim. Cosmetol.*, **9** (87), 137(1966); through *Chem. Abstr.*, **65**, 18423 (1966).
(481) Neuwald, F., *J. Soc. Cosmetic Chemists*, **17** (4), 213(1966).
(482) Boylan, J. C., *J. Pharm. Sci.*, **55**, 710(1966).
(483) Clark, R. C., *Chem. Ind. (London)*, **1966**, 489.
(484) Oriol Pascual, D. J., *Parfum., Cosmet., Savons*, **9** (4), 171(1966); through *Chem. Abstr.*, **65**, 8662(1966).
(485) Steggles, J. S., *Chem. Ind. (London)*, **1966**, 976.
(486) Rutgers, R., *Nature*, **209**, 391(1966).
(487) Woodman, M., and Marsden, A., *J. Pharm. Pharmacol.*, **18(S)**, 198S(1966).
(488) Kramer, A., and Hawbecker, J. V., *Food Technol.*, **20**, 111(1966).
(489) Lesshafft, C. T., Jr., *J. Pharm. Sci.*, **55**, 1371(1966).
(490) Powell, D. R., Swarbrick, J., and Banker, G. S., *ibid.*, **55**, 601(1966).
(491) Izmailova, V. N., Pchelin, V. A., and Ali, S. A., *Dokl. Akad. Nauk SSSR*, **164** (1), 131(1965); through *Chem. Abstr.*, **64**, 4308(1966).
(492) Sherman, P., *J. Soc. Cosmetic Chemists*, **17** (8), 439(1966).
(493) Richman, M. D., and Shangraw, R. F., *Aerosol Age*, **11** (7), 28(1966); through *Chem. Abstr.*, **65**, 12062(1966).
(494) Carpenter, D. K., *Encycl. Polymer Sci. Technol.*, **4**, 16 (1966); through *Chem. Abstr.*, **65**, 17741(1966).
(495) Banker, G. S., *J. Pharm. Sci.*, **55**, 81(1966).
(496) Munden, B. J., *Dissertation Abstr.*, **27**, 529-B(1966).
(497) Srinivas, R., DeKay, H. G., and Banker, G. S., *J. Pharm. Sci.*, **55**, 335(1966).
(498) Borisenko, Yu. B., Safiulin, R. M., and Nosovits'ka, S. A., *Farmatsevt. Zh. (Kiev)*, **21** (3), 36(1966); through *Chem. Abstr.*, **65**, 15165(1966).
(499) Subinskava, E. S., *Vopr. Eksperim. i Klinich. Endokrinol., Sb., Moscow*, **1965**, 169; through *Chem. Abstr.*, **65**, 8680(1966).
(500) Lamonde, A. M., DeKay, H. G., and Banker, G. S., *J. Am. Pharm. Assoc.*, **NS6**, 148(1966).

(501) Guillot, B., and Gattefosse, H. M., *Prod. Probl. Pharm.*, 20 (9), 444(1965); through *Chem. Abstr.*, 64, 7974 (1966).
(502) Banker, G. S., Gore, A. Y., and Swarbrick, J., *J. Pharm. Pharmacol.*, 18, 457(1966).
(503) *Ibid.*, 18(S), 205S(1966).
(504) Schumacher, G. E., *J. Am. Pharm. Assoc.*, NS6, 430 (1966).
(505) Siegenthaler, W., *et al., Schweiz. Apotheker-Ztg.*, 103 (23), 835(1965); through *Chem. Abstr.*, 64, 9506(1966).
(506) Friend, D. G., *Clin. Pharmacol. Therap.*, 7, 706 (1966).
(507) Wang, E. L., *et al., J. Antibiotics (Tokyo) A*, 19, 216 (1966).
(508) Hoshino, Y., *et al., ibid.*, 19, 30(1966).
(509) Shimura, M., *et al., ibid.*, 19, 51(1966).
(510) Asahi, K., Nagatsu, J., and Suzuki, S., *ibid.*, 19, 195 (1966).
(511) Miyairi, N., *et al., ibid.*, 19, 56(1966).
(512) Garrett, E. R., Miller, G. H., and Brown, M. R. W., *J. Pharm. Sci.*, 55, 593(1966).
(513) Nezval, J., Maskova, J., and Holan, D., *Farmakoterap. Zpravy*, 11 (1), 80(1965); through *Chem. Abstr.*, 64, 11025(1966).
(514) Irmscher, K., *et al., Arzneimittel-Forsch.*, 16 (8), 1019(1966); through *Chem. Abstr.*, 65, 19193(1966).
(515) Clausen, J., *J. Pharmacol. Exptl. Therap.*, 153 (1), 167(1966); through *Chem. Abstr.*, 65, 6088(1966).
(516) Spring, P., *Arzneimittel-Forsch.*, 16 (3), 346(1966); through *Chem. Abstr.*, 65, 2837(1966).
(517) Jardetzky, O., and Wade-Jardetzky, N. G., *Mol. Pharmacol.*, 1 (3), 214(1965); through *Chem. Abstr.*, 64, 5623 (1966).
(518) Rolinson, G. N., and Sutherland, R., *Brit. J. Pharmacol.*, 25 (3), 638(1965); through *Chem. Abstr.*, 64, 7213(1966).
(519) Quinn, E. L., *Postgrad. Med. J. (London), Suppl.*, 40, 23(1964); through *Chem. Abstr.*, 65, 9527(1966).
(520) Rolinson, G. N., *Postgrad. Med. J. (London), Suppl.*, 40, 20(1964); through *Chem. Abstr.*, 65, 9527(1966).
(521) Hinoi, H., *Hiroshima Daigaku Igaku Zasshi*, 13 (11–12), 637(1965); through *Chem. Abstr.*, 65, 2847(1966).
(522) Bononi, L. J., *et al., Antibiotica*, 3 (4), 263(1965); through *Chem. Abstr.*, 65, 19144(1966).
(523) Kunin, C. M., *Clin. Pharmacol. Therap.*, 7, 166(1966).
(524) *Ibid.*, 7, 180(1966).
(525) Hinoi, H., *Hiroshima Daigaku Igaku Zasshi*, 13 (11–12), 601(1965); through *Chem. Abstr.*, 65, 2846(1966).
(526) Barna, K., Sabad, F., and Barnova, E., *Antibiotiki*, 11 (2), 161(1966); through *Chem. Abstr.*, 64, 18224(1966).
(527) Soekeland, J., *Intern. Congr. Chemotherapy, Proc., 3rd, Stuttgart, 1963*, 2, 1453; through *Chem. Abstr.*, 65, 12726(1966).
(528) Yakovlev, V. P., *Antibiotiki*, 10 (10), 896(1965); through *Chem. Abstr.*, 64, 2595(1966).
(529) Naumann, P., *Arzneimittel-Forsch.*, 15 (8), 919 (1965); through *Chem. Abstr.*, 64, 18224(1966).
(530) Dimmling, Th., and Wagner, W. H., *Arzneimittel-Forsch.*, 15 (11), 1288(1965); through *Chem. Abstr.*, 64, 7975 (1966).
(531) Wagner, H. J., and Emmons, H., *U. S. At. Energy Comm., Symp. Ser., No. 6*, 1966, 1; through *Chem. Abstr.*, 65, 11218(1966).
(532) Charlton, J. C., *U. S. At. Energy Comm., Symp. Ser., No. 6*, 1966, 33; through *Chem. Abstr.*, 65, 11218(1966).
(533) Bayly, R. J., *U. S. At. Energy Comm., Symp. Ser. No. 6*, 1966, 51; through *Chem. Abstr.*, 65, 11218(1966).
(534) Nordine, J. H., *et al., Proc. Meeting Coll. Intern. Neuro-Psychopharmacol., 3rd, Munich*, 1962, 404 through *Chem. Abstr.*, 64, 18218(1966).
(535) Komissarov, I. V., *Farmakol. i Toksikol.*, 29 (2), 244(1966); through *Chem. Abstr.*, 65, 6080(1966).
(536) Jung, L., *Bull. Soc. Pharm. Strasbourg*, 8 (1), 47 (1965); through *Chem. Abstr.*, 65, 6075(1966).
(537) Beckett, A. H., *Dansk Tidsskr. Farm.*, 40 (7), 197 (1966); through *Chem. Abstr.*, 65, 15930(1966).
(538) Koppanyi, T., and Avery, M., *Clin. Pharmacol. Therap.*, 7 (2), 250(1966); through *Chem. Abstr.*, 64, 16459 (1966).
(539) Doluisio, J. T., and Swintosky, J. V., *Am. J. Pharm.*, 137 (5), 175(1965); through *Chem. Abstr.*, 64, 4099(1966).
(540) Kato, R., *Tampakushitsu Kakusan Koso*, 10 (9), 848(1965); through *Chem. Abstr.*, 65, 7804(1966).
(541) Uehleke, H., *Arch. Pharm.*, 299 (6), 113(1966); through *Chem. Abstr.*, 65, 7803(1966).
(542) Garrett, E. R., and Lambert, H. J., *J. Pharm. Sci.*, 55, 626(1966).
(543) Garrett, E. R., and Alway, C. D., *Intern. Congr. Chemotherapy, Proc., 3rd, Stuttgart*, 1963, 2; through *Chem. Abstr.*, 65, 9532(1966).
(544) Fleischli, G., and Cohen, E. N., *Anesthesiology*, 27 (1), 64(1966); through *Chem. Abstr.*, 64, 8798(1966).
(545) Lightfoot, R. W., Jr., and Christian, C. L., *J. Clin. Endocrinol. Metab.*, 26, 305(1966).
(546) DiCarlo, F. J., *et al., Proc. Soc. Exptl. Biol. Med.*, 120 (3), 705(1965); through *Chem. Abstr.*, 64, 8781(1966).
(547) Priestly, B. G., and O'Reilly, W. J., *J. Pharm. Pharmacol.*, 18, 41(1966).
(548) Gibaldi, M., and Weiner, N. D., *J. Pharm. Sci.*, 55, 769(1966).
(549) Libby, D. A., Schertel, M. E., and Loy, H. W., *J. Assoc. Offic. Agr. Chemists*, 48 (5), 981(1965); through *Chem. Abstr.*, 64, 4874(1966).
(550) Cuthbert, A. W., *J. Pharm. Pharmacol.*, 18, 561 (1966).
(551) Beckett, A. H., and Rowland, M., *ibid.*, 17(S), 109S(1965); through *Chem. Abstr.*, 64, 8787(1966).
(552) Beckett, A. H., and Wilkinson, G. R., *J. Pharm. Pharmacol.*, 17(S), 107S(1965); through *Chem. Abstr.*, 64, 8787(1966).
(553) Shibaki, H., *Nippon Kagaku Ryohogakukai Zasshi*, 12 (5), 398(1966); through *Chem. Abstr.*, 64, 14782(1966).
(554) Shibaki, H., *Nippon Kagaku Ryohogakukai Zasshi*, 12 (5), 390(1964); through *Chem. Abstr.*, 64, 14782(1966).
(555) Stevens, C. E., and Stettler, B. K., *Am. J. Physiol.*, 210, 365(1966).
(556) Gallo, D. G., Bailey, K. R., and Sheffner, A. L., *Proc. Soc. Exptl. Biol. Med.*, 120 (1), 60(1965); through *Chem. Abstr.*, 64, 1200(1966).
(557) Nakanishi, Y., *et al., Yakugaku Zasshi*, 86, 46(1966).
(558) Lammers, W., *Pharm. Weekblad*, 100 (37), 1069 (1965); through *Chem. Abstr.*, 64, 4100(1966).
(559) Krowczynski, L., *Polski Tygod. Lekar.*, 21 (5), 191 (1966); through *Chem. Abstr.*, 65, 2063(1966).
(560) Fisera, M., *Cesk. Dermatol.*, 40 (5), 340(1965); through *Chem. Abstr.*, 64, 4099(1966).
(561) O'Reilly, R. A., Nelson, E., and Levy, G., *J. Pharm. Sci.*, 55, 435(1966).
(562) Levy, G., Miller, K. E., and Reuning, R. H., *ibid.*, 55, 394(1966).
(563) Matsumoto, H., *Yakugaku Zasshi*, 86, 590(1966).
(564) Voigt, R., and Wolf, C., *Pharmazie*, 21 (4), 229 (1966); through *Chem. Abstr.*, 65, 15165(1966).
(565) Voigt, R., and Wolf, C., *Sci. Pharm.*, 34 (1), 26 (1966); through *Chem. Abstr.*, 65, 15166(1966).
(566) Yamada, H., *et al., Chem. Pharm. Bull. (Tokyo)*, 14, 786(1966).
(567) Yamada, H., and Yamamoto, R., *ibid.*, 13 (11), 1279(1965); through *Chem. Abstr.*, 64, 6411(1966).
(568) Neuwald, F., and Ackad, P., *Am. J. Hosp. Pharm.*, 23, 347(1966).
(569) Kakemi, K., *et al., Yakugaku Zasshi*, 86, 278(1966).
(570) Fincher, J. H., Entrekin, D. N., and Hartman, C. W., *J. Pharm. Sci.*, 55, 23(1966).
(571) Naito, S., *Yakuzaigaku*, 25 (2), 125(1965); through *Chem. Abstr.*, 64, 14034(1966).
(572) Krogerus, A. E., and Tolvi, M., *Acta Pharm. Suecica*, 2 (4), 327(1965); through *Chem. Abstr.*, 64, 1903 (1966).
(573) Scioli, C., and Ciaffi, G., *Antibiotica*, 3 (3), 250 (1965); through *Chem. Abstr.*, 65, 19192(1966).
(574) Scioli, C., and Auriti, R., *Antibiotica*, 3 (3), 245 (1965); through *Chem. Abstr.*, 65, 19192(1966).
(575) Scioli, C., and Auriti, R., *Antibiotica*, 3 (3), 240 (1965); through *Chem. Abstr.*, 65, 19151(1966).
(576) Brewton, E. S., *Dissertation Abstr.*, 26, 5383(1966).
(577) Lybrand, R. A., *ibid.*, 26, 6401(1966).
(578) Steinetz, B. G., *et al., Proc. Soc. Exptl. Biol. Med.*, 123, 163(1966).
(579) Sorby, D. L., and Liu, G., *J. Pharm. Sci.*, 55, 504 (1966).
(580) Aguiar, A. J., and Fifelski, R. J., *ibid.*, 55, 1387 (1966).
(581) Bond, J. M., *et al., Bull. World Health Organ.*, 33 (6), 817(1965); through *Chem. Abstr.*, 64, 18226(1966).
(582) Smith, D. L., Pulliam, A. L., and Forist, A. A., *J. Pharm. Sci.*, 55, 398(1966).
(583) Rosen, E., *et al., J. Am. Med. Assoc.*, 194 (11), 1203(1965); through *Chem. Abstr.*, 64, 8787(1966).
(584) Forth, W., *et al., Arch. Exptl. Pathol. Pharmakol.*, 252 (3), 242(1965); through *Chem. Abstr.*, 64, 8801(1966).
(585) Mima, H., *et al., Takeda Kenkyusho Nempo*, 24, 23(1965); through *Chem. Abstr.*, 64, 8789(1966).
(586) Gretskii, V. M., *Aptechn. Delo*, 15 (4), 14(1966); through *Chem. Abstr.*, 65, 15928(1966).
(587) Kastin, A. J., Arimura, A., and Schally, A. V., *Arch. Dermatol.*, 93 (4), 471(1966); through *Chem. Abstr.*, 64, 18246(1966).
(588) Wagner, J. G., Gerard, E. S., and Kaiser, D. G., *Clin. Pharmacol. Therap.*, 7, 610(1966).
(589) Singh, P., Guillory, J. K., Sokoloski, T. D., Benet, L. Z., and Bhatia, V. N., *J. Pharm. Sci.*, 55, 63(1966).
(590) Nielsen, J. J., *Arch. Pharm. Chemi*, 72 (25), 897 (1965); through *Chem. Abstr.*, 64, 7965(1966).
(591) Holt, P. R., *Am. J. Physiol.*, 210, 635(1966).
(592) Jacobs, P., Bothwell, T. H., and Charlton, R. W., *ibid.*, 210, 694(1966).
(593) Draper, E. B., *Dissertation Abstr.*, 26, 5984(1966).
(594) Palva, I. P., and Heinivaara, O., *Ann. Med. Internae Fenniae*, 54 (2), 37(1965); through *Chem. Abstr.*, 64, 2613(1966).
(595) Palva, I. P., Heinivaara, O., and Mattila, M., *Scand. J. Haematol.*, 3 (2), 149(1966); through *Chem. Abstr.*, 65, 6161(1966).
(596) Shumikhina, K. I., and Panarin, E. F., *Antibiotiki*, 11 (7), 628(1966); through *Chem. Abstr.*, 65, 12725(1966).
(597) Sobolev, V. R., and Molochaeva, I. S., *Antibiotiki*, 11 (7), 625(1966); through *Chem. Abstr.*, 65, 12725(1966).
(598) Nogami, H., Hanano, M., and Aruga, M., *Chem. Pharm. Bull. (Tokyo)*, 14, 166(1966).
(599) Wolff-Soerensen, E., *Nord. Med.*, 74 (43), 1077 (1965); through *Chem. Abstr.*, 64, 8829(1966).
(600) Davis, P. S., and Deller, D. J., *Lancet*, II, 470 (1966).

(601) Pantio, M., and Karki, N. T., *Arch. Interam. Rheumatol.*, **8** (3), 133(1965); through *Chem. Abstr.*, **64**, 18246(1966).
(602) Rewerski, W., and Wysolowski, J., *Polski Tygop. Lekar*, **20** (20), 735(1965); through *Chem. Abstr.*, **64**, 11706 (1966).
(603) Wagner, W. H., *Intern. Congr. Chemotherapy, Proc., 3rd, Stuttgart*, **2**, 1716(1963); through *Chem. Abstr.*, **64**, 8779(1966).
(604) Nelson, E., *Intern. Congr. Chemotherapy, Proc., 3rd Stuttgart*, **2**, 1657(1963); through *Chem. Abstr.*, **65**, 12726 (1966).
(605) Pliska, V., *Arzneimittel-Forsch.*, **16** (7), 886(1966); through *Chem. Abstr.*, **65**, 19144(1966).
(606) Levy, G., *Clin. Pharmacol. Therap.*, **7**, 362(1966).
(607) Kruger-Thiemer, E., and Eriksen, S. P., *J. Pharm. Sci.*, **55**, 1249(1966).
(608) Vignoli, L., Defretin, J. P., and Cristau, B., *Bull. Soc. Pharm. Marseille*, **14** (53), 43(1965); through *Chem. Abstr.*, **64**, 10240(1966).
(609) Vignoli, L., *et al., Therapie*, **20** (6), 1491(1965); through *Chem. Abstr.*, **64**, 8832(1966).
(610) Turco, G. L., *et al., Clin. Pharmacol. Therap.*, **7**, 603 (1966).
(611) Boschi, F., Clini, V., and Gialdroni-Grassi, G., *Giorn. Ital. Chemioterap.*, **12** (1), 67(1965); through *Chem. Abstr.*, **65**, 19152(1966).
(612) Gibaldi, M., and Kanig, J. L., *Arch. Intern. Pharmacodyn.*, **161** (2), 343(1966); through *Chem. Abstr.*, **65**, 6076(1966).
(613) Cummings, A. J., Martin, B. K., and Renton, R., *Brit. J. Pharmacol. Chemotherapy*, **26** (2), 461(1966); through *Chem. Abstr.*, **64**, 14779(1966).
(614) Nordqvist, P., Harthon, J. G. L., and Karlsson, R., *Nord. Med.*, **74** (43), 1074(1965); through *Chem. Abstr.*, **64**, 8788(1966).
(615) Cummings, A. J., and King, M. L., *Nature*, **209**, 620(1966).
(616) Nelson, E., Hanano, M., and Levy, G., *J. Pharmacol. Exptl. Therap.*, **153** (1), 159(1966); through *Chem. Abstr.*, **65**, 6134(1966).
(617) Elliott, H. C., *Proc. Soc., Exptl. Biol. Med.*, **121**, 861 (1966).
(618) Harrison, C. E., Jr., *et al., J. Lab. Clin. Med.*, **67**, 764 (1966).
(619) Borzelleca, J. F., and Lowenthal, W., *J. Pharm. Sci.*, **55**, 151(1966).
(620) Krueger-Thiemer, E., Diller, W., and Buenger, P., *Antimicrobial Agents Chemotherapy*, **1965**, 183; through *Chem. Abstr.*, **65**, 11131(1966).
(621) Kulkarni, A. B., *Dissertation Abstr.*, **26**, 5383(1966).
(622) Winkler, K., *Biliary System, Symp. NATO Advan. Study Inst., Newcastle-upon-Tyne, Engl.*, **1963**, 551; through *Chem. Abstr.*, **64**, 14778(1966).
(623) Beckett, A. H., and Tucker, G. T., *J. Pharm. Pharmacol.*, **18(S)**, 72S(1966).
(624) Levy, G., and Amsel, L. P., *Biochem. Pharmacol.*, **15**, 1033(1966).
(625) Harada, K., *et al., Bitamin*, **32** (5), 464(1965); through *Chem. Abstr.*, **64**, 2616(1966).
(626) Vaccari, G. L., *et al., Boll. Soc. Ital. Biol. Sper.*, **42** (2), 47(1966); through *Chem. Abstr.*, **65**, 6086(1966).
(627) Aso, K., *et al., Bitamin*, **33** (4), 399(1966); through *Chem. Abstr.*, **64**, 18225(1966).
(628) Gerhards, E., and Kolb, K. H., *Arzneimittel-Forsch.*, **15** (12), 1375(1965); through *Chem. Abstr.*, **64**, 18224(1966).
(629) Bell, S. A., Berdick, M., and Holliday, W. M., *J. New Drugs*, **6**, 284(1966).
(630) Portmann, G. A., McChesney, E. W., Stander, H., and Moore, W. E., *J. Pharm. Sci.*, **55**, 59(1966).
(631) *Ibid.*, **55**, 72(1966).
(632) Carpenter, O. S., Northam, J. I., and Wagner, J. G., *Antibiol., Advan. Res., Prod. Clin. Use, Proc. Congr., Prague*, **1964**, 165; through *Chem. Abstr.*, **65**, 20678(1966).
(633) Munies, R., *Dissertation Abstr.*, **26**, 5384(1966).
(634) Buhler, D. R., Harpootlian, H., and Johnston, R. L., *Biochem. Pharmacol.*, **15**, 1507(1966).
(635) Hecht, G., Christian, J. E., and Banker, G. S., *J. Pharm. Sci.*, **55**, 678(1966).
(636) Heinrich, H. C., *et al., Z. Naturforsch.*, **20b** (11), 1062(1965); through *Chem. Abstr.*, **64**, 8833(1966).
(637) Urakubo, G., and Kido, Y., *Eisei Shikensho Hokoku*, **82**, 53(1964); through *Chem. Abstr.*, **65**, 11140(1966).
(638) Calesnick, B., Christensen, J. A., and Richter, M., *Proc. Soc. Exptl. Biol. Med.*, **123**, 17(1966).
(639) Johnson, A. M., *Am. Perfumer Cosmet.*, **81** (12), 35 (1966).
(640) Ashbel, S. I., *et al., Klinich. Med.*, **44** (5), 69(1966); through *Chem. Abstr.*, **65**, 6089(1966).
(641) Berger, St., Gronowska-Senger, A., and Chabrowska, B., *Roczniki Technol. Chem. Zywnosci*, **12**, 157(1966); through *Chem. Abstr.*, **65**, 19187(1966).
(642) Levy, G., and Jusko, W. J., *J. Pharm. Sci.*, **55**, 285(1966).
(643) Kristensen, K., and Hansen, T., *ibid.*, **55**, 610(1966).
(644) Mima, H., *et al., Takeda Kenkyusho Nempo*, **24**, 9 (1965); through *Chem. Abstr.*, **64**, 8788(1966).
(645) Mima, H., *et al., Takeda Kenkyusho Nempo*, **24**, 30 (1965); through *Chem. Abstr.*, **64**, 8789(1966).
(646) Yoshida, Y., *Shika Igaku*, **27** (3), 89(1964); through *Chem. Abstr.*, **64**, 18284(1966).
(647) Symchowicz, S., *et al., Arch. Intern. Pharmacodyn.*, **158** (2), 360(1965); through *Chem. Abstr.*, **64**, 11703(1966).
(648) Ballard, B. E., *J. Pharm. Sci.*, **55**, 515(1966).
(649) Fischer, L. J., *Dissertation Abstr.*, **26**, 5732(1966).
(650) Kojima, S., Ichibagase, H., and Iguchi, S., *Chem. Pharm. Bull. (Tokyo)*, **14**, 959(1966).

A Mechanism for the Hydrolytic Deamination of Cytosine Arabinoside in Aqueous Buffer

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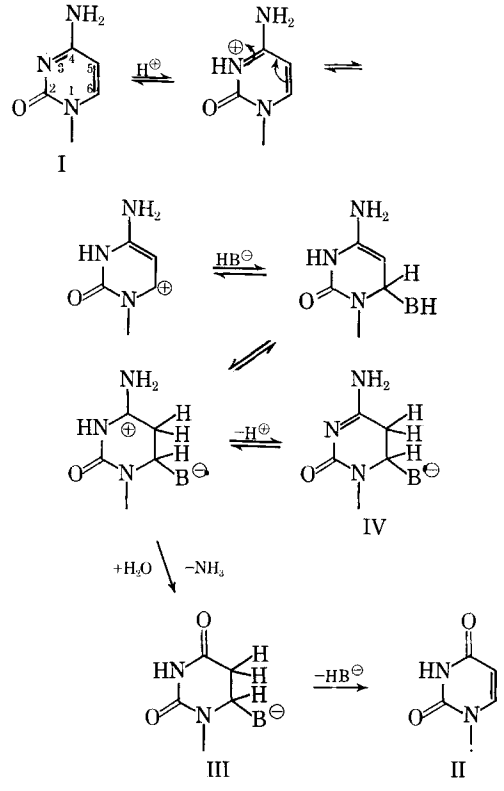
The kinetics of hydrolytic deamination of cytosine arabinoside have been studied as a function of pH, temperature, and buffer composition. The reaction components were isolated, identified, and quantified. The sole catalytic buffer species in the phosphate system is $H_2PO_4^-$. The rate of reaction between $NaHSO_3$ and cytosine arabinoside has been studied and the resulting products characterized. A mechanism for the hydrolytic deamination of cytosine nucleosides, catalyzed by hydrogen ion and general acid-base, HB^-, is hypothesized. This mechanism is shown to be consistent with existing literature data as well as the kinetic and $NaHSO_3$ reaction data reported here.

THE ROLE which chemical mutagens play in the deamination of cytosine nucleosides (I) to uracil derivatives (II) has been the subject of several investigations directed toward elucidation of *in vivo* mechanisms of mutagenic activity (1–5). Recently, Shapiro and Klein (5) have proposed two kinetically equivalent mechanisms for the deamination of cytidine (or cytosine) in aqueous buffers of pH 3 to 6. Both mechanisms combine specific hydrogen-ion catalysis with general base catalysis by buffer anion, B^-. However, the catalytic interaction by buffer was not unequivocally defined. It was hypothesized that an anion, B^-, increased deamination rate either by (*a*) removal of a proton from water or (*b*) partial saturation of the 5,6-double bond. Isolation of the components in reaction mixtures revealed only cytidine and uridine (or cytosine and uracil) and did not, therefore, differentiate between the two alternate routes.

The present investigation lends support to the proposed hydrogen-ion catalysis and eliminates the possiblility that proton exchange between anion, B^-, and water is the mechanism for general base catalysis. Evidence presented here demonstrates that general acid-base catalysis results primarily from complete saturation of the 5,6-double bond by a nucleophile, HB^-, and that catalysis by other buffer species such as B^-, B^{2-}, or B^{3-} is either absent or relatively unimportant. The prime criteria for catalysis by a nucleophile such as HB^-, are shown to be (*a*) sufficient nucleophilicity to attack the 6-position following the initial protonation at the 3-position and (*b*) the ability to subsequently donate a proton resulting in the complete saturation of the 5,6-double bond (Scheme I).



$HB^\ominus$ = $HSO_3^\ominus$; $H_2PO_4^\ominus$; H_2 citrate$^\ominus$; H oxalate$^\ominus$; H succinate$^\ominus$

Scheme I

Received December 27, 1966, from the College of Pharmacy, Ohio State University, Columbus, OH 43210

Accepted for publication March 7, 1967.

This investigation was conducted while the author served as the 1966 Summer Professor, Product Research and Development, The Upjohn Co., Kalamazoo, Mich.

The author wishes to acknowledge the fine technical assistance of Mr. Carl Zipple, who perfected and performed the analyses. Appreciation for helpful comments on the manuscript is expressed to Drs. W. J. Wechter and R. C. Kelley.

The name for cytosine arabinoside approved by USAN is cytarabine.

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EXPERIMENTAL

Quantitative Analysis of Cytosine Arabinoside and Uracil Arabinoside—Thin-layer plates, 20 × 20 cm., were coated with a 3:1 mixture of Silica Gel G and Silica Gel GF 254. After activating for 1 hr. at 105°, they were scored to form nine 1-cm. channels and washed with benzene–propanol (1:1). Reaction aliquots of 25 μl. were spotted, dried, and then developed with water-saturated butanol–propanol (3:1) for about 2.5 hr. Chromatograms were air-dried for 1 hr. and photographed under 254 mμ ultraviolet light.

The appropriate spot was then scraped from the plate and eluted with five 3-ml. aliquots of 0.01 *M* H_2SO_4 in ethanol. The pooled extract was brought to 50 ml. and its ultraviolet absorbance determined against a similarly prepared blank. A primary standard was run on each plate and the standard absorbance used to calculate the amount of the unknown. Recovery was generally 95% with excellent reproducibility. The wavelength of maximum absorption under these conditions is 285 mμ for cytosine arabinoside and 263 mμ for uracil arabinoside.

Kinetics of Cytosine Arabinoside Loss—Reactions were prepared with "Water for Injection" which had been boiled and cooled under nitrogen. The solutions were filtered under nitrogen through a Millipore filter (0.045) then dispensed into 5-ml. ampuls and sealed. Ampuls were placed in appropriate constant-temperature baths and analyzed as a function of time as described above. (See Tables I and II for experimental conditions.)

Preparation of Cytosine Arabinoside-Bisulfite Reaction Products—An aqueous solution containing 0.2 *M* cytosine arabinoside and 2.0 *M* $NaHSO_3$ was allowed to react for 85 hr. at 70°. The reaction was considered complete when a 254 mμ ultraviolet light no longer revealed a cytosine arabinoside spot on a thin-layer chromatogram. A similar solution was prepared with uracil arabinoside and treated in the same manner.

Characterization of the Components in Reaction Mixtures—Products resulting from the reaction of bisulfite with cytosine arabinoside or from the deamination of cytosine arabinoside were compared to standards on thin-layer chromatograms. The standards were: cytosine arabinoside, uracil arabinoside, cytosine, uracil, and D-arabinose. The following solvents were employed with silica gel support: *A*, butanol–propanol (3:1) saturated with water; *B*, 2-propanol–ammonia–water (7:1:2); *C*, chloroform–propanol (3:1); *D*, ethyl acetate–acetic acid–

TABLE I—EXPERIMENTAL CONDITIONS AND APPARENT FIRST-ORDER RATE CONSTANTS FOR THE DEAMINATION OF CYTOSINE ARABINOSIDE[a]

°C.	Observed pH	Buffer Compn. [$NaH_2PO_4 \cdot H_2O$]	Buffer Compn. [Na_2HPO_4]	Buffer Compn. [NaCl]	10^3k (hr.$^{-1}$)
70	6.15 ± 0.15	0.120	0.012	0.000	3.11
		0.048	0.0048	0.094	1.71
		0.024	0.0012	0.125	1.18
		0.000	0.000	0.153	0.705[b]
	6.90 ± 0.10	0.040	0.040	0.000	1.13
		0.029	0.029	0.043	0.872[c]
		0.016	0.016	0.092	0.619
		0.000	0.000	0.153	0.292[b]
	7.81 ± 0.05	0.0055	0.055	0.000	0.504
		0.0033	0.033	0.063	0.470
		0.0022	0.022	0.094	0.417
		0.000	0.000	0.153	0.379[b]
60	6.90 ± 0.10	0.029	0.029	0.043	0.390[c]
55	6.90 ± 0.10	0.029	0.029	0.043	0.273[c]
47	6.90 ± 0.10	0.029	0.029	0.043	0.153[c]

[a] The initial concentration of cytosine arabinoside was 4 × 10^{-2} *M*. All solutions contained the antimicrobial agent, benzyl alcohol (8.8 × 10^{-2} *M*) and had the ionic strength adjusted to 0.153. [b] Calculated from the intercept of the plot $k_{obsd.}$ *vs.* $NaH_2PO_4 \cdot H_2O$. (See Fig. 4.) [c] The heat of activation (ΔHa in Kcal./mole) for this system is 16.4. (See Fig. 5.)

TABLE II—MATERIAL BALANCE BASED ON ANALYSIS OF CYTOSINE ARABINOSIDE (CA) AND URACIL ARABINOSIDE (UA) PRESENT AFTER 425 hr., pH 6.9, 0.06 *M* PHOSPHATE BUFFER, μ 0.15, AND VARYING $NaHSO_3$ CONCENTRATION

°C.	Sample[a]	Total Initial moles	% Total as CA	% Total as UA	% Total Recovered	% Unacct. for
70	*A*	0.0402	68.7	22.2	90.9	− 9.1
	B	0.0410	64.6	20.6	85.2	−14.8
	C	0.0407	63.1	20.8	83.9	−16.1
	D	0.0406	56.2	14.1	70.3	−29.7
	E	0.0405	34.3	0.0	34.3	−65.7
60	*A*	0.0402	84.8	9.5	94.3	− 5.7
55	*A*	0.0402	91.2	9.2	100.4	+ 0.4
47	*A*	0.0402	94.8	3.2	98.0	− 2.0
	B	0.0410	88.3	4.1	92.4	− 7.6
	C	0.0407	92.6	3.7	96.3	− 3.7
	D	0.0406	74.1	3.2	77.3	−22.7
	E	0.0405	51.4	0.0	51.4	−48.6

[a] [$NaHSO_3$]: *A*, 0.000; *B*, 0.004; *C*, 0.010; *D*, 0.020; *E*, 0.040.

ethanol (23:1:1); *E*, butanol–water (86:14). One additional system, *F*, employed aluminum oxide gel and 2-propanol–ammonia–water (7:1:2). Both supports contained phosphors.

RESULTS

Effect of $NaHSO_3$ on Kinetics of Cytosine Arabinoside Loss—The effect of $NaHSO_3$ on the rate of loss of cytosine arabinoside in aqueous phosphate buffer, pH 6.9, 70°, μ = 0.15, is illustrated in Fig. 1. The relative order of the rates was the same at 40°. The 0.01 *M* $NaHSO_3$ solution contained the calculated amount of bisulfite necessary to react with the oxygen content in the headspace of a sealed ampul. Thus, there is an increased rate of cytosine arabinoside loss with increased [$NaHSO_3$] and a pronounced increase in rate when [$NaHSO_3$] exceeds that required for the atmospheric oxygen.

Rate of loss of cytosine arabinoside was found to be first order in the absence of $NaHSO_3$ (Fig. 2). Reactions were not first order in the presence of $NaHSO_3$. The change in order was attributed to $NaHSO_3$ reacting with cytosine arabinoside. (An earlier study demonstrated a loss of $NaHSO_3$ in excess of that consumed by the headspace oxygen.) Assuming a 1:1 reaction permits calculation of the time required for the oxygen and reactant to completely expend the $NaHSO_3$. It can be seen in Fig. 2 that first-order plots become both linear and parallel after the $NaHSO_3$ is depleted.

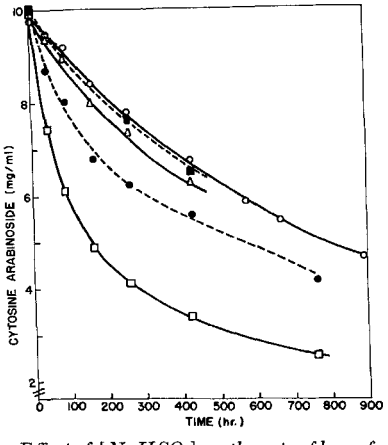


Fig. 1—Effect of [$NaHSO_3$] on the rate of loss of cytosine arabinoside in 0.06 M *phosphate buffer, pH 6.9, 70°, μ = 0.15. Key:* ○, A (*0.000*); ■, B (*0.004*); △, C (*0.010*); ●, D (*0.020*); □, E (*0.040*).

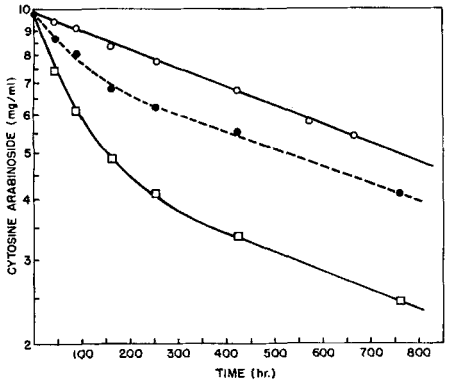


Fig. 2—Effect of [$NaHSO_3$] on the first-order plots for cytosine arabinoside loss in 0.06 M *phosphate buffer, pH 6.9, 70°, μ = 0.15. Key:* ○, A (*0.000*); ●, D (*0.020*); □, E (*0.040*).

A material balance based on the concentration of cytosine arabinoside and uracil arabinoside in several reaction mixtures at 70°, 425 hr., gives further support to the existence of an additional bisulfite reaction product (Table II). It is obvious, from these results, that another reaction product must exist and that its yield increases with increased $NaHSO_3$ concentration. For example, 66% of the initial cytosine arabinoside was converted to the alternate product in the presence of 0.04 *M* $NaHSO_3$ at 70°. In the absence of bisulfite all of the material was accounted for within the limit of experimental error; no other product was formed.

Thin-layer chromatograms, which were viewed under a 254 mμ ultraviolet light, revealed only two spots: cytosine arabinoside and uracil arabinoside (R_f values 0.42 and 0.70 using solvent system *A*). The preservative, benzyl alcohol, appeared as a spot near the solvent front and will be ignored for the purpose of this discussion. Charring with H_2SO_4 and heat revealed a third spot just above the origin. An aqueous solution containing 0.2 *M* cytosine arabinoside and 2.0 *M* $NaHSO_3$ produced no cytosine arabinoside or uracil arabinoside spot after 85 hr. at 70°. Charring with H_2SO_4 and heat again revealed a spot just above the origin.

Bisulfite is known to react with α,β-unsaturated ketones by a 1,4-addition to yield a sulfonate which reverses to starting material upon addition of NaOH (6). Addition of NaOH to the above 85-hr. bisulfite reaction gave the following chromatographic results in the solvents listed: (*a*) no uracil spot, *B,C,D,E;* (*b*) no arabinose spot, *A,B;* (*c*) definite uracil arabinoside spot, *A,B,C,E,F;* and (*d*) definite but minor cytosine arabinoside spot, *A,E,F*. It was assumed that no cytosine was present since the absence of uracil and arabinose was demonstrated.

It was concluded that the major bisulfite reaction product was the sulfonate of uracil arabinoside (III) and the minor reaction product was the sulfonate of cytosine arabinoside (IV). This conclusion is consistent with the following experimental observations: (*a*) the product of the bisulfite reaction has a low R_f value and is not visible under a 254 mμ ultraviolet light; (*b*) the rate of cytosine arabinoside loss is not first order in the presence of $NaHSO_3$; (*c*) there is an increased yield of reaction product other than II with increasing $NaHSO_3$ concentration; (*d*) there is a complete loss of I without formation of II in the presence of a tenfold excess of $NaHSO_3$; and (*e*) treatment of the bisulfite reaction product(s) with NaOH yields primarily II and traces of I.

The reaction between uracil arabinoside and $NaHSO_3$ did not result in an appreciable decrease in the size of the uracil arabinoside spot size during the 85-hr. reaction period. The reaction, thus, appeared to be slower than the corresponding cytosine arabinoside reaction. It seems unlikely, in light of this evidence, that the product III is formed by this route in the cytosine arabinoside reactions.

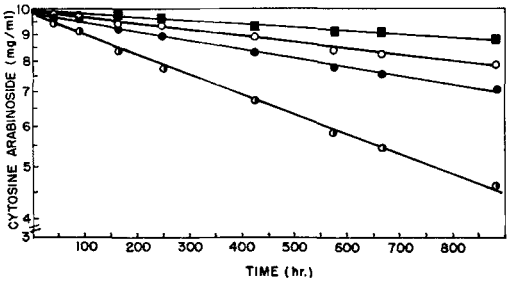


Fig. 3—First-order plots for deamination of cytosine arabinoside in 0.06 M phosphate buffer, pH 6.9, μ = 0.16. Key: ■, 47°; ○, 55°; ●, 60°; ◑, 70°.

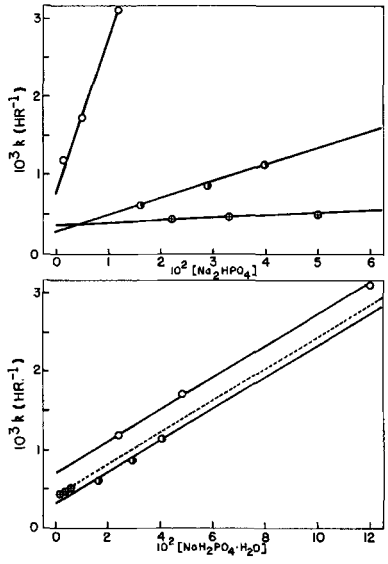


Fig. 4—Apparent first-order rate constants for cytosine arabinoside deamination at 70°, μ = 0.15, as a function of (top) disodium phosphate concentration and (bottom) sodium phosphate concentration. Key: ○, pH 6.2; ◑, pH 6.9; ⊕, pH 7.8.

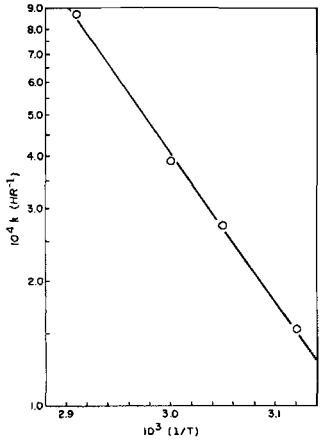


Fig. 5—Arrhenius plot of first-order rate constants for hydrolytic deamination of cytosine arabinoside in 0.06 M phosphate buffer, pH 6.9, μ = 0.15.

Kinetics of Cytosine Arabinoside Deamination in Buffer Solutions—Good first-order plots were obtained for deamination of cytosine arabinoside to uracil arabinoside in aqueous phosphate buffer solutions lacking $NaHSO_3$. Typical examples are shown in Fig. 3. Apparent first-order rate constants, calculated from the slopes of such plots, are tabulated in Table I.

The dependency of the observed first-order rate constants on the concentration of the buffer components is demonstrated in Fig. 4. The parallelism which is present in the plot of *k versus* $NaH_2PO_4 \cdot H_2O$ (Fig. 4, bottom) and absent in the corresponding plot for Na_2HPO_4 (Fig. 4, top) clearly demonstrates that the sole catalytic species in this buffer is $H_2PO_4^-$.

The intercept values, or the rate constants in the absence of buffer, are also reported in Table I. The observed increase in rate in going from pH 6.9 to 6.2 may be interpreted as specific hydrogen-ion catalysis in agreement with that previously reported (5). The failure of HPO_4^{2-} to catalyze the reaction demonstrates that removal of a proton from H_2O by base is not the mechanism by which general base catalysis is occurring. The very small difference between pH 6.9 and 7.8 suggests that specific hydroxyl-ion catalysis does not contribute to an appreciable extent in this pH region.

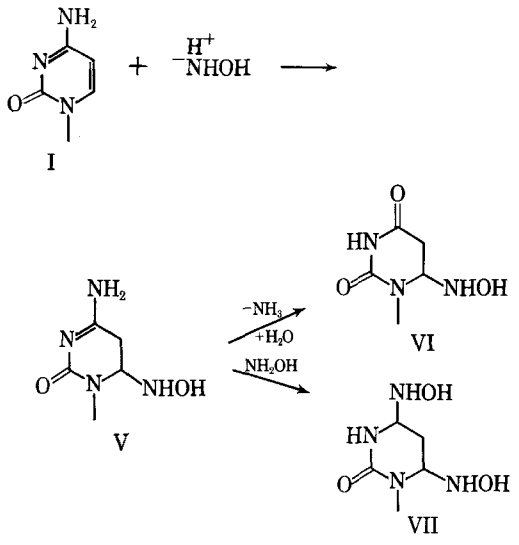
An Arrhenius plot of the apparent first-order rate constants for the deamination of cytosine arabinoside in 0.029 $NaH_2PO_4 \cdot H_2O$, 0.029 Na_2HPO_4, pH 6.9, $\mu = 0.15$ is given in Fig. 5. The value of ΔHa, in the presence of catalytic species, is 16.4 Kcal./mole.

DISCUSSION

Scheme I illustrates the proposed mechanism for either the hydrogen-ion, general acid-base catalysis of cytosine arabinoside (I) deamination or for 1,4-addition by HSO_3^-. The major bisulfite addition product is the sulfonate of uracil arabinoside (III), although some of the cytosine sulfonate (IV) is definitely present. It was concluded from thin-layer chromatographic and material balance data (Table II) that the product resulting from the deamination of cytosine arabinoside in the presence of general acid-base other than HSO_3^- (Scheme I) was uracil arabinoside (II).

There is precedence for both the 1,4-addition by HSO_3^- (6) and also the 5,6-addition to pyrimidine nucleosides. The reaction of NH_2OH with cytosine derivatives has been investigated by several workers (1–3). The reaction pathway involves initial conjugate addition in which NH_2OH saturates the 5,6-double bond to form V which can either deaminate to VI or exchange with a second molecule of NH_2OH at the 4-position (VII). The first addition was illustrated as attack by H^+–$NHOH$ by Schuster (1). (Scheme II.)

The compound V was not isolated since saturation at the 5,6-position results in rapid nucleophilic exchange at C-4 to form VI or VII. This predisposition to deamination which results from saturation of the 5,6-position has been observed on several occasions. The nucleophilic exchange reaction which occurs during hydrolytic deamination has been found to proceed very slowly for cytosine as compared to the corresponding 5,6-dihydro derivatives (3). The deamination of cytidylic acid hydrate was shown to



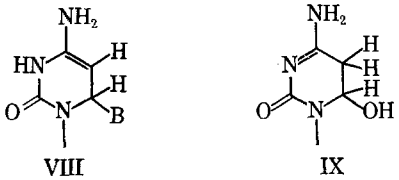
Scheme II

be rapid even at room temperature (4). In addition to this, the rate constants for the deamination of this 5,6-saturated species were independent of both the type and concentration of the buffer. The heat of activation for this deamination is the same as that reported here for the general acid-base catalyzed deamination, 16 Kcal./mole. In the absence of general acid-base species, HB^-, the value for ΔHa is 22 Kcal./mole (7).

An hypothesis explaining the observed increase in deamination rates of the 5,6-saturated cytosines is offered in steps 1–4, Scheme I. Furthermore, it is asserted that this represents the primary mechanism by which specific hydrogen-ion, general acid-base catalysis is occurring. Initial protonation at N-3 followed by saturation of the 5,6-position results in an electron deficiency at C-4 thus facilitating nucleophilic attack by water. Partial saturation to form VIII, as represented by Shapiro and Klein (5), would not result in electron deficiency at C-4.

Accordingly, $H_2PO_4^-$ is an effective catalytic species while HPO_4^{2-}, which will not supply a proton, is not (Fig. 4). This fact also excludes the suggestion that a possible catalytic mechanism is through abstraction of a proton from water by the base (5). If this were the case HPO_4^{2-} would be expected to exhibit a greater catalytic effect than $H_2PO_4^-$.

Catalysis by saturation of the 5,6-double bond is also consistent with the fact that cytidylic acid hydrate (IX) is not subject to buffer effects and has the same ΔHa for deamination as the $H_2PO_4^-$ catalyzed reaction reported here.



There is at present insufficient data available to allow the calculation of catalytic constants for buffer species other than the phosphate system which is defined in this paper. Studies are currently in progress, by the author, to quantitatively evaluate the catalytic effects of other buffers. However, the hypothesis that cytosine deamination is subject to general acid-base catalysis by HB^- can be shown to be consistent with the existing data of several workers. For example, the observed first-order rate constant for cytosine arabinoside deamination in the presence of citrate ion, pH 7.4, 70°, was found to be 3.77×10^{-4}/hr. (7). This agrees with the intercept value of the plot, *k versus* phosphate buffer concentration, pH 7.8, 70°, which is 3.79×10^{-4}/hr. (Fig. 4; Table I). Furthermore, the value of ΔHa for cytosine arabinoside deamination in the presence of $citrate^{3-}$ was shown to be 22 Kcal./mole (7). These data are consistent with the prediction that $citrate^{3-}$ does not meet the criteria for general acid-base catalysis as outlined here.

Table III—Concentrations of Each Buffer Species in 0.5 *M* Solutions at pH 4, the Corresponding First-Order Rate Constants for Cytidine Deamination at 97° ($k_{obsd.}$ in hr.$^{-1}$),[a] and the Portion of $k_{obsd.}$ Attributed to Catalysis by Buffer ($k_{cat.}[cat.]$)[b]

Buffer	HnB	$Hn_{-1}B^-$	$Hn_{-2}B^{2-}$	$10^2 k_{obsd.}$	$10^2 k_{cat.}$ [cat.]
Citrate	0.046	0.385	0.069	3.4	2.1
Oxalate	0.000	0.305	0.195	2.8	1.5
Succinate	0.293	0.202	0.005	2.0	0.7
Formate	0.198	0.306	...	1.4	0.1
Lactate	0.288	0.288	...	1.3	0.0
Acetate	0.432	0.068	...	1.3	0.0

[a] $k_{obsd.}$ = the observed first-order rate constants as reported by Shapiro and Klein (5). [b] These values are calculated from the equation $k_{cat.}[cat.] = k_{obsd.} - 1.26 \times 10^{-2}$ (hr.$^{-1}$), where 1.26×10^{-2} is the value for the intercept of the plot $k_{obsd.}$ *vs.* [H_2 citrate$^-$].

A re-examination of the data of Shapiro and Klein (5) also lends support to the mechanism proposed in Scheme I. Their observed first-order rate constants, $k_{obsd.}$, are listed in Table III. The concentrations of each individual buffer species were calculated and the results are also given. A plot of $[H_{n-1}B^-]$ *versus* $k_{obsd.}$ for the citrate buffer data under good first-order conditions ($[H_2 citrate^-]/[cytidine] \geq 4$) was found to be linear with an intercept value of 1.26×10^{-2} (hr.$^{-1}$). This value for the deamination of cytidine in the absence of buffer effects is comparable to the first-order rate constants reported for the formate, acetate, or lactate buffers under the same experimental conditions (Table III). It was also reported that cytidine deaminated only 2% after 21 hr. of refluxing in either water or 0.5 *M* sodium acetate at pH 7.2. It can be concluded that these buffers do not contain any major catalytic species for cytosine deamination.

Conversely, it is evident from Table III that the citrate buffer system does in fact catalyze cytidine deamination. Since it has been demonstrated that $citrate^{3-}$ is not catalytic, it follows that one of the protonated species must be the catalyst. As a further test of catalysis by HB^-, the derivative of [H_2 citrate$^-$] with respect to [H^+] was set equal to zero and the expression solved for the pH of maximum H_2 citrate$^-$ concentration. Results indicate that the pH of maximum HB^- concentration in a citrate buffer of fixed total concentration would be 3.9. This is consistent with the data which show a rate maximum between 3.5 and 4.0 for this system (5).

Attempts to correlate the catalytic effects of HB^- with that predicted from the Brönsted relation (8) are complicated by the dual nature of the catalyst which is defined here as participating both as an acid and a base. The values for the predicted catalytic constants, k_A (or k_B), in terms of G_A and α(or G_B and β) can be compared for each type of buffer species since the Brönsted relation is well obeyed within a group of similar catalytic species such as H_nB, $H_{n-1}B^-$, B^{2-}, *etc.* The value of the experimental catalytic constant for a potentially catalytic species, X, can also be calculated from $k_X = k_{cat.}$ [cat.]/(concn. X). (See Table III.) The assumption in this latter calculation is that one catalytic species predominates in each buffer as has been demonstrated for the phosphate system. The data were treated in this manner and the results, while not conclusive, are consistent with the present hypothesis. The catalytic constants as calculated from the data were compared to the catalytic constants as calculated from the Brönsted relation using the statistical correction and assigning a value of 0.5 to α or β. The only good correlation evident was that between k_{Hn-1B^-} and the values of k_A (in terms of G_A) for the species $H_{n-1}B^-$. This result is consistent with the choice of HB^- as the primary catalytic species. The fact that the correlation exists for k_A in this comparison and not for k_B implies that the catalytic species requires a donatable proton. The formate, lactate, and acetate ions, which exhibit no significant buffer effect as seen in Table III, also have no value for k_A since the anions cannot act as proton donors.

It can be concluded from the above discussion that the mechanism offered in Scheme I is consistent with the experimental data presented here as well as the related data of the other workers cited above.

SUMMARY

Consideration of the 1,4-addition products formed from the reaction between bisulfite and cytosine arabinoside and consideration of the effects of phosphate, oxalate, succinate, lactate, formate, and acetate buffers has demonstrated that cytosine deamination will be significantly catalyzed by a nucleophile bearing a donatable proton in the presence of hydrogen ions. The primary mechanism of hydrolytic deamination of cytosine nucleosides in aqueous catalytic buffer systems, as illustrated in Scheme I, is as follows: (*a*) protonation of N-3, (*b*) nucleophilic attack at C-6, (*c*) saturation at C-5 by proton addition, and (*d*) nucleophilic displacement at C-4 by H_2O with loss of NH_3.

REFERENCES

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Effect of Some Nonionic Surfactants on the Rate of Release of Drugs from Suppositories

By J. M. PLAXCO, JR., C. B. FREE, JR.*, and C. R. ROWLAND†

Twenty-eight nonionic surfactants were added to theobroma oil base suppositories. The amount of aminophylline, ephedrine alkaloid, and ephedrine hydrochloride released from these bases was determined by dialyzing the released drug through a cellophane membrane and determining the optical absorbance at the appropriate wavelength. Data obtained indicate that surfactants with HLB values of less than 11 had little effect on the rate of release. Surfactants with HLB values over 11 increased significantly, in most cases, the amount of drug dialyzed. Besides HLB values, other properties of surfactants such as chemical structure, composition, and melting point also affect the rate of release.

THE USE OF suppositories as a form of medication dates back to the time of Hippocrates. Many unsatisfactory agents were tried as bases, but it was not until the discovery and development of theobroma oil as a base in the 18th century that they received much attention. In more recent days much attention has been directed toward modifying and improving bases of theobroma oil, glycerinated gelatin, and polyethylene glycol polymers, and in developing new bases. Until fairly recently, as long as the suppository retained its shape on the shelf, melted or dissolved in the rectum, and showed no visible evidence of incompatibility it was considered satisfactory. Recently it has been recognized

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This investigation was supported in part by grant GM-11801-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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that many factors may affect the action of the drug following administration of the suppository.

From the literature we have a plethora of conflicting reports as to the efficacy of suppository medication. Most clinical work has been done with aminophylline, hence this part of the discussion will be limited mainly to reports concerning aminophylline or theophylline. Segal *et al.* (1) compared the protection against intravenously administered histamine by aminophylline administered by various routes. They found that suppositories gave slightly less relief than intravenous administration but that relief persisted for a longer period of time. Tablets were slower acting and afforded less relief than either of the other two dosage forms. Waxler and Schack (2), on the other hand, found blood levels of aminophylline from suppositories to be much lower than those obtained from intravenous or intramuscular injection, or from uncoated oral tablets. However, blood levels from suppositories were sustained for longer periods of time, except for intramuscular injection. Truitt *et al.* (3) found retention enemas gave highest levels, except for intravenous administration, and that suppositories gave lowest blood levels of all methods tested. Glass *et al.* (4) obtained satisfactory blood levels of theophylline following administration of suppositories of a mannitol or a buffered polyethylene glycol base. Lower blood levels were obtained from other bases.

Drugs may be absorbed from the intestinal tract by three processes: (*a*) active transport, (*b*) passive transport, and (*c*) specialized transport. Passive transport, or diffusion, has been shown to be the method by which most drugs are absorbed. Brodie *et al.* (5) showed that once a drug is in solution in the gastrointestinal tract its passage through the gastrointestinal fluid and across the lipoidal membrane is governed mainly by physical processes and is often predictable, based on the partition coefficient of the undissociated drug moiety and the dissociation constant of the drug. Riegelman and Crowell (6) in their reports on rectal absorption obtained results which agree with the assumption that the limiting factor is the diffusion of the drug to the absorption site. Suppositories, or other dosage forms, which release the drug fastest to the surrounding medium should, theoretically, afford more rapid absorption by the body and higher blood levels of the drug.

The literature contains numerous reports (7–19) of the effects of surfactants on the rate of release of various medicaments from oils, fats, *etc.* This study was to determine the effects of various factors, particularly nonionic surfactants, on the rate of release of drugs from different suppository bases, primarily those composed of theobroma oil.

Since most clinical reports concern aminophylline (theophylline ethylene diamine), it was selected as the primary test drug. For comparative purposes, ephedrine alkaloid and one of its more water-soluble salts, ephedrine hydrochloride, were selected.

Since many of the bases contained surfactants and emulsified when in contact with water, it was necessary to use some means of separating the drug from the emulsified base of theobroma oil. Dialyzing the liberated drug through a cellophane membrane accomplished this separation, permitting determination of the amount of drug, although some ingredients of the base, particularly the surfactants, may have dialyzed also.

EXPERIMENTAL

Materials—Where possible, official products were used. Nonofficial products were of food or drug grade and were used as received from the manufacturer or distributor with no further purification. All theobroma oil was purchased at one time and all dialyzing membrane was from the same manufacturer.

Preparation of Suppositories—After calibration of the mold, 140 mg. of aminophylline, or 700 mg. of ephedrine or ephedrine hydrochloride, sufficient for 14 suppositories, was incorporated by geometric dilution into the just-melted base of theobroma oil and surfactant. After thorough mixing the mass was poured immediately into an unlubricated mold for 12 suppositories. The excess mass was used to prepare the melting point tubes or discarded. Blank suppositories—containing no drug—were prepared of each base to determine the absorbance of each base. The suppositories and melting point tubes were placed in a refrigerator at 5° for 2 days, then stored at room temperature for at least 3 days before testing. All suppositories were satisfactory in appearance and physical stability with no evidence of leakage, blooming, or color change for 3 months. After that time, several batches began to show a mottled appearance but looked satisfactory otherwise.

Procedure for Aminophylline—Dialyzing bags were prepared from dialyzing cellophane tubing, tied with cotton thread, and soaked overnight in distilled water before use. After rinsing the bags twice, 25 ml. of distilled water was placed in each bag which was then placed in a 500-ml. wide-mouth bottle containing 175 ml. of distilled water. The bottle was placed in a constant-temperature water bath at 39° and allowed to reach that temperature. This temperature was selected to ensure being above the clear melting point for all suppositories.

One suppository was placed in each bag which was suspended so that the level of the water inside was even with the level of the water outside the bag. The water in the bottle was stirred by a slowly turning coated magnet and a magnetic stirrer. At intervals a sample was removed from the bottle

and the absorbance at 273 mμ was determined using a Beckman model DB spectrophotometer. The concentration of aminophylline was obtained from a standard curve with corrections made for the absorbance of each suppository base (theobroma oil plus surfactants).

Procedure for Ephedrine and Ephedrine Hydrochloride—Since ephedrine and its salts passed through the dialyzing membrane very rapidly, it was necessary to modify the technique used previously. Instead of using a dialyzing bag, a cellophane membrane, previously soaked overnight and rinsed in distilled water, was stretched firmly over the open end of a thistle tube 38 mm. in diameter and made watertight by a rubber band.

After a suppository had been placed in the tube and the end closed as described, the inverted tube was suspended so that the membrane was just below the surface of 200 ml. of distilled water in a 500-ml. wide-mouth bottle. Water rapidly passed through the membrane and dissolved the drug. The bottle was immersed in a constant-temperature water bath at 39° and stirred by a slowly turning coated magnet and a magnetic stirrer. At intervals a sample was removed from the bottle and the absorbance at 257 mμ determined using a Beckman model DU spectrophotometer. The concentration of ephedrine or ephedrine hydrochloride was determined from the respective standard curve with corrections made for the absorption of the particular suppository base.

RESULTS AND DISCUSSION

Aminophylline—Table I shows the composition and melting range of various types of suppository bases selected to establish variations in rate of release and dialysis of aminophylline. The results of dialyzing the suppositories, shown in Table II, are in general agreement with previously published reports (7–19) on the rate of release of water-soluble substances from suppository bases. The addition of the hydrogenated vegetable oil to stabilize the melting point of theobroma oil greatly reduced the rate of release of aminophylline. The degree of

Table II—Aminophylline Dialyzed from Various Types of Suppository Bases

Base	Time, min. 15	30	60	120	180
	Aminophylline Dialyzed, mg.				
Theobroma oil	0.1	0.35	1.0	2.5	4.5
Theobroma oil–hydrogenated vegetable oil	Neg.[a]	Neg.[a]	0.08	0.3	0.5
Polyethylene glycol	0.5	0.9	2.0	4.3	6.1
Polysorbate 61	...	1.5	3.0	5.0	6.0
Anhydrous liquid	0.2	0.4	0.7	1.2	...
Petrolatum–paraffin	Neg.[a]	Neg.[a]	0.1	0.3	0.5
Adeps solidus base[b]	0.1	0.3	0.5	1.0	...
Aqueous soln., no base 10 mg./25 ml.	0.7	1.5	2.8	5.3	6.1

[a] Neg., negligible. [b] Marketed as Witepsol H12 by Chemische Werke Witten, Germany.

Table I—Composition and Melting Range of Various Types of Suppository Bases

Compn.		Melting Range
Theobroma Oil Base		
Theobroma oil	100%	32–33°
Theobroma Oil–Vegetable Oil		
Theobroma oil	97%	36–39°
Completely hydrogenated vegetable oil[a]	3%	
Theobroma Oil–Surfactant Bases		
Theobroma oil	95%	32–34° for all suppositories.
Surfactant	5%	
Polyethylene Glycol Base[b]		
Polyethylene glycol 4000	53%	Did not melt, but dissolved in test.
Polyethylene glycol 6000	47%	
Polysorbate 61 Base		
Polyoxyethylene sorbitan monolaurate (polysorbate 61)[c]	90%	Did not melt, but emulsified in test.
Glyceryl monolaurate	10%	
Anhydrous Liquid Base[d]		
Emulsifier[e]	5%	Liquid at room temperature; emulsified in water in test.
Polysorbate 80[f]	5%	
Silica[g]	4%	
Centrofil SM[h]	0.5%	
Liquid petrolatum, heavy	85.5%	
Petrolatum–Paraffin Base		
White petrolatum	75%	51–53°
Paraffin	25%	Did not melt in test.
Adeps Solidus Base		
Adeps solidus	100%	32–33.5°

[a] Marketed as Fix-X by Procter & Gamble. [b] Other proportions tried but no significant difference in results. [c] Marketed as Tween 61 by Atlas Chemicals Industries, Wilmington, Del. [d] Formula from Atlas Chemical Industries, Inc., Pharmaceutical Bulletin LD-99. [e] Marketed as Atmos 300 by Atlas Chemical Industries. [f] Marketed as Tween 80 by Atlas Chemical Industries. [g] Marketed as Cab-O-Sil by Cabot Corp. [h] Marketed as Centrofil SM by Central Soya Co. [i] Marketed as Witepsol H12 suppository base by Chemische Werke Witten, Germany, and contains varied proportions of saturated natural fatty acids.

Table III—Aminophylline Dialyzed from Theobroma Oil Containing 5% Nonionic Surfactants

Surfactant Chemical Type or Compn.	HLB	Time, min. 15	30	60	90
		Aminophylline Dialyzed, mg.			
Theobroma Oil only	0	0.1	0.35	1.0	1.6
Sorbitan Fatty Acid Esters[a]					
Sorbitan trioleate	1.8	...	0.8	1.9	2.7
Sorbitan monooleate	4.3	...	0.9	2.1	4.0
Sorbitan monostearate	4.7	...	0.3	1.2	2.5
Sorbitan monopalmitate	6.7	...	0.6	1.7	2.7
Sorbitan monolaurate	8.6	...	1.5	2.1	3.6
Polyoxyethylene Sorbitan Fatty Acid Ester-Ethers[b]					
POES monostearate	9.6	...	1.5	2.9	4.6
POES monooleate	10.0	...	0.7	2.1	...
POES tristearate	10.5	...	1.5	2.7	3.6
POES trioleate	11.0	...	0.8	2.4	3.4
POES monolaurate	13.3	...	2.4	4.1	5.1
POES monostearate	14.9	...	1.5	3.3	3.7
POES monooleate	15.0	...	1.0	2.5	3.5
POES palmitate	15.6	...	1.2	2.4	3.5
POES monolaurate	16.7	...	1.2	2.5	3.6
Polyoxyethylene Fatty Acid Esters[c]					
POE (8) stearate	11.1	1.7	6.0	9.5	...
POE (40) stearate	16.9	1.0	1.5	7.4	...
POE (50) stearate	17.9	1.1	1.7	8.1	...
Polyoxyethylene Fatty Ethers[d]					
POE (4) lauryl ether	9.7	1.7	2.6	9.8	...
POE (23) lauryl ether	16.9	...	1.8	6.6	...
POE (2) cetyl ether	5.3	0.3	0.6	1.3	...
POE (10) cetyl ether	12.9	1.1	2.0	7.8	...
POE (20) cetyl ether	15.7	0.8	0.9	2.6	...
POE (2) stearyl ether	4.9	0.4	0.6	1.1	...
POE (10) stearyl ether	12.4	1.2	2.1	7.4	...
POE (20) stearyl ether	15.3	0.5	1.0	2.1	...
POE (2) oleyl ether	4.9	0.9	1.7	5.2	...
POE (10) oleyl ether	12.4	1.2	2.2	8.1	...
POE (20) oleyl ether	15.3	0.7	1.5	5.6	...

[a] Marketed as Span 85, 80, 60, 40, and 20, respectively; [b] marketed as Tween 61, 81, 65, 85, 21, 60, 80, 40, 20, respectively; [c] marketed as Myrj 45, 52, and 53, respectively; [d] marketed as Brij 30, 35, 52, 56, 58, 72, 76, 78, 92, 96, and 98 respectively, by Atlas Chemical Industries, Wilmington, Del.

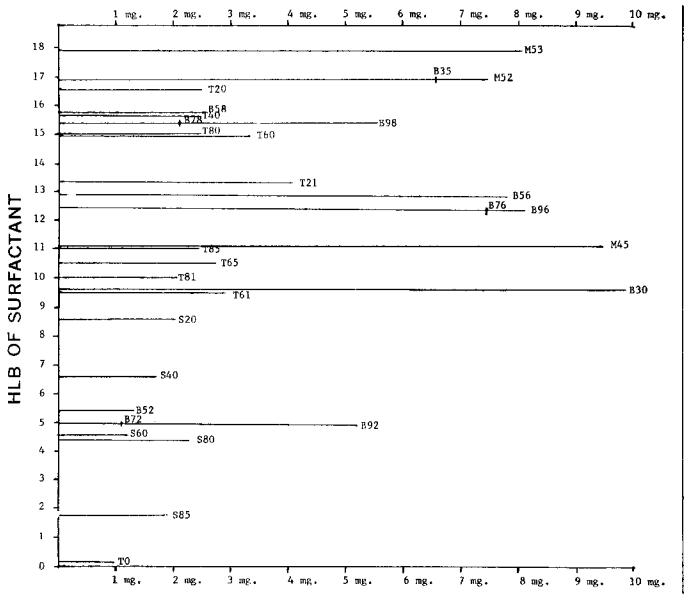


Fig. 1—Milligrams aminophylline dialyzed after 1 hr. Key: B, polyoxyethylene fatty ethers; M, polyoxyethylene fatty acid esters; S, sorbitan fatty acid esters; T, polyoxyethylene sorbitan fatty acid ester ethers; TO, theobroma oil.

interference with dialysis suggests that it was more than simply a matter of raised melting point.

Effect of HLB—To determine the effects of some nonionic surfactants in theobroma oil on the rate of release and dialysis, the various surfactants were incorporated as previously described. Table III shows the surfactants, grouped by chemical composition and type, which were added, and the amount of aminophylline dialyzed. Figure 1, with the surfactants arranged in descending order of HLB values, shows the amount of aminophylline dialyzed. In every case more aminophylline was dialyzed from the surfactant-containing base. Relatively uniform and moderate increases were noted from surfactants having an HLB value of less than 11, except for polyoxyethylene oleyl ether and polyoxyethylene lauryl ether.

Maximum drug was dialyzed from bases containing surfactants with HLB values of 11 to 14. Some surfactants with high HLB values (16–18) also released large amounts of drug. This agrees, in general, with other reports (9, 10, 12, 14, 19). Since the required HLB value of theobroma oil for an o/w emulsion is approximately 14 (20), it is to be expected that a surfactant with an HLB of about 14 would be most effective in emulsifying the theobroma oil, greatly increasing the oil–water interface.

Effect of Chemical Composition and Type—A comparison of those surfactants containing the C_{18} saturated moiety (stearic acid ester or stearyl alcohol ether) reveals that the polyoxyethylene fatty acid esters consistently dialyzed more aminophylline than the corresponding polyoxyethylene fatty ethers, sorbitan monostearate, or polysorbate 60, 61, or 65. As a group, the polyoxyethylene fatty acid esters gave best release even though their HLB values were not at 14. Part of the difference in amount dialyzed is undoubtedly due to HLB differences but apparently the ester linkage is superior to the ether linkage. Since stearate is the only radical common to all groups of surfactants, other groupings cannot be compared as completely.

A comparison of laurate or lauryl alcohol ether and oleate or oleyl alcohol ether reveals that the ethers were far superior to the sorbitans or polysorbates (esters or ester-ethers). The presence of sorbitan in the molecule consistently resulted in poor dialysis results. Under the conditions of the test (suppository bases emulsified) it was not possible to determine whether the poor results were due to slow release of the aminophylline from the suppository or whether it was released but not dialyzed. It is probable that the large sorbitan molecule hindered passage of the drug through the membrane.

Examination of the results from the surfactants containing fatty alcohols with equivalent-length polyoxyethylene chains and equivalent HLB values shows that the stearyl alcohol ethers dialyzed the released aminophylline most slowly, followed by cetyl, oleyl, and lauryl alcohol ethers, respectively. The melting range of each suppository base was determined by the U.S.P. XVI method for class II substances (21). All melting ranges were between 32–34°, well below the test temperature, with no consistent pattern due to surfactant. It appears from the results that the more saturated hydrocarbons with higher melting points inhibit the dialysis of aminophylline either by slightly raising the melting point or possibly by complexing with the drug. The former is in general agreement with the finding of Eckert and Muhlemann (15), who reported that at temperatures above the clear melting point of suppositories the rate of release increases enormously for water-soluble drugs, and the rate of diffusion reaches the values of a corresponding aqueous solution.

The increased rate of dialysis of aminophylline from theobroma oil suppositories to which the surfactants were added was probably due to several factors. Apparently the surfactants, acting as solubilizing agents, increased the solubility of aminophylline in water, or they increased the rate of solution by emulsifying the base, thereby increasing the

oil–water interface. It is also possible that they affected the dialyzing membrane, rendering it more permeable to the aminophylline.

It is probable that an intermolecular reaction or binding occurred between the aminophylline and the surfactant or that the drug was trapped within the micelles, especially those containing the sorbitan moiety. The evident difference in amounts dialyzed with those surfactants containing sorbitan indicates a slowdown, which is probably due to the formation of a large, slowly diffusible and dialyzable particle. Recently Kakemi *et al.* (22) have reported reduced absorption of several sulfonamides when nonionic surfactants were added to aqueous solutions of the sulfonamides. They theorized that the sulfonamide was trapped within the micelles of the surfactant and the large molecule passed slowly through the rectal membrane.

Ephedrine and Ephedrine Hydrochloride—Table IV summarizes the milligrams of both ephedrine and ephedrine hydrochloride dialyzed after 30 min. and 1 hr. For ephedrine hydrochloride, equilibrium was approached after 1 hr. For ephedrine, equilibrium was not reached after 24 hr. for all bases. For comparative purposes, the dialysis of the drugs from theobroma oil with no surfactant is shown at the top of the table.

Ephedrine Hydrochloride—Figure 2, with the surfactants arranged in descending order of HLB values, shows the amount of ephedrine hydrochloride dialyzed. The moderate increases noted were much less than the increases reported for aminophylline.

TABLE IV—EPHEDRINE AND EPHEDRINE HYDROCHLORIDE DIALYZED FROM THEOBROMA OIL CONTAINING 5% NONIONIC SURFACTANTS

Surfactant Chemical Type or Compn.[a]	HLB	Time, min. 30 Ephedrine HCl	60 Ephedrine HCl	30 Ephedrine	60 Ephedrine
		Drug Dialyzed, mg.			
Theobroma Oil	0	25	31	9	14
Sorbitan Fatty Acid Esters					
Sorbitan trioleate	1.8	32	35	9	13
Sorbitan monooleate	4.3	26	31	6	9
Sorbitan monostearate	4.7	15	27	5	8
Sorbitan monopalmitate	6.7	18	25	6	10
Sorbitan monolaurate	8.6	..	..	..	..
Polyoxyethylene Sorbitan Fatty Acid Ester-Ethers					
POES monostearate	9.6	35	41	12	17
POES monooleate	10.0	33	37	9	14
POES tristearate	10.5	29	35	7	11
POES trioleate	11.0	29	32	7	12
POES monolaurate	13.3	35	40	12	18
POES monostearate	14.9	36	42	13	18
POES monooleate	15.0	33	42	17	21
POES palmitate	15.6	22	27	17	23
POES monolaurate	16.7	37	41	14	19
Polyoxyethylene Fatty Acid Esters					
POE (8) stearate	11.1	41	42	10	15
POE (40) stearate	16.9	36	40	14	20
POE (50) stearate	17.9	32	35	14	20
Polyoxyethylene Fatty Ethers					
POE (4) lauryl ether	9.7	40	43	8	13
POE (23) lauryl ether	16.9	32	34	12	17
POE (2) cetyl ether	5.3	30	33	8	12
POE (10) cetyl ether	12.9	39	41	10	17
POE (20) cetyl ether	15.7	33	37	13	18
POE (2) stearyl ether	4.9	31	35	8	10
POE (10) stearyl ether	12.4	37	42	13	19
POE (20) stearyl ether	15.3	33	39	12	17
POE (2) oleyl ether	4.9	27	30	8	12
POE (10) oleyl ether	12.4	39	41	10	17
POE (20) oleyl ether	15.3	33	39	13	19

[a] See footnotes to Table III.

Those surfactants having an HLB value of less than 9 had little effect, some slightly decreased the amount dialyzed. It is possible that the surfactant complexed or reacted with the drug or trapped the drug within the micelles, but the evidence is not conclusive as three of the surfactants, sorbitan monopalmitate and sorbitan monostearate, and POE (2) oleyl ether decreased dialysis and four, sorbitan monooleate and sorbitan trioleate, and POE (2) cetyl ether and POE (2) stearyl ether, had no effect or slightly increased the amount dialyzed. Above an HLB value of 9, the surfactants, with the exception of polysorbate 40, increased slightly the amount dialyzed. The increases were probably due to either an increased solubility of ephedrine hydrochloride or, more probably, to an increased rate of solution of the salt due to the formation of an o/w emulsion, thus increasing the oil–water interface. Below an HLB value of 9, a w/o emulsion formed in which the dissolved or released drug remained inside the oil (external phase).

Ephedrine Alkaloid—Figure 3 shows the amount of ephedrine dialyzed. As expected, due to its more lipid solubility, much less alkaloid was dialyzed than the corresponding hydrochloride salt. Much the same general pattern of dialysis was noted as with ephedrine hydrochloride, with several exceptions. Maximum ephedrine dialyzed from the polysorbate 40-containing base but less ephedrine hydrochloride dialyzed than from theobroma oil alone. With the bases containing polysorbate 60 and POE (4) lauryl ether maximum amounts of ephedrine hydrochloride were dialyzed but essentially no increase was noted with ephedrine. Other minor discrepancies are noted. All surfactant-containing bases with HLB values of less than 9 dialyzed less ephedrine than the base composed only of theobroma oil.

Comparing the dialysis of aminophylline with the dialysis of ephedrine and ephedrine hydrochloride reveals little pattern of consistency. Those bases containing surfactants with HLB values of less than 9 had little effect on the rate of dialysis but surfactants with HLB values of greater than 9 had large increases in the amount dialyzed. This lack of consistency re-emphasizes the necessity for determining the rate of release of each individual drug in each base.

SUMMARY AND CONCLUSIONS

1. The rate of dialysis of aminophylline from various types of suppository bases was determined. Wide variations were found in the amount of aminophylline dialyzed.

2. The effect of the 28 nonionic surfactants added to theobroma oil was variable, although all increased the amount of aminophylline dialyzed. In general, surfactants with low HLB values afforded least increase, and surfactants with HLB values of 11 to 14 showed greatest increase.

3. Those surfactants containing sorbitan in the molecule dialyzed lesser amounts than corresponding surfactants without sorbitan (polyoxyethylene fatty acid esters and polyoxyethylene fatty ethers).

4. It is not clear what effect, if any, the type linkage of the surfactant (*e.g.*, ether, ester, *etc.*) has on the rate of release.

5. It appears that the degree of saturation of the

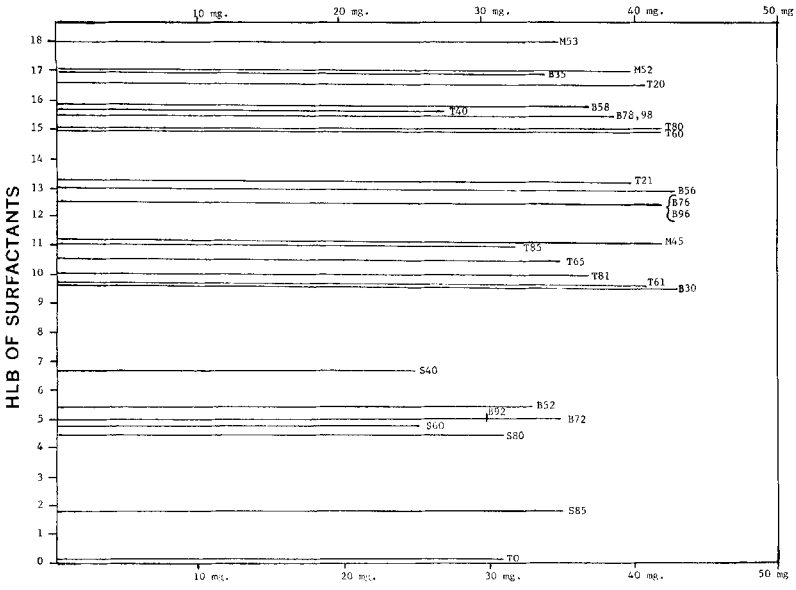


Fig. 2—Milligrams of ephedrine HCl dialyzed after 1 hr. Key same as in Fig. 1.

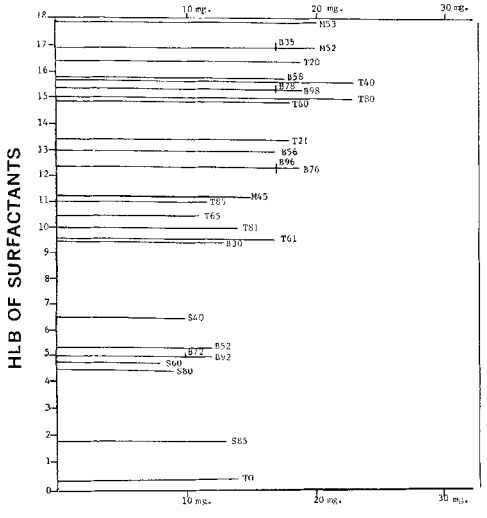


Fig. 3—Milligrams of ephedrine dialyzed after 1 hr. Key same as in Fig. 1.

surfactant, which should affect the melting characteristics of the suppository, has an effect.

6. The rate of dialysis of ephedrine and ephedrine hydrochloride from theobroma oil suppositories containing nonionic surfactants was also determined.

7. For ephedrine hydrochloride no consistent pattern was noted for surfactants having an HLB value of less than 9. Above an HLB value of 9, the surfactants increased moderately the amount of ephedrine hydrochloride dialyzed, but the increases were much less than noted with aminophylline.

8. For ephedrine alkaloid, essentially the same pattern of behavior was noted except that much less drug was liberated and dialyzed in the same period of time. For surfactants with an HLB value of less than 9 there was a decrease in the amount dialyzed.

9. The rate of release of drugs from suppositories containing nonionic surfactants remains unpredictable and should be determined for each drug in each base.

10. This study utilizing a relatively large number of nonionic surfactants points to the possibility of error in using only a small number of surfactants and drawing widespread conclusions.

REFERENCES

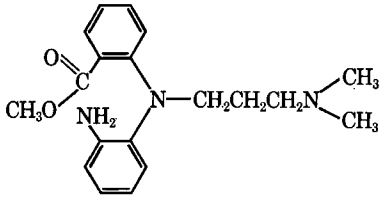
(1) Segal, M. S., Levinson, L., Bresnick, E., and Blakey, J., *J. Clin. Invest.*, **28**, 1190(1949).
(2) Waxler, S. H., and Schack, J. A., *J. Am. Med. Assoc.*, **143**, 736(1950).
(3) Truitt, E. B., McKusick, V. A., and Krantz, J. C., *J. Pharmacol. Exptl. Therap.*, **100**, 309(1950).
(4) Glass, G. B., Barowshy, H., Boyd, L. J., Rich, M., and Ebin, L., *Am. J. Med. Sci.*, **231**, 51(1956).
(5) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C., *J. Pharmacol. Exptl. Therap.*, **120**, 528(1957).
(6) Riegelman, S., and Crowell, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 115, 123, 127(1958).
(7) Gross, H. M., and Becker, C. H., *ibid.*, **42**, 96(1953).
(8) Peterson, C. F., Lee, C. O., and Christian, J. E., *ibid.*, **42**, 731(1953).
(9) Whitworth, C. W., and LaRocca, J. P., *ibid.*, **48**, 353 (1959).
(10) Spittle, R. Y., and Hartman, C. W., *ibid.*, **49**, 325 (1960).
(11) Allawalla, N. A., and Riegelman, S., *ibid.*, **42**, 267 (1953).
(12) Gross, H. M., and Becker, C. H., *ibid.*, **42**, 498(1953).
(13) Peterson, C. F., and Guida, A. J., *ibid.*, **42**, 537(1953).
(14) Ryne, J. W., Payne, W. J., and Hartman, C. W., *ibid.*, **49**, 234(1960).
(15) Eckert, V., and Muhlemann, H., *Pharm. Acta Helv.*, **33**, 649(1958).
(16) Pennati, L., and Steiger-Trippi, K., *ibid.*, **33**, 663 (1958).
(17) Muhlemann, H., and Neuenschwander, R. H., *ibid.*, **31**, 305(1956).
(18) Muhlemann, H., and Graffenried, D. U., *ibid.*, **36**, 186 (1961).
(19) Fincher, J. H., Entrekin, D. N., and Hartman, C. W., *J. Pharm. Sci.*, **55**, 23(1966).
(20) Abbott, W. K., Atlas Chemical Industries, Inc., Wilmington, Del., personal communication.
(21) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 925.
(22) Kakemi, K., Arita, T., and Muranishi, S., *Chem. Pharm. Bull. (Tokyo)*, **13**, 969, 976(1965).

Metabolism and Excretion of Methyl *N*-(*o*-Aminophenyl)-*N*-(3-dimethylaminopropyl) Anthranilate in the Dog and Human

By DAVID L. SMITH, ALBERT L. PULLIAM, and HOWARD KO

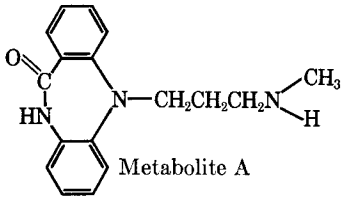
Four urinary metabolites of the title drug, excreted by both the dog and human, have been identified and quantitated. Metabolite A is 5-(3-methylaminopropyl)-5,10-dihydro-11H-dibenzo[*b*,*e*][1,4]diazepin-11-one, metabolite B is the methyl ester of *N*-(*o*-aminophenyl)-*N*-(3-methylaminopropyl)-anthranilic acid, metabolite C is 5-(3-dimethylaminopropyl)-5,10-dihydro-11H-dibenzo[*b*,*e*][1,4]diazepin-11-one, and metabolite D is 5-(3-aminopropyl)-5,10-dihydro-11H-dibenzo[*b*,*e*][1,4]diazepin-11-one. At least 46.3 ± 4.4 per cent (S.E.M.) of the dose was absorbed in the human, 8.6 ± 1.5 per cent was excreted as metabolite A, 30.5 ± 3.4 per cent as metabolite B, 2.4 ± 0.2 per cent as metabolite C, less than 0.2 per cent as metabolite D, and 4.8 ± 0.7 per cent as unchanged drug. The kinetics of excretion of the drug and metabolites indicates that absorption and metabolism take place rapidly. In the human, the average (±S.E.M.) half-lives of excretion of drug and metabolites A, B, and C were 3.0 ± 0.2, 6.5 ± 0.6, 6.2 ± 0.7, and 7.7 ± 1.2 hr., respectively. A comparison of urinary excretion after 10-mg./Kg. oral and intravenous doses of the drug to the same dog indicates efficient absorption of the oral dose. In the dog, the half-lives (±S.D.) for urinary excretion of the drug and metabolites A, B, and C were 5.5 ± 0.8, 6.6 ± 0.5, 4.3 ± 0.5, and 7.2 ± 0.6 hr., respectively. Biliary excretion of the drug and/or metabolites or the presence of undetected metabolites may be indicated from the low recovery after intravenous administration.

THIS STUDY was undertaken to determine the absorption, half-life, and metabolic fate of methyl *N*-(*o*-aminophenyl)-*N*-(3-dimethylaminopropyl) anthranilate (I), an orally active diuretic agent in the rat and dog (1). This report describes the quantitative determination of the drug and its metabolites in dog and human urine samples. Qualitative aspects of the metabolism of this drug in the dog and human have already been reported (2).

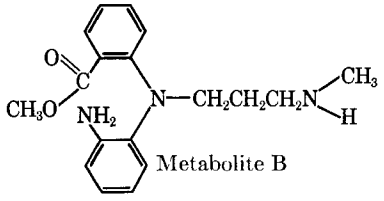


I

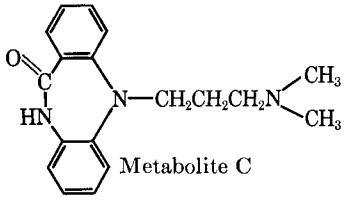
Thin-layer chromatography of chloroform extracts of dog and human urine revealed a zone corresponding to unchanged drug and four other drug-related zones (designated metabolites A, B, C, and D). Thin-layer chromatography in conjunction with the bromcresol purple procedure of Woods *et al.* (3) was used to determine individually the drug and three of its metabolites. Two of these metabolites (A and C) were synthesized to yield samples for analytical calibration purposes. Since standards of metabolites B and D were not available for calibration purposes, the calibration curves for the drug and metabolites A and C were averaged and used for these metabolites.



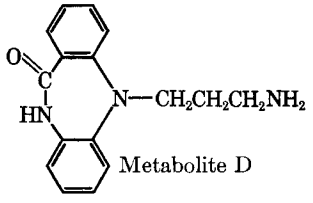
Metabolite A



Metabolite B



Metabolite C



Metabolite D

Received January 19, 1967, from The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication March 14, 1967.

The assistance of Dr. E. M. Schneider and Dr. J. G. Wagner with the design and execution of the human studies, of Dr. G. A. Elliott and Dr. F. S. Eberts with the dog studies, and of Mr. J. I. Northam with the statistical analyses is gratefully acknowledged.

TABLE I—THIN-LAYER CHROMATOGRAPHIC R_f VALUES[a]

Compd.	System A	System B	System C
Drug	0.61	0.96	0.20
Metabolite A	0.24	0.48	0.02
Metabolite B	0.30	0.79	0.04
Metabolite C	0.49	0.88	0.20
Metabolite D	0.24	0.48	0.45

[a] The zones used for analysis are underlined.

EXPERIMENTAL

Materials—Bromcresol purple (5′,5″-dibromo-*o*-cresolsulfonphthalein indicator) was obtained from Eastman Organic Chemicals. The bromcresol purple reagent was prepared by dissolving 50 mg. of the solid in 100 ml. of analytical reagent grade $CHCl_3$. The synthesis of the drug and metabolite C have been described previously (4). Metabolite A was prepared by Dr. R. S. P. Hsi, The Upjohn Co., using the procedures described by Hanze *et al.* (4).

Thin-Layer Chromatography (TLC)—Silica Gel G plates of 250 μ thickness, impregnated with $ZnSiO_3$ and $(ZnCd)_2S$ phosphors, were employed with the following three solvent systems: system *A*, C_6H_6:CH_3OH:NH_4OH (50:50:1); system *B*, EtOAc:DMF:NH_4OH (50:50:5); system *C*, CH_3OH:$(CH_3)_2C{=}O$:C_6H_6:7 *M* NaOH (50:50:10:1). The zones were located with a mercury lamp (254 mμ). System *A* was used for the isolation of I and metabolite C, system *B* for metabolites A and B, and system *C* for metabolite D. The R_f values are presented in Table I.

Procedure for Determination of Drug and Metabolites—To 5.0 ml. of urine or serum in a 35-ml. centrifuge tube was added 3.0 ml. of pH 10 buffer (20.0 Gm./L. Na_2CO_3 + 12.4 Gm./L. $Na_2B_4O_7$), followed by 25.0 ml. of $CHCl_3$. The mixture was shaken vigorously. After 30 min. of standing at room temperature, the phases were separated by centrifugation (2000 r.p.m. for 10 min.) and the aqueous layer removed by aspiration. A 20.0-ml. aliquot of the $CHCl_3$ was transferred to a 50-ml. open-mouth centrifuge tube and evaporated to dryness under a stream of nitrogen. The tube was washed down successively with 5, 3, and 1 ml. of $CHCl_3$, and evaporated to dryness each time. The residue was dissolved in a few drops of $CHCl_3$, and quantitatively transferred to a thin-layer plate. Standards were spotted and the plate was developed with the appropriate system (Table I). The drug and metabolite zones were individually scraped from the chromatogram and transferred to 15-ml. centrifuge tubes. Five milliliters of pH 10 buffer was added to each tube and, after shaking, the mixtures were centrifuged. Four-milliliter aliquots of the supernates were transferred to another set of 15-ml. centrifuge tubes, 5.0 ml. of $CHCl_3$ added, and the mixtures were shaken vigorously and centrifuged at 2000 r.p.m. for 5 min. The aqueous phases were aspirated and 3.0 ml. of the $CHCl_3$ phases transferred to 10-ml. volumetric flasks containing 0.3 ml. of bromcresol purple reagent. The absorbances were measured at 410 mμ with a Cary spectrophotometer, employing $CHCl_3$ in the reference cell. To correct for background, a zero-hour urine sample was subjected to the same procedure. The concentrations of drug and metabolites were calculated from calibration curves having the following slopes (mcg. drug equivalents/ml./absorbance unit): drug, 32.9; metabolite A, 30.7; metabolite C, 28.1. Since samples of metabolites B and D were not available for calibration, an estimated slope of 30.0 was used.

Dog Studies—To test the extent of absorption of the drug, dog *1* (10.0-Kg. female) was given a 105-mg.[1] oral dose on day 1, no drug on days 2–7, and a 105-mg. intravenous dose on day 8. To test the effect of pretreatment, dog *2* (10.5-Kg. male) was given a 50-mg. oral dose on both days 1 and 2 and dog *3* (9.5-Kg. male) was given a 50-mg. oral dose on day 1, and a 250-mg. oral dose on day 2. In the case of dog *1*, a single 0–24-hr. urine sample was collected and 10 ml. of blood was withdrawn at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 hr. after each dose. In the case of dogs *2* and *3*, urine was collected quantitatively during the intervals 0–1, 1–3, 3–5, 5–8, 8–12, 2–16, 16–24, 24–25, 25–27, 27–32, 32–36, 36–40, 40–49, 49–56, 56–74, and 74–80 hr. after the first dose of drug.

Human Studies—A single dose of 350 mg.[1] of the drug was administered to each of five male volunteer subjects after an overnight fast. Food was allowed 1 hr. after drug administration. Water intake was limited to 8 fl. oz. at the following times relative to the time of dosing: −1, 0, 1.5, 4, 6.5, 10, 14, 23, 24, 25.5, 28, and 30.5 hr. Urine was collected quantitatively during the intervals 0–1, 1–3, 3–5, 5–8, 8–12, 12–16, and 16–32 hr. after drug administration. Blood (10–12 ml.) was withdrawn from each subject at 0, 0.5, 1.5, 4, 6.5, 10, 14, and 24 hr.

RESULTS AND DISCUSSION

Identification of Metabolites—The isolation procedure developed for the drug employed chloroform extraction of pH 10 serum or urine followed by thin-layer chromatography (TLC). When this method was subsequently applied to the determination of the drug in dog and human urine samples, TLC revealed not only a zone corresponding to drug but also at least two other drug-related zones. Two additional TLC solvent systems (Table I) revealed that four metabolites were actually present. The metabolites were isolated from the urine and identified (2) as metabolites A, B, C, and D.

Both the dog and human convert the drug to these metabolites, but, as described below, the relative amounts and rates of excretion of the metabolites differed in the two species.

Quantitation of Drug and Metabolites—The drug and metabolites could be quantitatively extracted from pH 10 serum or urine with 5 vol. of chloroform. The diazotized drug did not react with *N*-1-naphthylethylenediamine, and since its spectral properties were not suitable for direct spectrophotometric determinations at microgram levels in serum or urine, the bromcresol purple procedure of Woods *et al.* (3) was investigated. Both the drug and its metabolites responded in this sensitive procedure. Thin-layer chromatography

[1] In all cases the drug was administered as the dihydrochloride.

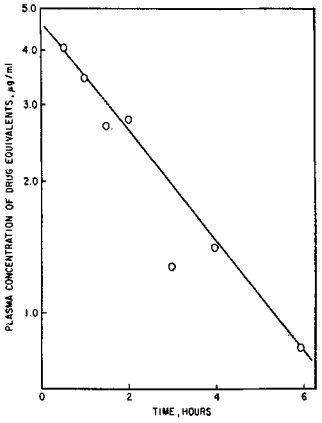


Fig. 1—Plasma levels of drug equivalents after a 10-mg./Kg. intravenous dose to dog 1.

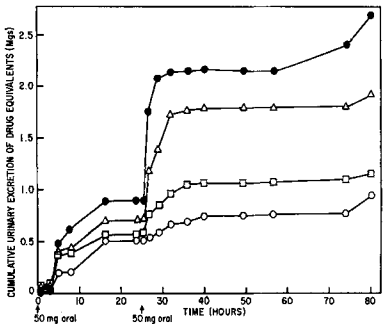


Fig. 2—Cumulative excretion of drug and metabolites after two 50-mg. oral doses to dog 2. *Key: ●, drug; ○, metabolite A; △, metabolite B; □, metabolite C.*

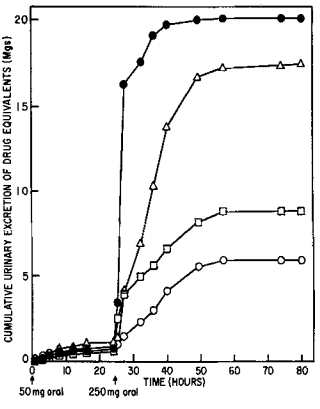


Fig. 3—Cumulative excretion of drug and metabolites after 50- and 250-mg. oral doses to dog 3. *Key the same as Fig. 2.*

was added to the procedure, since the bromcresol purple procedure lacks specificity. The final procedure, therefore, included the following steps. The serum or urine was adjusted to pH 10 and extracted with chloroform. A measured volume of the chloroform extract was evaporated to a smaller volume and quantitatively transferred to a silica gel thin-layer plate. The compounds were eluted from the silica gel support with pH 10 buffer, which was then extracted with chloroform to yield solutions for colorimetric measurement. Three thin-layer systems were required to resolve completely the drug and its four metabolites.

Serum Levels—*Dog Studies*—At a dose of 10 mg./Kg. to the dog, the serum levels after both oral and intravenous administration approached the lower limits of the analytical method; consequently, the half-life for the disappearance of drug-related material from the serum can only be estimated. Because of the extremely low serum concentrations, no attempt was made to quantitate the drug and metabolites individually. After the intravenous dose, the drug-related material disappears from the serum with a half-life of about 3 hr. (Fig. 1). Although the serum concentrations after oral administration were of the same order of magnitude as those after intravenous administration, the points were too scattered to yield a half-life estimate.

Human Studies—The concentrations of the drug and metabolites in the human serum samples were below detectable limits (< 1 mcg./ml.). Since an intravenous dose of 10 mg./Kg. of the drug to the dog had given only barely detectable serum levels, the observation of low serum levels in man was not surprising, and certainly did not prove poor absorption.

Urinary Excretion of Drug and Metabolites—*Dog Studies*—The distribution of drug and metabolites A, B, and C and the per cent of the dose recovered in the urine of each dog are presented in Table II. Since metabolite D was present only in trace amounts, no attempt was made to quantitate it.

The cumulative urinary excretion of drug and metabolites in the dogs which received the drug on 2 successive days is presented in Figs. 2 and 3.

TABLE II—URINARY EXCRETION OF DRUG AND METABOLITES IN THE DOG

Treatment Day	Dog	Rt.	Dose, mg.	% of Dose Excreted: Metabolite A[a]	Metabolite B	Metabolite C	Drug	Total
1	*1*	Oral	105	2.51	5.50	2.94	4.51	15.46
1[b]	*1*	i.v.	105	3.67	6.62	3.51	5.53	19.33
1	*2*	Oral	50	1.02	1.40	1.10	1.76	5.28
2[c]	*2*	Oral	50	0.86	2.42	1.18	3.58	8.04
1	*3*	Oral	50	1.72	2.18	1.10	1.60	6.60
2[c]	*3*	Oral	250	2.05	6.56	3.32	8.16	20.09

[a] Includes a small amount of metabolite D (<10% of the values given). [b] Received the 105-mg. oral dose 1 week prior to the i.v. dose. [c] Received the 50-mg. oral dose on the previous day.

TABLE III—CUMULATIVE EXCRETION OF DRUG AND METABOLITES IN THE HUMAN (DOSE = 350 mg.)

Subject	Time, hr.	Drug Equivalents Excreted, mg. Metab. A[a]	Metab. B	Metab. C	Drug	Total
1	1	1.5	6.7	0.6	2.1	10.9
	3	11.0	29.9	2.8	9.5	53.2
	5	17.9	48.0	4.3	13.2	83.4
	8	26.1	71.5	5.7	15.4	118.7
	12	34.1	103.7	7.4	17.3	162.6
	16	35.1	107.9	7.7	17.4	168.1
	32	42.3	129.3	9.8	18.5	199.9
2	1	0.2	1.6	0.1	0.6	2.5
	3	3.0	17.6	1.7	9.9	32.2
	5	5.3	27.7	2.9	14.7	50.5
	8	8.0	41.3	3.9	19.4	72.6
	12	12.0	54.9	5.5	22.1	94.5
	16	14.1	60.4	6.2	22.7	103.4
	32	18.9	76.7	8.3	23.8	127.7
3	1	1.0	7.2	1.0	2.5	11.7
	3	4.5	27.3	3.0	7.3	42.1
	5	9.2	41.7	4.5	10.5	65.9
	8	12.2	58.9	6.3	13.1	90.6
	12	17.1	72.8	8.1	14.3	112.3
	16	18.0	76.1	9.2	14.8	118.0
	32	21.8	85.6	11.1	15.4	133.9
4	1	0.4	2.0	0.1	0.5	2.9
	3	0.4	2.0	0.1	0.5	2.9
	5	7.7	32.7	1.4	6.1	47.9
	8	21.1	50.8	2.2	7.3	81.4
	12	27.2	74.4	3.1	8.2	112.9
	16	30.4	96.1	3.8	8.6	138.9
	32	42.4	138.3	6.6	9.4	196.8
5	1	0.5	4.1	0.3	1.1	6.0
	3	3.9	27.9	1.5	7.8	41.1
	5	7.8	41.9	2.5	11.3	63.4
	8	14.3	66.2	3.6	14.4	98.5
	12	18.8	85.3	4.8	15.4	124.3
	16	21.5	93.4	5.4	15.7	135.9
	32	25.3	103.6	6.6	16.0	151.6

[a] Includes a small amount of metabolite D (<2% of the values given).

The urinary excretion of the first dose seems to be essentially complete prior to the administration of the second one. In the dog which received the two 50-mg. doses, the urinary excretion of drug-related material was greater after the second dose than after the first. Also, in the dog which received the 50-mg. dose on the first day and the 250-mg. dose on the second day, the urinary excretion of drug and metabolites after the second dose—both individually and collectively—was greater than five times the excretion after the first dose. Therefore, in both dogs that received doses on successive days, the percentage of the first dose excreted in the urine was smaller than that of the second. These data illustrate the importance of crossover experiments when comparing different formulations of the same drug.

The intact drug accounts for a greater percentage of the total recovered in the urine on the second day of treatment than on the first, while metabolite A accounts for a much smaller percentage of the total on the second day. These data indicate either a decrease in metabolism or a change in excretion pathways with chronic administration, *e.g.*, the ratio of biliary to urinary excretion of metabolite A may vary with chronic administration in a different manner from that of the intact drug.

The distribution of drug and metabolites in the urine was essentially identical after oral and intravenous administration. An estimate of the oral efficiency can be obtained by comparing the total urinary excretion of drug and metabolites after

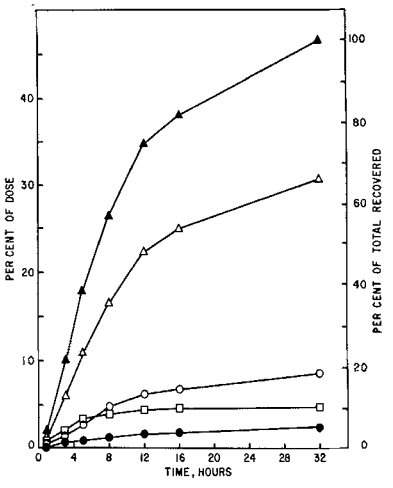


Fig. 4—Average cumulative excretion of drug and metabolites in five humans. Key: ▲, total excretion; ○, metabolite A; △, metabolite B; ●, metabolite C; □, drug.

oral and intravenous administration of the same dose. When the oral and intravenous doses were given on successive weeks to the same dog, the 24-hr. urinary excretion after the oral dose (first week) was 80% of the 24-hr. excretion after the intravenous dose. Assuming the first dose had no effect on the amount excreted after the second dose, the oral efficiency in this dog was about 80%. Actually, it is impossible to determine from these data whether the oral dose had any effect on the amount excreted in the urine after the intravenous dose 1 week later. However, since pretreatment tends to increase the percentage excreted in the urine, the 80% value is very likely a minimum estimate.

Not only does the percentage of the dose excreted in the urine increase with repeated administration, it also increases with increasing dose. The dogs given 50-mg. oral dose excreted an average of 6% of the dose in the urine, while the dog given the 105-mg. oral dose excreted 15.5%. These data support hypotheses such as (*a*) sufficiently high concentrations of the drug or its metabolites may inhibit nonurinary (*e.g.*, biliary) excretion, (*b*) saturation of binding sites of drug and metabolites, or (*c*) stimulation of urinary transport mechanisms.

The low recovery of the drug-related material in the urine after intravenous administration probably results from extensive biliary excretion. Other possibilities, considered to be less likely, include urinary excretion of undetected metabolites or binding of drug and/or metabolites by body constituents.

Human Studies—The cumulative excretion data for the drug and metabolites A, B, and C for each of the five subjects are presented in Table III. The average cumulative excretion is plotted in Fig. 4. Since the cumulative levels of metabolites do not appear to have reached their asymptotic values, excretion may not be complete in 32 hr. The average cumulative urinary excretion of intact drug and metabolites at each time interval, expressed as per cent of dose, is presented in Table IV. The distribution of drug and metabolites in the 0–32 hr. urine of each subject is presented in Table V.

Of the 350-mg. dose, at least 162.0 ± 15.3 mg. (S.E.M.), *i.e.*, 46.3 ± 4.4% (range: 36.5–57.1%), was absorbed. Expressed as drug equivalents, an average (±S.E.M.) of 30.2 ± 5.1 mg. was excreted as metabolite A, 106.7 ± 11.9 mg. as metabolite B, 8.5 ± 0.9 mg. as metabolite C, and 16.7 ± 2.3 mg. as unchanged drug. The percentage of drug-related compounds in each urine collection present as unchanged drug decreases from about 20% in the 0–1-hr. sample to about 3% in the 16–32-hr. sample, whereas beyond 1 hr., the percentage of the total as each metabolite increases only slightly.

Rate of Excretion of Drug and Metabolites—*Dog Studies*—The cumulative excretion data were used to estimate the over-all half-lives for the appearance in the urine of the drug and its metabolites. The half-lives were estimated by plotting the data in first-order fashion (Fig. 5). In the case

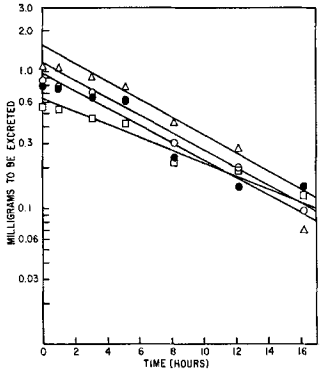


Fig. 5—First-order plots of drug and metabolite excretion after a 50-mg. oral dose to dog 3. Key the same as Fig. 2.

TABLE IV—AVERAGE CUMULATIVE URINARY EXCRETION OF DRUG AND ITS METABOLITE IN FIVE HUMANS

Time, hr.	% of Dose				
	Metabolite A	Metabolite B	Metabolite C	Drug	Total
1	0.20	1.23	0.12	0.39	1.94
3	1.31	5.97	0.52	2.00	9.80
5	2.74	10.97	0.89	3.19	17.79
8	4.67	16.51	1.23	3.98	26.39
12	6.24	22.35	1.65	4.42	34.66
16	6.80	24.79	1.85	4.52	37.96
32	8.62	30.48	2.42	4.75	46.27

TABLE V—DISTRIBUTION OF DRUG AND METABOLITES IN THE 0–32 hr. URINE OF EACH HUMAN (DOSE = 350 mg.)

Subject	mg. Drug Equivalents					% of Dose
	Metabolite A	Metabolite B	Metabolic C	Drug	Total	
1	42.3	129.3	9.8	18.5	199.9	57.1
2	19.0	76.7	8.3	23.9	127.9	36.5
3	21.8	85.6	11.1	15.5	134.0	38.3
4	42.4	138.2	6.6	9.4	196.6	56.2
5	25.3	103.6	6.6	16.1	151.6	43.3
Mean	30.2	106.7	8.5	16.7	162.0	46.3
S.E.M.	±5.1	±11.9	±0.9	±2.3	±15.3	±4.4

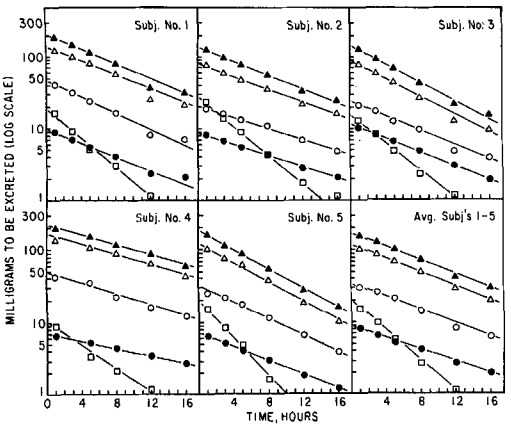


Fig. 6—First-order plots of drug and metabolite excretion after a 350-mg. oral dose of the drug (as the dihydrochloride) to five humans. Key same as Fig. 4.

TABLE VI—EXCRETION HALF-LIVES OF THE DRUG AND ITS METABOLITE IN EACH HUMAN

Subject	Half-Life, hr. Metabolite A	Metabolite B	Metabolite C	Drug
1	5.3	6.2	6.2	3.0
2	7.8	6.8	7.6	2.9
3	6.0	4.9	6.1	3.1
4	7.9	8.6	12.3	3.8
5	5.3	4.6	6.3	2.4
Mean	6.5	6.2	7.7	3.0
S.E.M.	0.6	0.7	1.2	0.2

of dog *3*, the half-lives (±S.D.) for the appearance in the urine of drug and metabolites A, B, and C after the first dose were 5.5 ± 0.8, 6.6 ± 0.5, 4.3 ± 0.5, and 7.2 ± 0.6 hr., respectively, and, after the second dose, 3.8 ± 0.4, 4.7 ± 0.5, 5.4 ± 0.6, and 4.6 ± 0.6 hr. The half-life for the appearance of total drug-related material was 5.0 ± 0.4 hr. after the first dose, and 5.3 ± 0.4 hr. after the second.

In the case of dog *2*, the scatter of the data does not justify the calculation of the individual half-lives. The appearance of total drug-related material in the urine after both doses also seemed to be consistent with a half-life of about 5 hr. Unlike that found in the human (*vide infra*), the rate constant for the appearance of drug in the urine is not significantly larger than for the metabolites. Therefore, the dog is probably not a satisfactory species for the study of the unusual excretion kinetics observed in the human.

Metabolite C was also administered to the dog. As expected, metabolites A and D were found in the urine. The average half-lives for the appearance of metabolites A, C, and D in the urine, after a single dose of metabolite C, were 4.1, 3.6, and 6.2 hr., respectively. These half-lives for metabolites A and C do not differ significantly from those obtained when I was administered, which indicates that, at least in the dog, the rate of excretion of these compounds is not limited by their rate of manufacture. Like the case of I, when the same dose of metabolite C was administered on 2 successive days to the same

TABLE VII—AVERAGE[a] FITTING PARAMETERS FOR URINARY EXCRETION OF DRUG AND ITS METABOLITES

Compd.	Method of Fitting[b]	Half-Life ($t_{1/2}$), hr.[c]	Total Excreted at t = ∞ hr. U_∞ (mg.)	Lag Time t_0 (hr.)
A	(*1*)	6.5	30.2	0
	(*2*)	9.1	33.7	0
	(*3*)	7.0	31.8	1.04
B	(*1*)	6.2	106.7	0
	(*2*)	8.2	116.3	0
	(*3*)	6.9	111.7	0.69
C	(*1*)	7.7	8.48	0
	(*2*)	8.7	9.2	0
	(*3*)	8.1	9.0	0.38
Drug	(*1*)	3.0	16.62	0
	(*2*)	3.6	16.9	0
	(*3*)	2.8	16.44	0.52
Total	(*1*)	6.1	162.0	0
	(*2*)	7.6	173.5	0
	(*3*)	6.3	167.2	0.73

[a] Average for five human adult males. [b] Methods: (*1*) fit of $U = U_{32}(1 - e^{-Kt})$; (*2*) fit of $U = U_\infty (1 - e^{-Kt})$; (*3*) fit of $U = U_\infty [1 - e^{-K(t-t_0)}]$ for $t > t_0$ hr. [c] $t_{1/2}$ (hr.) = $0.693/K$.

dog, the percentage of the second dose excreted in the urine was much more than twice that of the first dose. This was true even though cumulative urinary excretion plots indicated that excretion of the first dose was essentially complete prior to administration of the second dose.

Human Studies—As a first approximation, in order to estimate the excretion half-lives, the assumption was made that the amount excreted in 32 hr. was equal to the total amount excreted, *i.e.*, $U_{32} = U_\infty$ [method (*1*) of Table VII]. The data for the individual subjects and the average of the five subjects are plotted in first-order fashion in Fig. 6. It is apparent from these plots that first-order excretion kinetics are followed for both intact drug and metabolites. The excretion half-lives over the whole collection period, obtained from the slopes of Fig. 6, are presented in Table VI. The average half-lives (±S.E.M.) for drug and metabolites A, B, and C are 3.0 ± 0.2, 6.5 ± 0.6, 6.2 ± 0.7, and 7.7 ± 1.2 hr., respectively.

The half-lives for the appearance of drug and metabolites in the urine of the humans were also estimated by fitting the data by the method of least squares [methods (*2*) and (*3*) of Table VII] to the following first-order equations:

$$U = U_\infty [1 - e^{-Kt}] \qquad \text{(Eq. 1)}$$

$$U = U_\infty [1 - e^{-K(t-t_0)}] \qquad \text{(Eq. 2)}$$

where U is the milligrams of drug equivalents excreted to time t, U_∞ is the amount excreted at infinite time, K is the observed rate constant for excretion ($t_{1/2} = 0.693/K$), and t_0 in Eq. 2 is a "lag time" which corrects for delays caused by absorption, metabolism, and distribution. Employing these equations, the least squares fit of the data yields estimates of U_∞ as well as the half-life (Table VII). All of the average half-lives obtained from Eq. 2 are within 0.5 hr. of the values obtained from the slopes of the $U_{32} - U$ *versus* t plots of Fig. 6. The average half-lives indicate that the excretion of

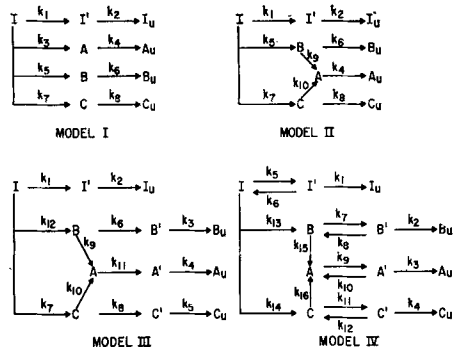


Fig. 7—Schematic models. Drug and metabolites in plasma: I, A, B, C; drug and metabolites in "compartment": I', A', B', C'; drug and metabolites excreted in urine: I_u, A_u, B_u, and C_u.

each of the metabolites is at least 95% complete in 32 hr., thus justifying the original approximation that $U_{32} = U_\infty$.

Rate of Absorption and Metabolism—Since simple first-order excretion appears to be followed for both drug and metabolites (Fig. 6) beginning with the first hour after administration, the drug which is absorbed by the human must be absorbed and metabolized very rapidly. In general, these data indicate that absorption and metabolism are so rapid that excretion of the metabolites is not limited by their rate of formation or by the rate of absorption of the drug.

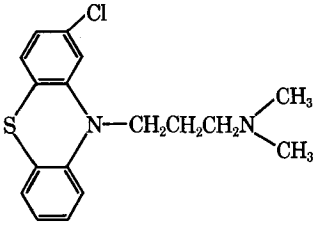
Kinetic Models for Urinary Excretion in the Human—An attempt was made to formulate a mathematical model or models consistent with all the available data. For simplicity, it was assumed that drug absorption had essentially ceased 1 hr. after oral administration and only the data after 1 hr. were considered.

The complexity of kinetic models and equations required to describe the metabolism and excretion of a drug is dependent upon the precision and discrimination of the data. In the present case, measurement by a nonspecific assay of total urinary drug and metabolites yields data which can be approximated by a single exponential function (plus a constant) and which suggest that a single rate constant is involved. However, the added information that three metabolites are formed and excreted at their individual rates requires a more complicated description.

The increasing complexity of mathematical models resulting from increasing discrimination of analytical results is demonstrated by the evolution of models I–IV (Fig. 7). In order to account for the observed urinary excretion, model I postulates that the rate of metabolism of the drug is much faster than the excretion rates of the drug and its metabolites, that each of the metabolites is formed *directly* from the drug (*i.e.*, metabolites B and C are not necessary intermediates in the formation of metabolite A), and that there is a compartment in which the drug is stored and no longer metabolized prior to excretion. Assumption of such a compartment would be consistent with the incomplete metabolism of the drug, even though its excretion rate is postulated to be relatively slow compared to its metabolism.

Once the chemical structures of the metabolites were known, however, model II was constructed to take into account the possibility that metabolites B and C may be chemical intermediates in the formation of metabolite A. In this model, the assumptions are made that the rate of metabolism of the drug is much faster than the excretion rates of the drug or its metabolites (B and C), or the formation rates of the metabolite A, that metabolite A is formed from B and C, and that there is a compartment in which the drug is no longer metabolized prior to excretion.

Since the drug and metabolites have similar chemical structures, it is likely that they enter into the same physiological compartments in which solubility probably plays the main selective role. Hence, in addition to the assumptions of model II, model III postulates a compartment in which the drug *and* its metabolites are no longer metabolized prior to excretion. Model IV is similar to model III except that equilibria are postulated. At present, model IV appears to be the most reasonable description of the chemical and physiological data but none of the models have been completely excluded. A mathematical discussion of these models will be the subject of a future communication.



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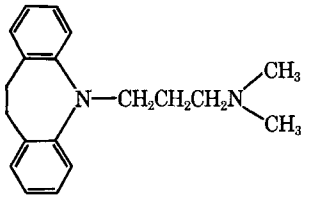

III

Metabolism of Structurally Related Drugs—The investigated drug, although noncyclic, has some structural similarity to the phenothiazine drugs *e.g.*, chlorpromazine (II) and imipramine (III). Both of these compounds are mono- and di-demethylated *in vivo* by several species (5).[2] However, both of these drugs are also ring-hydroxylated and then conjugated with glucuronic acid. Even after hydrolysis with β-glucuronidase, no evidence was found in the present study for ring-hydroxylated or conjugated metabolites. Although Fishman *et al.* (6) have reported the isolation of the *N*-oxides of chlorpromazine (0.7% of the dose) and imipramine (~2% of the dose), no evidence was found for *N*-oxide metabolites in the present study.

REFERENCES

(1) Graham, B. E., unpublished data.

[2] *Cf. Reference 5* for a review of phenothiazine metabolism. For imipramine, *cf.* Haflizer, F., and Burckhardt, V., in "Psychopharmacological Agents," vol. I, Gordon, M., ed. Academic Press Inc., New York, N. Y., 1964, chap. 3.

(2) Smith, D. L., and Grostic, M. F., *J. Med. Chem.*, presented to the Medicinal Division, American Chemical Society, September 1966, to be published.
(3) Woods, L. A., Cochin, J., Fornefeld, E. J., McMahon, F. G., and Seevers, M. H., *J. Pharmacol. Exptl. Therap.*, **101**, 188(1951).
(4) Hanze, A. R., Strube, R. E., and Greig, M. E., *J. Med. Chem.*, **6**, 767(1963).
(5) Emmerson, J. L., and Miya, T. S., *J. Pharm. Sci.*, **52**, 411(1963).
(6) Fishman, V., Heaton, A., and Goldenberg, H., *Proc. Soc. Exptl. Biol. Med.*, **109**, 548(1962); Fishman, V., and Goldenberg, H., *ibid.*, **110**, 187(1962).

Applications of Differential Scanning Calorimetry in Pharmaceutical Analysis

By RENATE REUBKE* and JOSEPH A. MOLLICA, JR.

The application of differential scanning calorimetry to several aspects of pharmaceutical analysis is presented. These include the utilization of this technique in the quantitative estimation of purity, in the relative determination of purity for a control procedure, and in the detection of polymorphism. The purities and heats of fusion of diallylbarbituric acid, naphthalene, glutethimide, dibucaine, and anthraquinone are given. A comparative analysis of several samples of methyl reserpate is presented and the detection of polymorphism in tripelennamine citrate is discussed.

THE AVAILABILITY of commercial instruments capable of performing various types of thermal analysis has led to an increasing number of publications of their application to organic compounds (1–4). One area of application which is of particular interest to a pharmaceutical analyst, and one which has not been extensively investigated, is the quantitative estimation of purity through thermal analysis. Phase solubility analysis is presently one of the most widely used techniques for purity determination (5, 6), since the equilibrium solubility of a pure compound is as characteristic a property as is the melting point. This method, however, suffers from certain disadvantages. Perhaps the most important of these is that the time required for equilibration may be several weeks. It was decided therefore to see if differential scanning calorimetry could overcome some of these disadvantages in determining the "absolute" purity of a compound. This determination is based on the other fundamental property of a compound, its melting point. This method would overcome some of the disadvantages of phase solubility in that the analysis could be completed in 1 day and since one determines the sum of the impurities, it would be possible to estimate purity to within $\pm 0.1\%$ when dealing with samples at the 99%+ level, even if errors of the order to 20% were introduced.

Two other areas of application were also investigated—namely, the utilization of this technique in the determination of relative purity and in the detection of polymorphism. It provides a rapid method for control procedures since one can compare the endotherm of the sample to endotherms of known purity without the necessity of a complete analysis. The presence of polymorphism in several samples of tripelennamine citrate is easily detected by comparing thermograms of different samples and this substantiated evidence that was obtained by spectroscopic methods.

Received January 31, 1967, from the Research Department, Ciba Pharmaceutical Co., Summit, NJ 07901
Accepted for publication April 10, 1967.
The authors are grateful to Miss Janet Conover for technical assistance.
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EXPERIMENTAL

A Perkin-Elmer model DSC-1B differential scanning calorimeter was used in this study (7). Aluminum sample pans and pan lids which fit the DSC-1B were used for all samples except the anthraquinone. Anthraquinone samples were sealed in a volatile sample sealer. Care was taken in sample handling and in covering the sample holders with the covers in the same relative position in order to minimize base line drift, which, as pointed out by Rogers and Morris (8), can be caused by differences and variations in thermal emissivity. All samples were run in a nitrogen atmosphere. The materials used were of the quality specified in the text.

A semimicro balance with a sensitivity of 0.01 mg. was used to weigh all samples. Areas were generally determined by planimeter, although in some cases areas were determined by cutting and weighing. In either case, the reproducibility of an area determination was to within 3%.

THEORY

Calorimetric methods for the determination of purity are well known (9, 10); hence, only a brief review of the theory will be given. The equation for the lowering of the freezing point in dilute solution is that developed by Van't Hoff:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f}(k/k' - 1) \qquad \text{(Eq. 1)}$$

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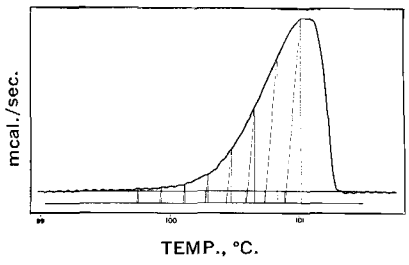


Fig. 1—Thermogram of p-*chlorophenoxyaniline; 1.74 mg.; 1 mcal./sec., full scale; scan rate, 0.625°/min.; chart speed, 4 in./min.*

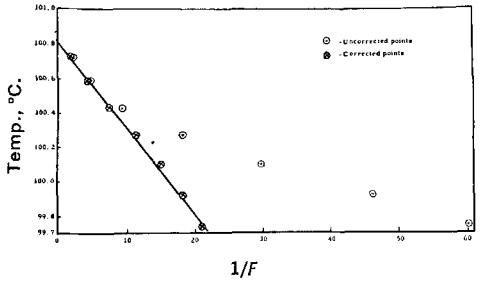


Fig. 2—Plot of T vs. *1/*F *for* p-*chlorophenoxyaniline from thermogram in Fig. 1.*

where T = temperature in °K.,
X_2 = mole fraction of minor component (solute; impurity),
ΔH_f = molar heat of fusion of the major component,
R = gas constant,
k/k' = the distribution of the solute between the solid and liquid phases.

If it is assumed that no solid solutions are formed, *i.e.*, $k/k' = 0$, then upon integration of Eq. 1, one obtains the most commonly used expression for relating mole fraction of impurity to the melting point depression:

$$X_2 = \frac{(T_0 - T_m)\ \Delta H_f}{RT_0^2} \qquad \text{(Eq. 2)}$$

where T_0 = melting point of the pure compound in °K., T_m = melting point of the sample in °K.

If there are no solid solutions formed, then the concentration of impurity in the liquid phase at any temperature during melting is given by:

$$X_2' = X_2/F \qquad \text{(Eq. 3)}$$

where X_2' = mole fraction of impurity in the liquid phase,
X_2 = mole fraction of impurity in the total sample,
F = fraction melted at temperature, T.

Since the melting point depression is directly proportional to the mole fraction of impurity, one obtains:

$$T_0 - T = 1/F\,(T_0 - T_m) \qquad \text{(Eq. 4)}$$

which predicts that a plot of the sample temperature *versus* the reciprocal of the fraction melted at temperature, T, should yield a straight line with a slope equal to $-(T_0 - T_m)$, the melting point depression and the intercept at $1/F = 0$ gives T_0, the melting point of the pure compound.

The value for ΔH_f, the molar heat of fusion, is obtained by determining the area under the melting curve which is proportional to the heat absorbed by the sample during fusion.

These three values, T_0, $T_0 - T_m$, and ΔH_f, may then be introduced into Eq. 2 to calculate the mole fraction of impurity.

RESULTS AND DISCUSSION

Although Eq. 4 predicts a linear relationship in a plot of T *versus* $1/F$, in practice, linear plots were not obtained. Neglecting deviation caused by experimental error, the curvature could result from two sources. [The use of slow scanning rates and small samples of relatively high purity makes errors negligible due to thermal resistance and deviations from "ideality" (11).] If the impurity forms a solid solution with the major component, then the concentration in the liquid phase will be less than that predicted by Eq. 3, and the corresponding equilibrium temperature will be too high; hence, the plot will be concave upward (12). Another factor, which will produce the same type curvature, is the sensitivity limit of the instrument.[1]

With respect to Fig. 1, the melting point depression, $T_0 - T_m$, is equal to 0.048° and T_0 is 100.8° C. If Eq. 4 is rearranged to:

$$F = \frac{T_0 - T_m}{T_0 - T}$$

it is readily seen that at 2° below the melting point, the sample is 2.4% melted, yet the signal at this point is indistinguishable from the base line. If the point at which the signal departs from the base line ($\approx$99.3°) is taken as the starting point for the area measurement, 3–4% of the total area will have been neglected. If the total measured area is designated A, and the partial measured areas are designated a_i, one then has $1/F_i = A/a_i$. The neglected portion could be considered negligible with respect to the total area, but it is significant with respect to the first few partial areas measured as they may be in error by as much as 50–100%. One may compensate for this by adding an adjustable parameter x to linearize the plot, which compensates for the neglected portion which was undetectable due to the sensitivity of the instrument. One then defines:

$$1/F_i = \frac{A + x}{A_i + x}$$

Figure 2 shows a plot of the data before and after correction. The correction in this case amounted to 3.4%. The value x is determined by successive approximation, and if the theory is correct, there should be but one value of x which will yield a linear plot.

However, since no *a priori* knowledge exists as to whether a solid solution is formed, it cannot be stated that the curvature is due entirely to instru-

[1] A complete discussion of the application of the theory to the detailed analysis of a melting curve obtained by a continuously scanning calorimeter was presented by Gray, A. P., in "Determination of Purity by Differential Scanning Calorimetry," presented to Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1966. He has also treated this subject in the "Thermal Analysis Newsletter," No. 5 and 6, published by the manufacturers of this instrument.

TABLE I—PURITY AND HEAT OF FUSION OF SEVERAL COMPOUNDS DETERMINED BY DIFFERENTIAL SCANNING CALORIMETRY

Sample	Wt., mg.	ΔH, cal./Gm.	T_0, °C.	Purity, mole %
Diallylbarbituric acid[a]	4.04	31.9 ± 1	173.3	99.7_6
	1.14		173.7	99.8_4
Naphthalene[b]	3.60	35.1 (lit. 35.6)[c]	80.3	99.6_4
Glutethimide[d]	4.05	28.7 ± 1	86.8	99.1_1
	4.55		87.0	99.1_1
p-Chlorophenoxyaniline[e]	1.74	32.0	100.8	99.8_3
Dibucaine: 1[f]	2.38	20.2 ± 1	63.6	99.5_5
2[g]			63.1	98.4
Anthraquinone: 1[h]	1.19	38.8 ± 2	285.8	99.9_9
2[i]	1.55	(lit. 37.5)[c]	285.3	99.9_7
2	0.89		285.1	99.9_4
3[j]	1.71		285.6	99.9_1
4[k]	2.18		285.1	99.6_9

[a] Reference standard which analyzed 100.0% by phase solubility. [b] Recrystallized from MeOH and dried *in vacuo*. [c] "Handbook of Chemistry and Physics," 43rd ed., Chemical Rubber Publishing Co., Cleveland, Ohio. [d] Reference standard. [e] Material purified by sublimation. [f] Reference standard. [g] Production sample. [h] Zone-refined material. [i] Synthetic sample for temperature calibration. [j] Chromatographically purified. [k] Production sample—minimum 99.5% by GC.

mental insensitivity. But, because the corrected value $A + x$ gave good results for heat of fusion in those cases where there was a literature value for comparison and the calculated purities were reasonable, it was assumed that this was the main cause of the curvature.

Samples were selected which would melt without decomposition, and in most cases if there was some independent determination of their purity. The first criterion, that the compound fuse without decomposition, eliminates many compounds of pharmaceutical interest, particularly salts which either melt with decomposition or do not behave ideally.

The anthraquinone samples presented an interesting problem. The attempt was made to distinguish among four samples, all of which were at least 99.5% pure. The calculated purities and the heat of fusion are given in Table I. The results agree with what one would predict semiquantitatively, *i.e.*, the zone-refined material should be the purest and the production sample the least pure. A difficulty encountered with the anthraquinone was that it started to sublime well below its melting point, which led to sloping base lines and endotherms which were not resolvable. This was remedied when the samples were enclosed in the volatile sample sealer. Figure 3 shows two of the endotherms, sample 1 (99.9_9%) and sample 3 (99.9_1%). One can detect a visual difference in these two endotherms, yet they differed in purity by approximately 0.1%.

As a control procedure, methyl reserpate is analyzed by the solubility temperature method. The method involves the determination of the temperature at which the material goes into solution and then a comparison with a standard curve of sample purity *versus* solubility temperature. The sample purity for the working curve is determined by phase solubility. The method is tedious and subject to error. It was decided to investigate whether one could differentiate among these samples by DSC. The purity of the samples listed is that obtained by the solubility temperature method. Figure 4 shows the results of these studies at two different scanning rates. It is seen that even at the relatively fast scanning rate of 10°/min. one can

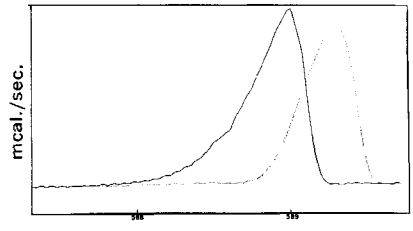


Fig. 3—Thermogram of two samples of anthraquinone; 2 mcal./sec., full scale; scan rate, 0.625°/min.; chart speed, 4 in./min. Key: ·····, zone refined; ———, chromatographically pure.

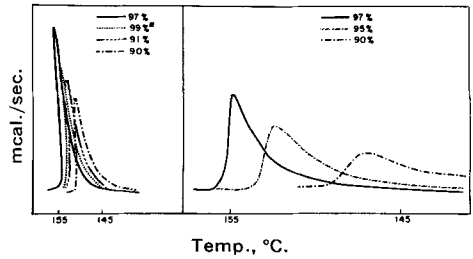


*Fig. 4—Thermograms of several samples of methyl reserpate at scan rates of 10°/min. and 2.5 min.; 8 mcal./sec., full scale; sample sizes were all about 2.5 mg. *Sample which analyzed 99% by temperature solubility method, but was later shown to have a high salt content.*

resolve the sample and is able to estimate the purity to ±1%, although it was found necessary to run at least two compounds of known purity with the unknown in addition to comparing the result to a previously determined working curve in order to compensate for any day-to-day instrumental variation. The method is therefore advantageous in those cases where an existing analytical method is particularly tedious or insensitive. To achieve reproducible results, however, in the authors'

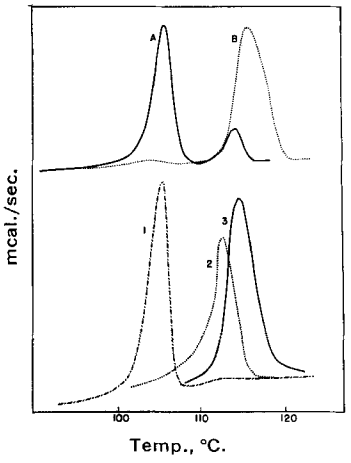


Fig. 5—Thermograms of two samples of tripelennamine citrate (A *and* B). *Curve* 1 *is form* B, *which had been recrystallized and seeded with form* A; *curve* 3 *is form A, which had been recrystallized and seeded with form* B; *curve* 2 *is form* A *after heating. Sample weights were all about 5.3 mg.; scan rate 5°/min.; 8 mcal./sec., full scale.*

opinion, a greater amount of care is necessary than one would have to utilize, for example, in making a spectrophotometric measurement.

During the investigation of various compounds by DSC, it was observed that tripelennamine citrate displayed some anomalous behavior. Two different sources of the material differed in their melting behavior, although chemical analysis showed no significant difference. The thermograms of these two samples are shown in the top half of Fig. 5. It was thought that the tripelennamine citrate existed in two different crystalline forms. The X-ray diffraction patterns and infrared spectra of the two substances also indicated this. If the behavior was caused by different crystalline modifications, then one should be able to transform one into the other. Some preliminary results of these attempts are shown in the lower half of Fig. 5. Form A was carefully recrystallized from acetone and seeded with crystals of form B. Form B was recrystallized from acetone and seeded with crystals of form A. The thermograms of the recrystallized material indicate transformation to forms B and A, respectively. It was also possible to transform A by heating it overnight at a temperature just below its melting point. The data presented above are some preliminary observations. Further investigation on the application of DSC in combination with X-ray analysis on this problem is under way.

SUMMARY

Differential scanning calorimetry provides a rapid and accurate method for purity determination. However, it is limited to those compounds which do not decompose on melting, and unfortunately, therefore, eliminates many compounds of interest. As such, it does not have as wide a range of applicability as does phase solubility analysis. There is also the possibility that the impurity may form a solid solution and thus invalidate the analysis. However, in this respect there are analogous interferences which may invalidate phase solubility analysis (6).

With a reasonable amount of care, the technique can be used to rapidly estimate the purity of a sample by comparison with samples of known purity as evidenced by the differentiation of several samples of methyl reserpate. Also, the technique can be utilized to provide evidence of polymorphic behavior which can complement or supplement spectroscopic analysis.

Differential scanning calorimetry is not the answer to all analytical problems, but it can offer evidence in many areas which is not obtainable by other methods, or it can provide evidence to support that obtained by other techniques.

REFERENCES

(1) Brancone, L. M., and Ferrari, H. J., *Microchem. J.*, **10,** 370(1966).
(2) Rosenheld, J. M., Loncrini, D. F., and Murphy, C. B., *Talanta,* **13,** 1129(1966).
(3) Perron, R., and Mathieu, A., *Chim. Anal.*, **46,** 293 (1964).
(4) Chiu, J., *Anal. Chem.*, **34,** 1841(1962).
(5) Mader, W. J., in "Organic Analysis," vol. 2, Interscience Publishers, Inc., New York, N. Y., 1954.
(6) Higuchi, T., and Connors, K. A., in "Advances in Analytical Chemistry and Instrumentation," vol. 4, Reilley, C. N., ed., Interscience Publishers, Inc., New York, N. Y., 1965.
(7) Watson, E. S., O'Neill, M. J., Justin, J., and Brenner, N., *Anal. Chem.*, **36,** 1233(1964); O'Neill, M. J., *ibid.*, **36,** 1238(1964).
(8) Rogers, R. N., and Morris, E. D., Jr., *ibid.*, **38,** 410 (1966).
(9) Tunnicliff, D. D., and Stone, H., *ibid.*, **27,** 73(1955).
(10) Sturtevant, J. M., in "Techniques of Organic Chemistry," vol. 1, 3rd ed., Weissberger, A., ed., Interscience Publishers, Inc., New York, N. Y., 1959.
(11) Wendlant, W., "Thermal Methods of Analysis," Interscience Publishers, Inc., New York, N. Y., 1964.
(12) Mastrangelo, S. V. R., and Dornte, R. W., *J. Am. Chem. Soc.*, **77,** 6200(1955).

Experimental Evidence for the Existence of an Adrenochrome–Adrenolutin Complex

By R. W. JANSSEN and C. A. DISCHER

Horizontal linear paper chromatography was utilized to achieve the semiquantitative separation of adrenochrome–adrenolutin mixtures. Data obtained by the spectrophotofluorometric assay of bands eluted from these chromatograms show evidence of the formation of a 1:1 complex of the two components.

As a facet of a continuing study of the photodegradative mechanisms of epinephrine, chromatographic separations of its degradation products were undertaken with a view toward quantitative estimation of the various components present. Heacock and his group used an adsorption method to separate the oxidative degradation products of epinephrine (1). Heacock and Mahon reported the separation of adrenochrome, adrenolutin, and 5,6-dihydroxy-*N*-methylindole on acetylated paper (2).

The accepted mechanism of oxidation and subsequent degradation of epinephrine was first elucidated about 1950 (3–6). An expanded scheme has been published by Harrison (7). Adrenolutin, one of the degradation products, is characterized by an intense apple-green fluorescence. This fluoresence has been utilized as the basis for the assay of epinephrine, adrenochrome, or adrenolutin (8–11).

EXPERIMENTAL

Adrenochrome Preparation—Adrenochrome was prepared by the silver oxide oxidation of epinephrine, using a modification of the method of Somkaite (12), which was derived from that of Sobotka and Austin (13). Oxygen was displaced from the adrenochrome solution during crystallization by placing a small piece of dry ice in the solution. In order to minimize the tendency of moisture from the air to condense in the −40° solution, a cover filled with dry ice was placed over the container holding the crystallizing solution, m.p. 125° dec.[1]

Adrenolutin Preparation—All operations were performed under a carbon dioxide atmosphere using solutions prepared with doubly distilled carbon dioxide saturated water unless otherwise stated. These solutions ranged in concentration from 0.05 to 1.0 mg./ml.

An amount of adrenochrome equivalent to the concentration of adrenolutin desired was placed in a carbon dioxide filled 10-ml. flask. Nitrogen saturated 0.05 *N* tertiary sodium phosphate solution (4.0 ml.) containing ascorbic acid (0.1 mg./ml.) was added. After 2 min., 0.1 *N* phosphoric acid (4.0 ml.) was introduced and the solution brought to volume with buffer (0.1 *M* sodium dihydrogen phosphate adjusted to pH 5.5 with 0.1 *N* sodium hydroxide). The resultant solution was always used immediately. It exhibited the characteristic fluorescence peak of adrenolutin at 518 mμ (corrected) when activated with 412 mμ (corrected) light.

Chromatographic Method—The chromatographic paper[2] (25-cm. strips) was suspended from a glass rod and washed with 2% acetic acid for 18 hr. The strips were next washed with two portions of distilled water and soaked in distilled water for 1 hr. The paper was then dried over silica gel for 6 hr. and stored in a 100% humidity chamber for a minimum of 48 hr. before use.

A modification of the chromatographic apparatus of Hauton (14) was employed. A continuous posterboard strip was placed between the edges of the glass plates to effect a slight separation and to serve as a vapor barrier. The saturation strips described by Hauton were omitted and his procedure of clamping the plates together during runs was found unnecessary.

All chromatographic procedures and elutions were performed in a dry box[3] under a positive carbon dioxide pressure. The running solvent used was normal saline solution saturated with carbon dioxide. Fifty-microliter samples were used in all cases.

Adrenochrome Assay—The sample was applied to the prepared paper as a band 5 cm. from one end. Chromatographic development was allowed to proceed in total darkness until the solvent front was a few centimeters from the terminal end. The section of R_f 0.6–0.9 including the red adrenochrome band at R_f 0.75 was cut from the strip, rolled into a cylinder, and inserted into a micro-filter tube-type funnel and this assembly placed in a 10-ml. flask. The adrenochrome band was eluted by dropwise addition (0.5 ml./min.) of bicarbonate buffer (8.0 ml.) (0.05 *M* sodium bicarbonate adjusted to pH 6.0 with 0.2 *N* hydrochloric acid.) Four per cent ascorbic acid (100 μl.) was added to the eluate and the flask filled to the mark with 10.0 *N* sodium hydroxide, stoppered, and inverted five times. An aliquot of the now pale yellow solution was placed in the spectrophotofluorometer[4] and its fluorescent intensity read at 518 mμ (corrected) exactly 5 min. after the time of addition of the sodium hydroxide. Light at 412 mμ (corrected) was used to activate the solution. The results were given the designa-

Received February 16, 1967, from the College of Pharmacy, Rutgers—The State University, Newark, NJ 07104

Accepted for publication April 24, 1967.

Abstracted from a dissertation submitted by Richard W. Janssen to the Graduate School, Rutgers—The State University, New Brunswick, N. J., in partial fulfillment of Master of Science degree requirements.

This investigation was supported in part by research grant H-6114 (Medicinal Chemistry) from the U. S. Public Health Service, Bethesda, Md.

[1] Lit. 125° dec. (12).

[2] Whatman No. 1, 1.5 in. wide strip.

[3] Manostat Dry Box, model 41-905.

[4] Aminco-Bowman spectrophotofluorometer. The No. 3 slit arrangement and the 0.3 M. multiplier setting were used for all fluorescence determinations.

tion (A). (All assays throughout this investigation utilized the same activation and emission wavelengths.)

To obtain a time zero fluorescent intensity reading, a 50-μl. sample of each original adrenochrome solution was placed in bicarbonate buffer (8.0 ml.). This solution was treated and read using the same procedure as that for the eluate from the adrenochrome band of the chromatogram. The time zero adrenochrome reading was designated (A_0).

Adrenolutin Assay—Pure adrenolutin solution was analyzed in a manner similar to that for adrenochrome at time zero. A 50-μl. sample was placed in a 10-ml. flask containing bicarbonate buffer (8.0 ml.) and the fluorescent intensity obtained as above; (L_0) was used to designate these results.

Preparation and Analysis of Adrenochrome–Adrenolutin Mixtures—Adrenolutin was prepared as previously described by the addition of 0.05 N tertiary sodium phosphate (4.0 ml.) to an amount of adrenochrome equivalent to the desired quantity of adrenolutin in a 10-ml. flask. The resulting adrenolutin solution was adjusted to a pH below 7 by the addition of 0.1 N phosphoric acid (4.0 ml.). The adrenochrome portion of the mixture was then added, adrenochrome being stable at pH's slightly below 7. The flask was then filled to the mark with pH 5.5 phosphate buffer and the contents mixed by inverting the flask for 1 min. A 50-μl. sample was streaked on the chromatogram and development begun. Upon completion of the chromatographic run, the segment of R_f 0.6–0.9 was cut from the strip and treated according to the procedure outlined for adrenochrome. The value of the fluorescent intensity found was given the designation (F'). (The adrenochrome band was located between R_f 0.70 and 0.80 and the adrenolutin band between R_f 0.40 and 0.50.)

While the chromatogram was being developed, a second 50-μl. sample of the same adrenochrome–adrenolutin mixture was taken and placed in a 10-ml. flask containing bicarbonate buffer (8.0 ml.). Subsequent treatment was identical to that used in the adrenochrome assay. Since this procedure should rearrange all adrenochrome present to adrenolutin and should merely stabilize the adrenolutin already present, the fluorescent intensity found should equal the sum of the values expected from each. This experimentally found total fluorescent intensity at time zero was designated (F_t).

The mixtures of adrenochrome and adrenolutin chromatographed varied in ratio but always totaled 1.5 mg./ml. in solute concentration.

RESULTS AND DISCUSSION

Calibration data are shown in Table I and in graphical form in Fig. 1. The three sets of calibration data gave straight line plots on log-log paper between 0.05 mg./ml. and 1.0 mg./ml. concentration (2.6×10^{-4} to 5.3×10^{-3} M) and were utilized to calculate the amount of each compound present in the mixtures studied.

Adrenochrome concentration in the mixture was found by extending a line from the value of its fluorescent intensity (F') to line (A). Its concentration was then taken as that point on the concentration axis. Adrenolutin concentration was determined by subtracting the fluorescent intensity value on line (A_0) at the above experimental concentration of adrenochrome from the total fluorescent intensity value at time zero (F_t) and extending a line from the fluorescent intensity calculated to line (L_0). The mg./ml. of adrenolutin was then read on the concentration axis directly below the point of intersection.

The results obtained for the adrenochrome–adrenolutin mixtures are represented graphically by Fig. 2. As seen from this figure, when mixtures of adrenochrome and adrenolutin are separated chromatographically, although a qualitative separation is achieved, theoretical recovery of either component was not obtained. The adrenochrome recovery was always above the theoretical line of slope unity, indicating greater than theoretical recovery. Adrenolutin, on the other hand, showed indicated recoveries well below theoretical for all mixtures. Considering the method of analysis used, these experimental results necessitate the conclusion that some adrenolutin or substance giving rise to it was being carried in the adrenochrome band.

The above data can be used to show the existence

TABLE I—FLUORESCENCE-CONCENTRATION CALIBRATION DATA[a]

Concn., mg./ml.	Fluorescence: Adrenochrome-Eluted from Chromatogram (A)	Fluorescence: Adrenochrome at Time Zero (A_0)	Fluorescence: Adrenolutin (L_0)
0.01	1.16	1.16	...
0.05	5.2	6.3	3.58
0.1	9.6	10.5	7.17
0.2	17.9	18.6	14.0
0.5	37.5	42.3	32.6
1.0	64.8	71.5	63.6
2.0	99.8	103.0	...

[a] All fluorescent values were obtained on an Aminco-Bowman spectrophotofluorometer with a No. 3 slit arrangement, a 412 mμ (corrected) activation wavelength, and a 518 mμ (corrected) emission wavelength. All fluorescent values are adjusted to the equivalent of a 0.3 M. multiplier setting. Each value is the average of 4–7 runs.

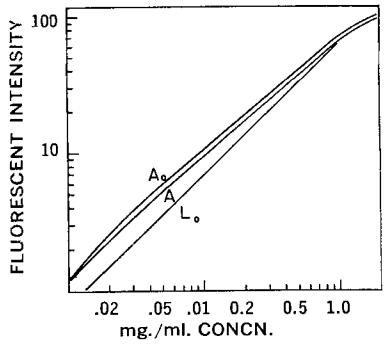


Fig. 1—Standard curves for the analysis of adrenochrome–adrenolutin mixtures. Key: A_0, *the fluorescent intensity obtained from adrenochrome assayed at time zero;* A, *the fluorescent intensity obtained from alkaline ascorbate treated adrenochrome after chromatography and elution;* L_0, *the fluorescent intensity obtained from adrenolutin assayed at time zero.*

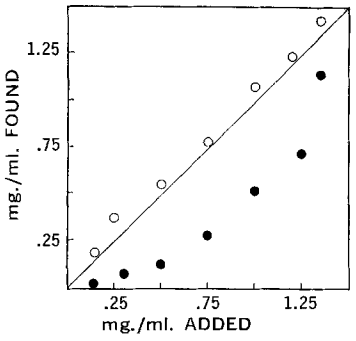


Fig. 2—Analysis results for adrenochrome–adrenolutin mixtures.

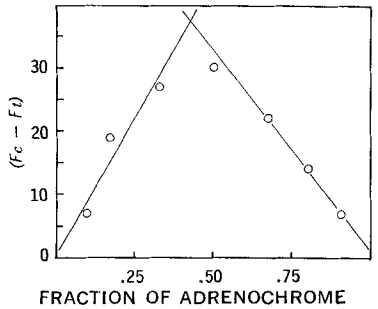
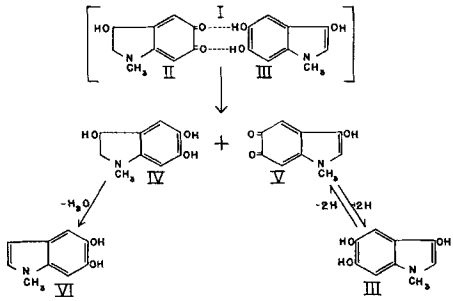


Fig. 3—Modified plot of continuous variation.

of an adrenochrome–adrenolutin complex in the mixture. By use of a modified method of continuous variation, the difference between the theoretical total fluorescent intensities (F_c) and the actual total fluorescent intensities (F_t) for the various solute ratios studied are plotted against the mole fraction of adrenochrome in the mixtures. Two lines are generated which intersect at approximately 45% adrenochrome (Fig. 3), indicating the greatest decrease in fluorescence near the 1:1 *M* ratio.

It was also observed that when a mixture of adrenochrome and adrenolutin was being separated chromatographically, a nonfluorescent yellow band appeared at the trailing edge of the adrenochrome band. When the chromatogram was sprayed with Ehrlich's reagent [*p*-dimethylaminobenzaldehyde (1 Gm.) dissolved in a mixture of concentrated hydrochloric acid (30 ml.) and ethanol (30 ml.) and the solution diluted to 100 ml. with water], a blue-violet band indicating 5,6-dihydroxy-*N*-methylindole formed at the location of the zone; adrenochrome and adrenolutin did not give the color with this reagent. The nonfluorescent yellow band did not appear when either adrenochrome or adrenolutin were chromatographed separately.

These observations can be explained by assuming a hydrogen bonded 1:1 complex (I) of adrenochrome (II) and adrenolutin (III). As a result of the complexation, the adrenolutin can transfer two hydrogens to the adrenochrome, giving an equimolar mixture of leucoadrenochrome (IV) and adrenolutin-quinone (V) (Scheme I). The latter substance should be stable but the former will dehydrate to 5,6-dihydroxy-*N*-methylindole (VI), in line with the



Adrenochrome–Adrenolutin Complex Degradation Scheme

Scheme I

indicated presence of this substance on the chromatogram. For every molecule of adrenochrome which gains two hydrogens and is transformed to leucoadrenochrome, one molecule of adrenolutin-quinone should also be obtained. When the adrenolutin-quinone is reduced to adrenolutin by the addition of the alkaline ascorbate solution, an amount of fluorescence equivalent to that obtainable from the same number of adrenochrome molecules should result. In addition, the fluorescence due to the now rearranged adrenochrome which originally did not complex is also read. Thus the developed fluorescence of the adrenochrome band should be the sum of the rearranged uncomplexed adrenochrome, adrenolutin carried as the complex, and reduced adrenolutin-quinone formed by degradation of the complex. This sum should be greater than the amount of fluorescence expected from the adrenochrome concentration originally present.

Additional conclusions may be drawn. Since part of the adrenolutin is traveling as a complex in the adrenochrome band, the fluorescent intensity of adrenolutin should be decreased, as observed. Experimental results also show that as expected from these interpretations, the sum (F_t) of the fluorescent intensity related to adrenochrome plus that of adrenolutin is always less than theoretical since part of the original adrenochrome has been converted to the nonfluorescent 5,6-dihydroxy-*N*-methylindole.

REFERENCES

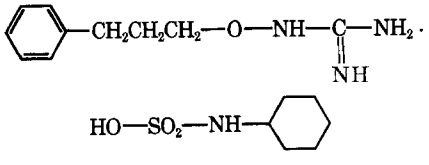
(1) Heacock, R. A., Nerenberg, C., and Payza, A. N., *Can. J. Chem.*, **36**, 853(1958).
(2) Heacock, R. A., and Mahon, M. E., *J. Chromatog.*, **6**, 91(1961).
(3) Bu'Lock, J. D., and Harley-Mason, J., *J. Chem. Soc.*, **1951**, 712.
(4) Falk, J. E., *Biochem. J.*, **44**, 369(1949).
(5) Lund, A., *Acta Pharmacol. Toxicol.*, **5**, 75(1949); through *Chem. Abstr.*, **43**, 5816*b*(1949).
(6) Ruiz-Gijon, J., *Nature*, **166**, 831(1950).
(7) Harrison, W. H., *Arch. Biochem. Biophys.*, **101**, 116 (1963).
(8) Anton, A. N., and Sayre, D. F., *J. Pharmacol. Exptl. Therap.*, **138**, 360(1962).
(9) Atkinson, R., and Wynne, N., *J. Pharm. Pharmacol.*, **14**, 794(1962).
(10) Bertler, A., Carlsson, A., and Rosengren, E., *Acta Physiol. Scand.*, **44**, 273(1958); through *Chem. Abstr.*, **53**, 9344*e*(1959).
(11) Cohen, G., and Goldenberg, M., *J. Neurochem.*, **2**, 58(1957).
(12) Somkaite, R., Ph.D. Thesis, Rutgers—The State University, New Brunswick, N. J., 1962.
(13) Sobotka, H., and Austin, J., *J. Am. Chem. Soc.*, **73**, 3077(1951).
(14) Hauton, J. C., *J. Chromatog.*, **7**, 341(1962).

Pharmacologic Studies with 3-(Phenylpropoxy)guanidine Cyclohexanesulfamate

By W. VELDKAMP, H. H. KEASLING, G. A. JOHNSON, W. A. FREYBURGER, and R. J. COLLINS

Some comparisons of the pharmacologic properties of 3-(phenylpropoxy)guanidine cyclohexanesulfamate (U-16,178F) and dextroamphetamine sulfate are reported. While less potent on a milligram basis than dextroamphetamine sulfate, U-16,178F is an effective anorexigenic agent in dogs, rats, and mice at nontoxic doses. In contrast to dextroamphetamine sulfate, U-16,178F, after a single intraperitoneal dose, does not increase motor activity in mice. However, when administered chronically, by admixing it in the diet, U-16,178F causes an increase in motor activity after 24 hr. on diet. U-16,178F, in contrast to amphetamine, causes an initial fall in blood pressure following intravenous administration in the anesthetized dog. It is about one-tenth as potent as dextroamphetamine sulfate in lowering brain catecholamine levels and, unlike dextroamphetamine sulfate, has no tryptamine potentiating activity but protects against maximal electroshock. It has much less antireserpine activity than dextroamphetamine sulfate. U-16,178F also differs from dextroamphetamine sulfate in that changes in ambient temperature have little effect on its acute toxicity.

THE CHEMISTRY and preliminary pharmacology of a series of aralkoxyguanidines was previously reported by Martin *et al.* (1). From these studies 3-(phenylpropoxy)guanidine nitrate was selected as a potential anorexigenic agent. However, the potential hazards involved in preparing and purifying larger quantities of the nitrate salt for further studies prompted the substitution of the cyclohexanesulfamate salt for the nitrate. The cyclohexanesulfamate salt was preferred over the hydrochloride since the latter compound retained a slight odor even after several recrystallizations. Additional pharmacologic studies and comparisons with dextroamphetamine sulfate have been completed and are the subject of this report. U-16,178F has the following chemical structure:



METHODS

A comparison was made of the acute effects of U-16,178F and dextroamphetamine sulfate on food intake in a group of 32 adult mongrel dogs. A crossover design for an 8-point assay was used (2). The dogs were housed in individual cages in a relatively constant-temperature (75° F.) dog room and were fed daily at the same hour. Dog pellets (Purina laboratory chow) were presented for a 30-min. period and the amount eaten was recorded. Drinking water was available at all times. When drugs were tested, they were given orally in a gelatin capsule 1 hr. before presentation of food. The dogs employed had been on this feeding schedule for at least 3 weeks before the assay started. Drug effect was measured as the per cent decrease in food intake for each dog. Control intake was the average of the 5 control days preceding the assay and the 3 control days between the crossover.

To obtain additional information concerning the influence of U-16,178F on food intake and body weight in the dog, it was also administered on a chronic basis. For comparison, dextroamphetamine sulfate was tested under the same conditions, although not simultaneously. Two groups of four mature mongrel dogs were used. These dogs were housed in individual cages in a relatively constant-temperature (75° F.) dog room. Food was available to the dogs from 8:00 a.m. until 3:00 p.m. daily with water available continuously. The dogs used had been on this feeding schedule for at least 1 month. Food consumption was measured daily. Body weight was determined before and after drug treatment. Drugs were administered orally (gelatin capsule) twice daily at 7:00 and 11:00 a.m. for 5 days. Drug effect was assessed as the per cent decrease in food intake for each dog and the decrease in body weight from the beginning to the end of the treatment period. Control food intake was the average of the 5 to 7 control days preceding the treatment period.

Anorexigenic activity was also determined in rats. Upjohn Wistar male rats weighing 190 to 210 Gm. were housed individually and fasted 22 hr. Six rats were used at each dose level. U-16,178F was tested at 3, 10, and 30 mg./Kg. and dextroamphetamine sulfate at 0.3, 1, and 3 mg./Kg. The drugs, dissolved in aqueous methylcellulose (0.25%) so that the dose was contained in a volume of 1 ml./100 Gm. body weight, were administered by oral intubation. Thirty minutes after dosing, a stock ground diet (Upjohn BA) was supplied and the animals were allowed to feed *ad libitum* for a 2-hr. period. The amount of food each rat consumed was recorded and the potency of U-16,178F relative to dextroamphetamine sulfate determined by means of a parallel line assay.

Received February 3, 1967, from the Pharmacology Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication March 22, 1967.

The authors gratefully acknowledge the technical assistance of Mr. D. M. Devendorf, Mr. R. D. Hamilton, Mr. R. R. Russell, Mr. A. P. Tazelaar, and Mr. H. J. Triezenberg.

TABLE I—EFFECT OF ACUTE SINGLE DOSE ADMINISTRATION 1 hr. BEFORE ON FOOD INTAKE IN DOGS

Compd.	Dose, mg./Kg.	% Inhib.	Compd.	Dose, mg./Kg.	% Inhib.
U-16,178F	1.25	59	Dextroamphetamine sulfate	0.0625	45
U-16,178F	2.5	55	Dextroamphetamine sulfate	0.125	80
U-16,178F	5.0	73	Dextroamphetamine sulfate	0.25	87
U-16,178F	10.0	75	Dextroamphetamine sulfate	0.5	93

The effects of U-16,178F and dextroamphetamine sulfate on food intake, body weight, and motor activity were determined in mice. Groups of eight Carworth Farm male mice (22–24 Gm.) were weighed and then housed together and fed a stock ground diet (Upjohn BA) containing various concentrations of the test compound. The control group received ground diet only. After 24 hr. on this diet, food consumption and body weight changes were determined. Motor activity was measured in actophotometers[1] after the method of Dews (4). After a 10-min. acclimation period in the actophotometers, motor activity of individual mice was recorded for 1 hr. An 8 × 8 Latin square design balanced with respect to treatments, actophotometers, and time of day was used. The mice were left on test diet until they were placed in the actophotometers. The dose of drug ingested by each group of mice was calculated.

Motor activity effects of U-16,178F and dextroamphetamine sulfate were also compared when the compounds were administered intraperitoneally to groups of eight mice. Immediately after injection, the mice were placed in individual actophotometers, allowed to acclimate for a 10-min. period, and then motor activity was recorded for 1 hr. The same 8 × 8 Latin square as described above was used.

To study the effects of U-16,178F and dextroamphetamine sulfate on the overt behavior of cats, groups of eight mature cats were placed together in a small room. Their behavior was evaluated subjectively using a modification of the rating scale devised by Norton (5). This rating system consists of 35 evaluations of cat behavior grouped in six categories: stimulation, sociability, defensive hostility, aggressive hostility, contentment, and a miscellaneous group. The behavior of the cats was scored 7 times at hourly intervals. After the second rating each cat received a dose of one of the drugs, administered orally in a gelatin capsule. Each trial included all dose levels of each drug plus a lactose control. All dose levels of each drug were tested 6 times with the doses randomized among the 16 cats used. No animals were used less than twice or more than 4 times. In all but the first trial, the observer was unaware of the dosing schedule used.

Blood pressure effects of intravenous doses of U-16,178F were determined in 10–14-Kg. mongrel dogs. The dogs were anesthetized with sodium thiopental, 20 mg./Kg. intravenously, followed by sodium barbital, 250 mg./Kg. intravenously. The trachea of each dog was cannulated to ensure an adequate airway, and a femoral artery was cannulated to record blood pressure. Pressure tracings were obtained on a Grass polygraph through a Statham P-23AC transducer. Injections were made into an exposed femoral vein. Responses to epinephrine, acetylcholine, and histamine standards were determined before and after administration of U-16,178F. In two of the four dogs, a 1-min. period of bilateral carotid artery occlusion was added as a test for sympathetic reactivity.

Brain norepinephrine and serotonin levels were determined in the mouse 2 hr. after intraperitoneal administration of U-16,178F and dextroamphetamine sulfate. The method used for extraction of norepinephrine and serotonin from the homogenates was essentially that of Shore and Olin (6) as utilized by Mead and Finger (7). Norepinephrine was determined by the method of Shore and Olin (6) and serotonin was assayed using a modification of the method of Snyder, Axelrod, and Zweig (8).

Tryptamine potentiation, reserpine antagonism, and maximal electroshock protecting activities of U-16,178F and dextroamphetamine sulfate were determined using groups of six Carworth Farm male mice weighing 18–22 Gm. U-16,178F and dextroamphetamine sulfate were administered intraperitoneally in aqueous methylcellulose (25%) at multiple dose levels distributed at 0.3 log intervals. In the tryptamine potentiation test the mice were pretreated with U-16,178F or dextroamphetamine sulfate. Thirty minutes later they were challenged with 25 mg./Kg. of tryptamine hydrochloride intravenously and observed for tryptamine-like symptoms. The 25-mg./Kg. dose of tryptamine hydrochloride does not produce symptoms in control mice while animals treated with an effective dose (*e.g.*, 100 mg./Kg. i.v.) show the following symptoms: hind leg spread, arched back, pawing, head weave, and tremors (9).

In the reserpine antagonism tests one group of mice was treated with 2.5 mg./Kg. of reserpine intraperitoneally and 2 hr. later challenged with the test compound intraperitoneally. Thirty minutes later the degree of ptosis was noted using the 4-point scale of Rubin (10). Another group of mice was treated with the test compound intraperitoneally and after 30 min. challenged with 2.5 mg./Kg. of reserpine intraperitoneally. In each situation control reserpine groups were tested simultaneously. All mice receiving only reserpine had ptosis scores of 4 (complete closure). In the electroshock test mice were treated with the test compounds and 1 hr. later were shocked *via* ear clip electrodes with a 60 c. current for 0.2 sec. at a current intensity of 25 ma. (11).

The influence of ambient temperature on the acute toxicity of U-16,178F and dextroamphetamine sulfate was determined in mice. Groups of six Carworth Farm male mice weighing 18–22 Gm. were injected either intraperitoneally or orally with the compound dissolved in aqueous methylcellulose (0.25%). After injection, the mice were placed in mouse cages[2] that had been divided in half by a plastic wall making compartments 28 × 18 × 14

[1] Metro Industries, Mineola, L. I., N. Y.

[2] Keystone Plastics Co., Media, Pa.

TABLE II—EFFECTS OF CHRONIC DOSING ON FOOD INTAKE AND BODY WEIGHT IN DOGS

	U-16,178F	Dextroamphetamine Sulfate
Dose, mg./Kg. b.i.d.	5.0	0.5
Av. % inhibition of food intake ± S.E.	68.3 ± 4.0	83.8 ± 6.9
Av. wt. loss in Kg. ± S.E.	1.25 ± 0.4	0.98 ± 0.06

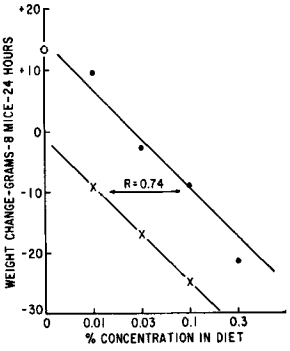


Fig. 1—Parallel bioassay on weight change in mice. U-16,178F (●) vs. dextroamphetamine sulfate (×); control (○).

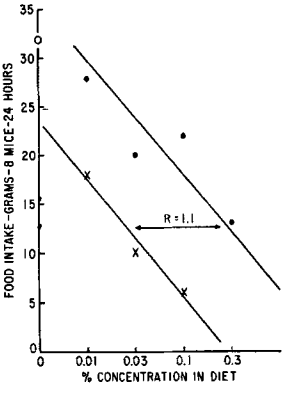


Fig. 2—Parallel bioassay on inhibition of food consumption in mice. U-16,178F (●) vs. dextroamphetamine sulfate (×); control (○).

cm. Standard perforated metal tops were used. Cages with one group of mice at each dose level were placed in a cabinet (120 × 54 × 56 cm.) under the laboratory bench at 22° C., similar groups were placed in a constant-temperature oven (74 × 46 × 46 cm.) at 34° C., and in a refrigerator at 4° C. The two compounds were run in parallel. The high dose used for the oral test with U-16,178F was 400 mg./Kg. and the high dose for the intraperitoneal test for both U-16,178F and dextroamphetamine sulfate was 200 mg./Kg. Decreasing at 0.3 log intervals, the lowest dose of U-16,178F both orally and intraperitoneally was 25 mg./Kg. and the lowest dose of dextroamphetamine sulfate administered was 1.56 mg./Kg. Although the mice were examined at 15-min. intervals for 2 hr., the LD_{50}'s were computed by the method of Spearman and Karber (see *Reference 3*) for the 1 and 2-hr. period.

TABLE III—EFFECT ON MOTOR ACTIVITY OF U-16,178F AND DEXTROAMPHETAMINE SULFATE WHEN ADMIXED WITH DIET

Compd.	Dose Ingested, mg./Kg.	Total Counts 8 Mice for 60 min.
Control	...	4,883
U-16,178F	478	8,437
U-16,178F	251	8,301
U-16,178F	66	7,121
U-16,178F	29	5,975
Dextroamphetamine sulfate	77	11,024
Dextroamphetamine sulfate	36	9,202
Dextroamphetamine sulfate	20	8,681

RESULTS

Acute Effects on Food Intake in Dogs—The results of the comparison of U-16,178F and dextroamphetamine sulfate are shown in Table I. The potency of U-16,178F relative to dextroamphetamine sulfate in this test on a mg./Kg. basis was 0.025 with 95% confidence limits of 0.002–0.078. In this analysis the dog to dog standard deviation for per cent inhibition of food intake was 37%. The dose response slope for U-16,178F was 25%/tenfold increase, while that of dextroamphetamine sulfate was 50%/tenfold increase, but this difference was not statistically significant.

Chronic Effects on Food Intake and Body Weight in Dogs—U-16,178F was tested at 5.0 mg./Kg. b.i.d. and dextroamphetamine sulfate at 0.5 mg./Kg. b.i.d. for 5 days. Body weight loss with the two compounds was about the same, while dextroamphetamine sulfate caused a somewhat greater inhibition of food intake (Table II).

Anorexigenic Activity in Rats—Analysis of the food intake data obtained in rats treated with U-16,178F and dextroamphetamine sulfate showed that on a mg./Kg. basis the potency of U-16,178F relative to dextroamphetamine sulfate was 0.030 with 95% confidence limits of 0.007–0.058.

Effects of Body Weight Change, Food Intake, and Motor Activity in Mice When Admixed with Diet—U-16,178F was tested at 0.01, 0.03, 0.1, and 0.3% concentrations in the diet, while dextroamphetamine sulfate was tested at 0.01, 0.03, and 0.1% concentrations. Effects on body weight change and on food intake are shown in Figs. 1 and 2. On a mg./Kg. basis, U-16,178F was about $^1/_5$ as potent as dextroamphetamine sulfate in causing body weight loss and $^1/_{12}$ as potent in inhibiting food intake in this procedure.

Effects on motor activity after the mice had been on the diet containing the test compound for 24 hr. are shown in Table III. Analysis of variance of motor activity data showed no significant difference between the control and the mice on the diet containing U-16,178F, while the motor activity of the mice on the diet containing dextroamphetamine sulfate was significantly higher than that of the controls. However, when covariance analysis was used to compare motor activity at doses which caused a comparable inhibition of food intake or weight loss, it was found that the motor stimulant effects of U-16,178F were somewhat less than those of dextroamphetamine sulfate, but the difference was not statistically significant.

TABLE IV—EFFECT ON MOTOR ACTIVITY OF U-16,178F AND DEXTROAMPHETAMINE SULFATE WHEN ADMINISTERED 10 min. BEFORE BY INTRAPERITONEAL INJECTION

Compd.	Dose, mg./Kg.	Total Counts 8 Mice for 60 min.
Control	...	6,863
U-16,178F	40	6,616
U-16,178F	20	5,309
U-16,178F	10	6,206
U-16,178F	5	5,763
Dextroamphetamine sulfate	5	18,138
Dextroamphetamine sulfate	2.5	16,690
Dextroamphetamine sulfate	1.2	8,918

Motor Activity in Mice After Intraperitoneal Administration—The effects of U-16,178F and dextroamphetamine sulfate on motor activity in mice after intraperitoneal administration are summarized in Table IV. Analysis of variance of these data showed that while dextroamphetamine sulfate at the 5 and 2.5 mg./Kg. doses caused a statistically significant increase in motor activity compared to controls, U-16,178F had no effect on motor activity at any of the doses tested.

Effect on Overt Behavior in Cats—U-16,178F was administered orally at 1, 3, 10, and 30 mg./Kg. and dextroamphetamine sulfate at 1, 3, and 10 mg./Kg. Both U-16,178F and dextroamphetamine sulfate increased the incidence of head turning, head bob, piloerection, and dilated pupils. However, U-16,178F was only $^1/_{10}$ to $^1/_{30}$ as potent as dextroamphetamine sulfate. Both compounds decreased overt signs interpreted as contentment while sociability was reduced only by dextroamphetamine sulfate. Measures of hostility were not increased by either compound, while spontaneous motor activity was decreased by both. Emesis, diarrhea, hallucinations, and poor contact were not observed in any of the animals, although a slight increase in the incidence of salivation was noted with U-16,178F. A time course evaluation of the data indicated that the maximum activity of both compounds was seen during the first and second hourly observations with a gradual decrease in activities thereafter.

Effects on Blood Pressure, Autonomic Standards, and Carotid Occlusion Response in Anesthetized Dogs—U-16,178F was tested intravenously in four dogs. Doses of 0.5, 1.0, 2.0, 8.0, and 16.0 mg./Kg. were employed in the first pair of animals and 4.0, 8.0, and 16.0 mg./Kg. in the second pair. It produced a brief dose-related fall in arterial pressure reaching −70 mm. Hg for 3 min. at 16 mg./Kg. The hypotensive response to U-16,178F was not blocked by a dose of propranolol (1 mg./Kg.) which completely blocked the depressor action of 10 mcg. of isoproterenol. This would indicate that the hypotensive effect of U-16,178F is not the result of β-adrenergic stimulation. The blood pressure response to carotid occlusion was unchanged at the lower doses of U-16,178F; however, following the 16-mg./Kg. dose, the blood pressure response to carotid occlusion was depressed. Responses to histamine and acetylcholine standards were not changed in any of the four dogs. The pressor responses to epinephrine after all doses of U-16,178F were increased; however, the effect was small (average 11% greater) and was not dose related. The duration of the epinephrine induced pressor response was also somewhat increased (17%–54% longer) and the effect was directly related to the U-16,178F dose.

Catecholamine Depleting Activity—The effects of U-16,178F and dextroamphetamine sulfate on brain catecholamine levels in mice are summarized in Table V. These results show that the norepinephrine values were slightly decreased at each dosage level of U-16,178F with the effect of the 100 mg./Kg. dose approximating the decrease produced by 10 mg./Kg. of dextroamphetamine sulfate. Neither U-16,178F nor dextroamphetamine sulfate altered the serotonin levels.

Effects Against Tryptamine, Resperine, and Electroshock—U16,178F at doses up to 100 mg./Kg. caused no potentiation of tryptamine activity in the mouse while the ED_{50} of dextroamphetamine sulfate for this activity was 18 mg./Kg. U-16,178F

TABLE V—EFFECT OF U-16,178F AND DEXTROAMPHETAMINE SULFATE ON BRAIN AMINE LEVELS IN MICE

Compd.	Dose, mg./Kg.	Norepinephrine: Determinations, No.	Norepinephrine: Concn., mcg./Gm. ± S.E.M.	Norepinephrine: % of Control	Serotonin: Determinations, No.	Serotonin: Concn., mcg./Gm. ± S.E.M.	Serotonin: % of Control
Saline	...	14	0.53 ± 0.06	100	6	0.94 ± 0.05	100
U-16,178F	10	8	0.46 ± 0.05	87	7	0.88 ± 0.08	94
U-16,178F	30	8	0.44 ± 0.05	83	8	0.86 ± 0.05	93
U-16,178F	100	8	0.43 ± 0.06	81	8	0.96 ± 0.06	102
Dextroamphetamine sulfate	1	4	0.55 ± 0.05	103	4	0.86 ± 0.03	92
Dextroamphetamine sulfate	3	6	0.52 ± 0.06	98	6	0.87 ± 0.07	93
Dextroamphetamine sulfate	10	8	0.42 ± 0.04	79	8	0.96 ± 0.06	102

TABLE VI—EFFECTS OF AMBIENT TEMPERATURE ON ACUTE TOXICITY IN MICE

Temp., °C.	LD_{50}, mg./Kg.: Dextroamphetamine Sulfate (i.p.) 1 hr.	Dextroamphetamine Sulfate (i.p.) 2 hr.	U-16,178F (i.p.) 1 hr.	U-16,178F (i.p.) 2 hr.	U-16,178F (p.o.) 1 hr.	U-16,178F (p.o.) 2 hr.
34°	4.5	4.5	79	79	284	224
22°	>12.5	5.6	126	100	400	284
4°	142.0	142.0	142	142	400	356

showed weak antireserpine activity at 100 mg./Kg. On a mg./Kg. basis, U-16,178F was about $^1/_{50}$ as active as dextroamphetamine sulfate when given after reserpine and $^1/_{16}$ as active when given before reserpine. U-16,178F had an ED_{50} of 89 mg./Kg. against maximal electroshock while dextroamphetamine sulfate at doses up to 50 mg./Kg. gave no protection.

Ambient Temperature on Acute Toxicity in Mice—Variations in temperature caused a significant change in the toxicity of dextroamphetamine sulfate but had little influence on the toxicity of U-16,178F. Comparing the LD_{50}'s at 4° and 34° C. there was a thirtyfold change in toxicity with dextroamphetamine sulfate while U-16,178F had less than a twofold change. The results of this experiment are summarized in Table VI.

DISCUSSION

U-16,178F is structurally different from the present marketed anorexigenic agents. As originally pointed out by Modell (12) all of the available anorexigenic agents to date have a phenethylamine moiety in the molecule. This moiety is not present in U-16,178F.

U-16,178F is an effective anorexigenic agent in dogs, rats, and mice. This activity is considered to be due to its central appetite depressant effect and not due to nauseant or anticholinergic activity. No emesis was observed with U-16,178F in dogs or cats at any of the doses tested, and no anticholinergic activity was seen when it was tested against blood pressure effects of intravenous acetylcholine in the anesthetized dog.

When the acute and chronic effects of U-16,178F on anorexigenic activity in the dog are compared, the data suggest that U-16,178F is more effective when given repeatedly than when given in a single dose. This might indicate that U-16,178F has a slower onset of effect than does dextroamphetamine sulfate and/or that there is a less rapid development of tolerance to U-16,178F than to dextroamphetamine sulfate.

A difference between the acute and chronic effects of U-16,178F was demonstrated in the motor activity measurements in mice. An intraperitoneal dose of U-16,178F caused no increase in motor activity when measured for a 1-hr. period 10 min. after administration. However, when administered chronically by admixing it in the diet, it caused an increase in motor activity after 24 hr. on the diet. In contrast, dextroamphetamine sulfate causes an increase in motor activity when given acutely or chronically.

When given intravenously in the anesthetized dogs, U-16,178F produced a brief fall in arterial pressure, followed by a modest rise while the amphetamine is known to cause a definite pressor effect as previously reported (13). The hypotensive effect of U-16,178F was not blocked by propranolol. The secondary rise in pressure seen with U-16,178F coupled with the observation that the blood pressure response to carotid occlusion was depressed following the 16 mg./Kg. dose suggests that U-16,178F has some propensity for releasing norepinephrine and lowering catecholamine stores. This is in agreement with the finding that U-16,178F causes some decrease in brain norepinephrine.

Other qualitative and quantitative differences between U-16,178F and dextroamphetamine sulfate suggest that U-16,178F may be a different type of anorexigenic agent. It is about $^1/_{10}$ as potent as dextroamphetamine sulfate in depleting brain catecholamine levels. Unlike dextroamphetamine sulfate, it had no tryptamine potentiating activity and has much less antireserpine activity. U-16,178F gave protection against maximal electroshock while dextroamphetamine sulfate did not exhibit this activity. The toxicity of U-16,178F was not appreciably increased when the LD_{50} was determined at 30° C. instead of 4° C., while the LD_{50} of dextroamphetaine sulfate increased thirtyfold in this situation.

REFERENCES

(1) Martin, D. G., Schumann, E. L., Veldkamp, W., and Keasling, H. H., *J. Med. Chem.*, **8**, 456(1965).
(2) Finney, D. J., "Statistical Methods in Biological Assay," Hafner Publishing Co., New York, N. Y., 1952, pp. 278–280.
(3) *Ibid.*, pp. 524–530.
(4) Dews, P. B., *Brit. J. Pharmacol.*, **8**, 46(1953).
(5) Norton, S., "Psychotropic Drugs," Elsevier Publishing Co., New York, N. Y., 1957, pp. 73–82.
(6) Shore, P. A., and Olin, J. S., *J. Pharmacol. Exptl. Therap.*, **122**, 295(1958).
(7) Mead, J. A. R., and Finger, K. F., *Biochem. Pharmacol.*, **6**, 52(1961).
(8) Snyder, S. H., Axelrod, J., and Zweig, M., *ibid.*, **14**, 831(1965).
(9) Keasling, H. H., Russell, R. R., and Triezenberg, H. J., *Federation Proc.*, **22**, 2771(1963).
(10) Rubin, B., Malone, M. H., Waugh, M. H., and Burke, J. C., *J. Pharmacol. Exptl. Therap.*, **120**, 125(1957).
(11) Swinyard, E. A., Brown, W. C., and Goodman, L. S., *ibid.*, **106**, 319(1952).
(12) Modell, W., *J. Am. Med. Assoc.*, **173**, 1131(1960).
(13) Veldkamp, W., Tazelaar, A. P., Freyburger, W. A., and Keasling, H. H., *Toxicol. Appl. Pharmacol.*, **6**, 15(1964).

Identification and Differentiation of Organic Medicinal Agents V

Quaternary Ammonium Compounds

By LESLIE G. CHATTEN, ANN C. NAPPER, and PAMELA JANE BARRY

Identification tests have been presented for 23 compounds containing either one or two quaternary ammonium functional groups. The reactions of these compounds with ammonium reineckate, picric acid, and chloroplatinic acid have been studied and 54 adducts have been isolated and their melting ranges presented. In addition, microcrystal tests showed that many adducts possessed distinctive crystalline morphology. Photomicrographs are presented as a supplemental and additional parameter for characterization purposes.

MEDICINAL AGENTS containing a quaternary ammonium function comprise several pharmacologically heterogeneous groups such as cationic surface-active agents, parasympathomimetics, parasympatholytics, skeletal muscle relaxants, or antagonists of curariform drugs, just to mention a few. Despite the broad scope and importance of these compounds, satisfactory methods for their identification are not available. Although a number of the compounds investigated in this report have official status in either the B.P. (1), the U.S.P. (2), or the N.F. (3), in general, the identification tests employed in these compendia are nonspecific.

It is the purpose of this investigation to develop identification tests of greater specificity, in order to permit differentiation between the various quaternary ammonium compounds.

EXPERIMENTAL

Apparatus—Thomas-Hoover melting point apparatus (calibrated with melting point reference standards); Leitz Laborlux-Pol. microscope equipped with a Zeiss Ikon 35 mm. camera.

Received January 13, 1967, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.

Accepted for publication March 14, 1967.

Mrs. Napper and Mrs. Barry are indebted to the National Research Council of Canada for financial support during the course of this investigation.

The authors gratefully acknowledge the generous samples of quaternary ammonium compounds from the following drug manufacturers: Abbott Laboratories, Montreal, Quebec, Canada (hexocyclium methylsulfate); Ayerst, McKenna & Harrison, Montreal, Quebec, Canada (valethamate bromide); Ayerst Laboratories, New York (echothiophate iodide); Ciba Company Ltd., Dorval, Quebec, Canada (chlorisondamine chloride, oxyphenonium bromide, and domiphen bromide); Hoffmann-LaRoche Ltd., Montreal, Quebec, Canada (edrophonium chloride, pyridostigmine bromide); Lakeside Laboratories, Milwaukee, Wis. (mepenzolate bromide, pipenzolate bromide); Eli Lilly and Co., Indianapolis, Ind. (dithiazinine iodide); Merck Sharp & Dohme, Montreal, Quebec, Canada, and West Point, Pa. (bethanechol chloride, demecarium bromide); Wm. S. Merrell Co., Cincinnati, Ohio (cetylpyridinium chloride); Parke Davis & Co., Ltd., Brockville, Ontario, Canada (benzethonium chloride); Searle & Co., Chicago, Ill. (methantheline bromide, propantheline bromide); Smith Kline & French, Inter-American Corp., Montreal, Quebec, Canada (isopropamide iodide); Sterling-Winthrop Research Institute, Rensselaer, N. Y. (ambenonium chloride, penthienate bromide); Warner-Chilcott Co. Ltd., Toronto, Ontario, Canada (hexamethonium chloride); John Wyeth and Brother, Walkerville, Ontario, Canada (trimethidinium chloride); Wyeth Laboratories Inc., Philadelphia, Pa. (pentolinium tartrate).

Quaternary Ammonium Compounds—Drugs investigated are listed in Table I.

Reagents and Solutions—All reagents used were A.C.S. grade, or reagent grade if the former was not commercially available. Ammonium reineckate 1% aqueous solution; picric acid saturated ethanolic solution; chloroplatinic acid 10% in diluted HCl; solutions of quaternary ammonium compounds, ammonium reineckate, picric acid, and chloroplatinic acid for microcrystallography are listed in Table II.

Formation of Derivatives

All derivatives were dried *in vacuo* over phosphorus pentoxide for 24 hr. prior to determining their respective melting points on a Thomas-Hoover melting point apparatus. All melting points were corrected by reference to a calibration curve.

Reineckates—The reineckates were prepared according to the following modified procedure of Chatten and Levi (4). With the exception of isopropamide iodide, mepenzolate bromide, and dithiazinine iodide, 1 mmole of quaternary ammonium salt was dissolved in a minimum amount of water. While stirring vigorously, 1 mmole of ammonium reineckate in aqueous solution was added for each quaternary ammonium functional group present in the parent compound. Isopropamide iodide and mepenzolate bromide were dissolved in a minimum of ethanol, while acetone was selected as the most satisfactory solvent for dithiazinine iodide.

After cooling for 2 hr. or until precipitation was complete, the reineckate crystals were filtered, dried, and then recrystallized by dissolving in a minimum of acetone and adding water to the cloud point. The solution was cooled until recrystallization was complete. For certain reineckates, *i.e.*, those of cetylpyridinium, pyridostigmine, edrophonium, propantheline, methantheline, chlorisondamine, demecarium, and domiphen, compounds of high purity could only be obtained by washing them with ethanol prior to drying.

Elemental analyses (C, H, N) confirmed the purity and identity of the reineckates.

Picrates—To 1 mmole of the quaternary ammonium salt, dissolved in water, 1 mmole of picric acid in 10 ml. of ethanol was added for each quaternary ammonium functional group. The mixture was allowed to stand in the refrigerator for 30 min. and the crystals were removed by suction filtration. Recrystallization was accomplished by dissolving the

TABLE I—MELTING POINTS OF DERIVATIVES OF QUATERNARY AMMONIUM COMPOUNDS

Parent Compd.	Reineckates, °C.	Picrates, °C.	Chloroplatinates, ° C.
Ambenonium chloride	168–169	205 dec.	206–207
Benzethonium chloride	158–160	Oil	165.5–167
Bethanechol chloride	160 dec.	126–130	194.5–196.5
Cetylpyridinium chloride	131–133	50–50.5	187–188
Chlorisondamine chloride	183–188	246–247 dec.	Carbon analysis not theoretical
Demecarium bromide	180–185	Oil	...
Dithiazinine iodide	...	220	...
Domiphen bromide	126–127.5	Oil	197–198
Echothiophate iodide	129–140 dec.	Oil	Black mass
Edrophonium bromide	...	161–161.5	194–194.5 dec.
Hexamethonium chloride	272–275 dec.	239.5–241 240 (1)	282–293 dec.
Hexocyclium methylsulfate	191–192	189–190	224–226 dec.
Isopropamide iodide	154–155	140–140.5	Black mass
Mepenzolate bromide	166–167	160–161	Carbon analysis not theoretical
Methantheline bromide	131.5–135	114.5–115	187–188.5
Oxyphenonium bromide	130–132	101.5–102	198–200 dec.
Penthienate bromide	142–146	Oil	149
Pentolinium tartrate	272–276 dec.	270–271 dec. 270 (1)	233.5–234.5 dec.
Pipenzolate methylbromide	158–159 dec.	160.5–162	193–196
Propantheline bromide	134–137	Oil	165–167
Pyridostigmine bromide	140–143 153–156 (2)	115–115.5	160–162
Trimethidinium methosulfate	250–251	300–301	255
Valethamate bromide	128–130	Oil	174.5–177.5

TABLE II—QUATERNARY AMMONIUM COMPOUNDS CHARACTERIZED BY MICROCRYSTALLOGRAPHY[a]

Parent Compd.	Ammonium Reineckate Parent:Reineckate	Picric Acid Parent:Picric Acid	Chloroplatinic acid Parent:Chloroplatinic Acid
Ambenonium chloride	Oil globules	0.125:0.125	0.125:0.25
Benzethonium chloride	Oil globules	Oil globules	Brownish mass
Bethanechol chloride	4:0.3	Oil globules	...
Cetylpyridinium chloride	0.5:0.025	Oil globules	Brownish mass
Chlorisondamine chloride	1:1	0.062:0.125	Oil globules
Demecarium bromide	Oil globules	Oil globules	Oil globules
Dithiazinine iodide	Dithiazinine crystals	Dithiazinine crystals	Dithiazinine crystals
Domiphen bromide	1:1	Oil globules	Oil globules
Echothiophate iodide	2:0.1	Oil globules	Black mass
Edrophonium bromide	10:1	1.0:0.5	1.0:1.0
Hexamethonium chloride	0.007:0.025	0.5:0.5	0.5:0.5
Hexocyclium methylsulfate	1:1	Oil globules	Oil globules
Isopropamide iodide	Oil globules	Oil globules	Oil globules
Mepenzolate bromide	1:0.25	Oil globules	0.25[b]:0.125
Methantheline bromide	Oil globules	Oil globules	Brownish mass
Oxyphenonium bromide	5:0.1	Oil globules	Amorphous
Penthienate bromide	0.015:0.3	Oil globules	Oil globules
Pentolinium tartrate	0.5:1	0.5:0.5	0.5:0.5
Pipenzolate methylbromide	0.25:0.15	Oil globules	Oil globules
Propantheline bromide	5:0.1	Oil globules	Brownish mass
Pyridostigmine bromide	2:0.5	2.0:2.0[b]	4.0:1.0
Trimethidinium methosulfate	2:0.5	0.25:0.125	0.25:0.125
Valethamate bromide	0.05:0.3	Oil globules	Amorphous

[a] Concentrations of solutions are expressed in percentages. Time when picture was taken: 1–5 min. [b] Ethanol (95%) solution.

derivative in a minimum of ethanol or acetone and adding water slowly until the cloud point was reached. The mixture was set aside in the refrigerator to recrystallize. Elemental analyses (C, H, N) verified the purity and identity of the picrates. For the three quaternary ammonium parent compounds which are insoluble in water, solvents utilized are outlined under *Reineckates*.

Chloroplatinates—To 1 mmole of the quaternary ammonium salt, dissolved in a minimum of water, 1 mmole of chloroplatinic acid in dilute HCl was added for each quaternary ammonium functional group in the molecule. The resulting mixture was cooled in an ice bath for 20 min. or overnight in the refrigerator. The fine precipitate was filtered off, washed with cold water, and recrystallized from

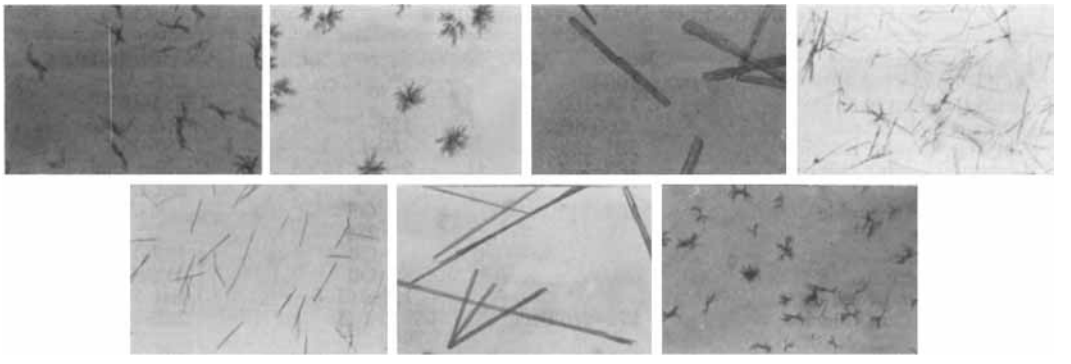


Fig. 1—Photomicrographs of the picrates. Top: ambenonium, chlorisondamine, edrophonium, hexamethonium. Bottom: pentolinium, pyridostigmine, trimethidinium. (Left to right.)

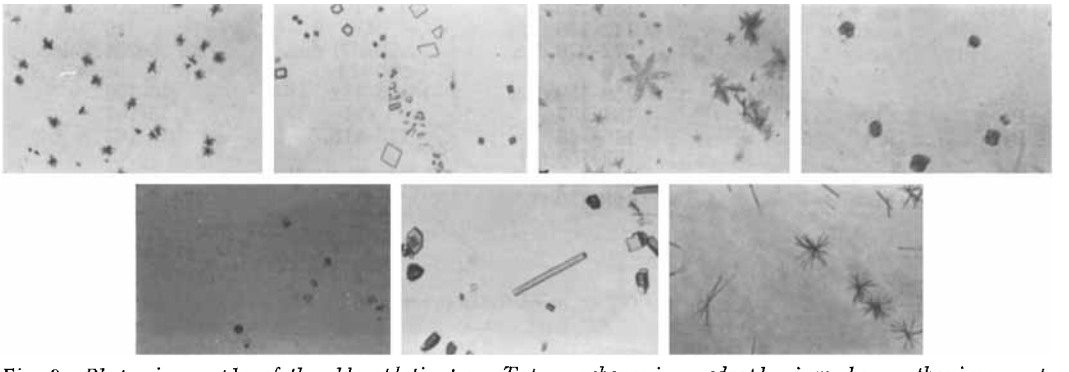


Fig. 2—Photomicrographs of the chloroplatinates. Top: ambenonium, edrophonium, hexamethonium, mepenzolate. Bottom: pentolinium, pyridostigmine, trimethidinium. (Left to right.)

boiling ethanol. If the adduct was insoluble in hot ethanol, it was washed well with cold methanol. In those three instances where the quaternary ammonium parent compound was not soluble in water, they were dissolved in ethanol and the chloroplatinic acid was added as an aqueous methanolic solution.

Elemental analyses (C, H, N) confirmed the purity and identity of the chloroplatinates.

Photomicrographs

To 1 drop of an aqueous or alcoholic solution of each quaternary ammonium salt placed on a microscope slide, 1 drop of reagent solution (ammonium reineckate, picric acid, or chloroplatinic acid) was added. The solutions were mixed well and covered with a cover glass. One to five minutes was allowed for the formation of crystals, and the pictures were taken at a magnification of approximately 100×.

RESULTS AND DISCUSSION

Examination of the official compendia (1-3) reveals that 13 of the 23 quaternary ammonium compounds are official in one or more compendia. In only three monographs are derivatives used as a means of identification. Two are picrates and one a reineckate. Monographs of the other compounds rely, to a large extent, upon identification by a series of relatively nonspecific color and precipitation tests. All adducts prepared in this investigation are presented in Table I, together with their melting ranges and previously reported literature values.

Reineckates—In general, ammonium reineckate proved to be a very useful reagent for the preparation of derivatives, although products of satisfactory purity could not be obtained for either dithiazinine or edrophonium. For cetylpyridinium, chlorisondamine, demecarium, methantheline, propantheline, and pyridostigmine repeated crystallization failed to yield products which gave acceptable elemental analysis. However, adducts of high purity were obtained after these materials were well washed with ethanol prior to drying. According to the official compendia (1, 2) pyridostigmine reineckate melts at 153–156°. The results of this investigation revealed that only that derivative which melted at 140–143° gave satisfactory elemental analysis.

Examination of Table I reveals that a certain amount of overlap of melting points exists between several derivatives. Therefore, reineckates cannot be used as the sole criterion for identification purposes.

Picrates—Sixteen of the 23 quaternary ammonium compounds gave adducts of high purity with picric acid. For each of the remaining seven compounds, a viscous oil was obtained which failed to crystallize. Examination of Table I reveals that the picrates are highly satisfactory derivatives in that a minimum of overlapping of melting ranges occurs between compounds.

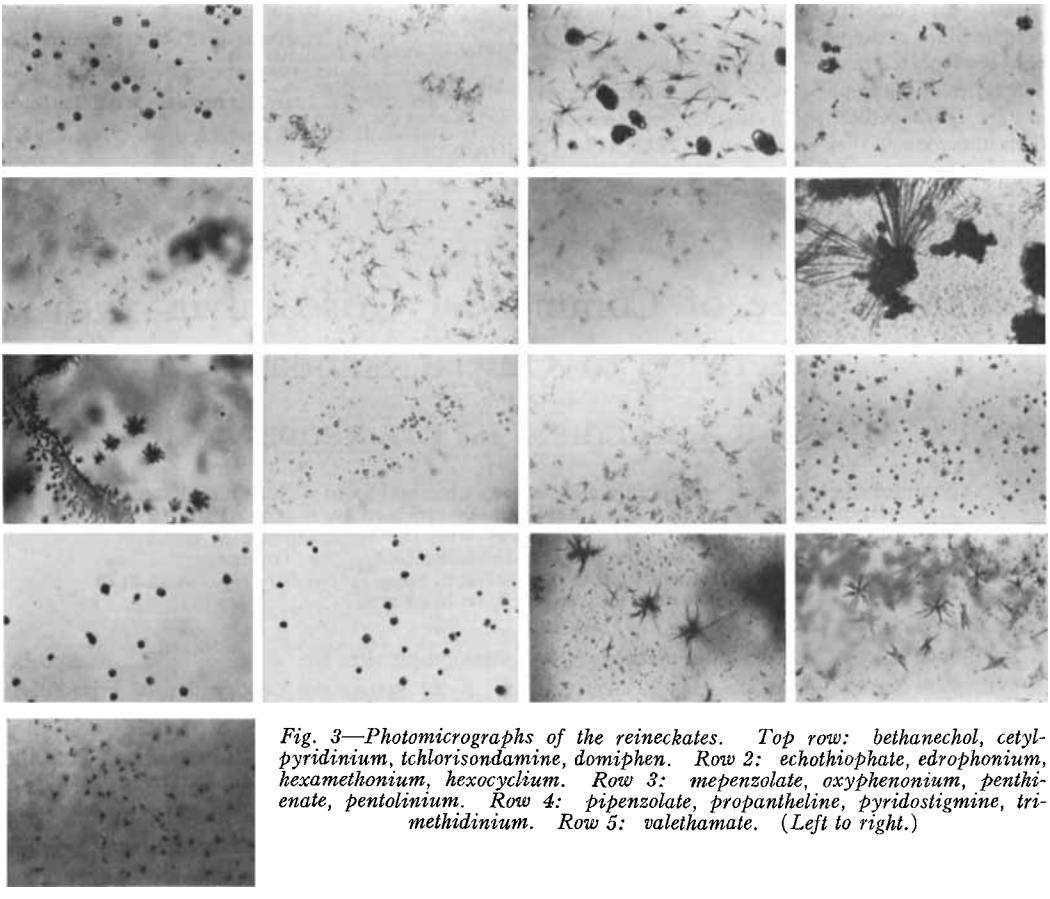


Fig. 3—Photomicrographs of the reineckates. Top row: bethanechol, cetylpyridinium, tchlorisondamine, domiphen. Row 2: echothiophate, edrophonium, hexamethonium, hexocyclium. Row 3: mepenzolate, oxyphenonium, penthienate, pentolinium. Row 4: pipenzolate, propantheline, pyridostigmine, trimethidinium. Row 5: valethamate. (Left to right.)

Chloroplatinates—Although some overlapping of melting ranges occurs in a few instances, the 17 derivatives that resulted with this reagent are valuable aids in the identification of the corresponding parent compound.

Attempted preparation of the chloroplatinate from isopropamide iodide and echothiophate iodide resulted immediately in a black decomposed mass from which no satisfactory product could be isolated. Demecarium bromide and dithiazinine iodide gave impure products which could not be purified by recrystallization. Chlorisondamine and mepenzolate chloroplatinates were crystalline compounds in which the carbon analysis did not correspond with theoretical values, even after repeated crystallizations.

Photomicrography

The three reagents employed in the preparation of the derivatives were utilized in the characterization studies by microcrystallography. Table II, a compilation of the experimental results, shows the percentage concentration of quaternary ammonium compound to that of reagent when crystals occurred. Other results such as oil globules or amorphous mass are recorded in the appropriate columns. In some instances, each of the three reagents produced crystals with a particular quaternary ammonium compound. Generally, success was noted with at least one of the reagents employed. However, exceptions to this were benzethonium chloride, demecarium iodide, isopropamide iodide, methantheline bromide, oxyphenonium bromide, and dithiazinine iodide. With the last named material, crystals of the parent compound appeared repeatedly.

Figure 1 shows the photomicrographs of those compounds which gave crystalline picrates, while Fig. 2 illustrates the chloroplatinates. While each of these reagents was useful in only a limited number of instances, the crystalline morphology of the derivatives depicted is remarkable. Figure 3 presents the photomicrographs of the 17 reineckates and most of these offer unique crystalline habits. Thus the photomicrographs of these three figures would be of high utility in differentiating the parent compounds. Like other physicochemical criteria, however, one should not rely upon one single criterion such as crystalline morphology for identification. It is an aid to identification just as color tests or derivatives are.

It is interesting to note the crystals of ambenonium reineckate and isopropamide reineckate were obtained once, but since neither could be repeated, their photographs were not included in Fig. 3.

SUMMARY

A series of physical chemical criteria, by which 23 quaternary ammonium compounds can be identified and differentiated, has been presented.

Fifty-four derivatives of these drugs have been prepared in a systematic manner, of which 52 have not been reported to date in the literature.

A series of photomicrographs have been included in Figs. 1–3, together with a summary of the conditions under which they were formed (Table II).

REFERENCES

(1) "The British Pharmacopoeia," The Pharmaceutical Press, London, England, 1963.
(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(3) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.
(4) Chatten, L. G., and Levi, L., *Anal. Chem.*, **31**, 1581 (1959).

Particle Size of Commercial Griseofulvin with Reference to Official Standards

By B. A. MATTHEWS and C. T. RHODES

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GRISEOFULVIN (7-chloro-4,6-dimethoxycoumaran - 3 - one - 2 - spiro - 1′ - [2′ - methoxy-6′-methylcyclohex-2′-en-4′-one]) has gained widespread acceptance in the oral treatment of fungal infections in man (1). It is the subject of monographs in a number of pharmacopeias (2–6). Early work on serum and tissue levels of the drug in rats, by Bedford *et al.* (7), and in humans, by McNall (8), suggested that the drug is poorly absorbed from the gastrointestinal tract. Further confirmation of this was published by Sharpe and Tomich (9), who were unable to determine the lethal dose of orally administered griseofulvin in rats and mice and by Gonzales-Ochoa *et al.* (10), who attempted to treat dermatophytoses in man.

All drugs, whether readily or sparingly soluble, dissolve more rapidly when finely divided, and Nelson (11, 12) showed that for theophylline salts and tetracycline, the rate of appearance of the drug in the blood stream following oral administration was determined by the solution rate. Griseofulvin is almost insoluble in water (13) and several attempts have been made to determine the effect of particle size on blood levels in animals (14) and man (15–17). Kraml *et al.* (17) found that a 0.5-Gm. dose of micronized griseofulvin produced serum levels indistinguishable from those produced by a 1.0-Gm. dose of nonmicronized griseofulvin. The monograph in the "British Pharmacopoeia" 1963 (2) recognizes two grades of griseofulvin called, respectively, "coarse particle" and "fine particle," whereas the "United States Pharmacopeia" (4) specifies one called "microsize," which corresponds approximately to the "fine particle" of the "British Pharmacopoeia." In each case some particle size requirements are given. The Food and Drug Administration monograph on microsize griseofulvin specifies additional particle size requirements based on specific surface area measurements by an air permeability technique. The limits are 1.3 to 1.6 sq. M./Gm. The proposed monograph for griseofulvin in the Volume of Specifications of the second edition of the "International Pharmacopoeia," to be published later this year, has a particle size specification based on a sieve technique. A limit of 5% by weight is allowed to be retained on a 300-mesh sieve (aperture size 53 μ).

The authors have obtained samples of commercial griseofulvin from several sources and by means of the Coulter counter (18, 19) have examined these to determine their particle size distribution. They have also utilized microscopy and specific surface area measurements to check results and have compared these with the particle size requirements of the pharmacopeias.

EXPERIMENTAL

Apparatus—A Coulter counter model B[1] with 50-μ and 140-μ orifices was used to determine the particle size. The electrolyte was 1% sodium

Received December 27, 1966, from the Physical Pharmacy Department, School of Pharmacy, Portsmouth College of Technology, Portsmouth, Hampshire, England.
Accepted for publication April 19, 1967.
The authors acknowledge the generosity of Glaxo Laboratories Ltd., Imperial Chemical Industries Ltd., and Ayerst Laboratories Division of American Home Products Corp. for supplying samples of griseofulvin.

[1] Marketed by Coulter Electronics Ltd., Dunstable, Bedfordshire, England.

Fifty-four derivatives of these drugs have been prepared in a systematic manner, of which 52 have not been reported to date in the literature.

A series of photomicrographs have been included in Figs. 1–3, together with a summary of the conditions under which they were formed (Table II).

REFERENCES

(1) "The British Pharmacopoeia," The Pharmaceutical Press, London, England, 1963.
(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(3) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.
(4) Chatten, L. G., and Levi, L., *Anal. Chem.*, **31**, 1581 (1959).

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TABLE I—RESULTS OF A SURVEY OF THE MANUFACTURERS OF GRISEOFULVIN IN THE UNITED KINGDOM, EUROPE, AND THE U. S.

Mfr.	Name	Griseofulvin, Coarse	Griseofulvin, Fine
A	Glaxo Laboratories Ltd., Middlesex, England	Mfd.	Mfd.
B	Imperial Chemicals Limited, Pharmaceuticals Division, Cheshire, England	Mfd.	Mfd.
C	Schering Corp., Bloomfield, N. J.	Obtained from	mfr. *A*
D	McNeil Laboratories, Inc., Fort Washington, Pa.	Obtained from	mfr. *A*
E	Ayerst Laboratories Division of American Home Products Corp., New York, N. Y.	Obtained from mfr. *B*	Mfd. from coarse material obtained from mfr. *B*
F	Rhein-Pharma Arzneimittel G.m.b.H., Heidelberg, Germany	Obtained from	mfr. *B*
G	Laboratories Clin-Comar, Paris, France	Obtained from	mfr. *A*
H	Laboratori Glaxo S.p.A., Verona, Italy	Obtained from	mfr. *A*

chloride and polysorbate 80[2] was used as a dispersing agent, in conjunction with ultrasonic vibration.[3] The 50-μ orifice was calibrated with 1.305-μ diameter polystyrene-latex[4] and also by 4.6-μ diameter puff-ball spores. Good agreement was achieved between the respective calibration constants. The 140-μ orifice tube, which was used for the coarse material, was calibrated with 13.6-μ paper mulberry pollen. The electrolyte was filtered through a 0.45-μ Millipore[5] filter, and background counts at all settings of the Coulter were always well below the recommended limits.

Materials—Samples of fine and coarse griseofulvin were requested from two manufacturers in the United Kingdom, three in the United States, and one in France, West Germany, and Italy. The results of the inquiries are given in Table I. The authors subsequently obtained several samples of different batches of griseofulvin from manufacturers *A*, *B*, and *E* and submitted these to analyses on the Coulter counter.

Method—For each measurement a concentrated suspension was prepared in electrolyte, which had been previously saturated with griseofulvin, and this was subjected to ultrasonic dispersion. Dilutions were made in saturated electrolyte to a suitable working strength and counts were taken at the lowest settings at the beginning and end of the determination to ensure that flocculation did not occur.

Mounts were prepared for microscopy by using ultrasonic dispersion to prepare a concentrated suspension of discrete particles and this was mounted in glycerin containing polysorbate 80. Photographs were taken as a check on particle size. Specific surface area measurements were made on the Fisher sub-sieve sizer[6] which is based on the work of Gooden and Smith (20).

The data from Kek Ltd. was supplied in the form of tables of mean particle diameter against porosity. Since the mean particle diameters reached a minimum at a porosity of 0.425 in each case, it was considered justifiable to make comparisons of specific surface at this value using the formula given by Pendleton (21).

[2] Marketed as Tween 80 by Honeywill-Atlas Ltd., London, England.
[3] M.S.E. Ultrasonic Disintegrator, model 60w, marketed by Measuring and Scientific Equipment Ltd., London, England.
[4] Kindly supplied by Mr. B. J. Lippie, Dow Chemical Co., Midland, Mich.
[5] Marketed by Millipore Ltd., Middlesex, England.
[6] The authors are grateful to Mr. P. D. Hines, Kek Ltd., Manchester, England, for specific surface area measurements.

RESULTS AND DISCUSSION

Fine Particle Griseofulvin—Three determinations were performed on separate samples of batch 1 of fine particle griseofulvin from manufacturer *B*, to determine the reproducibility of repeated measurements. The results of this experiment are given in Table II. Since the count was still increasing at the lowest settings of the instrument, the 100% level was determined by extrapolating the curve of log $\Sigma(\Delta n)\ \bar{v}$ against diameter d for the last six points as recommended in the Coulter instruction manual (22).

These results show good agreement especially at the middle and fine end of the particle size distribution. There is more variation at the coarse end, where only a few particles are counted at each size, and consequently sampling errors tend to be greater. Counts were then performed on three separate batches from manufacturer *A*, three from manufacturer *B*, and one from manufacturer *E*. These are shown in Table III. Extrapolation was performed on the data from all the batches from manufacturer *B* and for that from *E* for the reasons given above. The size distribution of the material obtained from

TABLE II—REPRODUCIBILITY OF REPLICATE DETERMINATIONS OF PARTICLE SIZE DISTRIBUTION ON BATCH 1 OF FINE PARTICLE GRISEOFULVIN FROM MANUFACTURER *B*

Equivalent Spherical Diam., μ	Cumulative Wt. % above Stated Size 1	2	3
16.96	2.25	1.71	2.98
13.46	5.64	3.43	5.96
10.69	9.03	6.43	7.45
8.48	10.16	9.00	10.43
6.73	13.83	11.03	13.60
5.34	16.44	14.62	16.58
4.24	20.50	18.82	20.17
3.37	27.50	27.66	27.18
2.67	42.19	45.03	42.01
2.12	65.71	67.00	64.19
1.68	85.15	82.57	82.14
1.34	92.83	88.17	89.91
1.06	97.15	95.00	95.17
0	100.0	100.0	100.0

TABLE III—PARTICLE SIZE DISTRIBUTION OF DIFFERENT BATCHES OF FINE PARTICLE GRISEOFULVIN FROM MANUFACTURERS *A*, *B*, AND *E*

Equivalent Spherical Diam., μ	Cumulative Wt. % Above Stated Size						
	Mfr. *A*			Mfr. *B*			Mfr. *E*
	1	2	3	1	2	3	1
16.96	0.72	0.75	0.57	2.25	0	2.21	2.46
13.46	2.15	1.18	1.72	5.64	2.12	6.62	9.43
10.69	3.58	3.20	3.29	9.03	4.94	7.73	14.97
8.48	5.99	5.75	5.52	10.16	9.70	11.86	25.85
6.73	10.10	9.94	9.06	13.83	13.24	14.75	35.49
5.34	18.17	19.48	16.90	16.44	17.56	19.79	46.70
4.24	36.10	41.76	37.54	20.50	23.51	28.45	59.25
3.37	61.46	66.62	64.98	27.50	37.83	43.80	70.57
2.67	82.17	84.67	85.56	42.19	59.05	46.65	81.20
2.12	93.09	94.27	95.24	65.71	78.93	71.40	89.21
1.68	98.43	98.35	99.01	85.15	91.02	87.29	95.58
1.34	99.38	99.52	99.74	92.83	94.85	92.70	97.89
1.06	100.0	100.0	100.0	97.15	97.61	96.82	99.27
0	...	...	...	100.0	100.0	100.0	100.0

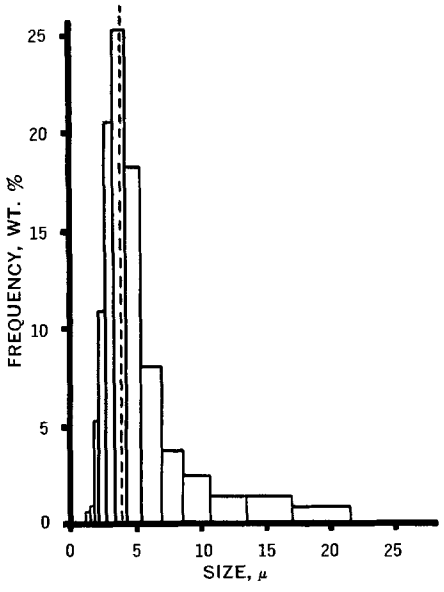

Fig. 1—Histogram plot of particle size distribution of batch 1 of fine particle griseofulvin from manufacturer A.

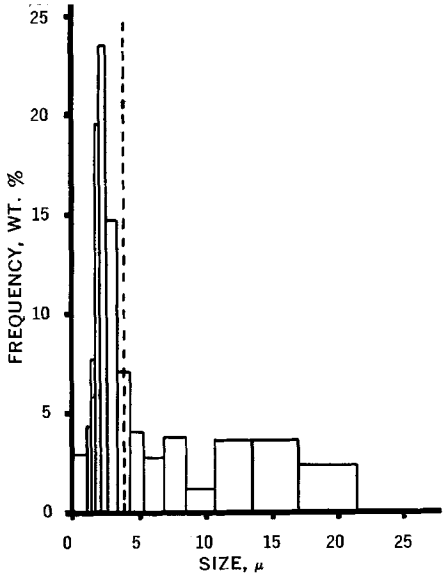

Fig. 2—Histogram plot of particle size distribution of batch 1 of fine particle griseofulvin from manufacturer B.

manufacturer *A* appeared to cut off at around 1 μ, which corresponded to the lowest setting on the instrument, and extrapolation was not considered necessary in this case. The size distribution of the three batches from manufacturers *A* and *B* appears to fall into a definite and distinctive pattern with little between-batch variation, suggesting that good control is achieved by each manufacturer.

Histograms of representative batches of material from manufacturers *A* and *B* and of the sample from manufacturer *E* are given in Figs. 1–3. In addition, plots of cumulative weight per cent above stated size against size using the mean of the values for the three batches from manufacturers *A* and *B* are given in Fig. 4. The "British Pharmacopoeia" monograph on griseofulvin (2) states that for the fine particle material "the particles are mostly less than 4 μ in diameter with only occasional particles exceeding 5 μ." The "United States Pharmacopeia" (4) states that "particles of the order of 4 μ pre-

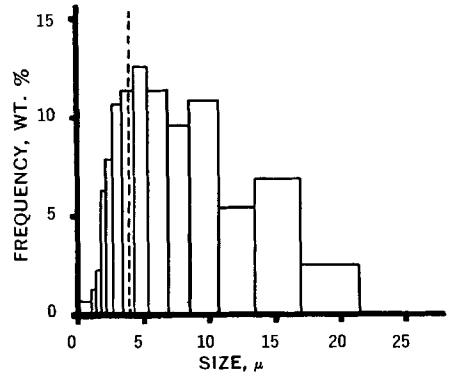

Fig. 3—Histogram plot of particle size distribution of batch 1 of fine particle griseofulvin from manufacturer E.

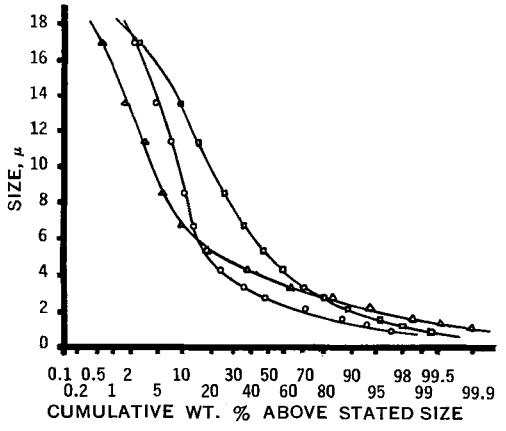

Fig. 4—Plot of cumulative weight per cent above stated size against size for fine particle griseofulvin. Key: △, mean of three batches from manufacturer A; ○, *mean of three batches from manufacturer* B; □, *batch from manufacturer* E.

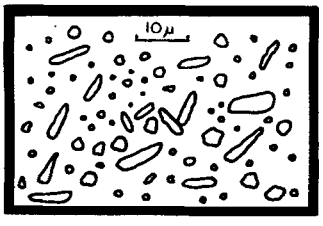

Fig. 5—Diagram of a typical field of fine particle griseofulvin taken from a photomicrograph.

TABLE IV—SPECIFIC SURFACE AREA OF FINE PARTICLE GRISEOFULVIN

	Mfr. *B* Sample 1	Mfr. *A* Sample 1	Mfr. *E*
Specific surface area, sq. M./Gm.	1.54	1.32	1.23
Peak of size distribution histogram	2.5 μ	3.5 μ	5 μ

TABLE V—PARTICLE SIZE DISTRIBUTION OF COARSE PARTICLE GRISEOFULVIN

Equivalent Spherical Diam., μ	Cumulative Wt. % Above Stated Size Mfr. *A*	Mfr. *B*
67.9	0	4.43
53.8	0	8.86
42.8	7.26	14.40
33.9	12.70	19.96
26.9	16.33	28.82
21.4	23.13	39.34
16.9	31.98	51.12
13.5	42.81	63.84
10.7	52.10	74.56
8.5	62.92	82.08
6.7	71.12	87.55
5.3	80.00	91.51
4.2	87.39	94.70
3.4	93.15	97.03
2.7	95.60	98.07
2.1	98.22	99.65
0	100.0	100.0

dominate." It is not entirely clear from these statements whether the specifications refer to a number distribution or a weight distribution. For powdered drugs the latter is obviously more important since a very few particles at the upper end of the distribution can affect the weight per cent distribution considerably.

When the results in Figs. 1–3 are compared with the above requirements, it is seen that the peak of the size distribution curve is at about 2.5 μ for the material from manufacturer *B*, 3.5 μ for that from *A*, and 5 μ with that from *E*. It was found that almost 60% by weight of the sample from this latter manufacturer was above the 4 μ limit. The B.P. requirement that only an occasional particle should be above 5 μ may be true on a number basis, but the results show that the samples from manufacturers *A* and *B* showed about 20% by weight above 5 μ and that from *E* almost 50%.

Shape Factor—Since the diameter obtained with the Coulter counter is a mean spherical diameter and the authors knew that many of the particles in fine particle griseofulvin were acicular, photographs of several samples of this material were taken. A drawing of a typical field taken from a photograph is shown in Fig. 5. From this it may be observed that the average length/breadth ratio of the needles is about 3. No mention of shape is made in the size requirements of the pharmacopeias and one dimension of the particle may be much larger than the Coulter diameter. For example a sphere of 4 μ diameter would have approximately the same volume as a cylinder of diameter 2.5 μ and length 7.5 μ. Allowing for this the results of microscopic measurements were in good agreement with the Coulter data. The length of the acicular particles is small compared with the dimensions of the sensitive volume of the orifice, and we have been informed (23) that despite the shape factor the pulse height will be a true measure of the particle volume.

Specific Surface Area Measurements—The papers (15–17) which have related the absorption of griseofulvin to its particle size have used the parameter, specific surface area. This was therefore determined for a sample of griseofulvin from each manufacturer. The results are given in Table IV.

The shape of the histograms in Figs. 1–3 are reflected in the results of these specific surface area measurements. The lower value of the modal particle size of the material from manufacturer *B* is to some extent counterbalanced by the existence of another peak between 10 and 20 μ, and so the difference in specific surface area between samples *A* and *B* is not as would otherwise have been expected. The coarsest sample *E* had a specific surface area of 80% of that of the finest *B*. Atkinson *et al.* (15) have shown a close correlation between specific surface area of griseofulvin and absorbability, but it is not possible to say whether the differences found in the samples above would be clinically significant.

Coarse Particle Griseofulvin—Owing to the relative economy of treatment with fine particle griseofulvin, both manufacturers *A* and *B* have ceased to market the coarse particle material. The authors have, however, obtained samples from each and the results of size distribution analyses with the Coulter counter are given in Table V and Fig. 6.

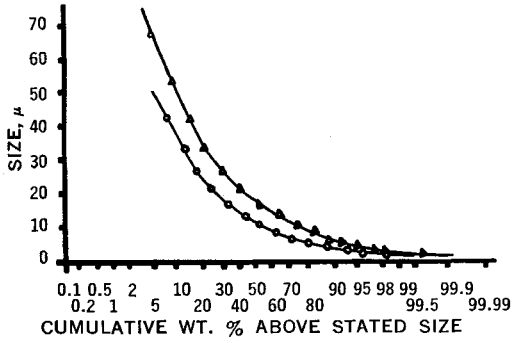

Fig. 6—Plot of cumulative weight per cent above stated size against size for coarse particle griseofulvin. Key: O, manufacturer A; △, manufacturer B.

TABLE VI—SPECIFIC SURFACE AREA OF COARSE PARTICLE GRISEOFULVIN

	Mfr. *A*	Mfr. *B*
Specific surface area, sq. M./Gm.	0.38	0.48

No specification is given in the U.S.P. for this material, but the B.P. states that the particles are "mostly about 30 μ in diameter with occasional particles up to 60 μ." The proposed monograph for the 2nd "International Pharmacopoeia" (24) does not specify whether the type of material referred to is coarse or fine. From the particle size test which allows 5% by weight to be retained by a sieve with a mesh size of 53 μ, it appears that the material is similar to coarse particle griseofulvin of the B.P.

The sample from manufacturer *A* has no particles with an equivalent spherical diameter greater than the maximum permitted value but the peak of the size distribution is around 15 μ. The sample from manufacturer *B* has a few particles coarser than the permitted maximum but its size distribution peak is also around 15 μ. The greater percentage of coarse material in this sample is reflected in the specific surface area measurements given in Table VI.

The sample from manufacturer *A* has no particles with a Coulter diameter greater than the 53-μ mesh diameter specified in the "International Pharmacopoeia," whereas that from manufacturer *B* had 8.9% above this limit. However, as mentioned earlier, the particles are often acicular and consequently such particles with an equivalent spherical diameter greater than 53 μ could pass through a sieve of this mesh size.

SUMMARY

Samples of griseofulvin available commercially from different sources exhibit considerable differences in particle size distribution. Because of the imprecise nature of the official standards in the pharmacopeias, a wide range of size distributions can be considered as meeting their requirements. The authors have shown that the Coulter counter can be used to determine the particle size distribution of this drug and because of the shape factor feel that the methods used should be specified by the pharmacopeias. Because of the relationship between blood levels and particle size, it is felt that more stringent specifications should be given. It is suggested that a modal diameter of the distribution expressed as a weight percentage with upper and lower limits controlling the amount outside them would be more effective in controlling the particle size of this material. Because of the relationship between specific surface and blood levels, it is felt that all pharmacopeias should also include some limits of this parameter.

REFERENCES

(1) *A.M.A. Arch. Dermatol.*, **81**, 650(1960).
(2) "The British Pharmacopoeia," The Pharmaceutical Press, London, England, 1963, p. 348.
(3) "The British Pharmaceutical Codex," The Pharmaceutical Press, London, England, 1963, p. 344.
(4) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(5) "Pharmacopoeia Nordica," Addendum 1965, Arnold Busck, Copenhagen, Denmark, 1965, p. 331.
(6) "Deutsches Arzneibuch," 7th ed., Ackedemie Verlag Berlin, Germany, 1965.
(7) Bedford, C., Busfield, D., Child, K. J., MacGregor, I., Sutherland, P., and Tomich, E. G., *A.M.A. Arch. Dermatol.*, **81**, 735(1960).
(8) McNall, E. G., "Antibiotics Annual 1959–1960," Antibiotica, Inc., New York, N. Y., 1960, p. 674.
(9) Sharpe, H. M., and Tomich, E. G., *Toxicol. Appl. Pharmacol.*, **2**, 44(1960).
(10) Gonzales-Ochoa, A., and Ahumada-Padilla, M., *A.M.A. Arch. Dermatol.*, **81**, 833(1960).
(11) Nelson, E. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 607(1957).
(12) Nelson, E., *ibid.*, **48**, 96(1959).
(13) "Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 499.
(14) Kraml, M., and Dubuc, J., *Can. J. Biochem. Physiol.*, **40**, 1449(1962).
(15) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Nature*, **193**, 588(1962).
(16) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Antibiot. Chemotherapy*, **12**, 232(1962).
(17) Kraml, M., Dubuc, J., and Guadry, R., *ibid.*, **12**, 239(1962).
(18) Coulter, W. H., *Proc. Natl. Electron. Conf.*, **12**, 1034 (1956).
(19) Kubitschek, H. E., *Research (London)*, **13**, 128(1960).
(20) Gooden, E. L., and Smith, C. M., *Ind. Eng. Chem., Anal. Ed.*, **12**, 479(1940).
(21) Pendleton, A. G., *Chem. Process Eng.*, **41**, 147(1960).
(22) "Instruction Manual for Coulter Counter Model A Industrial," Coulter Electronics Ltd., Dunstable, Bedfordshire, England, p. 10.
(23) Coulter Electronics Ltd., Dunstable, Bedfordshire, England, private communication.
(24) Wallen, O., World Health Organization, Geneva, Switzerland, personal communication.

Effect of Complex Formation on Drug Absorption IV

Role of Intragastric Diffusion in the Absorption of Free and Caffeine-Complexed Salicylic Acid from the Rat Stomach

By RICHARD H. REUNING* and GERHARD LEVY†

The effect of complex formation on salicylic acid absorption from the rat stomach has been determined under conditions where the drug solution in the stomach was either stirred by means of a microstirrer or left unstirred. Complexation of part of the salicylic acid with caffeine resulted in a significant decrease in the over-all salicylic acid absorption rate under both conditions. Stirring had no significant effect on the absorption rate of salicylic acid, nor did it affect the absorption rate of salicylic acid in the presence of caffeine. This indicates that diffusion of salicylic acid and/or of the salicylic acid–caffeine complex to the absorbing membranes is not the rate-limiting step in the gastric absorption of salicylic acid under the experimental conditions. It appears, therefore, that the decreased absorption rate of salicylic acid in the presence of caffeine is due to the decreased thermodynamic activity of salicylic acid resulting from complex formation, with the complex being either unabsorbed or more slowly absorbed than free salicylic acid.

IN A RECENT publication in this series (1) it has been reported that the absorption rate of salicylic acid from the *in situ* ligated rat stomach is decreased significantly in the presence of the complexing agent caffeine. It was suggested that this decrease may be due to the following mechanism(s): (*a*) a difference in the partition coefficients of salicylic acid and the salicylic acid–caffeine complex between the aqueous gastric fluids and the lipoid phase of the mucosal barrier, causing the salicylic acid–caffeine complex to be either unabsorbed or more slowly absorbed than free salicylic acid, and/or (*b*) a decreased diffusion rate of the complex through the gastric fluids to the gastric mucosa. The first mechanism was suggested by the relative values of the apparent *in vitro* partition coefficients of the different species (salicylic acid > salicylic acid–caffeine complex > caffeine), while the second mechanism derives support from the quantitative agreement between the results of the absorption experiments and the calculated effect of caffeine on the over-all[1] diffusion rate of salicylic acid in the system (1). The possibility that diffusion to the absorbing membranes is rate-limiting in the absorption of rapidly absorbed weak acids from the ligated rat stomach was suggested originally by Schanker *et al.* (2).

Two experimental approaches were utilized in an attempt to distinguish between these two different mechanisms which could account for the effect of caffeine on salicylic acid absorption. One of these approaches was to reverse the conditions of the original experiment and to determine the effect of salicylic acid on caffeine absorption. If the first mechanism (*i.e.*, modification of the apparent *in vivo* partition coefficient) were operative, one would expect salicylic acid to enhance the over-all absorption rate of caffeine since the apparent partition coefficient of the caffeine–salicylic acid complex is appreciably higher than that of caffeine alone. This approach was tried, but had to be abandoned due to the limited solubility of salicylic acid (which precluded extensive complexation of caffeine) and due to its damaging effect on the gastric mucosa when nearly saturated solutions of the drug were used. The second experimental approach, which is the subject of this report, was to stir the gastric contents by means of a specially designed microstirrer so that diffusion to the absorbing membranes would definitely not be rate-limiting in the absorption process. Thus, if diffusion to the gastric mucosal membrane is a rate-limiting step in the absorption of salicylic acid under unstirred conditions, one would expect the absorption rate of free salicylic acid (*i.e.*, in the absence of caffeine) to increase when the gastric content is stirred. One would also expect that the magnitude of the effect of caffeine on salicylic acid absorption would be changed when the gastric content is stirred because a step other than diffusion to the membranes will then become absorption rate-limiting. On the other hand, if diffusion to the absorbing membrane is not rate-limiting, one would expect that stirring would

Received February 6, 1967, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication April 7, 1967.

This investigation was supported in part by grant RO1 AM 08753-03 PET from the U. S. Public Health Service, Bethesda, Md.

The valuable assistance of Miss Judith A. Anello is acknowledged.

Previous paper: Levy, G., Miller, K. E., and Reuning, R. H., *J. Pharm. Sci.*, **55**, 394(1966).

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[1] In the context used in this paper, "over-all" refers to the combined effect or behavior of free and complexed drug.

have no effect on the absorption rate of salicylic acid in the absence of caffeine, and that the decrease in salicylic acid absorption rate caused by complexation with caffeine will be of equal magnitude under stirred and unstirred conditions.

EXPERIMENTAL

Absorption Rate Measurements—Female Wistar rats weighing 210 to 295 Gm. were used. Food was withheld for 18–24 hr. prior to the experiment. The rats were anesthetized with urethan (1.25 Gm./Kg. i.p.) and remained under anesthesia throughout the absorption experiment. Three different experimental procedures were used; these will be referred to as "unstirred," "static stirrer," and "rotating stirrer." In each experiment 6 ml. of one of the following solutions was introduced into the stomach: (*a*) ethanol 2%, (*b*) salicylic acid 0.1%, ethanol 2.0%, or (*c*) salicylic acid 0.1%, ethanol 2.0%, caffeine 2.75%. All concentrations refer to per cent weight in volume. One-tenth *N* HCl was used as the solvent for each solution.

In the experiments utilizing the "unstirred" procedure a midline incision was made in the abdominal region of the rat and the stomach was exposed. The esophagus was then ligated tightly immediately adjacent to the cardiac sphincter, care being taken not to occlude major blood vessels. A transverse incision was made in the intestine extending about half-way across the lumen diameter and located approximately 0.8 cm. from the pyloric sphincter.[2] The stomach was rinsed once with about 5 ml. of 0.9% sodium chloride solution (37°) by inserting a 16-gauge ball-tipped needle attached to a 5-ml. syringe into the stomach through the incision in the intestine. Six milliliters of the drug solution, initially at 37°, was then injected into the stomach *via* the intestinal incision by means of a 10-ml. syringe fitted with a 23-gauge silver lacrimal cannula.[3] A ligature was placed around the pyloric sphincter and needle prior to the injection in order to prevent leakage. This ligature was tightened further as the cannula was withdrawn. After completion of the above procedure the midline incision was closed with wound clips. One hour after injection the entire stomach was excised and homogenized.[4] The homogenate was then assayed for unabsorbed salicylic acid and ethanol.

The methodology used in the "static stirrer" experiments was similar to that described above, except for the following modifications. A ligature was not initially placed around the esophagus. After rinsing the stomach as previously described, a specially designed microstirrer,[5] shown in Fig. 1, was inserted through the intestinal incision into the stomach. The microstirrer was then secured by tying a ligature around the small intestine at the pyloric sphincter, since this was located directly over

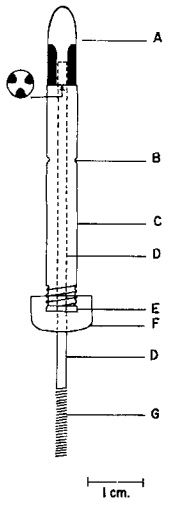

Fig. 1—Cylindrical microstirrer, longitudinal section. Key: A, *revolving Teflon tip with three indentations (shaded portion) which result in a paddlewheel design;* B, *groove for attachment at pyloric sphincter;* C, *Teflon sleeve;* D, *stainless steel shaft;* E, *neoprene O-ring;* F, *plastic screw cap;* G, *flexible shaft. Insert: cross-section of the Teflon tip.*

a groove in the stirrer shaft (Fig. 1, B). The drug solution was then introduced by the technique previously described, except that the lacrimal cannula was inserted into the stomach through a small incision in the esophagus about 0.5 cm. cephalad to the cardiac sphincter. The midline incision through the skin and body wall of the rat was then closed with suture except for an opening through which the stirrer protruded, as shown in Fig. 2. In these "static-stirrer" experiments the microstirrer was not rotated during the absorption period. At the end of this period the stomach with the remaining drug solution and the microstirrer was excised, the stirrer was detached, the stomach and its contents were homogenized, and the homogenate was assayed for unabsorbed salicylic acid and ethanol.

The "rotating stirrer" experiments were performed in the same manner as the "static stirrer" experiments except that after completion of the surgical procedure, the externally located Teflon sleeve of the microstirrer was secured by a clamp, the flexible shaft was attached to a precision laboratory motor,[6] and the stirrer was revolved at 290 ± 10 r.p.m. Stirring was initiated 5 to 7 min. after injection of the solution into the stomach and was continued over the remainder of the 1-hr. absorption period. Observation (through the somewhat transparent gastric wall) of the movement of the small air bubbles within the stomach indicated that adequate mixing of the gastric contents was achieved.

Analytical Methods—Salicylic acid in the gastric homogenate was assayed as described previously (1). Assays were done in duplicate using two 20-ml. portions of the 50 ml. of homogenate. The average recovery of known quantities of drug added to homogenized stomach tissue was 95%. Ethanol was determined by the method of Hoult and Pawan (3). Two aliquots of each homogenate were diluted and assayed, with duplicate determinations being carried out on each diluted aliquot. Standard curves were prepared simultaneously with each set of assays. The average recovery of ethanol from homogenized stomach tissue was 98%.

Statistical Analysis[7]—The salicylic acid and

[2] This incision was needed only for the insertion of the microstirrer in the other two procedures. The same methodology was used when the microstirrer was not inserted in order to obtain a proper control.

[3] This is a soft, blunt-tipped needle which was used to prevent gastric damage.

[4] Multi-mix Homogenizer, Lourdes Instrument Corp., Brooklyn, N. Y.

[5] The authors are indebted to Mr. William Tanski, Jr., Health Sciences Fabrications and Maintenance Laboratory, UNYB, for construction of the microstirrer.

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

[7] The authors acknowledge the helpful consultation with Dr. Roger L. Priore concerning the statistical analysis.

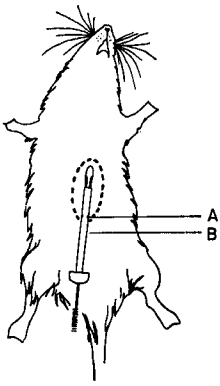

Fig. 2—Approximate position of the stirrer in relation to the rat after completion of surgical and injection procedures. The stippled line indicates the position of the stomach. Key: A, pyloric sphincter tied over groove in Teflon sleeve; B, stirrer sleeve protruding through midline incision.

ethanol absorption data (Table I) were analyzed separately using a 3 × 2 analysis of variance (three experimental conditions, absence and presence of caffeine) with subsequent *F*-tests (4, 5) on the six categories in which both ethanol and salicylic acid were present in the drug solution. The Student *t* test was used to compare the category in which ethanol alone was present with the category in which ethanol and salicylic acid were employed (unstirred condition).

Exclusion of Data—Experiments with the microstirrer occasionally caused pronounced gastric damage. The results of these experiments were not included in the tabulated data. The basis for the exclusion of data was the magnitude of ethanol absorption observed in the "ethanol alone-unstirred" category. All experiments in which ethanol absorption was less than the lowest individual value observed in this category (26%) were excluded.

RESULTS

The results of this study are summarized in Table I. The over-all absorption rate of salicylic acid was decreased by caffeine under all experimental conditions (unstirred, static stirrer, and rotating stirrer). The effect of caffeine was highly significant ($p < 0.01$) in each instance. The absorption rate of ethanol, which was included in the drug solutions as a noncomplexed marker, was not significantly affected by the presence of caffeine under any of the three experimental conditions ($p > 0.05$). Stirring itself had no significant effect on the absorption rate of free salicylic acid, salicylic acid complexed with caffeine, or ethanol (all static stirrer *versus* rotating stirrer experiments, $p > 0.1$). Thus, the decrease in the over-all absorption rate of salicylic acid caused by caffeine was of similar magnitude under both static stirrer and rotating stirrer conditions. Salicylic acid apparently increased the absorption rate of ethanol slightly. This effect was barely significant ($p \sim 0.05$ by *t* test of ethanol-unstirred *versus* salicylic acid, ethanol-unstirred data). It could also be concluded that the presence of the stirrer (whether rotating or not) in the stomach resulted in a significant decrease in the absorption rate of both ethanol and salicylic acid ($p < 0.01$).

Experiments with the microstirrer occasionally caused gastric damage (opaque appearance of the mucosa and/or bleeding). This resulted in a pronounced decrease in the absorption rates of ethanol and salicylic acid. Based on the criterion described under *Experimental*, the following data were excluded: salicylic acid–ethanol, static stirrer, 1; salicylic acid–ethanol–caffeine, static stirrer, 1; salicylic acid–ethanol, rotating stirrer, 7; salicylic acid–ethanol–caffeine, rotating stirrer, 2. It is apparent that the highest incidence of gastric mucosal damage occurred with salicylic acid–ethanol under rotating stirrer conditions. This is probably due to the well known mucosal damaging effect of salicylic acid which may have been enhanced by the mechanical effect of stirring. Some of the instances of mucosal damage may have been due to accidental contact of the rotating part of the stirrer with the gastric mucosa. The average absorption rate of ethanol in the discarded experiments was 19.5 (4.6)%[8] while that in the control experiments was 34.6 (6.7)%.[8] The effect of caffeine on salicylic acid absorption was evident even in the experiments which were discarded because of gastric damage; the difference between the absorption rates of salicylic acid in the absence and presence of caffeine was statistically significant (*t* test, $p < 0.01$) even when none of the collected data were excluded from the analysis.

TABLE I—EFFECT OF STIRRING ON THE ABSORPTION RATE OF SALICYLIC ACID WITH OR WITHOUT CAFFEINE

Initial Compn. of Soln.,[a] %		Condition	% Absorbed[b] in 1 hr. Salicylic Acid	Ethanol
Salicylic acid	0.100	Unstirred	58.1 (6.7)[c]	43.6 (9.3)[c]
Ethanol	2.00			
Salicylic acid	0.100	Unstirred	38.5 (4.9)	42.6 (8.4)
Ethanol	2.00			
Caffeine	2.75			
Salicylic acid	0.100	Static stirrer	46.5 (5.3)	34.4 (5.8)
Ethanol	2.00			
Salicylic acid	0.100	Static stirrer	36.9 (6.6)	41.3 (4.6)
Ethanol	2.00			
Caffeine	2.75			
Salicylic acid	0.100	Rotating stirrer	45.6 (6.1)	34.5 (5.9)
Ethanol	2.00			
Salicylic acid	0.100	Rotating stirrer	32.6 (3.2)	38.1 (6.1)
Ethanol	2.00			
Caffeine	2.75			
Ethanol	2.00	Unstirred		34.6 (6.7)

[a] 0.1 *N* HCl served as the solvent. [b] Mean of 8 animals per group. [c] Standard deviation in parentheses.

DISCUSSION

The decrease in the gastric absorption rate of salicylic acid caused by caffeine was of similar magnitude to that observed in a previous study from this laboratory (1).[9] Also, as in the previous study, caffeine had no significant effect on the absorption rate of ethanol. Ethanol, which is not complexed by caffeine, was included in all drug solutions as a marker to detect any possible changes in the gastric

[8] Standard deviation in parentheses.

[9] It should be noted that the volume of solution introduced into the rat stomach in this study was 6 ml., while the volume used in the previous study was 5 ml. The body weight of the rats used in the previous study was 100–150 Gm. Larger animals had to be used in the present study in order to accommodate the microstirrer.

mucosa and/or its blood supply which might affect the absorption rate of the drugs. Since caffeine did not affect the absorption rate of ethanol but reduced the over-all absorption rate of salicylic acid, it may be concluded that the observed effect is due to an interaction of caffeine with salicylic acid and not to a direct effect of caffeine on the permeability of the gastric mucosa. The complex formation between salicylic acid and caffeine is well known (6) and it has been estimated that about 84% of the salicylic acid in the salicylic acid–caffeine solutions used in this study is complexed with caffeine (1).

Stirring had no significant effect on the absorption rate of ethanol nor on the absorption rate of salicylic acid, with or without caffeine. It appears, therefore, that diffusion of drug through gastric fluids to the membrane is not the rate-limiting step in the absorption of a rapidly absorbed drug such as salicylic acid or a somewhat more slowly absorbed drug, such as ethanol. This conclusion is in agreement with the theoretical considerations outlined by Laidler (7) and Danielli (8), who assumed that when a molecule is diffusing through a nonporous membrane separating two aqueous phases, diffusion through the aqueous phases ordinarily is sufficiently rapid relative to diffusion through the membrane that the former can usually be neglected in the kinetic analysis. Our results indicate that fluid mixing due to body movement of the breathing, although anesthetized, animal is sufficient so that gastric absorption, even without artificial stirring, will not be rate limited ordinarily by intragastric diffusion. Since the magnitude of the caffeine effect on the absorption rate of salicylic acid was similar under "static stirrer" and "rotating stirrer" conditions, it may also be concluded that the absorption of salicylic acid existing in complexed form is not rate limited by the rate of diffusion of the salicylic acid–caffeine complex through the gastric fluids to the absorbing membranes. It should be mentioned, however, that movement of drug to the gastric membranes can be the absorption rate-limiting step if the viscosity of the drug solution is increased considerably (9).

These results are of further interest since they may shed some light on an important aspect of the gastrointestinal absorption of drugs by passive diffusion. Schanker *et al.* (2) have observed an "upper limit" in the relative absorption rate (*i.e.*, per cent per hour) of rapidly absorbed drugs such as salicylic acid, benzoic acid, and *p*-hydroxypropiophenone from the rat stomach. These investigators have suggested that this "upper limit" may be the result of drug diffusion through gastric fluids to the biologic membrane (rather than diffusion through the membrane itself) becoming the rate-limiting step in the gastric absorption of sufficiently rapidly absorbed drugs. They were able to show that gastric blood flow was not a limiting factor in the absorption process. The results of the present investigation, which involved experimental conditions similar to those of the study by Schanker *et al.* (2), show that the rate of movement of drug molecules through the gastric fluids is not rate limiting and cannot account for the upper limit in the gastric absorption of drugs as observed by Schanker and co-workers. A review of the available evidence leads to the conclusion that the rate of transfer of drug from aqueous gastric fluids into the lipoid barrier (real or hypothetical) of the gastric membranes will no longer be absorption rate limiting when the *in vivo* partition coefficient of the drug exceeds a certain value. The quantitative similarity in the maximum relative absorption rate (or, more exactly, the absorption rate constant) of diverse passively absorbed drugs suggests that the rate-limiting step is one of diffusion; the results of the present study indicate that the locus of the rate-limiting diffusion is somewhere within the gastric membrane barrier or possibly in the mucous layer immediately adjacent to the gastric epithelium (particularly in the gastric pits), rather than the gastric fluids. From a practical point of view, it appears that increasing the lipoid–water partition coefficient of drugs by molecular modification may be of limited value in increasing absorption rate when the drug itself already has a relatively high partition coefficient. It must be realized, of course, that when a drug is given in solid form rather than in solution, it is dissolution, rather than membrane permeation, which will usually be rate limiting in the absorption process.

The reason for the effect of salicylic acid on ethanol absorption is not readily apparent. This relatively small effect, which was only of borderline statistical significance, was not observed in a previous study (1) and also was not apparent in the "static stirrer" and "rotating stirrer" experiments of the present study. It is likely to have been due to chance, particularly since ethanol absorption appears to have been more variable than salicylic acid absorption.

The decrease in the absorption of salicylic acid and ethanol in the presence of the stirrer may have been due to the manipulation involved while inserting the stirrer into the stomach. It does not appear to be due to the extra distention of the stomach by the added volume of the stirrer ($\sim$0.75 cm.^{3}) and a resulting decrease in blood flow through capillaries in the gastric mucosa, since Schanker *et al.* (2) found that aniline clearance from blood to stomach was essentially constant over a range of gastric volumes encompassing the volume used in the present study.

Certain theoretical calculations outlined previously (1) suggest that the salicylic acid–caffeine complex may be absorbed as such and at a rate intermediate between that of free salicylic acid and free caffeine. These calculations had to be based on assumptions which, unfortunately, are rather speculative. The possible presence of other than 1:1 complexes of salicylic acid and caffeine, the possible existence of caffeine dimers and larger aggregates, the possibility of competitive binding of one or both components of the complex by membrane constituents, and the likelihood of partial dissociation of the complex at the surface of the biologic membranes (10) make it impossible to reach a definitive conclusion with respect to the absorption of the salicylic acid–caffeine complex as such. A relatively conclusive experiment, namely the determination of the effect of complexation with salicylic acid on the absorption rate of caffeine, could not be carried out because of the technical difficulties outlined in the introductory discussion. Studies are now in progress with systems which, it is hoped, do not involve these difficulties.

REFERENCES

(1) Levy, G., and Reuning, R. H., *J. Pharm. Sci.*, **53**, 1471(1964).
(2) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **120**, 528 (1957).
(3) Hoult, H. W., and Pawan, G. L. S., *Biochem. J.*, **87**, 15P(1963).
(4) Goldstein, A., "Biostatistics: An Introductory Text," The Macmillan Co., New York, N. Y., 1964, pp. 63–83.
(5) Snedecor, G. W., "Statistical Methods Applied to Experiments in Agriculture and Biology," 5th ed., Iowa State College Press, Ames, Iowa, 1956, pp. 329–355.
(6) Higuchi, T., and Zuck, D. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 138(1953).
(7) Laidler, K. J., and Shuler, K. E., *J. Chem. Phys.*, **17**, 851(1949).
(8) Danielli, J. F., in "The Permeability of Natural Membranes," Davson, H., and Danielli, J. F., Cambridge University Press, Cambridge, England, 1943, chap. V.
(9) Levy, G., and Jusko, W. J., *J. Pharm. Sci.*, **54**, 219 (1965).
(10) Levy, G., and Matsuzawa, T., *ibid.*, **54**, 1003(1965).

Effect of Polymorphism on the Absorption of Chloramphenicol from Chloramphenicol Palmitate

By ARMANDO J. AGUIAR, JOHN KRC, JR., ARLYN W. KINKEL, and JOSEPH C. SAMYN

The effect of polymorphism on the availability of chloramphenicol from chloramphenicol palmitate was studied. *In vivo* absorption studies in humans, following single oral doses of the ester, indicate that the polymorphic state of chloramphenicol palmitate influences significantly the blood levels obtained. The use of enzymatic hydrolysis as an *in vitro* method for predicting blood levels is evaluated. The utility of X-ray diffractometry, melting point determinations, and infrared spectroscopy in qualitative and quantitative analysis of the polymorphic forms is discussed. In addition to the two previously reported polymorphs of this drug designated as polymorphs A and B, a third polymorph, form C, is described.

PHYSICAL PROPERTIES such as particle size, solubility, rates of solution, aggregation of primary particles, and wettability have been shown by various workers (1–7) to influence the absorption and therapeutic efficacy of relatively insoluble drugs. The polymorphic state of a drug can also be an important factor (8) since differences in the free energy of polymorphs influence some of these properties. Higuchi (9) has suggested that these differences may appreciably affect their physiological activity.

The present study is concerned with an evaluation of the effect of polymorphic state on the absorption of chloramphenicol from chloramphenicol palmitate. The study was also designed to determine the usefulness and limitations of commonly applied analytical techniques such as X-ray diffractometry, infrared spectroscopy, and melting point techniques in identifying and characterizing the polymorphs of chloramphenicol palmitate. Furthermore, the utility of enzymatic hydrolysis as an *in vitro* criterion for predicting *in vivo* blood levels is examined.

This report deals with the properties and absorption characteristics of polymorphs A and B of chloramphenicol palmitate. Data on the third polymorph (form C) will be presented in a subsequent publication.

Chloramphenicol palmitate was synthesized by Edgerton (10) as a tasteless derivative of chloramphenicol. Glazko *et al.* (11) showed that the intact esters are poorly absorbed from the intestinal tract and must first be hydrolyzed by the esterases in the small intestine before any significant absorption can take place. The rate of hydrolysis of the ester is governed by the rate of solution which, in turn, is dependent on factors such as primary particle size, state of aggregation of primary particles, but most important, as this study shows, on the polymorphic form.

PAST WORK ON THE POLYMORPHISM OF CHLORAMPHENICOL ESTERS

Milosovich (12), studying the physical stability of chloramphenicol palmitate suspensions, suggested the existence of two polymorphs of this drug. Subsequently, Tamura and Kuwano (13) and Maruyama *et al.* (14) reported their work on the two polymorphs and an additional amorphous glassy phase.

Almirante and his co-workers (15) showed that chloramphenicol stearate also exists in two forms, one of which gave good blood levels regardless of particle size. These authors also showed that the presence or absence of a wetting agent had no appreciable effect on the blood levels obtained.

Recently Menachemoff (16) reported that the hydrolysis time of chloramphenicol palmitate is the

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Accepted for publication May 4, 1967.
The authors are grateful to Dr. W. Kitto, Dr. J. Fitzsimmons, and Mr. J. Conlin for the human experiments; Dr. W. Alegnani and Dr. A. Glazko for the colorimetric assays; Mr. R. B. Scott for help in the X-ray analysis; Dr. D. Szulczewski for the I.R. measurements; and Miss C. Gryczko for the enzymatic hydrolysis determinations. Special appreciation is also expressed to Dr. L. M. Wheeler and Dr. L. M. Lueck for their suggestions and constructive criticism.

REFERENCES

(1) Levy, G., and Reuning, R. H., *J. Pharm. Sci.*, **53**, 1471(1964).
(2) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **120**, 528 (1957).
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(8) Danielli, J. F., in "The Permeability of Natural Membranes," Davson, H., and Danielli, J. F., Cambridge University Press, Cambridge, England, 1943, chap. V.
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Accepted for publication May 4, 1967.
The authors are grateful to Dr. W. Kitto, Dr. J. Fitzsimmons, and Mr. J. Conlin for the human experiments; Dr. W. Alegnani and Dr. A. Glazko for the colorimetric assays; Mr. R. B. Scott for help in the X-ray analysis; Dr. D. Szulczewski for the I.R. measurements; and Miss C. Gryczko for the enzymatic hydrolysis determinations. Special appreciation is also expressed to Dr. L. M. Wheeler and Dr. L. M. Lueck for their suggestions and constructive criticism.

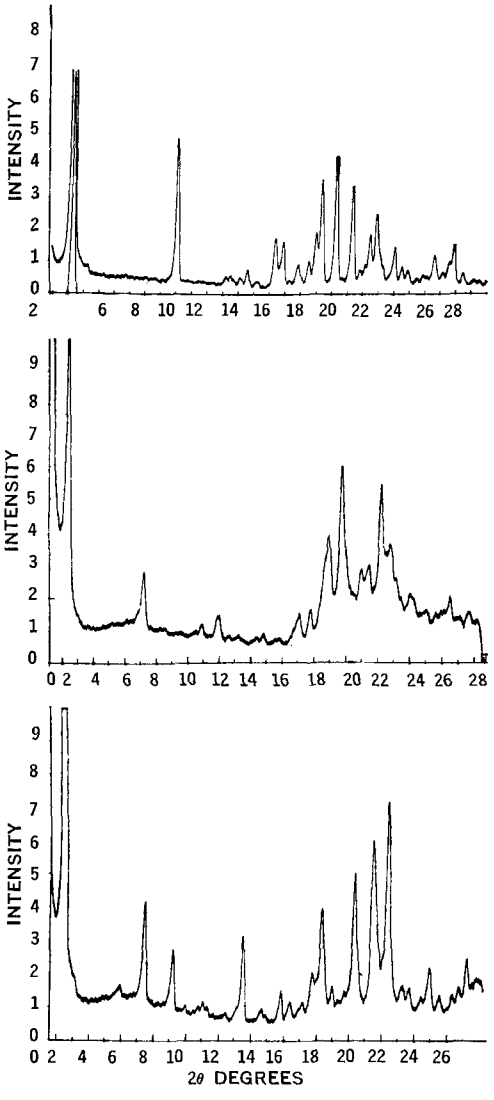

Fig. 1—X-Ray diffractograms for polymorphs A (top), B (middle), and C (bottom) of chloramphenicol palmitate.

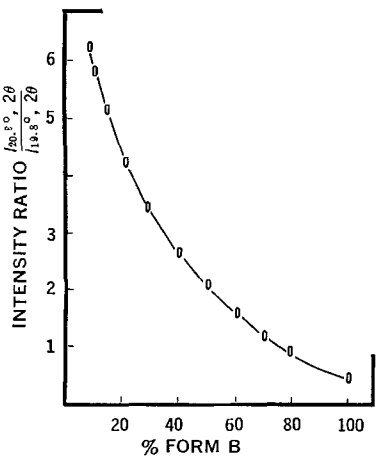

Fig. 2—Intensity ratio as a function of composition of forms A and B of chloramphenicol palmitate.

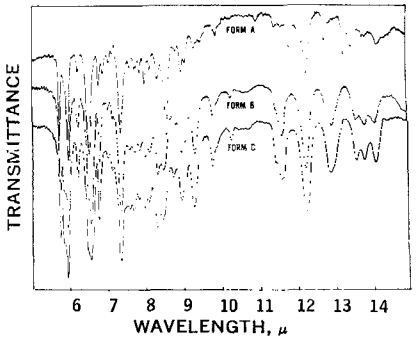

Fig. 3—Infrared spectra of polymorphs A, B, and C of chloramphenicol palmitate.

essential factor for the rate of absorption, and the amount of hydrolysis is related to the crystalline structure of the palmitate.

ANALYTICAL METHODS

The analytical methods commonly applied to confirm the existence of polymorphic states are X-ray diffractometry, optical crystallography, infrared spectroscopy, and fusion behavior (17–20). When polymorphs can be prepared separately and their physical characteristics established, distinguishing features can be accurately chosen for their identification and analysis in mixtures. In these laboratories, X-ray diffractometry provided a dependable method for initial identification and for quantitative analysis of polymorphs of chloramphenicol palmitate.

Once the characteristic diffraction criteria for the polymorphic forms were established by X-ray diffraction, it was found that other methods, such as infrared spectroscopy, could be conveniently used for routine determinations of the quantity of polymorph A present in any samples containing mixtures of the polymorphic forms.

X-Ray Diffractometry—The X-ray diffraction patterns for polymorphs A, B, and C of chloramphenicol palmitate are summarized in Fig. 1. It is evident that these patterns differ.

X-Ray diffractometry was used successfully to measure the quantities of polymorphs A and B present in chloramphenicol palmitate suspensions over the whole range of composition. The relative intensities of two diffraction lines—one at 19.8° (2θ, CuKα) associated with form B, and the other at 20.3° (2θ), associated with form A—were used. A working curve, in which composition was plotted against the ratio of intensities, $I_{20.3°}/I_{19.8°}$ (Fig. 2) was prepared by reading intensities of known mixtures of forms A and B.

It should be noted that an analysis of this type is based on the assumption of a binary system of A and B polymorphs. The presence of form C or amorphous material in the sample can cause errors. The interference of form C was obviated by applying a suitable correction to the intensity at 20.3° (2θ), determined on the basis of the intensity of form C at 9.2° (2θ). Amorphous chloramphenicol palmi-

Table I—Comparison of Values of Polymorph A Obtained by X-Ray and Infrared Procedures in Samples Containing Known Mixtures of Polymorphs A and B

% A (Theoretical)	% A (by I.R.)	% A (by X-Ray)
5	4	8
7	7	7
15	14	13
50	50	49
75	79	72

tate did not affect the analysis of A and B with regard to proportion, but only with regard to the absolute amount of each.

X-Ray Diffraction Instrumentation and Procedure—A Norelco diffractometer was used. The instrument was equipped with a flat specimen spinner, which rotated the packed powder in the plane of its surface. The sample was packed into an aluminum planchet having a depression 1.5 mm. deep and 22 mm. in diameter. The flat side of a spatula was used to pack the powder and smooth it so that a uniform level surface was presented to the X-ray beam. Coarse or lumpy powder was first ground in a mortar or mechanical blender.

The instrumental variables of the diffractometer were set as follows: (*a*) 1° divergence and scatter slits, and 0.006 in. receiving slits; (*b*) CuKα radiation, 35 Kv., 20 ma., Ni filtered; (*c*) detector: scintillation counter with pulse height discrimination. Intensities were measured by recording the time necessary to count a fixed number of pulses, usually 25,600.

Infrared Spectroscopy—The method used for the infrared determination of chloramphenicol palmitate polymorphs followed closely that of Maruyama *et al.* (14) using a Beckman IR-4 spectrophotometer.

Thus far, two infrared spectra of solid chloramphenicol palmitate have been encountered, one of which is specific for polymorph A, while the other shows minor variations for any of the "non-A" phases. The infrared spectra for polymorphs A, B, and C are shown in Fig. 3. It is readily apparent that while the spectrum for form A is characteristic, the spectra for forms B and C are indistinguishable.

The infrared procedure can therefore be used to determine quantitatively the concentration of polymorph A present in a sample of chloramphenicol palmitate. However, it cannot be used to either distinguish or estimate the quantity of polymorph B in the presence of C or other "non-A" phases.

To compare the accuracy of X-ray diffractometry and infrared spectroscopy in estimating quantities of polymorph A in chloramphenicol palmitate powders, known mixtures of polymorphs A and B were prepared and analyzed by these methods. The results are given in Table I. The values obtained are close and it is obvious that these methods can be used interchangeably for estimating the quantity of polymorph A.

Melting Point—The use of melting point determinations is of limited value for identification of polymorphic forms of chloramphenicol palmitate or as a dependable criterion of their purity. It was found that regardless of the techniques used for determining the melting point, form C was transformed into form B, and the melting point observed (about 86°) was that of form B. Similar transformations also occurred when attempts were made to determine the melting point of the amorphous phase.

The presence of impurities also affected the melting point. Analytical difficulties precluded the quantitative determination of homologous impurities in chloramphenicol palmitate. However, it was found that the extent and nature of such impurities had a marked and varying effect on the melting point.

Table II—Summary of Properties of Suspensions Used in the Absorption Studies

Designation	% Polymorph B Theoretical	% Polymorph B by X-Ray	Dispersion	Av. Particle Size, μ
K[a]	100	100	W.D.[b]	1
L	100	95	H.A.[c]	5
M	0	0	H.A.	5
N	25	24	H.A.	5
O	50	50	H.A.	5
P	75	75	H.A.	5
Q	100	99	H.A.	25

[a] Polysorbate 60 added. [b] Well dispersed. [c] Highly aggregated. [d] Microscopic evaluation.

ABSORPTION STUDIES

The absorption studies were carried out to: (*a*) evaluate and compare the blood levels obtained with suspensions of form B of chloramphenicol palmitate in the presence and absence of a surface-active agent, (*b*) determine the absorption properties of suspensions containing varying concentrations of polymorphs A and B, (*c*) ascertain the type of blood levels obtained from suspensions containing 100% polymorph B with a larger (25 μ) particle size.

Experimental—For the purpose of the absorption studies, experimental suspensions of chloramphenicol palmitate were prepared in an aqueous vehicle containing sweetening agents, gums, a preservative, and flavoring agents. For convenience of identification, these suspensions were labeled K, L, M, N, O, P, and Q.

Suspension K was prepared by suspending polymorph B with a uniform particle size of about 1 μ. The drug was well dispersed by adding polysorbate 60 to the vehicle. This preparation was used as a "reference standard" with which the absorption patterns of the other suspensions were compared.

Suspension L contained 100% polymorph B. No surface-active agent was added to the vehicle. The drug was highly aggregated. The average particle size, determined microscopically, was approximately 5 μ.

Suspension M was made from 100% form A chloramphenicol palmitate powder. The powder was prepared by dissolving the drug in hot xylene and allowing to cool overnight. The suspension was highly aggregated. The average particle size was about 5 μ. No surface-active agent was added to the vehicle.

[1] Marketed as Tween 60 by Atlas Powder Co., Wilmington, Del.

Suspension N, containing 75% of form A and 25% of form B, was prepared by mixing suspensions M and L in these proportions.

Suspension O was prepared by mixing equal quantities of suspensions L and M to obtain a mixture of 50% form A and 50% form B.

Suspension P, containing 25% of form A and 75% form B, was prepared by mixing 75 parts of L with 25 parts of M.

Suspension Q contained 100% form B with an average particle size of 25 μ. The drug was crystallized from ethanol. No surface-active agent was used, and the drug was highly aggregated in suspension.

The properties of the suspensions used in the absorption studies are summarized in Table II.

Methodology—To ascertain the effect of polysorbate 60 on the absorption of chloramphenicol from suspensions of chloramphenicol palmitate containing 100% polymorph B, a double-blind crossover type of study was used.

Six normal adult male human volunteers were used. These ranged in weight from 134 to 199 lb. with an average weight of 154 lb. Three of the subjects were given oral doses of suspension K, equivalent to 1.5 Gm. chloramphenicol, and three other subjects received the same dose of suspension L. After 1 week, each subject received the opposite suspension.

Blood specimens were taken at 0, 1, 2, 4, 8, 12, and 24 hr. after dosage. Urine collections were made for 24 hr. before medication, and at 0–2, 2–4, 4–6, 6–8, 8–12, and 12–24 hr. after dosage. Total urine volumes were measured for each collection period and a sample of each specimen was retained for analysis.

All samples were assayed for total nitro compounds, using Glazko's (21) colorimetric procedure. All values are reported in terms of chloramphenicol equivalents and are the average of the value obtained for six individuals in each instance.

Thirty normal male volunteers were used to study the absorption of chloramphenicol from suspensions containing varying quantities of polymorphs A and B (suspensions L, M, N, O, and P). Their weights ranged from 150 to 170 lb. Their average weight was 160 lb.

The volunteers were divided into groups of six individuals, and each group was given one of the suspensions. Each individual in the group received oral doses of the suspension equivalent to 1.5 Gm. of chloramphenicol. The procedure and intervals of time used to collect the blood and urine samples were the same as those described for the first study. The samples were also assayed in the same manner.

The absorption characteristics of chloramphenicol palmitate suspension Q were determined in five normal male volunteers. Their weights ranged from 143 to 220 lb. and their average weight was 182 lb. The dose, urine and blood collection periods, and assay procedure were identical to the ones described previously for the other suspensions.

RESULTS AND DISCUSSION

Results of blood levels obtained in the first study, designed to evaluate the absorption characteristics of polymorph B with and without polysorbate 60 (suspensions K and L), are shown in Fig. 4. This

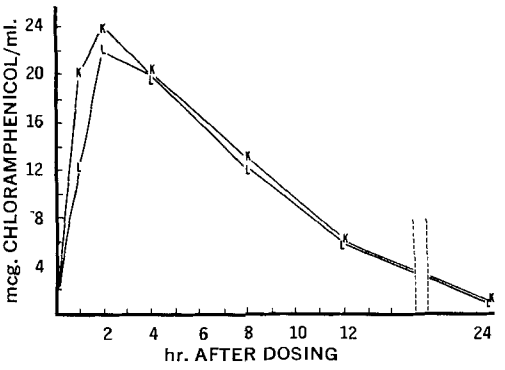

Fig. 4—Comparison of mean blood serum levels in normal humans, obtained with suspensions of 100% form B chloramphenicol palmitate. (K, with polysorbate 60; L, no surface-active agent.) Single oral dose equivalent to 1.5 Gm. chloramphenicol.

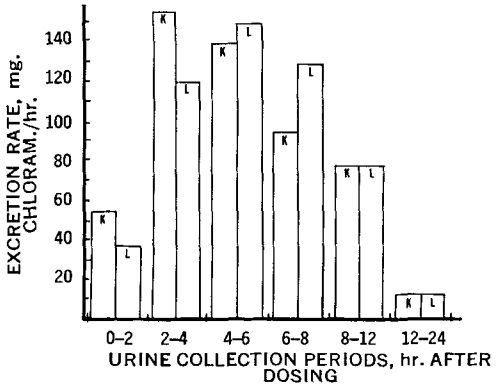

Fig. 5—Urinary excretion rate of total nitro compound (chloramphenicol equivalent) following single oral dose of 100% form B chloramphenicol palmitate suspension. (K with and L without polysorbate 60.)

plot of mean blood levels *versus* time shows that the suspension L without the surfactant appears to give comparable blood levels to suspension K with polysorbate 60.

The absorption from suspension L is, however, slightly slower initially as indicated by the lower plasma levels 1 hr. after dosage. However, no apparent differences occur at later time periods. The slower absorption is indicated also by the urinary excretion data shown in Fig. 5. The maximum excretion rate with suspension K was found in the 2–4-hr. collection period after the drug was administered, while the highest excretion rate with suspension L was found to be 4 to 6 hr. after dosage.

The delay in the absorption of suspension L can perhaps be ascribed to the slightly larger primary particle size (than that of suspension K), but it seems more likely that it is due to the highly aggregated state of the primary particles in the suspension. It is reasonable to assume that the presence of aggregates in the suspension in the initial stages will decrease the surface area presented for dissolution and hydrolysis of the ester. At later times, when the drug is dispersed in the gastric media, there is no

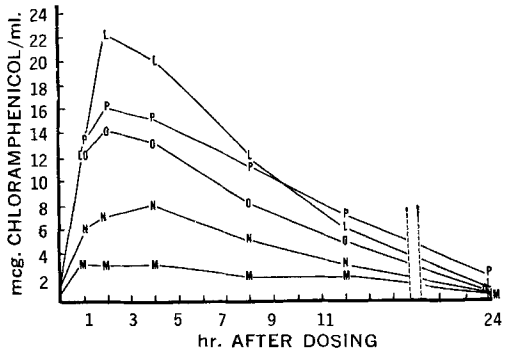

Fig. 6—Comparison of mean blood serum levels obtained with chloramphenicol palmitate suspensions containing varying ratios of A and B polymorphs, following single oral dose equivalent to 1.5 Gm. chloramphenicol. (Per cent polymorph B in the suspension: M, 0%; N, 25%; O, 50%; P, 75%; L, 100%.)

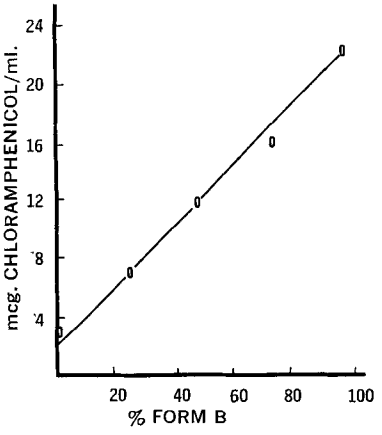

Fig. 8—Correlation of "peak" blood serum levels (2 hr.) vs. per cent concentration of polymorph B.

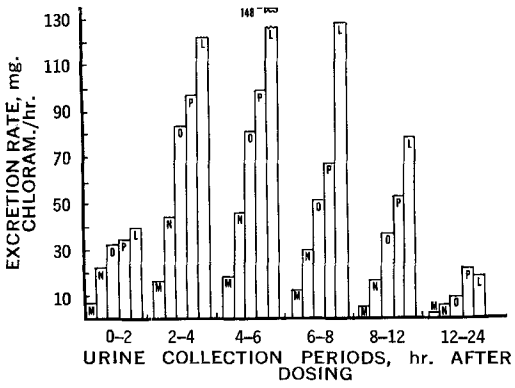

Fig. 7—Urinary excretion rate of total nitro compound (chloramphenicol equivalent) following single oral dose of chloramphenicol palmitate suspensions containing varying quantities of polymorphs A and B. Dose equivalent to 1.5 Gm. of chloramphenicol. (Per cent polymorph B in the suspension: M, 0%; N, 25%; O, 50%; P, 75%; L, 100%.)

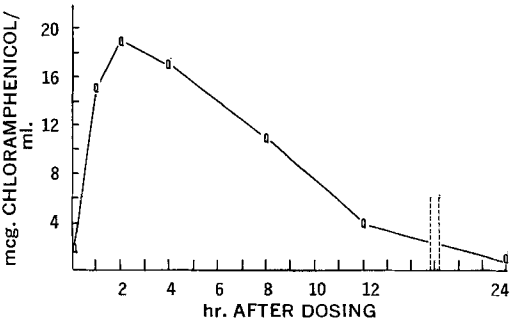

Fig. 9—Mean blood serum levels obtained with suspensions of chloramphenicol palmitate prepared from 100% polymorph B with average particle size of 25 μ.

longer any difference in the absorption patterns of the two suspensions.

The first study agrees with the observation of Almirante (15), who showed that good blood levels of chloramphenicol stearate could be obtained without polysorbate and the levels depend primarily upon the crystal form present.

Mean blood levels obtained in the second study intended to determine the absorption properties of chloramphenicol palmitate suspensions containing varying ratios of polymorphs A and B (suspensions L, M, N, O, P) are shown in Fig. 6. Urinary excretion data are given in Fig. 7.

In these single dose studies the highest mean blood levels were obtained with the suspensions containing only form B. The blood levels decreased proportionately as the concentration of form A increased. These results demonstrate that the levels are influenced by the type and concentration of the crystal polymorph present.

The differences in the absorption are even more evident from Fig. 8 where the absorption at 2 hr. ("peak" blood levels) is plotted *versus* per cent form B present in the suspension. A linear relationship apparently exists between the "peak" levels and concentration of polymorph B, the levels increasing in a direct relationship to the increase of polymorph B.

The results of the third study, shown in Fig. 9, indicate that good blood levels can be obtained with polymorph B having a larger particle size. Although the average primary particle size of the drug in suspension Q was approximately 25 μ, the absorption pattern is not significantly different from that observed with suspension L containing smaller particle size polymorph B.

It appears from these absorption studies that the polymorphic form is of much greater significance than the particle size. The greater availability of the metastable polymorph of chloramphenicol palmitate is apparently related to its greater thermodynamic activity.

Some measure of the difference in the free energy content between polymorphs A and B is obtained from solubility measurements carried out at different temperatures. From these measurements it was found that the heat of solution for polymorph A was 21.8 Kcal./mole, while that for polymorph B

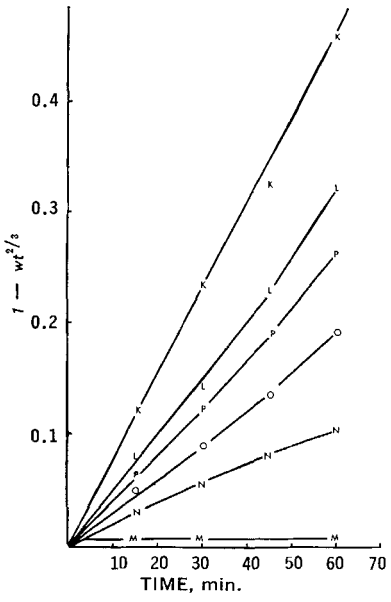

Fig. 10—Enzymatic hydrolysis rates of chloramphenicol palmitate suspensions plotted in accordance with Eq. 5. (K, 100% polymorph B with polysorbate 60) (L, 100%; P, 75%; O, 50%; N, 25%; M, 0%, polymorph B).

was 15.4 Kcal./mole. The details and results of these experiments are being presented in another publication.

USE OF ENZYMATIC HYDROLYSIS TO PREDICT BLOOD LEVELS

Glazko *et al.* (22) showed that chloramphenicol palmitate can be hydrolyzed *in vitro* at 37° by pancreatin at pH 4.5. They also indicated that the *in vitro* hydrolysis rate could be used to predict the development of blood levels in humans.

If proper conditions are selected for the experimental determinations of the hydrolysis rate, it can be shown that the rate of dissolution of the chloramphenicol palmitate particles in the suspension is a diffusion controlled process. Following Higuchi's work (23) on the dissolution rates of finely divided drug powders, one can then write Eq. 1 to describe the dissolution rate for a single particle as a function of time:

$$\frac{dw}{dt} = -\frac{4\pi D w^{1/3}}{\left(\frac{4}{3}\pi\rho\right)^{1/3}}\Delta c \qquad \text{(Eq. 1)}$$

where

Δc = concentration gradient from surface of particle to boundary of diffusion layer,
D = diffusion constant,
w = weight of single particle of chloramphenicol palmitate,
ρ = density.

For n particles in a uniform suspension of spheres, one then has:

$$\frac{dw_t}{dt} = \left(\frac{dw_1}{dt} + \frac{dw_2}{dt} + + + + \frac{dw_n}{dt}\right)$$

$$= -\frac{4\pi D\Delta c}{\left(\frac{4}{3}\pi\rho\right)^{1/3}}\left(w_1^{1/3} + w_2^{1/3} + + + + w_n^{1/3}\right) \qquad \text{(Eq. 2)}$$

Integrating Eq. 2, between the limits w_0 (total weight of particles at time zero) and w_t (total weight of particles at time t) one obtains:

$$-\int_{w_0}^{w_t}\frac{dw_t}{w_t^{1/3}} = \frac{4\pi D\Delta c n^{2/3}}{(4/3\pi\rho)^{1/3}}\int_{t=0}^{t=t} dt \qquad \text{(Eq. 3)}$$

which resolves to:

$$\frac{3}{2}\left(w_0^{2/3} - w_t^{2/3}\right) = \frac{4\pi D\Delta c n^{2/3}}{\left(\frac{4}{3}\pi\rho\right)^{1/3}}t \qquad \text{(Eq. 4)}$$

Now $w_0 = \frac{4}{3}\pi r_0^3\, nd$, where n = number of particles per 1 ml., and r_0 = initial radius of particles.

Equation 4 can be divided by the initial weight of chloramphenicol palmitate raised to the two-thirds power ($w_0^{2/3}$) and simplified to obtain Eq. 5.

$$\frac{w_0^{2/3} - w_t^{2/3}}{w_0^{2/3}} = \frac{2D\Delta c}{\rho r_0^2}t = Kt$$

or

$$1 - fw_t^{2/3} = Kt \qquad \text{(Eq. 5)}$$

where $K = \frac{2D\Delta c}{\rho r_0^2}$ and $fw_t^{2/3}$ = fraction total weight raised to the two-thirds power.

Therefore, a plot of $1 - fw_t^{2/3}$ *versus* t should give a straight line with a positive slope which is directly proportional to the solubility of chloramphenicol palmitate in the vehicle, and inversely proportional to the initial surface area of a single particle for a suspension containing one of the polymorphic forms.

In Fig. 10 representative data plotted according to Eq. 5 are shown. It is apparent from this plot that when dealing with suspensions containing either polymorph A or polymorph B (suspensions K, L, and M) the data appear to fit well with the theoretical concepts presented. The value of K can then be determined from the slope of these lines. When dealing with suspensions containing mixtures of polymorphs (suspensions P, O, and N) the lines initially appear to follow approximately Eq. 5. However, the authors have not been able to derive equations to deal quantitatively with suspensions containing such mixtures.

The experimental conditions can be selected to ensure that the determining factor in the initial rate of hydrolysis of chloramphenicol palmitate is the rate of solution or the drug. The factors which control rate of solution of chloramphenicol palmitate in suspensions are solubility of the different polymorphic forms and specific surface area.

Suspension K with 100% polymorph B well dispersed, having a particle size of about 1 μ, is used as a control. This control preparation is arbitrarily assigned as having a 100% hydrolysis rate. The values obtained with other suspensions are then compared with the control preparation and the results are tabulated as per cent relative hydrolysis rate with reference to the control preparation.

The rate of hydrolysis test values has paralleled blood level values with the exception of suspensions containing 100% polymorph B which are in a highly aggregated state. These suspensions gave lower hydrolysis rates but satisfactory blood levels.

Examples of the per cent relative hydrolysis rate values obtained with different suspensions are given in Table III together with the "peak" blood levels and the per cent mean urinary excretion 24 hr. after administration of the suspension.

From the data given in Table III it is apparent that the suspensions can be effectively deaggregated by the *in vivo* mechanisms of the gastrointestinal tract, thus increasing the surface area available for hydrolysis, which is not evident from the *in vitro* method.

It appears that although the *in vitro* enzymatic hydrolysis test could predict blood levels satisfactorily in most cases, certain refinements still have to be made in the technique to study the effect of aggregation, *etc.*, on the hydrolysis rates.

TABLE III—COMPARISON OF "PEAK" BLOOD LEVELS WITH THE PER CENT RELATIVE HYDROLYSIS RATE DETERMINED BY ENZYMATIC HYDROLYSIS TESTS

% Relative Hydrolysis Rate	Peak Blood Levels, mcg. CM/ml.	% Mean Urinary Excretion	% Polymorph B
85	15	69	100
65	22	93.6	95
50	16	69	75
35	14	49.4	50
18.21	8	29.5	25
16.9	9	29	7
14[a]	19	84.4	100
7.9[a]	18	67.7	100
1	3	9.7	0

[a] Suspensions with highly aggregated primary particles.

CONCLUSIONS AND SUMMARY

In using analytical methods for identifying polymorphs, careful consideration, as shown in this study, should be given to their usefulness and limitations. For identification and analysis of chloramphenicol palmitate polymorphs, X-ray diffractometry was found to be the most accurate method. The infrared spectra are useful in distinguishing form A from the "non-A" phases. Melting point *per se* is of limited value in identifying the polymorphs of chloramphenicol palmitate.

These studies demonstrate the influence of polymorphic state of chloramphenicol palmitate on its physiological availability. The suspension containing only form B gives higher blood levels following single oral doses than suspensions containing only form A. The blood levels decrease proportionately as the concentration of polymorph B decreased in the suspension.

Addition of a surface-active agent to the suspension has a very limited influence on the availability of chloramphenicol palmitate, and high blood levels are obtained in its absence.

It is also shown that increasing the particle size of chloramphenicol palmitate to 25 μ had little effect on the blood levels. The enzymatic hydrolysis test can be used for predicting blood levels from chloramphenicol palmitate suspensions if the particles are not highly aggregated.

This study emphasizes the effect of polymorphic form on the availability of chloramphenicol palmitate. In view of these findings, it would appear that other slightly soluble drugs should be carefully examined both for the existence and effect of polymorphs.

REFERENCES

(1) Boyd, E. M., and Dingwall, R. W., *Am. J. Med. Sci.*, **213**, 549(1947).
(2) Reinhold, J. G., Phillips, F., Flipper, H. Y., and Pollack, L., *ibid.*, **210**, 141(1945).
(3) Atkinson, R. M., Bedford, C., Child, K. J., and Tomichi, E. G., *Antibiot. Chemotherapy*, **12**, 232(1962).
(4) Kraml, M., Dubuc, J., and Gaudry, R., *ibid.*, **12**, 239 (1962).
(5) Nelson, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 607 (1957).
(6) *Ibid.*, **48**, 96(1959).
(7) Nelson, E., and Schaldemose, I., *ibid.*, **48**, 489(1959).
(8) Higuchi, W. I., Lau, P. K., Higuchi, T., and Shell, J. W., *J. Pharm. Sci.*, **52**, 150(1963).
(9) Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 659 (1958).
(10) Edgerton, W. H., U.S. pat. 2,662,906 (December 15, 1953).
(11) Glazko, A. J., Edgerton, W., Dill, W., and Lenz, W., *Antibiot. Chemotherapy*, **2**, 234(1952).
(12) Milosovich, G., Parke, Davis & Co., unpublished data.
(13) Tamura, G., and Kuwano, H., *J. Pharm. Soc., Japan*, **81**, 755(1961).
(14) Maruyama, M., Hayashi, N., and Kishi, M., *Takamine Kendyusho Nempo*, **13**, 176(1962).
(15) Almirante, L., DeCarneri, I., and Coppi, G., *Farmaco (Pavia), Ed. Pract.*, **15**, 471(1960).
(16) Menachemoff, E., *Harokeach Haivri*, **10**, 300(1964).
(17) Kofler, L., and Kofler, A., "Mikromethoden Zur Kemizeichung Organischer Stoffe und Stoffegemische," Innsbruck Universitatsverlag, Wagner, Austria, 1948.
(18) McCrone, W. C., "Fusion Methods in Chemical Microscopy," Interscience Publishers, Inc., New York, N. Y., 1957.
(19) Chamot, E. M., and Mason, C. W., "Handbook of Chemical Microscopy," vol. I, 3rd ed., John Wiley & Sons, Inc., New York, N. Y., 1958.
(20) Hartshorne, N. A., and Stuart, A., "Crystals and the Polarizing Microscope," 2nd ed., Longmans, Green & Co., New York, N. Y., 1950.
(21) Glazko, A. J., Wolf, L. M., and Dill, W. A., *Arch. Biochem.*, **23**, 411(1949).
(22) Glazko, A. J., Dill, W. A., Kazenko, A., Wolf, L. M., and Carnes, N. E., *Antibiot. Chemotherapy*, **8**, 516(1958).
(23) Higuchi, W. I., and Hiestand, E. N., *J. Pharm. Sci.*, **52**, 67(1963).

Biosynthesis of Tropic Acid in *Datura innoxia*

By C. A. GIBSON* and H. W. YOUNGKEN, JR.†

Phenylalanine-UL-^{14}C, phenylalanine-1,3-^{14}C, and shikimic acid-UL-^{14}C were fed to mature *Datura innoxia* Mill. plants, and the radioactive hyoscyamine and scopolamine were extracted and degraded to determine the position of the radioactive carbon of the tropic acid moiety. The results indicated that all the carbons of tropic acid are derived from the carbons of phenylalanine and the carboxyl carbon of tropic acid is derived from the carboxyl carbon of phenylalanine by way of some intramolecular shift mechanism.

IN THE PAST, several compounds have been studied as possible precursors of the branched chain aromatic acid, tropic acid. Among these phenylalanine, sodium acetate, phenylacetic acid, propionic acid, and tryptophan were employed. Although phenylacetic acid (1) and tryptophan (2) can be incorporated into tropic acid, more significant results have been obtained with phenylalanine as the labeled precursor (3–7).

When phenylalanine-3-^{14}C was fed to mature *Datura stramonium* plants, the hyoscyamine and scopolamine were labeled in carbon 2 of the tropic acid moiety (1, 3, 7). The hydroxymethyl group (carbon 3) was labeled when phenylalanine-2-^{14}C (4) or phenylacetic acid-1-^{14}C (1) was the radioactive precursor. The above labeling patterns have led to the formulation of a proposed pathway of biosynthesis in which an active 1-C unit serves as the precursor of the carboxyl group of tropic acid (1). Since ^{14}C-labeled carbonate, formate, formaldehyde, methionine, and serine do not significantly label the carboxyl carbon of tropic acid (1, 6), considerable interest has been focused upon the origin of this carbon atom.

Louden and Leete (5, 6) reported that tropic acid was labeled in the carboxyl carbon after *D. stramonium* plants were fed with L-phenylalanine-1-^{14}C. These results indicate the involvement of the carboxyl carbon of phenylalanine as a precursor of the carboxyl group of tropic acid. These workers have proposed a pathway of biosynthesis involving the equivalent of an intramolecular shift of the carboxyl carbon of phenylalanine to the position occupied in the tropic acid molecule. The work reported here was accomplished in order to add to the evidence in support of the latter pathway.

If a phenylpropanoid molecule, such as phenylalanine or phenylpyruvic acid, is actually involved in the biosynthesis of tropic acid, then labeled shikimic acid should label only the aromatic ring portion of this acid. Also, generally labeled phenylalanine should label tropic acid in such a way that benzoic acid, obtained by oxidation of tropic acid, would contain 7/9 the specific activity of its parent tropic acid. A third precursor, phenylalanine-1,3-^{14}C in which the ratio of the specific activities of the two radioactive carbon atoms is known, should label the carbons 1 and 2 tropic acid in the same ratio of specific activities as that of the parent compound if the "carboxyl shift" pathway is functioning.

EXPERIMENTAL

Growth and Processing of Plant Material—*D. innoxia* Mill. plants were grown from seeds selected from plants previously grown in the Drug Plant Garden, University of Rhode Island. The seedlings were potted in sandy loam and were grown in a Hotpack environment control room, model 1288-13, using the following conditions: light period, 16 hr. at a temperature of 32° ± 1° and a relative humidity of 40% ± 5%; dark period, 8 hr. at a temperature of 23° ± 1° and a relative humidity of 60% ± 5%.

The plants were maintained under the above conditions exposed to approximately 1800 f.-c. of light during the "light" period until the plants were 3 months old. The radioactive compounds were administered to the 3-month-old plants by means of cotton wicks pulled through their stems according to a modification of the procedure given by Comar (8). Details of the amount of tracer fed and the activities of the alkaloids are shown in Table I. The alkaloids were diluted prior to degradation.

Extraction and Separation of the Alkaloids—The plants were harvested 14 days after the administration of the ^{14}C-labeled compounds and the alkaloids extracted according to the procedure reported by Leete (9). Further purification of the total alkaloid extract was accomplished by immiscible solvent extraction in a separator. The hyoscyamine and scopolamine were separated by way of the column chromatography method reported by Leete (10) using diatomaceous earth[1] as the stationary phase. Final purification of the individual alkaloids was carried out by means of thin-layer chromatography. Glass thin-layer chromatography plates

Received March 13, 1967, from the †Department of Pharmacognosy, College of Pharmacy, University of Rhode Island, Kingston, RI 02881

Accepted for publication April 19, 1967.

Abstracted in part from a thesis submitted by Coy A. Gibson to the College of Pharmacy, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.

Supported in part by funds from the American Foundation for Pharmaceutical Education, Washington, D. C., and in part by grant NH-06511 from the National Institutes of Mental Health, U. S. Public Health Service, Bethesda, Md.

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[1] Marketed as Celite 545 by the Johns-Manville Corp., New York, N. Y.

TABLE I—*Datura innoxia* MILL.: ^{14}C-LABELED COMPOUNDS AND ALKALOIDS

	L-Phenylalanine-UL-^{14}C	DL-Phenylalanine-1,3-^{14}C[a]	Shikimic Acid-UL-^{14}C[b]
Wt., mg.	0.009	2.01	1.2
Activity, μc.	20.	60.5	2.82
Fresh wt. of plants, Gm.	70.5	289.0	73.4
Specific act., μc./mM	3.59×10^5	2.7×10^3	4.12×10^2
Hyoscyamine and Its Degradation Products (Act. in μc./mM)			
Incorp., %	0.1	0.28	0.3[c]
Hyoscyamine	0.437	1.66	0.101[c]
Tropine[d]	0.02[d]	0.04[d]	0.00[cd]
Tropic acid (TA)	0.416	1.62	0.1[c]
Benzoic acid (BA)	0.365	0.889	0.091[c]
Ratio, $\frac{\text{sp. act. TA}}{\text{sp. act. BA}}$	1.22	1.82	1.09[c]
Scopolamine and Its Degradation Products			
Incorp., %	0.07	0.08	...
Scopolamine	0.058	0.43	...
Scopine[d]	0.001[d]	0.03[d]	...
Tropic acid	0.058	0.397	...
Benzoic acid	0.047	0.159	...
Ratio, $\frac{\text{sp. act. TA}}{\text{sp. act. BA}}$	1.21	2.4	...

[a] Obtained by mixing known quantities of DL-phenylalanine-1-^{14}C and DL-phenylalanine-3-^{14}C. [b] Biosynthesized by Dr. B. A. Bohm, College of Pharmacy, University of Rhode Island. [c] Total alkaloids calculated as hyoscyamine. [d] Calculated by difference.

(20 cm. × 20 cm.) were prepared with a spreader[2] using Silica Gel G[2] as the adsorbent (25 Gm. Silica Gel G in 50 ml. water). The alkaloids were applied streakwise to the plates and chromatographed in sealed glass chambers using dimethylformamide–diethylamine–ethanol–ethyl acetate (1:1:6:12) as the solvent (11). The thin-layer chromatograms were air dried and kept in a vacuum desiccator for 24 hr. in order to remove the volatile solvents, particularly diethylamine and dimethylformamide. Dragendorff spray reagent, as modified by Munier and Macheboeuf (12), was used to locate the alkaloid zones by spraying a small strip across the plate perpendicular to the solvent front. Since dimethylformamide interferes with the Dragendorff color reaction (13), the strip had to be sprayed first with a 0.25% solution of sodium nitrate in 0.5% hydrochloric acid and dried before spraying with the Dragendorff reagent.

The zones of the thin-layer chromatograms containing the respective alkaloids were carefully scraped from the plates, and the alkaloids were eluted with absolute ethanol and rechromatographed repeating the above procedure. Each alkaloid, hyoscyamine and scopolamine, purified by this procedure, showed only a single spot on thin-layer chromatograms using either the system given above or with chloroform–diethylamine (9:1) as the solvent. The amount of the purified alkaloid was determined by colorimetric assay (14).

Hydrolysis of Alkaloids—Sufficient nonradioactive alkaloid was added to the purified alkaloids to provide a sample of known weight between 125 and 150 mg. Complete hydrolysis was accomplished by dissolving the alkaloid in 35 ml. of warm water and adding 10 ml. of 2 N sodium hydroxide. The mixture was evaporated on a water bath to 10 to 15 ml. The aminoalcohol, tropine or scopine, was extracted with five 25-ml. portions of chloroform, each chloroform portion being washed with 10 ml. of water. No further purification of the aminoalcohol was attempted.

Following acidification with dilute hydrochloric acid, the aqueous hydrolysate was extracted with several portions of isopropanol–chloroform (1:3). Crude tropic acid was obtained by evaporation of the isopropanol–chloroform solvent. Purification of the tropic acid was accomplished by repeated recrystallization from a mixture of hot benzene and petroleum ether yielding glistening white plates, m.p. 116.5–118°.

Degradation of Tropic Acid—Several workers (1, 3, 7) have shown that phenylalanine-3-^{14}C yields tropic acid labeled only in the carbon adjacent to the ring, and Leete (4, 6) has shown that L-phenylalanine-1-^{14}C labels only the carboxyl carbon of tropic acid. In no case has it been reported that either phenylalanine-3-^{14}C or phenylalanine-1-^{14}C can label the hydroxymethyl carbon of tropic acid. It was therefore determined that the specific radioactivity of tropic acid, along with that of the benzoic acid obtained from tropic acid, would yield the relative specific radioactivities of the two labeled carbons of tropic acid.

Tropic acid (40 to 50 mg.) was refluxed with 250 mg. of potassium permanganate in 5 ml. of water for 2 hr. The hot solution was filtered and, after cooling, acidified with hydrochloric acid. The acidic solution was then extracted with ether for 10 hr. using continuous liquid–liquid extraction. Removal of the ether yielded a light brown residue which, upon sublimation at 100–105° for 1 hr., yielded 10–12 mg. of colorless needles of benzoic acid, m.p. 121–122°.

Preparation of ^{14}C Samples and Determination of Radioactivity—Radioactive samples were assayed by combustion of the material to carbon dioxide and collecting the evolved gas in an ion chamber. The radioactivity of the gas was then determined on the Dynacon[3] model 6000 vibrating capacitor elec-

[2] Thin-layer chromatography kit, supplied by Research Specialties Co., Richmond, Calif.

[3] Nuclear Chicago Corp., Des Plaines Ill.

trometer using the rate of charge method of determining radioactivity.

The apparatus used for combustion of the organic material was the van Slyke wet combustion apparatus designed for use with the Dynacon model 6000. The reagents were prepared according to van Slyke *et al.* (15).

RESULTS

While all the alkaloid samples obtained after feeding with the three radioactive compounds were radioactive, the samples obtained from shikimic acid-fed plants were not sufficiently active to permit separation of the hyoscyamine and scopolamine. The low activity was due to the relatively very low specific activity of the shikimic acid sample. The alkaloids obtained from the plants fed L-phenylalanine-UL-^{14}C and DL-phenylalanine-1,3-^{14}C, respectively, were separated and the hyoscyamine and scopolamine purified before degradation.

If all the carbon atoms of L-phenylalanine-UL-^{14}C are incorporated in tropic acid, then the tropic acid should possess 1.28 times the activity present in the benzoic acid derived from it by oxidation. The results reported here support this theory since the tropic acid/benzoic acid specific activity ratios were 1.22 and 1.21, respectively (Table I). Shikimic acid-UL-^{14}C labels only the aromatic ring of tropic acid, as evidenced by the fact that almost all (91%) of the specific activity of the tropic acid obtained from the total alkaloid fraction was recovered in the benzoic acid obtained by oxidation.

When DL-phenylalanine-1,3-^{14}C, prepared by mixing known quantities of DL-phenylalanine-1-^{14}C and DL-phenylalanine-3-^{14}C, was fed and the values were determined by the following formula:

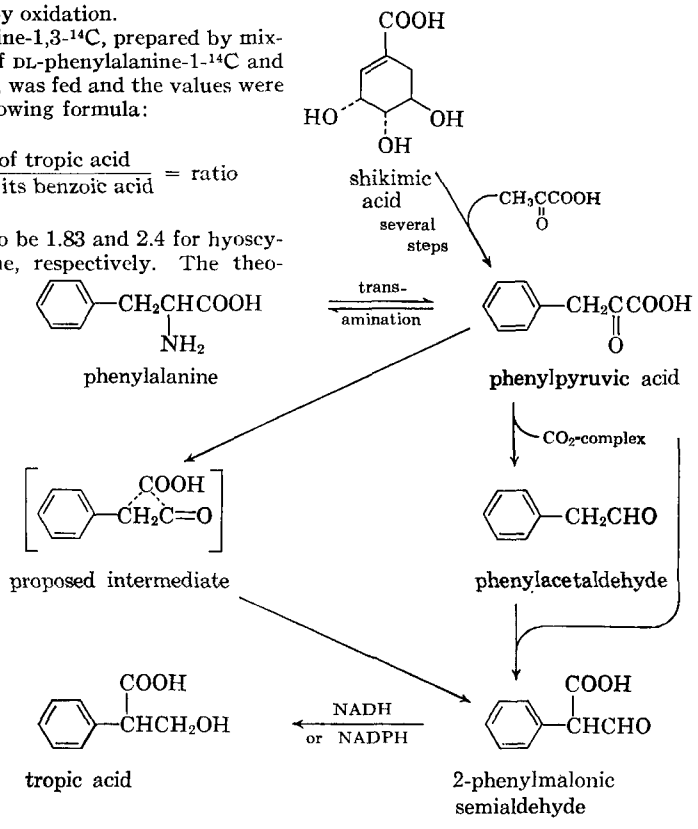
$$\frac{\text{specific activity of tropic acid}}{\text{specific activity of its benzoic acid}} = \text{ratio}$$

the ratios were found to be 1.83 and 2.4 for hyoscyamine and scopolamine, respectively. The theoretical value based upon the DL-phenylalanine-1,3-^{14}C is 1.74. The value found for hyoscyamine is a little higher than expected, but this may be explained by a more rapid hyoscyamine turnover or by losses incurred due to chemical manipulation. However, the ratio value for scopolamine (2.4) is significantly high and cannot be reasonably explained without further study.

DISCUSSION

Two general pathways have been proposed for the biosynthesis of tropic acid in *Datura*. Briefly, these may be stated as follows: (*a*) phenylalanine, after transamination to phenylpyruvic acid, undergoes decarboxylation to phenylacetic acid (or possibly its coenzyme A derivative); recarboxylation at the α-carbon of this acid followed by reduction of the original carbonyl group yields tropic acid; (*b*) phenylalanine, after transamination to phenylpyruvic acid, undergoes an intramolecular shift of the carboxyl group to the carbon adjacent to the ring; reduction at the carbonyl carbon yields tropic acid. The results of this research support the second pathway.

The total number of carbon atoms present in the molecule is the same for both phenylalanine and tropic acid. Several experiments (1, 3–7) have shown that phenylalanine can serve as the precursor of at least carbons 2 and 3 of tropic acid, *i. e.*, DL-



Proposed Biosynthetic Pathway for Tropic Acid

Scheme I

phenylalanine-3-^{14}C labels carbon 2 of tropic acid whereas DL-phenylalanine-2-^{14}C labels carbon 3 of this acid. One experiment (6) has indicated that L-phenylalanine-1-^{14}C can label the carboxyl carbon (carbon 1) of this aromatic branched-chain acid. Since no variations in this labeling pattern have been observed, the incorporation of phenylalanine-1,-3-^{14}C into tropic acid, wherein the ratio of the specific activity of carbons 1 and 2 of tropic acid equals that of carbons 1 and 3 of the phenylalanine precursor, strongly supports the hypothesis that the carboxyl carbon of phenylalanine is a precursor of the carboxyl carbon of tropic acid.

Since benzoic acid, which was obtained by oxidation of the tropic acid from *D. innoxia* plants that were fed phenylalanine-UL-^{14}C, possesses a specific activity equal to 80% of that of its parent tropic acid, phenylalanine appears to be a precursor of all the carbon atoms of the tropic acid. The incorporation of shikimic acid-UL-^{14}C in only the aromatic ring structure of tropic acid further supports the involvement of a C_6-C_3 compound. Phenylalanine is a C_6-C_3 compound biosynthesized by way of the so-called shikimic acid pathway (16). The incorporation of shikimic acid into tropic acid does not directly implicate phenylalanine in the biosynthesis of tropic acid, but it does implicate the involvement of the shikimic acid pathway by which phenylalanine is formed.

Scheme I shows a biosynthetic pathway for tropic acid [essentially the pathway proposed by Leete (6)] based upon the results given above and previous reports in the literature. It may best be described in the following three major steps.

Synthesis of Phenylalanine—This compound is formed by transamination of phenylpyruvic acid (16) which is formed by way of the shikimic acid pathway. [An excellent review on shikimic acid has been published by Bohm (17).] In the plant, tropic acid may well be formed from phenylpyruvic acid without the actual conversion of this acid to phenylalanine. However, the ease with which these two compounds undergo interconversion makes this point very difficult to confirm.

Formation of 2-Phenylmalonic Semialdehyde—The carboxyl group of this compound is derived from the carboxyl group of phenylpyruvic acid (phenylalanine). This transfer or shift of the carboxyl group may occur *via* an intramolecular shift, analogous to the methyl malonyl coenzyme A to succinyl coenzyme A isomerization (18). A second possible route by which this shift may occur is *via* an enzyme-bound decarboxylation–recarboxylation sequence. These two possible pathways for the carboxyl shift have also been suggested by Leete (6). Since no attempts have been made to determine the form in which the 2-phenylmalonic aldehyde occurs, *i. e.*, coenzyme A derivative, phosphate derivative, *etc.*, no suggestions are proposed for this pathway.

Formation of Tropic Acid—Reduction of the aldehyde group of 2-phenylmalonic semialdehyde would yield tropic acid. This reaction could be accomplished by NADH or NADPH mediated by an enzyme analogous to alcohol dehydrogenase.

The results of this work support the pathway given in Scheme I for the following reasons.

(*a*) The incorporation of shikimic acid into the ring-carbons of phenylalanine is in agreement with the known pathway of phenylalanine biosynthesis (16). (*b*) The results are in agreement with the results of other experiments (1, 3, 7) with respect to the incorporation of carbon-1 of the amino acid. (*c*) The ratio of the radioactivity of carbons 1 and 2 of tropic acid equals that of carbons 1 and 3 of phenylalanine as shown by degradation of the tropic acids obtained from plants fed DL-phenylalanine-1,3-^{14}C, as well as those fed L-phenyalanine-UL-^{14}C.

These results do not necessarily contradict the entire pathway proposed by Underhill and Youngken (1), but do contradict a sequence of decarboxylation of phenylpyruvic acid (from phenylalanine) followed by a separate recarboxylation step. However, a similar sequence during which all the reactants remain enzyme-bound so that the carbon dioxide removed in the first step is utilized in the recarboxylation step cannot be ruled out.

REFERENCES

(1) Underhill, E. W., and Youngken, H. W., Jr., *J. Pharm. Sci.*, **51**, 121(1962).
(2) Goodeve, A. M., and Ramstad, E., *Experientia*, **17**, 124(1961).
(3) Leete, E., *J. Am. Chem. Soc.*, **82**, 612(1960).
(4) Leete, E., and Louden, M. L., *Chem. Ind.*, **1961**, 1404.
(5) Louden, M. L., and Leete, E., *J. Am. Chem. Soc.*, **84**, 1510(1962).
(6) *Ibid.*, **84**, 4507(1962).
(7) Gross, D., and Schutte, H. R., *Arch. Pharm.*, **296**, 1(1963).
(8) Comar, C. L., "Radioisotopes in Biology and Agriculture," McGraw-Hill Book Co., Inc., New York, N. Y., 1955, p. 151.
(9) Leete, E., *J. Am. Chem. Soc.*, **84**, 55(1962).
(10) *Ibid.*, **82**, 612(1960).
(11) Randerath, K., "Thin-Layer Chromatography," translated by Libmann, D. D., Academic Press (Verlag Chemie), New York, N. Y., 1964, p. 78.
(12) *Ibid.*, p. 129.
(13) *Ibid.*, p. 79.
(14) Freeman, F. M., *Analyst*, **80**, 520(1955).
(15) van Slyke, R. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.*, **191**, 299(1951).
(16) West, E. S., and Todd, W. R., "Textbook of Biochemistry," The Macmillan Co., New York, N. Y., 1961, p. 1102.
(17) Bohm, B. A., *Chem. Rev.*, **65**, 435(1965).
(18) Stjernholm, R., and Wood, H. G., *Proc. Natl. Acad. Sci.*, **47**, 303(1961).

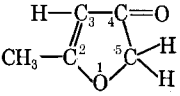
5-Methyl-3(2H)-furanone from Acid-Catalyzed Solvolysis of 2-Deoxy-D-ribose

By JOACHIM K. SEYDEL, EDWARD R. GARRETT*, WERNER DILLER, and KLAUS-JÜRGEN SCHAPER

5-Methyl-3(2H)-furanone (VIII) is produced from 2-deoxy-D-ribose (I) under defined conditions of acid, temperature, and time. Its production is a direct function of sugar concentration and its ultraviolet absorbance serves as a sensitive assay for the sugar. The compound VIII was identified by spectral data (NMR, U.V., I.R.), thin-layer chromatography R_f values in three different solvent systems, and chemical reactivities. The compound VIII obtained from deoxyribose and synthesized by a different route was the same. A mechanism dependent on the direction of water elimination after cyclization, dehydration, and rehydration can explain the formation of VIII and the previously obtained levulinic acid whose production from furfuryl alcohol (III) was explained by a similar mechanism. The spectrally observed kinetic intermediate with a $\lambda_{max.}$ at 225 mμ is consistent with the proposed mechanism.

THE THERMAL degradation of 2-deoxy-D-ribose in acid solution under specifically defined conditions produces an ultraviolet chromophore and the absorbance at $\lambda_{max.}$ 261 mμ is directly proportional to the sugar concentration (1, 2). The chromophore is extractable by chloroform and can easily be destroyed by treatment with mild alkali at room temperature. A direct sensitive spectrophotometric assay method for 2-deoxy-D-ribose in the presence of other sugars and other stable chromophores was developed on the basis of these properties (1). This method was used subsequently by Byvoet (3) for the determination of the deoxyribose content of deoxyribonucleic acid (DNA).

The isolation and identification of the compound which produces the chromophore as a 5-methyl-3(2H)-furanone [2-methyl-Δ^2-furenidone-(4)] (VIII) and its mechanism of formation are described in this paper.



VIII

EXPERIMENTAL

Apparatus—A Cary model 14 recording ultraspectrophotometer, a Varian HA 100 high resolution spectrometer with electronic counter, a Leitz infrared recording spectrometer with accessories for micro-techniques, and an Atlas mass spectrometer were used in these studies.

Materials—2-Deoxy-D-ribose (Carl Roth OHG, Karlsruhe, Germany) and diacetylethylacetate (Theodor Schuchardt G.m.b.H., Munchen, Germany), furfuryl alcohol (Eastman Organic Chemicals, Rochester, N. Y.), and ribitol (Nutritional Biochemical Corp., Cleveland, Ohio). All other chemicals were of analytical reagent grade.

Procedure for the Production, Isolation, and Purification of 5 - Methyl - 3(2H) - furanone [2 - Methyl - Δ^2 - furenidone - (4)] from Acid-Degraded 2 - Deoxy - D - ribose—2 - Deoxy-D-ribose (670 mg.) was dissolved in 1 L. of 1.0 *N* HCl and heated in a thermostated bath at 80.0° for 10 hr. The acid-degraded products were extracted with small portions of chloroform until no absorbance at 261 mμ was observed. The combined chloroform extracts were dried over anhydrous sodium sulfate and concentrated to about 10 ml. The concentrated mixture was purified by thin-layer chromatography.

The plates for thin-layer chromatography were prepared with 0.4-mm. or 1.0-mm. (preparative) layers of Silica Gel GF_{254} (E. Merck AG, Darmstadt). The mixture was spotted, or applied as a line with a syringe in the preparative case, at the origin of the plates. Chromatograms were developed for 12 cm. using as solvent systems: (*a*) chloroform–methanol, 80:15; (*b*) benzene–methanol, 9:1; (*c*) benzene–ether, 9:1. After development and drying, the spots were viewed under short wavelength ultraviolet light (254 mμ) or sprayed with 2,4-dinitrophenylhydrazine. Purification was effected by means of 1-mm. preparative thin-layer plates.

The compounds were scraped after development and extracted with chloroform. The silica gel was separated by centrifugation and filtration. When necessary for refining, the procedure was repeated. The R_f values of 5-methyl-3(2H)-furanone for the respective solvent systems were: (*a*) 0.72, (*b*) 0.37, and (*c*) 0.14. The purified substance was used to obtain the various spectrophotometric data.

Spectrophotometric Data—U.V.: $\lambda_{max.}$ 258 mμ, ϵ = 14,100, chloroform; $\lambda_{max.}$ 258 mμ, ϵ = 12,400, ethanol; $\lambda_{max.}$ 261 mμ, HCl; $\lambda_{max.}$ 295 mμ, NaOH.

Received February 17, 1967, from the Chemische Abteilung des Forschungsinstituts Borstel, Institut fur Experimentelle Biologie und Medizin, Borstel, Germany, and the *College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication April 24, 1967.

The excellent technical assistance of Miss S. Bockelmann is gratefully acknowledged. The authors are indebted to the Department of Organic Chemistry, Kiel University, Kiel, Germany, for mass spectrum analysis.

Edward R. Garrett acknowledges support in part by research grant GM-09864-03,04,05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and in part by an institutional grant from the American Cancer Society to the University of Florida.

I.R. (film): 2930, 2870, 2720, 1750, 1710, 1608, and 1150 cm.$^{-1}$. NMR: chemical shift δ-values are given in parts per million (p.p.m.) from an internal tetramethylsilane standard; coupling constants are in Hz; spectra were run at room temperature in $CDCl_3$ as solvent. NMR data were H-(3), 5.48 (Q), J_{3,CH_3} 0.8; H-(5), 4.50 (Q), J_{5,CH_3} 0.9; =C—CH_3, 2.24 (D), J 0.8.

Synthesis of 5-Methyl-3(2H)-furanone (VIII)—The compound was synthesized in a four-step reaction sequence in accordance with the methods of Becker (4) and Rosenkranz *et al.* (5).

Diacetylethylacetate, b.p. 103°, n_D 1.4663, was prepared from acetylacetate. γ,γ'-Dibromo-diacetyl-ethylacetate, m.p. 49–50° corrected, yield 88%, was prepared from diacetylethylacetate. [Lit. values: 38%, m.p. 54–55°, 52–54° (4).] 4-Carbethoxy-5-methyl-3(2H)-furanone was prepared subsequently by a method used by Becker (4), yield 37%, and purified by vacuum sublimation, m.p. 72–73°. (Lit. values: 55%, m.p. 74–76°.) The I.R., U.V., and NMR data were identical with those reported by Rosenkranz *et al.* (5) and are consistent with their structure assignments. The data do not support the structure assigned by Becker (4).

5-Methyl-3(2H)-furanone, b.p. about 60° (12 mm.), was prepared from 4-carbethoxy-5-methyl-3(2H)-furanone in accordance with the literature (5, 6). The final compound was purified by preparative thin-layer chromatography [plate 1.0 mm. Silica Gel GF_{254} (E. Merck AG), solvent: benzene–methanol 9:1] R_f: 0.37. The NMR data were H-(3), 548 [5, 41] (Q), J_{3,CH_3}0.8 [about 1]; H-(5), 4.50 [4.60] (Q), J_{5,CH_3}0.9 [0.9]; =C—CH_3, 2.24 [2.24] (D), J 0.8. Figures in brackets are the data reported by Hofmann *et al.* (6).

Degradation of Furfuryl Alcohol and Ribitol—Furfuryl alcohol and ribitol, 1×10^{-4} M, in 1.00 N HCl were maintained at 80.0° and aliquots analyzed on the Cary recording ultraviolet spectrophotometer as a function of time.

RESULTS AND DISCUSSION

When an aqueous solution of 2-deoxy-D-ribose is heated, a chromophore appears with a U.V. absorption at 261 mμ. The rate of appearance of this chromophore is not strictly first order as shown by preliminary kinetic studies (1). It is similar to an autocatalytic reaction with an initial lag before the first-order decay in the appearance of the chromophore. It is probable that the 261 mμ chromophore is associated with a final product of a sequential series of reactions. Prior to the 261 mμ chromophore a band, $\lambda_{max.}$ 225 mμ, appears by an apparent first-order rate process, which is proportional to hydrogen ion activity at a constant temperature. It is possible that this 225 mμ chromophore is a precursor to the compound responsible for the 261 mμ chromophore. The rate of appearance of the 261 mμ chromophore is proportional to the hydrogen ion activity at a constant temperature. At 80° and in 1 N HCl the final absorbance at 261 mμ is reached after 5 hr. and remains constant for at least 24 hr. The 261 mμ absorbance was found to be directly proportional to the deoxyribose concentration and serves as an assay method for deoxyribose (1, 3).

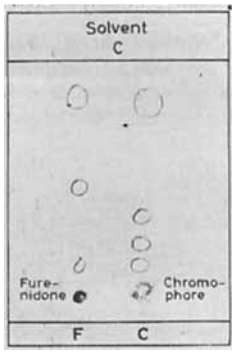
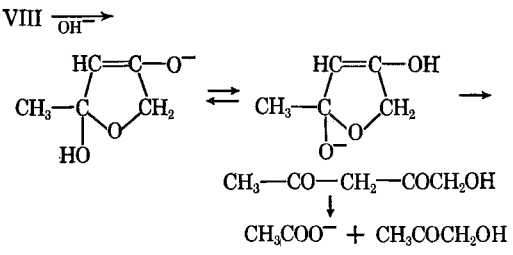


Fig. 1—Typical thin-layer chromatograms of a crude reaction mixture of synthesized 5-methyl-3(2H)-furanone, F, *and a chloroform extract of the acid-degraded 2-deoxy-*D*-ribose,* C. *The solvent system was benzene–ether, 9:1.*



Scheme I

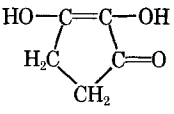
The chromophore-containing compound is extractable from acid solutions with chloroform and is unstable in alkaline solutions (1). The half-life at 28° in NaOH ($8.5 \times 10^{-4}M$) is 61 min. The compound reduces Fehling's solution and changes the color of a $FeCl_3$ solution to a red-brown. The oily colorless compound yellows and resinifies on standing in air.

Only a small fraction of the deoxyribose degrades to form the 261 mμ chromophoric compound. On the assumption that this compound is VIII, a maximum yield of 15% can be calculated from ultraviolet absorbance. Since many other compounds are present, isolation and purification were difficult. A characteristic thin-layer chromatogram of the chloroform extract of degraded 2-deoxy-D-ribose as compared with VIII is shown in Fig. 1. The spots were observed by spraying with 2,4-dinitrophenylhydrazine. The spots closest to the origin are VIII and the chromophoric compound, respectively. The hydrazones of both were red in color, indicative of compounds with unsaturated ketones.

The infrared spectrum of the purified isolates of degraded deoxyribose containing the ultraviolet chromophore had strong absorption bands at 1740, 1710, and 1608 cm.$^{-1}$. No hydroxyl group was indicated. Although the hydroxyl group absence and the presence of a conjugated ketone with a five carbon skeleton derived from a pentose would be consistent with an "angelica-type" lactone [X, XI (Scheme II)], the infrared spectra of X denied the hypothesis of such 4-hydroxy pentenic acid lactones which are formed on heating levulinic acid (7).

Several other substances are known to be products of the acid degradation of sugars. Reichstein and Oppenauer (8) reported the isolation of reductic acid (XIII) from strenuous acid treatment of pen-

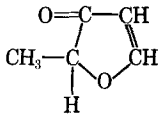
toses. The presence of hydroxyl groups in this compound denies this structure as our compound. The known products of sugar degradation such as levulinic acid (XII) (9) and ω-oxylevulinic aldehyde (10) are denied from their recorded ultraviolet absorption spectra.



XIII

The consideration of α-pyrone and furfural (12) as possible degradation products can be excluded by the wavelengths of their ultraviolet absorption and by the results of NMR and mass spectra.

The possible structures from the considered information are VIII and its structural isomer, XIV.



XIV

The three signals observed in the NMR are at 5.48, 4.50, and 2.24 p.p.m. and the intensities are in the ratio of 1:2:3 protons (Fig. 3). These signals can be assigned to a methyl group attached to a double bond, a methylene group attached to an ether oxygen, and a vinyl proton. They are inconsistent with XI and exclude XIV. The mass spectrum showed a peak consistent with a molecular weight of 98.

The 5-methyl-3(2H)-furanone (VIII) has been described in the literature (5, 6) and the NMR data were consistent. The comparison of I.R. (Fig. 2), U.V., NMR (Fig. 3), and thin-layer chromatograms in three different solvent systems (Fig. 4) demonstrated that VIII synthesized for comparative purposes and the isolated and characterized compound which gives rise to a 261 mμ chromophore from the thermal acid degradation of deoxyribose were identical. The chemical properties of structure VIII (5, 6) are the same as those of the acid degradation product of deoxyribose. Both compounds reduced Fehling's solution, gave a red-brown color with $FeCl_3$, formed phenylhydrazones, were stable in acidic solutions, and unstable in alkali with a shift of the 261 mμ chromophore to 295 mμ. The probable structures of compound VIII in alkali and its subsequent breakdown in analogy with the literature (5) are as shown in Scheme I.

Mechanism—Supportive evidence of the mechanism for the formation of VIII from deoxyribose through the pathway I–VIII (Scheme II) is available from the literature.

Birkofer and Beckmann (13) have demonstrated

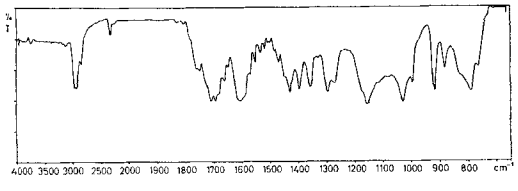


Fig. 2—Infrared spectrum of the isolated 261 mμ chromophoric compound obtained from the acid degradation of 2-deoxy-D-ribose which is the same as the spectrum of synthesized 5-methyl-3(2H)-furanone (film with microtechnique accessories).

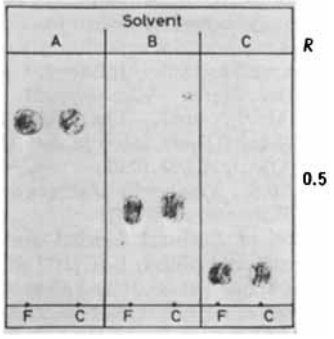


Fig. 4—Typical thin-layer chromatograms of synthesized 5-methyl-3(2H)-furanone, F, and the purified isolated 261 mμ chromophoric compound from degraded 2-deoxy-D-ribose, C, in several solvent systems. Key: A, chloroform–methanol, 80:15; B, benzene–methanol, 9:1; C, benzene–ether, 9:1.

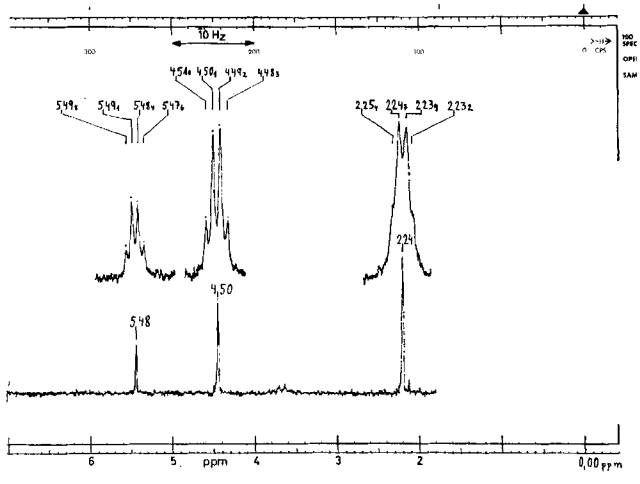
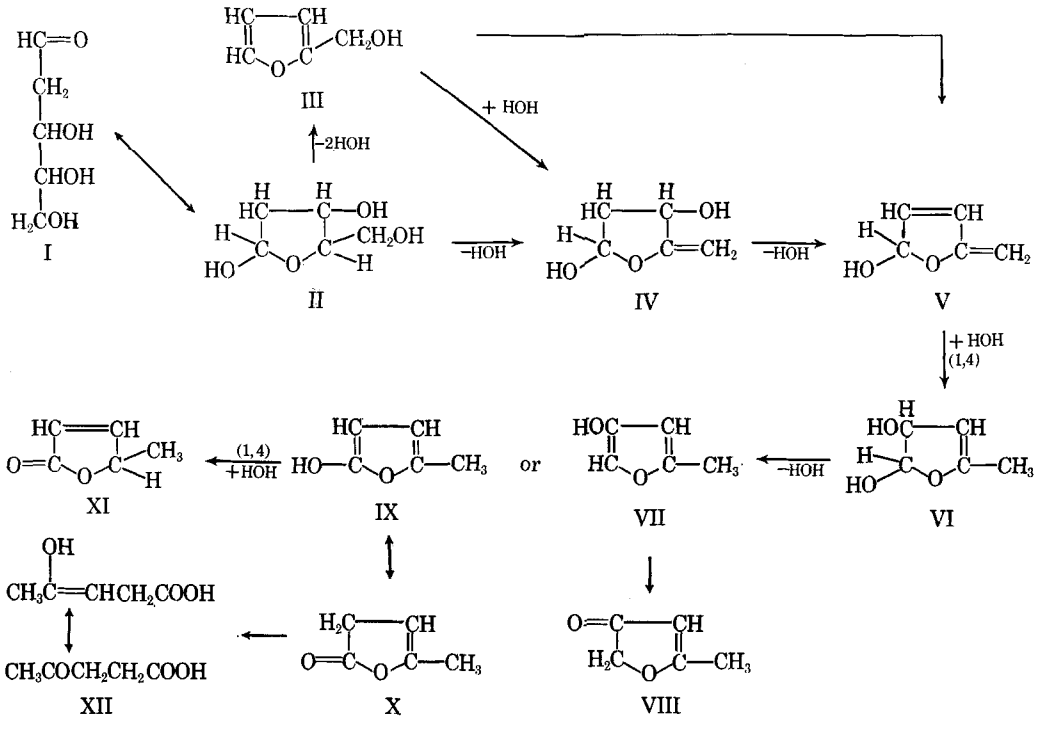


Fig. 3—NMR spectrum of the isolated 261 mμ chromophoric compound obtained from the acid degradation of 2-deoxy-D-ribose which is the same as the spectrum of synthesized 5-methyl-3(2H)-furanone.



Scheme II

that levulinic acid (XII) is a product of acid treatment of furfuryl alcohol (III) and passes through the isolated intermediate, 4,5-dihydroxy-4,5-dihydrosilvan (VI). They postulated the formation of this intermediate (VI) from 1,4-hydration of the precursor (V) which was produced from acid catalyzed rearrangement of furfuryl alcohol (III). This could occur just as readily from dehydration and rehydration of furfuryl alcohol (III) to form the postulated product (IV) of deoxyribose dehydration. This product (IV) can also form V by a subsequent dehydration.

On further dehydration, the 4,5-dihydroxy-4,5-dihydrosilvan (VI) will produce either the enolic form of the lactone of levulinic acid (XII) or VIII. Since the yield favors levulinic acid (XII) [about 50% (13)] rather than VIII [about 15% (1)], removal of the hydroxyl from the 3 position from VI is favored over the removal of the hydroxyl from the 2 position.

Recently, Anet (14) has shown that the methyl ether of the furanose form of aldoses produces a 5-substituted -3(2H)-furanone, on treatment with 0.1 *N* HCl at 70° in 30 min. These are conditions approximately equivalent to those used in the formation of VIII from deoxyribose. It was also reported that VIII rapidly polymerized in nonaqueous solvents containing traces of acid (5, 14) consistent with our observed resinification of chloroform-extracted VIII from deoxyribose degradation. This polymerization may be the explanation of the slow decrease in absorbance of the developed 261 mμ chromophore of degraded deoxyribose when held in acidic solution for long periods of time (1). The reported shifting (13) of the $\lambda_{max.}$ 261 mμ (acid solution) to 296 mμ in 0.01 *N* NaOH is the same as reported for the compound from degraded deoxyribose.

The sequence of reactions from deoxyribose through the pathway I–VI to the products VIII and levulinic acid (XII) is a plausible one consistent with all available information and identifications. It is also consistent with the observed kinetics of the formation of VIII by acid treatment of deoxyribose (1). The S-shaped curve for the formation of VIII implies that the reaction proceeds through a relatively stable intermediate or intermediates. It is possible that the 225 mμ absorbance which precedes the 261 mμ absorbance assigned to VIII represents an intermediate. In Scheme II, a plausible assignment of the 225 mμ $\lambda_{max.}$ by Woodward's rules is structure V, calculated $\lambda_{max.}$ 224 mμ (15).

The degradation of furfuryl alcohol (III) in 1.00 *N* HCl at 80.0° initially produced a 225 mμ absorbance that disappeared in 1 hr. while a 261 mμ absorbance appeared. The 261 mμ absorbance reached its maximum value, 0.62, after 5 hr. and was constant for 24 hr. Addition of alkali at room temperature shifted the $\lambda_{max.}$ to 293 mμ and a rapid loss of this new chromophore was observed. As predicted by Scheme II, furfuryl alcohol showed degradative properties similar to deoxyribose and produced the same chromophore with the same properties under the same conditions which were consistent with VIII. When ribitol was treated in the same manner, no significant chromophore was observed.

REFERENCES

(1) Seydel, J. K., and Garrett, E. R., *Anal. Chem.*, **37**, 271 (1965).

(2) Garrett, E. R., Seydel, J. K., and Sharpen, A. J., *J. Org. Chem.*, **31**, 2219(1966).
(3) Byvoet, P., *Anal. Biochem.*, **13**, 170(1965).
(4) Becker, A., *Helv. Chim. Acta*, **32**, 1114(1949).
(5) Eugster, C. H., Allner, K., and Rosenkranz, R. E., *Chemia*, **15**, 516(1961); Rosenkranz, R. E., Allner, K., Good, R., Philipsborn, W. V., and Eugster, C. H., *Helv. Chim. Acta*, **46**, 1259(1963).
(6) Hofmann, A., Philipsborn, W., and Eugster, C. H., *Helv. Chim. Acta*, **48**, 1322(1965).
(7) Olsen, S., and Russwurn, H., *Liebigs Ann. Chem.*, **639**, 1(1961).
(8) Reichstein, T., and Oppenauer, R., *Helv. Chim. Acta*, **16**, 988(1933); **17**, 390(1934).
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(10) Deriaz, R. E., Stacey, M., Teece, E. G., and Wiggins, L. F., *J. Chem. Soc.*, **1949**, 1222.
(11) Thiele, J., *Liebigs Ann. Chem.*, **319**, 144(1901).
(12) Stones, W. E., and Tollens, B., *ibid.*, **249**, 227(1888).
(13) Birkofer, L., and Beckmann, F., *Ann.*, **620**, 21(1959).
(14) Anet, E. F. L. J., *Tetrahedron Letters*, **15**, 1649(1966).
(15) Fieser, L. F., and Fieser, M., "Advanced Organic Chemistry," Reinhold Publishing Co., New York, N. Y., 1961, p. 204.

Preparation of Tritium-Labeled Compounds III

Lincomycin by Exposure to Tritium Gas and Partial Determination of Intramolecular Distribution of Tritium

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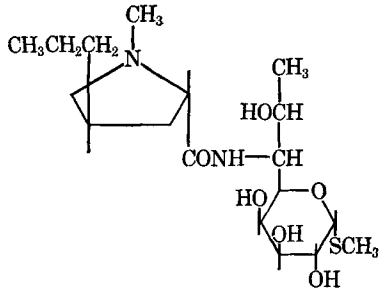
Lincomycin was tritiated more satisfactorily by its exposure to tritium in the presence of platinum black catalyst than by its exposure alone under conventional Wilzbach conditions. Incorporation of tritium and radiochemical purity, prior to purification, of the catalytically exposed lincomycin were far greater than those of the material exposed without catalyst. The intramolecular distribution of tritium was influenced by the exposure conditions. Catalytically exposed lincomycin contained virtually all its tritium in the propylhygric acid moiety, whereas the noncatalytically exposed sample contained approximately two-thirds of its tritium in this portion of the molecule; the remainder was in the methyl thiolincosaminide moiety.

LINCOMYCIN[1] (I) is a new antibiotic whose discovery and biological properties (1), isolation and characterization (2), and structure (3–7) have been reported.



I

Clinical use has shown it to be an effective agent for treatment of infections in man (8). This paper is concerned with preparation of radioactive lincomycin by two tritium-gas-exposure methods and a comparative determination of the resulting intramolecular distributions of tritium.

Radioactive lincomycin was required for metabolism studies in animals and man. Tritium labeling by the gas-exposure method of Wilzbach (9) was undertaken as the most attractive route to radioactive lincomycin. Incorporation of tritium, however, was poor and radiation-induced degradation was extensive; so, even though a usable product was obtained, attention was shifted to modifications of Wilzbach's method. The use of an electric discharge, as well as other sources of radiation, to promote ionization and excitation of the tritium gas was not particularly attractive since the authors had previously observed increased radiation-induced decomposition in applying this method to sulfonylurea compounds (10). Meshi and Takahashi (11), however, reported a modification of the Wilzbach method in which the compound, intimately mixed with platinum black catalyst, is exposed to tritium gas. Although this technique appears to have been little used since its introduction, it is reported to give better tritium incorporation than the conventional Wilzbach method. This was indeed found to be the case when the catalytic technique was applied to lincomycin.

Meshi and Sato (12) have shown that, in the cases of several compounds, the catalytic modification gives a different intramolecular distribution of tritium than does the Wilzbach method. A partial determination of the intramolecular distribution of tritium in lincomycin, labeled by

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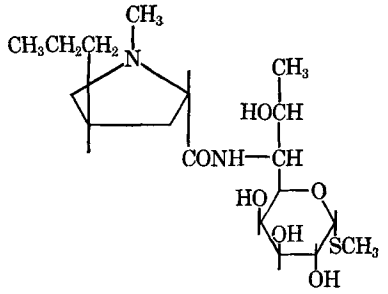

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the two methods, was therefore made. Virtually all the tritium was accounted for in the propylhygric acid moiety of the catalytically tritiated sample whereas only two-thirds was in this moiety in the sample tritiated without catalyst.

EXPERIMENTAL

Determination of Radioactivity—All counting was performed with a Tri-Carb[2] model 314X or 314-EX2A liquid scintillation spectrometer at −8° under conditions suitable for measuring tritium. Appropriate aliquots of samples were dissolved in 15 ml. of diotol scintillator (13) [toluene–dioxane–methanol (350:350:210 by volume) containing 73 Gm. naphthalene, 4.6 Gm. 2,5-diphenyloxazole, and 0.08 Gm. 1,4-bis-2-(5-phenyl-oxazolyl) benzene per liter]. The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of tritium-labeled toluene and results then expressed as microcuries (μc.).

Paper chromatograms were scanned for radioactivity with a Vanguard[3] model 880 radiochromatogram scanner. Thin-layer chromatograms were scanned for radioactivity by scraping sequential 1-cm. sections of the silica gel support into individual vials and counting in the usual manner with a liquid scintillation spectrometer.

Paper and Thin-Layer Chromatography—Paper chromatograms were developed by the descending method using Whatman No. 2 paper in the following systems: (*a*) 1-butanol–water (84:16 by volume), (*b*) 1-butanol–acetic acid–water (2:1:1 by volume), (*c*) 5% citric acid in water made to pH 7.0–7.5 with ammonia, and (*d*) 2-propanol–2 *N* HCl (65:35 by volume). Thin-layer chromatography was carried out on 250-μ films of Silica Gel GF[4] using the following systems: (*a*) methanol and (*b*) methyl ethyl ketone–acetone–water (15:5:5 by volume). Chemical detection was accomplished by spraying the developed paper sheets or thin-layer plates with a mixture of four parts 2% aqueous $NaIO_4$ and one part 1% $KMnO_4$ in 2% aqueous Na_2CO_3. Yellow spots appear on a purple background.

Column Chromatography—A 2.8-cm. diameter column was packed to a height of 55 cm. with 200–400 mesh acidic cationic exchange resin.[5] The column was washed consecutively with 4 *N* HCl, saturated NaCl solution, 4 *N* NaOH solution, 1 *N* HCl, and 0.1 *N* HCl prior to each use. The lincomycin sample to be purified was added to the column in 25 ml. of 0.1 *N* HCl. The column was then washed with 1 L. of 0.1 *N* HCl followed by 1 L. of water. Lincomycin was eluted by passing 1.5 L. of 2 *N* NH_4OH through the column at a flow rate of approximately 100 ml./hr. while collecting 20-ml. fractions.

Exposure of Lincomycin to Tritium Gas—(*a*) A 1.0-Gm. sample of finely pulverized lincomycin base was exposed to 4 c. of carrier-free tritium gas at room temperature in the dark for 8 days under approximately 0.2 Atm. tritium pressure. Following removal of the tritium gas, the crude sample was dissolved in 20 ml. of water and the resulting solution was lyophilized. Labile tritium was completely removed by repeating this procedure three times. The crude sample had a specific activity of approximately 11 μc./mg. and was grossly impure, radiochemically, as shown by the previously mentioned paper and thin-layer chromatography systems.

(*b*) Another 1.0-Gm. sample of finely pulverized lincomycin base was thoroughly mixed with 1.0 Gm. of platinum black[6] and exposed to 4 c. of carrier-free tritium gas under the conditions mentioned previously. In this case, however, the tritium gas was entirely adsorbed on the catalyst so the pressure was approximately 10^{-5} mm. At the end of the 8-day exposure period, the mixture was added to 20 ml. of water and the catalyst was removed by filtration through diatomaceous earth.[7] The filtrate, containing the lincomycin, was lyophilized and labile tritium was completely removed as previously described. The crude sample had a specific activity of approximately 15 μc./mg. Its radiochemical purity was approximately 80% as determined by paper and thin-layer chromatography.

Purification of Tritiated Lincomycin—In each case the crude lincomycin was taken up in 25 ml. of 0.1 *N* HCl and subjected to ion-exchange chromatography as previously described. Fractions containing lincomycin (as determined by counting individual column fractions and subjecting selected ones to paper chromatography and scanning for radioactivity) were combined and lyophilized to a residue *in vacuo*. The residue was recrystallized as the hydrochloride salt by taking it up in 3 ml. of water, adding 30 ml. of acetone, and adjusting the pH to 2 with 12 *N* HCl. This procedure was repeated until constant specific activity was attained.

In the case of lincomycin subjected to conventional Wilzbach tritiation, a constant specific activity of 0.60 μc./mg. was attained in three crystallizations with a yield of 0.317 Gm. (as the hydrochloride salt). A constant specific activity of 10.5 μc./mg. with a yield of 0.629 Gm. was obtained for the catalytically exposed material after two crystallizations. Both samples were shown to be radiochemically pure by paper and thin-layer chromatography, followed by scanning for radioactivity, in the previously mentioned systems. In each case the radioactive zone corresponded to the colored zone, produced by the spray reagent, which in turn corresponded to the migration of authentic lincomycin hydrochloride.

Both tritiated samples had infrared spectra corresponding to that of authentic lincomycin hydrochloride.

Anal.[8]—Calcd. for $C_{18}H_{34}N_2O_6S \cdot HCl \cdot H_2O$: C, 46.90; H, 7.87; Cl, 7.69; N, 6.08; S, 6.96; eq. wt., 461. Found (noncatalytically tritiated sample): C, 46.86; H, 8.11; Cl, 7.89; N, 6.10; S, 7.09; eq. wt., 458. Found (catalytically tritiated sample): C, 47.09; H, 8.09; Cl, 7.78; N, 6.32; S, 7.21; eq. wt., 462.

Acid Hydrolysis of Tritiated Lincomycin (4)—(*a*)

[2] Packard Instrument Co., Downers Grove, Ill.
[3] Vanguard Instruments, Division of Technical Measurements Corp., North Haven, Conn.
[4] Brinkmann Instrument Co., Great Neck, N. Y.
[5] Marketed as Dowex 50X8 by the Dow Chemical Co., Midland, Mich.
[6] Engelhard Industries, Inc., Newark, N. J.
[7] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.
[8] Another and larger sample of tritiated lincomycin hydrochloride, purified, recrystallized, and dried by the methods described, contained 1 mole of water as determined by Karl Fischer titration.

TABLE I—INTRAMOLECULAR DISTRIBUTION OF TRITIUM IN TRITIATED LINCOMYCIN

Component Counted	from Noncatalytically Tritiated Lincomycin		from Catalytically Tritiated Lincomycin	
	μc./m*M*	%	μc./m*M*	%
Lincomycin·HCl[a]	2.48	100	12.5	100
Propylhygric acid·HCl	1.53	62	11.9	95
Methyl thiolincosaminide	0.83	33.4	0.23	1.85
HCl distillate[b]	0.01	0.40	0.10	0.99
Methyl mercaptan[b]	0.08	3.22	0	0
Hydrazine distillate[b]	0.34	13.7	3.06	24.5

[a] The lincomycin hydrochloride was diluted with nonradioactive carrier for degradation. (See *Footnote 9.*) [b] Based on total lincomycin hydrochloride subjected to HCl hydrolysis or hydrazinolysis.

A solution of 1.252 Gm. of noncatalytically tritiated lincomycin hydrochloride[9] in 15 ml. of 1 *N* HCl was refluxed for 20 hr. while passing the evolved gases through a trap containing 10 *N* NaOH to trap methyl mercaptan liberated during hydrolysis. The reaction mixture was evaporated to dryness *in vacuo* and the distillate was collected for radioactivity determination. The residue was leached with boiling acetonitrile and the resulting solution was treated with charcoal[10] and evaporated to a small volume. The propylhygric acid hydrochloride was allowed to crystallize in the refrigerator overnight, filtered, and dried at room temperature *in vacuo*. Yield, 0.163 Gm.; m.p. 184–185° (capillary, uncorrected). The infrared spectrum was identical to that of anhydrous authentic propylhygric acid hydrochloride.

Anal.—Calcd. for $C_9H_{17}NO_3 \cdot HCl$: C, 52.05; H, 8.74. Found: C, 51.82; H, 8.90.

The radioactivities of the lincomycin hydrochloride, the contents of the NaOH trap, the distillate, and the propylhygric acid hydrochloride were determined and are listed in Table I.

(*b*) A 1.268-Gm. sample of catalytically tritiated lincomycin hydrochloride[9] was hydrolyzed as described above to yield 0.030 Gm. of propylhygric acid hydrochloride after three recrystallizations, m. p. 188–189° (capillary, uncorrected). The infrared spectrum of the sample corresponded to that of anhydrous authentic propylhygric acid hydrochloride.

Anal.—Calcd. for $C_9H_{17}NO_2 \cdot HCl$: C, 52.05; H, 8.74. Found: C, 51.74; H, 8.88.

The radioactivities of the various fractions are presented in Table I.

Hydrazinolysis of Tritiated Lincomycin (5)—(*a*) A solution of 0.400 Gm. of noncatalytically tritiated lincomycin hydrochloride[9] in 2 ml. of hydrazine hydrate was refluxed for 70 hr. The reaction mixture was evaporated to dryness *in vacuo* and the distillate was collected for radioactivity determination. The residue was triturated with hot acetonitrile, cooled, and filtered. The crystalline material (0.214 Gm.) was recrystallized from dimethylformamide, filtered, washed with cold methanol, and dried at room temperature *in vacuo* to yield 0.092 mg. of methyl thiolincosaminide, m.p. 222–223° (capillary, uncorrected). Its infrared and mass spectra corresponded to those of the authentic compound.

The radioactivities of the distillate and the methyl thiolincosaminide are presented in Table I.

[9] The tritiated lincomycin hydrochloride samples were diluted with nonradioactive carrier lincomycin hydrochloride by recrystallization from water–acetone prior to degradation.

[10] Darco G60.

(*b*) A 1.27-Gm. sample of catalytically tritiated lincomycin hydrochloride[9] was subjected to hydrazinolysis as described above. The crystalline residue (0.734 Gm.) from trituration was recrystallized to yield 0.296 Gm. of methyl thiolincosaminide, m.p. 223–224° (capillary, uncorrected). Its infrared and mass spectra corresponded to those of the authentic compound.

The radioactivities of the distillate and the methyl thiolincosaminide are presented in Table I.

RESULTS AND DISCUSSION

The radiochemical purity of the crude, catalytically tritiated lincomycin was far greater than that of lincomycin tritiated by the conventional Wilzbach method; 80% and 6%, respectively. It appears that either less radiation decomposition occurred under the catalytic conditions or that if high specific activity impurities were formed they were largely adsorbed on the platinum-black catalyst and not removed in the washing operation. In either event, the catalytic sample was easier to purify and radiochemically pure product was obtained in greater yield.

The incorporation of stably bound tritium in lincomycin by the catalytic method was 17 times that obtained by the conventional method. This effect of platinum black catalyst was found and reported by Meshi and Takahashi (11) in the cases of salicylic acid, valine, prednisolone, benzoic acid, inositol, and thiamine and by Meshi and Sato (12) in the cases of benzene, naphthalene, dinitrobenzene, phthalic anhydride, and benzoyl valine. They ascribe this increased incorporation to catalytic exchange of hydrogen rather than to increased absorption of decay energy by the system.

The intramolecular distributions of tritium in lincomycin prepared by the two methods were quite different. As shown in Table I, the noncatalytic sample contained 62% of its tritium in the propylhygric acid moiety and 33.4% in the methyl thiolincosaminide portion. Corresponding values for the catalytic sample were 95% and 1.84%, respectively. The origin of the small amount of tritium lost to the solvent during HCl hydrolysis is uncertain. The methyl mercaptan, cleaved from methyl thiolincosaminide during acid hydrolysis, contained 3.22% of the molecular tritium in the case of the noncatalytic sample and no tritium in the case of the catalytic sample. The rather large amount of tritium lost to the solvent during hydrazinolysis had to originate predominantly in the propylhygric acid moiety in the case of the catalytic sample and most likely did in the case of the noncatalytic sample. It has been shown (6) that during

hydrazinolysis the number 2 position of propylhygric acid is completely racemized. It, therefore, is likely that the tritium lost to the hydrazine solvent originated in this position of the lincomycin. Thus, 13.7% and 24.5% of the molecular tritium can be assigned to the number 2 position of the propylhygric acid moiety in the cases of the noncatalytic and catalytic samples, respectively.

In regard to ease of purification, yield of product, and incorporation of stable tritium, the catalytic method was superior to the conventional Wilzbach method for tritiating lincomycin. On the other hand, the noncatalytically tritiated lincomycin had a more uniform distribution of tritium making it more suitable for drug metabolism studies. If metabolism resulted in hydrolysis of lincomycin at the amide linkage, the methyl thiolincosaminide moiety could not be traced in the case of the catalytic sample because of its lack of tritium.

REFERENCES

(1) Mason, D. J., Dietz, A., and DeBoer, C., *Antimicrobial Agents Chemotherapy-1962*, **1962**, 554.
(2) Herr, R. R., and Bergy, M. E., *ibid.*, **1962**, 560.
(3) Hoeksema, H., Bannister, B., Birkenmeyer, R. D., Kagan, F., Magerlein, B. J., MacKellar, F. A., Schroeder, W., Slomp, G., and Herr, R. R., *J. Am. Chem. Soc.*, **84**, 4223 (1964).
(4) Herr, R. R., and Slomp, G., *ibid.*, to be published.
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(8) Daikos, G. K., Kontomichalou, P., Papadoyannakis, N., and Tourkantonis, A., *Antimicrobial Agents Chemotherapy-1963*, **1964**, 197; Holloway, W. J., Kahlbaugh, R. A., and Scott, E. G., *ibid.*, **1964**, 200; Harnecker, J., Contreras, J., Gilabert, B., and Ubilla, V., *ibid.*, **1964**, 205; Walters, E. W., Romansky, M. J., and Johnson, A. C., *ibid.*, **1964**, 210; Trakas, J. C., and Lind, H. E., *ibid.*, **1964**, 216.
(9) Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013(1957).
(10) Thomas, R. C., and Ikeda, G. J., *J. Pharm. Sci.*, **55**, 112(1966).
(11) Meshi, T., and Takahashi, T., *Bull. Chem. Soc. Japan*, **35**, 1510(1962).
(12) Meshi, T., and Sato, Y., *ibid.*, **37**, 683(1964).
(13) Herberg, R. J., *Anal. Chem.*, **32**, 42(1960).

Synthesis of Tetrose Nucleosides I

Adenine Nucleosides of Erythrose and Threose

By DANIEL H. MURRAY and JOHN PROKOP

D- and L-Erythrose and D- and L-threose were individually converted to their triacetates which were condensed with chloromercuri-6-benzamidopurine in the presence of titanium tetrachloride. After deacylation, the four crude mixtures of anomeric nucleosides were each resolved on a strong anion exchange resin, leading to the isolation of all eight possible 9-tetrafuranosyladenines. The anomeric configurational assignments were made by consideration of the mechanism of nucleoside condensation (Baker's *trans* rule) and by Hudson's rules of isorotation. Preliminary results of tests for biological activity with *Streptococcus faecalis* 8043 and with adenosine deaminase are reported.

In recent years substantial attention has been directed toward the synthesis of potential inhibitors of nucleic acid metabolism. This has led to the preparation of a wide variety of 5- and 6-carbon sugar nucleosides, of which several of the compounds containing pentose sugar moieties have exhibited antibacterial and/or antitumor activity, for example, cordycepin, xylofuranosyladenine, and spongoadenosine. The majority of such nucleosides have been shown to act as nucleotides *via* phosphorylation at the 5′-position. On the other hand, some compounds have been shown to act in their nucleoside form, for example, decoyinine (1) and 5′-deoxyxylofuranosyladenine (2). In these, lack of a hydroxyl group at a terminal position removes the possibility of nucleotide formation. The 9-tetrofuranosyladenines are further examples of this class of nucleosides. Only the 9-β-D-erythrofuranosyladenine (III) has been prepared previously (3). It has been of interest in the present study to prepare all eight of the possible 9-tetrosyladenines. The biological activity of these is reported in a separate communication (2).

Received January 4, 1967, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication April 24 1967.

DISCUSSION

The general method of synthesis involved coupling of an acetylated sugar with chloromercuri-6-benzamidopurine (II) (Scheme I) in the presence of titanium tetrachloride. Previous experience with this method (4, 5) has indicated that higher yields of nucleoside are frequently obtained as compared with the more usual procedure of condensation in which the acylated sugar is first converted to the corresponding halide. Furthermore, it has been reported that the use of titanium tetrachloride has led to the formation of significant quantities of the *cis* nucleoside[1] (6, 7). It was anticipated that at least one of the *cis* nucleosides in the present series, 9-β-D-threofuranosyladenine (X), might have significant biological activity. Hence, the authors

[1] The terminology *cis* or *trans* nucleoside refers to the configuration of substituents on carbons 1 and 2 of the sugar moiety.

hydrazinolysis the number 2 position of propylhygric acid is completely racemized. It, therefore, is likely that the tritium lost to the hydrazine solvent originated in this position of the lincomycin. Thus, 13.7% and 24.5% of the molecular tritium can be assigned to the number 2 position of the propylhygric acid moiety in the cases of the noncatalytic and catalytic samples, respectively.

In regard to ease of purification, yield of product, and incorporation of stable tritium, the catalytic method was superior to the conventional Wilzbach method for tritiating lincomycin. On the other hand, the noncatalytically tritiated lincomycin had a more uniform distribution of tritium making it more suitable for drug metabolism studies. If metabolism resulted in hydrolysis of lincomycin at the amide linkage, the methyl thiolincosaminide moiety could not be traced in the case of the catalytic sample because of its lack of tritium.

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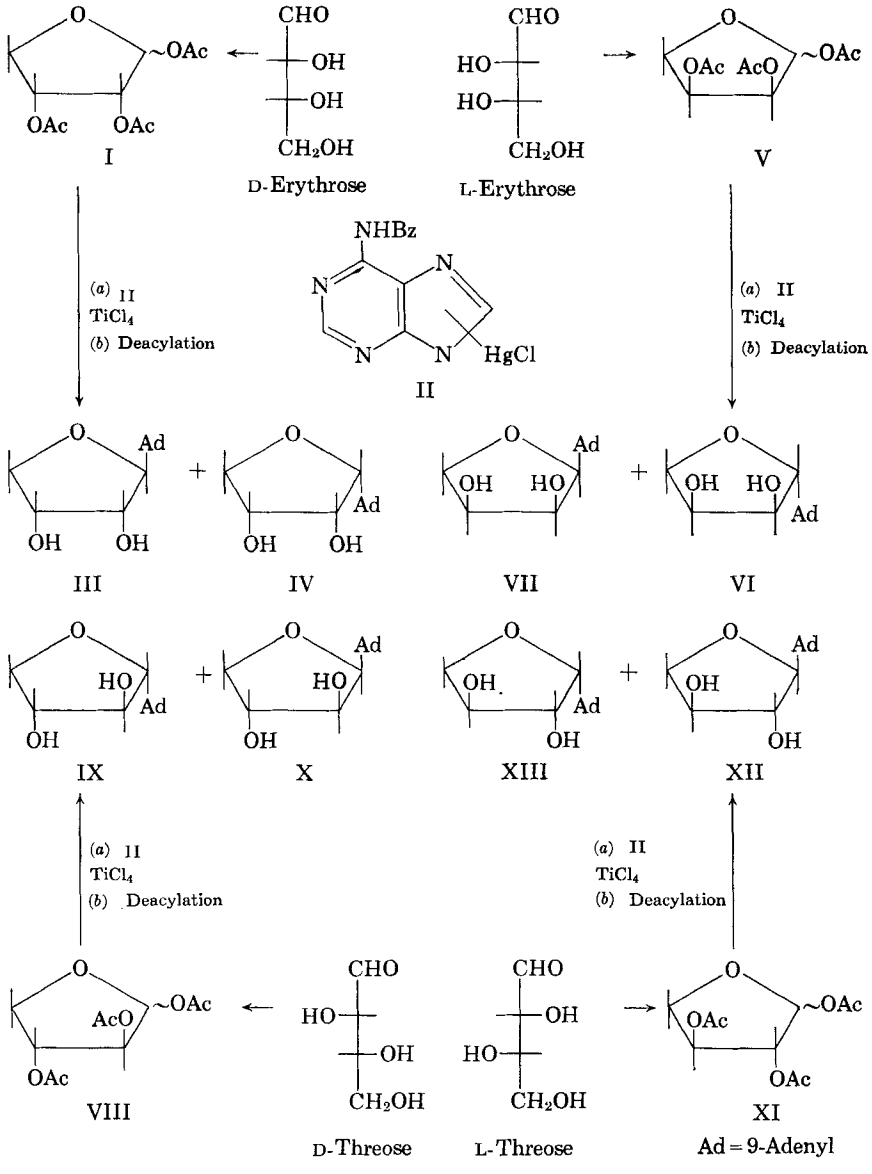


Scheme I

have sought a procedure which would yield sufficient of a desired *cis* nucleoside for limited biological testing. The titanium tetrachloride coupling step was a desirable element in the procedure.

A second desirable element was a routine method for separating the anomeric pairs of nucleosides. Although such anomeric mixtures of nucleosides, particularly those resulting from condensation of 2-deoxysugars, have been resolved satisfactorily through fractional crystallization procedures, this method is tedious and becomes of more limited use when one of the anomers in the mixture is present in only a relatively small proportion. Chromatographic separations involving adsorption techniques are also limited because of their inability to handle practical amounts of compounds, and, in some instances, because of poor resolution necessitating rechromatography. A recent method, which is reported to give good resolution and has large capacity, involves chromatography on a strong anion exchange resin, and utilizes the differences in pKa's of the sugar hydroxyl groups at high pH (8). Application of this procedure to the mixtures of nucleosides obtained by the condensation of acetylated tetroses led to the isolation of all eight possible 9-tetrafuranosyladenines.

The four tetroses required for these syntheses were obtained through established routes. D-Erythrose was prepared by periodate cleavage of 4,6-*O*-ethylidene-D-glucose followed by acid hydrolysis (9). The resulting syrupy sugar was acetylated using acetic anhydride in pyridine to give the syrupy acetate (I) which was probably an anomeric mixture. Condensation of I with chloromercuri-6-benzamidopurine (II) in the presence of titanium tetrachloride, deacylation with methanolic ammonia,

TABLE I—MELTING POINTS AND SPECIFIC ROTATION FOR THE NUCLEOSIDES

Nucleoside	M. p.	$[\alpha]_D^{20}$
III	239–240° dec.	−114°
VI	238.5–239.5° dec.	+112°
IV	217–218°	+45°
VII	216.5–218°	−44°
IX	214.5–215°	+66°
XII	214.5–215°	−66°
X	239.5–240.5° dec.	−5°
XIII	240–241° dec.	+5°

and chromatography on anion exchange resin of the quaternary ammonium type[2] led to the isolation of crystalline 9-β-D-erythrofuranosyladenine (III) and 9-α-D-erythrofuranosyladenine (IV) in yields of 31 and 13%, respectively. Sixty per cent aqueous methanol[3] was used as eluent throughout these separations.

L-Erythrose was obtained by the procedure starting from rhamnose (10). The sugar was acetylated as before to give the syrupy triacetate (V) in 81% yield. Similar condensation with II and deacylation with methanolic sodium methoxide, followed by chromatographic separation on the anion exchange resin gave the crystalline β- and α-anomers (VI and VII) in yields of 36 and 7.4%, respectively, based on the crude triacetate (V). That these nucleosides (VI and VII) were the enantiomers of III and IV can be seen by comparison of their melting points and specific rotations as shown in Table I. In addition, the infrared spectra of these mirror-image pairs were superimposable.

D- and L-Threose were obtained by periodate oxidation of 1,3-*O*-benzylidene-D- and -L- arabitol, respectively, followed by acid hydrolysis (11). These sugars were readily converted to their triacetates using acetic anhydride in pyridine. The product in each case consisted of both a crystalline isomer and a noncrystallizing syrup obtained from the liquors, and were probably the anomeric forms (12). Either of these products could be converted to the desired nucleosides. Crystalline D-threose triacetate (VIII) was converted to the anomeric adenine nucleosides as described above for D- and L-erythrose. Chromatography of the crude product on the anion exchange resin gave only two ultraviolet absorbing peaks which proved to be 9-α-D-threofuranosyladenine (IX) and 9-β-D-threofuranosyladenine (X) in yields of 55 and 10%, respectively, based on the triacetate. When the crude, syrupy triacetate (VIII), obtained from the mother liquors, was condensed and chromatographed, additional α- (IX) and β- (X) nucleosides resulted in yields of 31 and 3.1%, respectively, along with several minor ultraviolet absorbing peaks which were not characterized.

Crystalline L-threose triacetate (XI) was similarly converted to 9-α-L-threofuranosyladenine (XII) and 9-β-L-threofuranosyladenine (XIII) in yields of 57% and 12%, respectively. When the syrupy triacetate (XI) was employed, the respective yields of α- and β-anomers were 34 and 4.3%. That the α-nucleosides (IX and XII) and the β-nucleosides (X and XIII) represented enantiomeric pairs can be seen by comparison of the constants shown in Table I. The infrared spectra of these pairs were superimposable.

Periodate consumption studies established the furanoside structure of these nucleosides. In addition, the D-erythrosides (III and IV) oxidized rapidly with 1 *M* equivalent of periodate being consumed within 5 min. In contrast, oxidation of the L-threosides (XII and XIII) was considerably slower with 1 *M* equivalent of periodate being consumed only after 5 days. These results are consistent with those reported earlier for the periodate consumption of 2′,3′-*cis* and -*trans* hydroxyl groups in nucleosides containing furanosyl sugars (13).

The anomeric configurations of these nucleosides could be implied by their relative yields. So far as is known, the *trans* nucleoside predominates in all nucleoside condensations including those involving the titanium tetrachloride procedure (6, 7, 14). Application of Hudson's rules of isorotation (Table I) supports these assignments.

Biological Activity—The effect of these nucleosides on growth was examined in *Streptococcus faecalis* 8043. Only compounds X and XII were inhibitory, preventing 50% of growth at 2×10^{-5} *M*. All the others were negative. Compounds X, XII, and VII were significantly inhibitory in an adenosine deaminase system. These results are discussed in detail elsewhere (2).

EXPERIMENTAL[4]

D-Erythrose Triacetate (I)—To a stirred solution of 7.09 Gm. (54.8 mmoles) of D-erythrose syrup (9) in 50 ml. of reagent pyridine was added 23.5 ml. (250 mmoles) of acetic anhydride dropwise below 20°. The flask was stoppered and the reaction was allowed to remain at room temperature overnight. The dark amber reaction was then poured into 200 ml. of an ice water mixture with stirring and, after 30 min., the mixture was extracted with chloroform (3 × 100 ml.). These extracts were combined and washed with aqueous saturated sodium bicarbonate (3 × 100 ml.) and water (100 ml.). After drying over magnesium sulfate, the extract was treated with activated charcoal[5] and filtered through diatomaceous earth[6] giving a pale yellow filtrate which was evaporated to dryness *in vacuo* (60°). The last traces of pyridine were removed by evaporative distillation *in vacuo* with 25 ml. of toluene yielding 12.0 Gm. (89%) of a light amber syrup which was sufficiently pure for condensation. For analysis, a sample was distilled in a sublimation tube and a clear, colorless liquid was collected at 105–110°/0.06 mm.; $[\alpha]_D^{28}$ −66.2° (c 4.8, $CHCl_3$); $\nu_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1375 (methyl), 1220 (acetate C—O—C).

Anal.—Calcd. for $C_{10}H_{14}O_7$: C, 48.78; H, 5.73. Found: C, 48.55; H, 5.81.

[2] Marketed as Bio-Rad AG1×8(OH) by Bio-Rad Laboratories, Richmond, Calif.

[3] Practical grade methanol was entirely satisfactory for this purpose.

[4] Melting points were obtained using a Mel-Temp instrument and are uncorrected. The infrared and ultraviolet spectra were determined on Perkin-Elmer models 337 and 202, respectively, and optical rotations were obtained using a Perkin–Elmer 141 polarimeter. The column chromatograms were monitored with an ISCO model UA analyzer. Galbraith Laboratories, Inc., Knoxville, Tenn., performed the elemental analyses.

[5] Marketed as Norit by American Norit Co., Inc., Jacksonville, Fla.

[6] Marketed as Celite by Johns-Manville, New York, N. Y.

9-α- and β-D-Erythrofuranosyladenine (IV and III, Respectively)—A mixture of 9.27 Gm. (38 mmoles) of crude erythrose triacetate (I), 17.9 Gm. (38 mmoles) of chloromercuri-6-benzamidopurine (II) (4, 5), 18 Gm. of diatomaceous earth, and 650 ml. of ethylene dichloride was distilled until 80 ml. of distillate had been collected. After cooling to room temperature, a solution of 4.13 ml. (38 mmoles) of titanium tetrachloride in 45 ml. of ethylene dichloride was added dropwise and the mixture was then heated under reflux overnight while protected from moisture. The mixture was cooled to room temperature and 40 ml. of aqueous saturated sodium bicarbonate was added while stirring vigorously. Additional solid sodium bicarbonate was added until a neutral reaction was obtained. The mixture was filtered through diatomaceous earth and the cake was washed with additional ethylene dichloride (3 × 35 ml.). After combining the filtrate and washings and removal of the solvent *in vacuo*, the residue was redissolved in 300 ml. of chloroform, filtered free of some insolubles, and washed with 300 ml. of 30% aqueous potassium iodide and 300 ml. of water. The organic solution was dried over magnesium sulfate, treated with activated charcoal, and filtered, and the filtrate was evaporated to dryness *in vacuo* (60°) leaving crude 2',3'-di-*O*-acetyl-9-α- and β-D-erythrofuranosyl-6-benzamidopurine as an amber glass; yield, 11.3 Gm. (71%); $\bar{\nu}_{max.}^{KBr}$ (cm.$^{-1}$) 1750 (acetate C=O), 1700 (amide C=O), 1375 (methyl), 1220 (acetate C—O—C), 710 (monosubstituted benzene).

A solution of 11.2 Gm. of the above blocked nucleosides in 360 ml. of anhydrous methanol was saturated with ammonia gas while maintaining the temperature at about 5°. After standing in a stoppered flask at 5° overnight, the ammonia and solvent were removed *in vacuo* and the residue partitioned between 300 ml. of water and 100 ml. of chloroform. The organic phase was separated and the aqueous phase was washed further with chloroform (2 × 100 ml.). The organic phases were combined and back-washed with 100 ml. of water which was then added to the original aqueous phase. A small amount of insolubles was removed by filtration and the aqueous phase was then concentrated *in vacuo* to about 100 ml. After addition of 120 ml. of methanol, the nucleoside-containing solution was transferred to the top of a column (8.0 × 50 cm.), packed with the anion exchange resin, which was previously equilibrated with 60% aqueous methanol. The nucleosides were eluted with the same solvent at a rate of approximately 475 ml./hr. with the fractions collected at 1-hr. intervals. Fractions 23–28 were combined and evaporated to dryness giving the α-anomer (IV) as needles; yield, 1.15 Gm. (13%), m.p. 212–217°. Two recrystallizations from 95% ethanol gave the analytical sample, m.p. 217–218°; $[\alpha]_D^{20}$ + 45° (c 0.67, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 257.5 (ε 14,500); $\lambda_{max.\ 259.5}^{pH\ 7\ and\ 13}$ (ε 15,000); $\nu_{max.}^{KBr}$ (cm.$^{-1}$) 3400–3100 (broad OH and NH), 1605, and 1575 (C=C and C=N). Periodate consumption (moles of periodate/mole of compound) (13, 17, 18): 1.00 (5 min. and constant).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.54; H, 4.60; N, 29.70.

The β-anomer (III) was obtained by combining fractions 42–58 and removing the solvent *in vacuo* to give a nearly white, crystalline solid; yield, 2.74 Gm. (31%), m.p. 224.5–225.5° dec. The analytical sample was obtained by recrystallizing twice from 50% aqueous ethanol, m.p. 239–240° dec.; $[\alpha]_D^{20}$ −114° (c 0.40, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 257.5 (ε 14,000); $\lambda_{max.}^{pH\ 7\ and\ 13}$ 260 (ε 14,500); $\bar{\nu}_{max.}^{KBr}$ (cm.$^{-1}$) 3355, 3130 (OH and NH), 1605, 1575 (C=C and C=N). Periodate consumption (moles of periodate/mole of compound) (13, 17, 18): 0.95 (5 min.), 0.99 (1 hr.), 1.00 (48 hr.).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.69; H, 4.78; N, 29.54.

L-Erythrose Triacetate (V)—To a stirred solution of 3.64 Gm. (30 mmoles) of syrupy L-erythrose (10) in 30 ml. of reagent pyridine was added 12.9 ml. (136 mmoles) of acetic anhydride dropwise while maintaining the temperature below 20°. The reaction was allowed to remain at room temperature in a stoppered flask overnight, then poured into 100 ml. of an ice water mixture and worked up as described above for the D-isomer; yield of light amber syrup was 6.05 Gm. (81%) and was sufficiently pure for condensation. For analysis, a sample was distilled in a sublimation tube and a clear, colorless liquid was collected at 105°/0.06 mm.; $[\alpha]_D^{28}$ +63.7° (c 3.6, $CHCl_3$); $\bar{\nu}_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1375 (methyl), 1220 (acetate C—O—C).

Anal.—Calcd. for $C_{10}H_{14}O_7$: C, 48.78; H, 5.73. Found: C, 49.29; H, 6.04.

9-α- and β-L-Erythrofuranosyladenine (VII and VI, Respectively)—A mixture of 5.59 Gm. (23 mmoles) of crude L-erythrose triacetate (V), 10.8 Gm. (23 mmoles) of chloromercuri-6-benzamidopurine, 11 Gm. of diatomaceous earth, and 400 ml. of ethylene dichloride was distilled until 60 ml. of distillate had been collected. The reaction was cooled to room temperature and a solution of 2.5 ml. (23 mmoles) of titanium tetrachloride in 25 ml. of ethylene dichloride was added dropwise. The reaction was then heated under reflux for 18 hr. while protected from moisture. After cooling the mixture to room temperature, 25 ml of aqueous saturated sodium bicarbonate was added followed by additional solid sodium bicarbonate while stirring vigorously until a neutral reaction was obtained. The mixture was then processed as described for the D-isomers leaving crude 2',3'-di-*O*-acetyl-9-α- and β-L-erythrofuranosyl-6-benzamidopurine as a light amber glass; yield, 7.05 Gm. (73%); $\bar{\nu}_{max.}^{KBr}$ (cm.$^{-1}$) 1750 (acetate C=O), 1700 (amide C=O), 1375 (methyl), 1220 (acetate C—O—C), 710 (monosubstituted benzene).

A solution of 6.82 Gm. of the crude, blocked nucleosides in 160 ml. of 0.1 *N* methanolic sodium methoxide was allowed to stand at room temperature in a stoppered flask for 24 hr. After neutralizing the reaction with glacial acetic acid and removal of the solvent *in vacuo*, the residue was partitioned between 100 ml. of water and 100 ml. of chloroform. The organic phase was separated and the aqueous phase washed further with chloroform (2 × 50 ml.), filtered free of insolubles, and evaporated to near dryness *in vacuo*. The residue was redissolved in 100 ml. of 50% aqueous methanol and transferred to the top of a column (3.8 × 50 cm.) packed with the anion exchange resin. The nucleosides were eluted using 60% aqueous methanol (to which the column had been equilibrated originally) at a rate of approximately 80 ml./fraction. Fractions were

collected at 1-hr. intervals. The α-anomer (VII) was obtained by combining fractions 31–38. These fractions also contained a small amount of a contaminating substance which immediately preceded the desired peak from which it was incompletely separated. Subsequent rechromatography of these combined fractions did allow complete removal of the interfering substance. Removal of the solvent *in vacuo* left the desired nucleoside (VII) as a crystalline solid; yield 385 mg. (7.4% from L-erythrose triacetate), m.p. 207–211°. Three recrystallizations from 95% ethanol gave the analytical sample, m.p. 216.5–218°; $[\alpha]_D^{20}$ −44° (c 0.61, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 257.5 (ε 14,200), $\lambda_{max.}^{pH\ 7\ and\ 13}$ 259.5 (ε 14,800). The infrared spectrum of this substance was superimposable with that of the D-isomer (IV). Mixed melting point with IV was depressed.

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.81; H, 4.75; N, 29.74.

Combination of fractions 55–74 and removal of the solvent *in vacuo* gave the β-anomer (VI) as a crystalline solid; yield, 1.88 Gm. (36% from L-erythrose triacetate), m.p. 229–233°. For analysis, a sample was recrystallized twice from 50% aqueous ethanol, m.p. 238.5–239.5° dec.; $[\alpha]_D^{20}$ +112° (c 0.42, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 257.5 (ε 14,000), $\lambda_{max.\ 260}^{pH\ 7\ and\ 13}$ (ε 14,300). The infrared spectrum was superimposable with that of the D-isomer (III). Mixed melting point with III was depressed.

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.62; H, 4.80; N, 29.71.

D-Threose Triacetate (VIII)—To a stirred solution of 3.52 Gm. (29 mmoles) of syrupy D-threose (11) in 30 ml. of reagent pyridine was added dropwise 20 ml. of acetic anhydride below room temperature. The reaction was allowed to remain at room temperature in a stoppered flask overnight, then poured into 100 ml. of an ice water mixture to decompose the remaining anhydride. The mixture was extracted with chloroform (3 × 50 ml.) and the combined extracts were washed with aqueous saturated sodium bicarbonate (4 × 50 ml.) and water (50 ml.). The extract was dried over magnesium sulfate, treated with activated charcoal, and filtered, and the filtrate evaporated to dryness *in vacuo* (50°). The last traces of pyridine were removed by repeated distillations *in vacuo* of toluene (3 × 25 ml.) yielding 6.79 Gm. (94%) of a partially crystalline syrup. After trituration with 30 ml. of absolute ethanol, the crystalline portion was collected on a filter, washed with absolute ethanol, and dried; yield, 1.43 Gm. (20%), m.p. 115.5–117°. One recrystallization from absolute ethanol gave m.p. 117–118.5°; $[\alpha]_D^{28}$ +35.3° (c, 4.1, $CHCl_3$). [Lit. (12) m.p. 117–118°, [α] +35.55 (c 4, $CHCl_3$).]

The above filtrate was evaporated to dryness *in vacuo* (50°) yielding 5.31 Gm. (74%) of the noncrystalline anomer of D-threose triacetate. This material was also suitable for condensation.

9-α- and β-D-Threofuranosyladenine (IX and X, Respectively)—A mixture of 1.23 Gm. (5 mmoles) of crystalline D-threose triacetate, 2.37 Gm. (5 mmoles) of chloromercuri-6-benzamidopurine (II), 3 Gm. of diatomaceous earth, and 100 ml. of ethylene dichloride was distilled until 25 ml. of distillate had been collected. The reaction was cooled to room temperature and a solution of 0.55 ml. (5 mmoles) of titanium tetrachloride in 6 ml. of ethylene dichloride was added dropwise. The mixture was then heated under reflux overnight while protected from moisture. To the cooled mixture was added 16 ml. of aqueous saturated sodium bicarbonate with vigorous stirring, followed by additional solid sodium bicarbonate until a neutral reaction was obtained. After filtration through diatomaceous earth, the cake was washed with 35 ml. of ethylene dichloride, the washing combined with the filtrate, and the solvent removed *in vacuo*. A solution of the residue in 50 ml. of chloroform was filtered free of some insolubles, then washed with 50 ml. of 30% aqueous potassium iodide and 50 ml. of water. The chloroform solution was dried over magnesium sulfate, treated with activated charcoal and filtered through diatomaceous earth, and the filtrate evaporated to dryness *in vacuo* (50°) leaving crude 2′, 3′-di-*O*-acetyl-9-α and β-D-threofuranosyl-6-benzamidopurine as an amber glass; yield 1.77 Gm. (83%); $\bar{\nu}_{max}^{KBr}$ ($cm.^{-1}$) 1750 (acetate C=O), 1700 (amide C=O), 1375 (methyl), 1220 (acetate C—O—C), 710 (monosubstituted benzene).

A solution of 1.75 Gm. of the above blocked nucleosides in 40 ml. of 0.1 *N* methanolic sodium methoxide was allowed to stand at room temperature in a stoppered flask for 24 hr. Without further processing, the solution was transferred to the top of a column (3.8 × 50 cm.) containing the anion exchange resin which had previously been equilibrated with 60% aqueous methanol. The nucleosides were eluted using 60% aqueous methanol at a rate of approximately 80 ml./hr. Fractions were collected at 1-hr. intervals. The β-isomer (X) was isolated by evaporation of fractions 60–70 which yielded 121 mg. (10.4% from D-threose triacetate) of a crystalline solid, m.p. 234–236° dec. Two recrystallizations from 95% ethanol gave the analytical sample, m.p. 239.5–240.5° dec.; $[\alpha]_D^{20}$ −5° (c 0.40, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 257.5 (ε 14,000), $\lambda_{max.\ 260}^{pH\ 7\ and\ 13}$ (ε 14,600); $\bar{\nu}_{max.}^{KBr}$ ($cm.^{-1}$) 3400–3100 (broad OH and NH), 1605, 1575 (C=C and C=N).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.66; H, 4.71; N, 29.66.

The α-isomer (IX) was isolated from fractions 74–91 as a crystalline solid; yield, 641 mg. (54.6% from D-threose triacetate), m.p. 213–215°. A pure sample was obtained by recrystallizing twice from 95% ethanol and thorough drying at 100°, m.p. 214.5–215°; $[\alpha]_D^{20}$ + 66° (c 0.58, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 258 (ε 14,000), $\lambda_{max.\ 260}^{pH\ 7\ and\ 13}$ (ε 14,500); $\bar{\nu}_{max.}^{KBr}$ ($cm.^{-1}$ 3500–3150 (broad OH and NH), 1605, 1575 (C=C and C=N).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.84; H, 4.82; N, 29.72.

When the crude, noncrystallizing portion of D-threose triacetate (above) was condensed, deacylated, and chromatographed in a similar manner, additional nucleosides were obtained amounting to 31% of the α- and 3.1% of the β-isomer.

L-Threose Triacetate (XI)—To a stirred solution of 3.75 Gm. (31 mmoles) of syrupy L-threose (15) in 35 ml. of reagent pyridine was added 24 ml. (255 mmoles) of acetic anhydride dropwise below 30°. After standing overnight at room temperature in a stoppered flask, the solvents were removed *in vacuo* (50°). The remaining traces of pyridine and acetic acid were removed by repeated distillation *in vacuo*

of toluene (3 × 30 ml.). A solution of the residue in 200 ml. of chloroform was treated with activated charcoal, filtered through diatomaceous earth, and the filtrate evaporated to dryness *in vacuo* (60°) leaving a partially crystalline, amber syrup; yield, 7.18 Gm. (93%). The crystalline anomer was obtained by triturating the residue with 10 ml. of absolute ethanol and collecting the product on a filter; yield 2.22 Gm. (29%), m.p. 114–117°. One recrystallization from 10 ml. of absolute ethanol gave 2.17 Gm. of the pure compound, m.p. 117–118°; $[\alpha]_D^{28}$ −35.5° (c 4.2, $CHCl_3$). [Lit. (12) m.p. 117–118°, $[\alpha]_D$ +35.55° (c 4, $CHCl_3$) for the D-isomer.]

Evaporation of the filtrate to dryness gave 4.95 Gm. (64%) of the crude, noncrystalline anomer of L-threose triacetate which was also suitable for condensation.

9-α- and β-L-Threofuranosyladenine (XII and XIII, Respectively)—Crystalline L-threose triacetate (XI), 1.23 Gm. (5 mmoles), was condensed with chloromercuri-6-benzamidopurine (II) in exactly the same manner as described for the D-isomer. This resulted in the isolation of 1.70 Gm. (80%) of crude 2′,3′-di-*O*-acetyl-9-α- and β-L-threofuranosyl-6-benzamidopurine as a yellow glass; $\bar{\nu}_{max.}^{KBr}$ (cm.$^{-1}$) 1750 (acetate C=O), 1700 (amide C=O), 1375 (methyl), 1220 (acetate C—O—C), 710 (monosubstituted benzene).

A solution of 1.03 Gm. of the crude, blocked nucleosides in 25 ml. of 0.1 *N* methanolic sodium methoxide was allowed to remain at room temperature for 24 hr. in a stoppered flask. Without further processing, the solution was transferred to the top of a column (3.8 × 50 cm.) of the anion exchange resin previously equilibrated with 60% aqueous methanol. The elution procedure was identical to that described for the D-isomers except that the rate was somewhat slower, approximately 70 ml./hr. Fractions 68–81 were combined and evaporated to dryness *in vacuo* to give 84 mg. (11.7% from L-threose triacetate) of crystalline β-anomer (XIII), m.p. 236.5–238° dec. The analytically pure compound was obtained after two recrystallizations from 95% ethanol, m.p. 240–241° dec.; $[\alpha]_D^{20}$ +5° (c 0.49, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 258 (ε 14,100), $\lambda_{max.\ 260}^{pH\ 7\ and\ 13}$ (ε 14,500). The infrared spectrum was superimposable with that obtained for the β-D-isomer (X). Mixed melting point with the D-isomer (X) showed a depression. Periodate consumption (moles of periodate/mole of compound) (13, 17, 18): 0.05 (5 min.), 0.12 (1 hr.), 0.60 (24 hr.), 0.83 (72 hr.), 0.91 (96 hr.), 0.98 (120 hr.).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.61; H, 4.70; N, 29.46.

The crystalline α-isomer (XII) was obtained by combining fractions 85–107 and evaporating to dryness *in vacuo;* yield, 409 mg. (57.0% from L-threose triacetate), m.p. 211.5–213°. Two recrystallizations from 95% ethanol and thorough drying *in vacuo* at 100° for at least 24 hr. gave the pure compound, m.p. 214.5–215°; $[\alpha]_D^{20}$ −66° (c 0.60, H_2O); $\lambda_{max.}^{pH\ 1}$ (mμ) 258 (ε 13,800); $\lambda_{max.}^{pH\ 13}$ 260 (ε 14,200), $\lambda_{max.}^{H_2O}$ 260 (ε 14,200). The infrared spectrum was superimposable with that obtained for the α-D-isomer (IX). Mixed melting point with IX was depressed. Periodate consumption (moles of periodate/mole of compound) (13, 17, 18): 0.02 (5 min.), 0.08 (1 hr.), 0.59 (24 hr.), 0.80 (48 hr.), 0.89 (72 hr.), 0.94 (96 hr.), 0.98 (120 hr.).

Anal.—Calcd. for $C_9H_{11}N_5O_3$: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.43; H, 4.58; N, 29.72.

Condensation of crude, noncrystalline L-threose triacetate (above), deacylation, and chromatography as described above gave the α-anomer (XII) in 34% yield and the β-anomer (XIII) in 4.3% yield.

REFERENCES

(1) Bloch, A., and Nichol, C. A., *Biochem. Biophys. Res. Commun.*, **16**, 400(1964).
(2) Murray, D. H., Prokop, J., and Bloch, A., unpublished data.
(3) Shah, R. H., Schaeffer, H. J., and Murray, D. H., unpublished data.
(4) Prokop, J., and Murray, D. H., *J. Pharm. Sci.*, **54**, 359(1965).
(5) Murray, D. H., and Prokop, J., *ibid.*, **54**, 1468(1965).
(6) Baker, B. R., Schaub, R. E., and Kissman, H. M., *J. Am. Chem. Soc.*, **77**, 5911(1955).
(7) Goldman, L., Marsico, J. W., and Angier, R. B., *ibid.*, **78**, 4173(1956).
(8) Dekker, C. A., *ibid.*, **87**, 4027(1965).
(9) Schaffer, R., *ibid.*, **81**, 2838(1959).
(10) Baxter, J. N., and Perlin, A. S., *Can. J. Chem.*, **38**, 2217(1960).
(11) Neish, A. C., *ibid.*, **32**, 334(1954).
(12) Hockett, R. C., *J. Am. Chem. Soc.*, **57**, 2260(1935).
(13) Fox, J. J., Yung, N., Davoll, J., and Brown, G. B., *ibid.*, **78**, 2117(1956).
(14) Baker, B. R., "Chemistry and Biology of Purines," in "Ciba Foundation Symposium," Wolstenholme, G. E. W., and O'Connor, C. M., eds., Little, Brown and Co., Boston, Mass., 1957, p. 120.
(15) Steiger, M., and Reichstein, T., *Helv. Chim. Acta*, **19**, 1016(1936).
(16) Gätzi, K., and Reichstein, T., *ibid.*, **21**, 195(1938).
(17) Jackson, E. L., and Hudson, C. S., *J. Am. Chem. Soc.*, **59**, 994(1937).
(18) Lythgoe, B., and Todd, A. R., *J. Chem. Soc.*, **1944**, 592.

Irreversible Enzyme Inhibitors LXXXVII

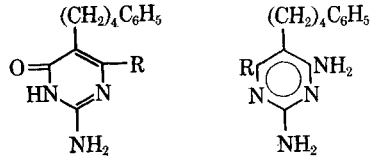
Hydrophobic Bonding to Dihydrofolic Reductase IX. Mode of Binding of *m*-Aryloxyalkyl Groups on 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazine

By B. R. BAKER and GERHARDUS J. LOURENS

The relative binding to dihydrofolic reductase by 16 *m*-substituted 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazines—nine of which are newly synthesized—has been measured. Strong evidence is presented to indicate that the increased binding by terminal phenyl groups on a *m*-substituent is due to complexing to a relatively hydrophilic area since terminal benzyl and terminal phenoxy groups give nearly the same increments in binding. The possible conversion of such compounds to candidate active-site-directed irreversible inhibitors of dihydrofolic reductase is discussed.

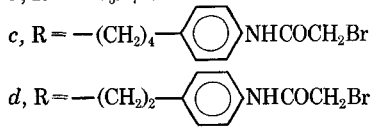
A HYDROPHOBIC BONDING area on an enzyme can have considerable utility in the design of potent and selective inhibitors, particularly if this area is adjacent to the active site (1). Since a hydrophobic bonding area on an enzyme that uses a polar substrate, such as dihydrofolic reductase, is probably nonfunctional with respect to binding of the substrate (1), large differences in the hydrophobic bonding area of this enzyme from different species would be expected to occur by evolution (2); such differences in the hydrophobic bonding area between pigeon liver and *E. coli* B dihydrofolic reductases (3), or between T_2-phage induced and *E. coli* B dihydrofolic reductases (4), or in other species (5) have been detected. In the case of dihydrofolic reductase, binding to the hydrophobic region can lead to complications; for example, if hydrophobic bonding is sufficiently strong, different rotomers of pyrimidine-type inhibitors may be bound where the hydrophobic group on the inhibitor is determinate for the rotomer (6).

The evidence for rotomers was initially based on differences in reversible binding with a variety of inhibitors (6, 7), then further verified with candidate irreversible inhibitors (8, 9). It was noted in the 2-amino-5-phenylbutyl-4-pyrimidinol series (I) that the reversible binding varied considerably with the R-substituent at the 6-position (6); the 6-methyl derivative (I*a*) was about 30 times more effective than the 6-propyl derivative (I*b*) (6), and the 6-phenylethyl derivative (I*d*) was in between in effectiveness (10). In contrast, in the 2,4-diamino series (II), the binding was independent of the 6-R-group; R = CH_3 (II*a*) (11), R = *n*-propyl (II*b*) (6), and R = the phenethyl derivative (II*d*) (8). All showed nearly identical reversible inhibition of the dihydrofolic reductase from pigeon liver. These results were rationalized on the basis that (*a*) the hydrophobic region is not between the pyrimidyl and phenyl moieties of dihydrofolate (III) (12, 13), but is near where the 4-position (or 8-position) of III resides on the enzyme (6)—as indicated by rotomer I; (*b*) the 4-pyrimidinols projected the 6-R group back toward the active site as indicated in rotomer I; and (*c*) the 2,4-diaminopyrimidines projected the 6-R group away from the active site (6) with the R-group probably not even in contact with the enzyme. That the alkylating function of I*d* and II*d* was not positioned the same way within their complexes with dihydrofolic reductase was indicated by the facts that the 4-pyrimidinol (I*d*) was an effective irreversible inhibitor of the enzyme (10), but the

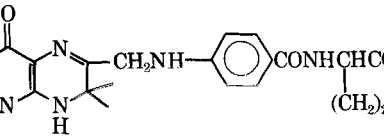


I II

a, R=CH_3
b, R=$-C_3H_7$-*n*



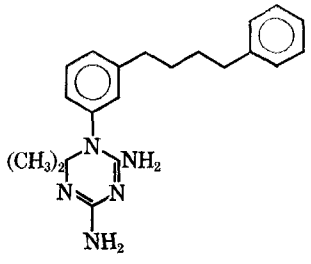
c, R=$-(CH_2)_4-$ NHCOCH$_2$Br



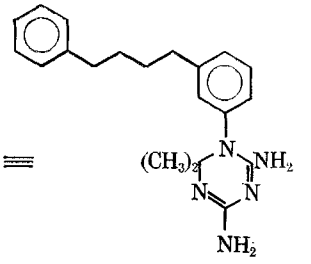
d, R=$-(CH_2)_2-$ NHCOCH$_2$Br

III

Received January 5, 1967, from the Department of Chemistry, University of California, Santa Barbara, CA 93106
Accepted for publication March 2, 1967.
This work was supported in part by grant CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
G. J. Lourens thanks the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition scholarship.
Previous paper: Baker, B. R., Ho, B.-T., and Lourens, G. J., *J. Pharm. Sci.*, **56**, 737(1967).



IV*A*



IV*B*

corresponding 2,4-diaminopyrimidine (II*a*) was not (8); although this result with II*d* was not unanticipated, it did create a serious problem in the design of irreversible inhibitors effective at low physiological concentration (8).

The effectiveness of an active-site-directed irreversible enzyme inhibitor is dependent upon two major factors: (*a*) the rate of inactivation is dependent upon the concentration of reversible enzyme–inhibitor complex which is in turn dependent upon the concentration of the inhibitor and the binding constant of the inhibitor, and (*b*) the rate of reaction of the neighboring group reaction between the leaving group on the inhibitor juxtaposed to a nucleophilic site on the enzyme (14, 15). Thus, in order to have an irreversible inhibitor effective at a physiological concentration of 10^{-8} *M*, it would be necessary that the inhibitor have a reversible binding constant of 10^{-7} *M* or less (8, 15). The 2-amino-4-pyrimidinol (I*d*) meets one of the two criteria for an effective irreversible inhibitor: I*d* rapidly alkylates a nucleophilic site on the enzyme with a half-life of about 12 min. at a concentration sufficient to convert 50% of the enzyme to a complex, but this concentration (4×10^{-5} *M*) is much too high for *in vivo* use. The 2,4-diaminopyrimidine (II*d*) also meets only one of the two criteria for an effective irreversible inhibitor since at 4×10^{-9} *M* it can complex 50% of the enzyme, but unfortunately does not inactivate the enzyme in a rotomeric conformation believed to be II. One possible solution to this enigma has been previously explored (16); the exploration of another possible solution is the subject of this paper.

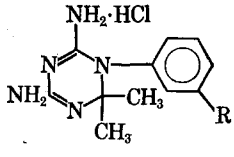
DISCUSSION

The 4,6-diamino-1-phenyl-*s*-triazines are believed to complex to dihydrofolic reductase in a rotomeric configuration similar to the 2,4-diamino-5-phenylbutylpyrimidines (II). It was recently observed that additional binding to the enzyme can occur with aralkyl groups that are substituted on the *m*- or *p*-position of the 1-phenyl moiety (17) (see Table I). Since the 1-phenyl group does not have a restricted rotation and must be nearly coplanar with the triazine ring when complexed to the dihydrofolic reductase from most species (5, 7, 18–21), a substituent such as the phenylbutyl group of IV can be pointed to the left (IV*B*) away from the active site or pointed to the right (IV*A*) toward the enzyme active site when complexed to the enzyme. Since the area on the right approaches the area that binds the polar dihydrofolate (III) it should be more polar than the hydrophobic region and be more apt to have polar nucleophilic groups present, which could be subject to attack by active-site-directed irreversible inhibitors; therefore, the mode of binding of the *m*-phenylalkyl substituents were investigated, and the results are listed in Table I.

Two series of side chains were investigated with terminal aryl or aryloxy groups—namely, (*a*) *m*-alkyl substituents and (*b*) *m*-alkoxy substituents. The latter class has the advantage that there is a greater variety of ground-state conformations for the bridge between the two phenyl rings; this is of considerable importance in the ability of a leaving group of the inhibitor to bridge to a nucleophilic site on the enzyme within the enzyme–inhibitor reversible complex.

Introduction of an ether linkage such as methoxy (VIII) on the 1-phenyl moiety leads to a fivefold loss in binding (Table I); such a loss could be

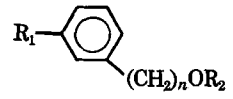
TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Concn. × 10^9 *M* for 50% Inhib.[a]	Inhibition Ratio: V/ Compd.	Inhibition Ratio: VI/ Compd.
V	—C_4H_9-*n*	30[b]	1	
VI	—OC_3H_7-*n*	1500[b]		1
VII	H	110[c]		
VIII	—OCH_3	540		
IX	—$(CH_2)_2C_6H_5$	24[c]	8.8[d]	
X	—$CH_2OC_6H_5$	55	3.8[d]	
XI	—CH_2OCH_3	210		
XII	—$(CH_2)_3C_6H_5$	5.9[b]	5.1	
XIII	—$O(CH_2)_2C_6H_5$	280		5.3
IV	—$(CH_2)_4C_6H_5$	2.7[b]	11	
XIV	—$O(CH_2)_3C_6H_5$	88		17
XV	—$O(CH_2)_2OC_6H_5$	75		20
XVI	—$(CH_2)_5C_6H_5$	7.1[b]	4.7	
XVII	—$(CH_2)_4OC_6H_5$	3.2	10	
XVIII	—$O(CH_2)_4C_6H_5$	74		20
XIX	—$O(CH_2)_3OC_6H_5$	110		14

The technical assistance of Maureen Baker, Barbara Baine, Pepper Caseria, and Ann Jaqua is acknowledged.
[a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction prepared from pigeon liver and assayed with 6 μM dihydrofolate and 12 μM TPNH at pH 7.4 as previously described (27). [b] Data from *Reference 17*. [c] Data from *Reference 18*. [d] Ratio of XI to compound.

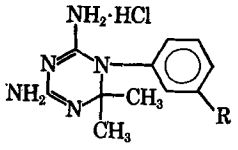
TABLE II—PHYSICAL CONSTANTS FOR



Compd.[a]	R_1	R_2	n	Method	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
XXII*b*	NO_2	$C_6H_5(CH_2)_2-$	0	*A*	23	46–48[b]						
XXII*c*	NO_2	$C_6H_5(CH_2)_3-$	0	*A*	62	27–28[c]	70.2	5.65	5.60	70.0	5.87	5.44
XXII*d*	NO_2	$C_6H_5O(CH_2)_2-$	0	*B*	64	94–95[d]	64.9	5.05	5.40	65.0	5.11	5.44
XXII*e*	NO_2	$C_6H_5(CH_2)_4-$	0	*B*	64	Oil						
XXII*f*	NO_2	$C_6H_5O(CH_2)_3-$	0	*B*	73	Oil						
XXIV*b*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_2-$	0	*C*	56	126–128[e]	67.3	6.46	5.61	67.4	6.48	5.47
XXIV*c*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_3-$	0	*C*	95	140–143[f]	68.3	6.88	5.31	68.2	7.02	5.39
XXIV*d*	$NH_2\cdot HCl$	$C_6H_5O(CH_2)_2-$	0	*C*	57	167–170[e]	63.3	6.07	5.27	63.2	6.29	5.13
XXIV*e*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_4-$	0	*C*	22[g]	110–111[e]	69.2	7.26	5.04	68.9	7.36	4.83
XXIV*f*	$NH_2\cdot HCl$	$C_6H_5O(CH_2)_3-$	0	*C*	43[g]	161–164[e]	64.4	6.49	5.01	64.2	6.70	4.98
XXVII*a*	NO_2	CH_3-	1	*B*[h]		Oil						
XXVII*b*	NO_2	C_6H_5-	1	*B*	82	Oil[i]						
XXIX*a*	$NH_2\cdot HCl$	CH_3-	1	*C*	98[j]	111–112[e]	55.3	6.97	8.07	55.1	7.10	8.27
XXIX*b*	$NH_2\cdot HCl$	C_6H_5-	1	*C*	41[j]	143–146[e]	66.2	5.99	5.94	66.0	6.27	6.13
XXXIII	$NH_2\cdot HCl$	C_6H_5-	4	*D*	47	150–152[e]	69.2	7.26	5.04	69.2	7.38	5.14

[a] All compounds had infrared spectra in agreement with their assigned structures. [b] Melting point of 50° and 29% yield previously reported (25) for this compound prepared by a different method. [c] Recrystallized from *n*-propanol. [d] Recrystallized from isopropyl alcohol. [e] Recrystallized from isopropyl alcohol–petroleum ether (b.p. 40–60°). [f] Recrystallized from isopropyl alcohol–ether. [g] Over-all yield for two steps from *m*-nitrophenol. [h] See Mann, F. G., and Stewart, F. H. C., *J. Chem. Soc.*, **1954**, 4127. [i] A b.p. of 182°/3 mm. for this oil has been reported (26). [j] Over-all yield for two steps from *m*-nitrobenzyl chloride.

TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	R	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
VIII	$-OCH_3$	77	189–190[b]	50.8	6.39	24.7	50.5	6.52	24.7
X	$-CH_2OC_6H_5$	76	136–138[c]	60.1	6.16	19.5	59.9	6.27	19.3
XI	$-CH_2OCH_3$	88	192–193[c]	52.4	6.77	23.5	52.4	6.75	23.6
XIII	$-O(CH_2)_2C_6H_5$	83	175–176[d]	61.0	6.47	18.7	61.1	6.70	18.5
XIV	$-O(CH_2)_3C_6H_5$	60	175–178[c]	61.9	6.75	18.0	62.1	6.68	18.0
XV	$-O(CH_2)_2OC_6H_5$	65	173–174[d]	58.5	6.20	18.0	58.3	5.91	17.7
XVII	$-(CH_2)_4OC_6H_5$	88	174–175[c]	62.8	7.02	17.4	62.6	6.99	17.3
XVIII	$-O(CH_2)_4C_6H_5$	80	170–171[d]	62.8	7.02	17.4	62.5	7.21	17.6
XIX	$-O(CH_2)_5OC_6H_5$	88	171–172[d]	59.5	6.49	17.3	59.4	6.66	17.3

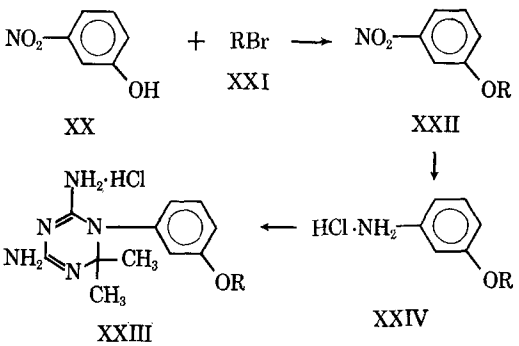
[a] All compounds were prepared by method *E* and had infrared and ultraviolet spectra in agreement with their assigned structures. [b] Recrystallized from ethanol. [c] Recrystallized from ethanol–ether. [d] Recrystallized from ethanol–petroleum ether (b.p. 40–60°).

anticipated since introduction of other polar groups on the 1-phenyl moiety led to decreased binding, presumably due to repulsion from the hydrophobic region on the enzyme (22). Surprisingly, another threefold loss occurred in binding when the chain was extended to *n*-propoxy (VI). Of more importance was the fact that introduction of a terminal phenyl on the *n*-propoxy group, as in XIV, gave a seventeenfold increase in binding; this result compares favorably with the elevenfold increase in binding when a terminal phenyl group is introduced on the *n*-butyl group as in IV (17). Similar increments in terminal phenyl binding were observed in IX *versus* X and XII *versus* XIII.

It was previously observed that the binding of a 5-phenylbutyl group on the pyrimidine (I*a*) was thirtyfold better than the 5-phenoxypropyl group (23); this result has been attributed to the increased hydrophobic bonding of a terminal benzyl group compared to a terminal phenoxy group plus some possible repulsion of the ether oxygen from the hydrophobic region (7). A similar repulsion of the ether group of VIII has just been discussed. Therefore, the replacement of a methylene group by an ether oxygen can be used as a test for the relative hydrophobic character of a binding region. Note that exchange of benzyl in XIV for phenoxy in XV gave no appreciable change in binding; similar comparisons are XVI *versus* XVII and XVIII *versus* XIX. Therefore, the nearly identical binding by phenoxy and benzyl indicates that the terminal phenyl moiety may well be complexed to a relatively hydrophilic region on the enzyme surface, as suggested earlier for the conformation of IV*B*. If indeed these terminal phenyl groups of molecules such as IV and XV are complexed in a relatively polar region of the enzyme, then introduction of a bromoacetamido group on the terminal phenyl group should make logical candidate active-site-directed irreversible inhibitors of dihydrofolic reductase that could be operational in the 10^{-7}–10^{-8} *M* region; such studies are being vigorously pursued.

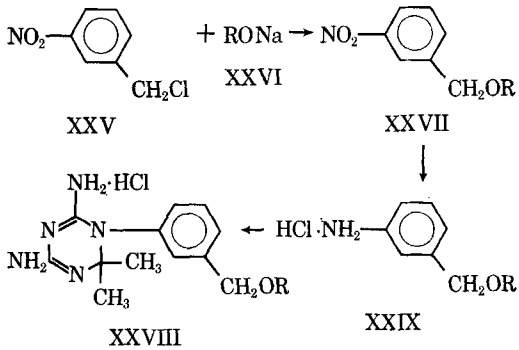
CHEMISTRY

The 1,2-dihydro-1-phenyl-*s*-triazines in Tables I and III were made from the appropriate aryl amine hydrochloride and cyanoguanidine in acetone—the three-component method of Modest (20). The synthetic methods for the required amines could be divided into three classes. The first class contained an ether linkage to the 1-phenyl moiety of type XXIII. *m*-Nitrophenol (XX) was converted to the sodium salt with sodium hydride then alkylated with the appropriate bromide (XXI), except for XXIIe where 4-phenylbutyl chloride was employed. The nitro groups were reduced catalytically with a palladium-charcoal catalyst in ethanol in the presence of a slight excess of hydrochloric acid; the amine hydrochlorides (XXIV) were isolated in good yield. Condensation of XXIV with cyanoguanidine in acetone afforded the triazines (XXIII); of course, commercial XXIV*a* base was employed. (Scheme I.)



Series *a*, R = CH_3
b, R = $C_6H_5(CH_2)_2$—
c, R = $C_6H_5(CH_2)_3$—
d, R = $C_6H_5O(CH_2)_2$—
e, R = $C_6H_5(CH_2)_4$—
f, R = $C_6H_5O(CH_2)_3$—

Scheme I



Series *a*, R = CH_3
b, R = C_6H_5

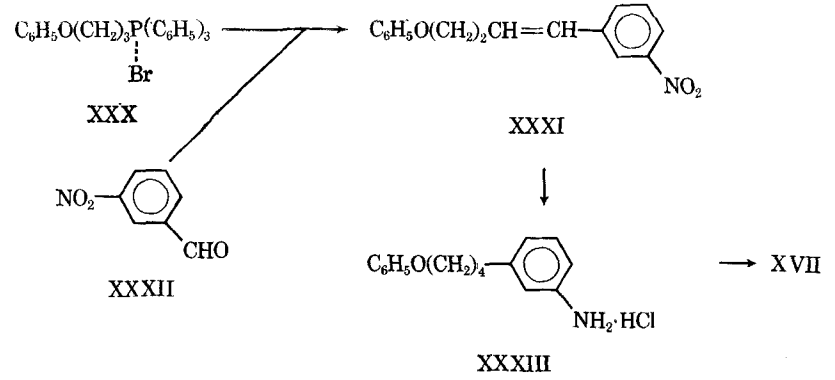
Scheme II

The second series contained the oxymethylene bridge as in XXVIII; these were prepared *via* *m*-nitrobenzyl chloride (XXV). Reaction of XXV with phenol in acetone in the presence of potassium carbonate afforded XXVII*b*. The methyl ether (XXVII*a*) was prepared from XXV with sodium methoxide. The remainder of the sequence for XXVIII was the same as for XXIII. (Scheme II.)

The third type is represented by XVII (Table III). Condensation of the Wittig reagent (XXX) from phenoxypropyl bromide (24) with *m*-nitrobenzaldehyde (XXXII) in tetrahydrofuran in the presence of potassium *tert*-butoxide afforded XXXI as an oil. Hydrogenation with platinum oxide catalyst gave crystalline XXXIII in 40% over-all yield from XXXII. Condensation with cyanoguanidine afforded the desired dihydro-*s*-triazine (XVII). (Scheme III.)

Methods—Melting points were determined in capillary tubes with a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by iodine vapor or by visual examination under ultraviolet light.

m-Nitrophenyl γ-Phenylpropyl Ether (XXIIc)—*Method A*—A solution of 3.83 Gm. (27.5 mmoles) of *m*-nitrophenol in 10 ml. of dimethylsulfoxide was added dropwise over a period of about 10 min. to a magnetically stirred mixture of 10 ml. of dimethylsulfoxide and 1.27 Gm. (26 mmoles) of a 53.5% dispersion of sodium hydride in mineral oil, protected from moisture. The red solution was warmed to 80°, then 4.98 Gm. (25 mmoles) of 3-



Scheme III

phenylpropyl bromide was added; the temperature rose to 95°. After being stirred in a bath at 80–85° for 90 min., the mixture was cooled, diluted with 75 ml. of benzene, and washed successively with 100 ml. of water, 25 ml. of water, 5% aqueous sodium hydroxide (3 × 15 ml.), and finally water (2 × 25 ml.). The benzene solution, dried with magnesium sulfate, was evaporated *in vacuo*. Crystallization from *n*-propanol gave 4.02 Gm. (62%) of product, m.p. 27–28°. $\nu_{max.}$ (film): 1610, 1580 (C=C); 1530, 1345 (NO_2); 1245, 1030 (C—O—C); 810, 795 (*m*-C_6H_4); 735, 698 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data and for other compounds prepared by Method *A*.)

m-Nitrophenyl Phenoxyethyl Ether (XXIId)—*Method B*—To a solution of 1.41 Gm. (26 mmoles) of sodium methoxide in 10 ml. of reagent methanol was added 3.83 Gm. (27.5 mmoles) of *m*-nitrophenol (XX), followed by 5.03 Gm. (25 moles) of β-bromophenetole. After being refluxed with magnetic stirring for 18–22 hr., the mixture was spin-evaporated *in vacuo*. The residue was partitioned between 50 ml. of ether and 30 ml. of water. The separated ether layer was washed with 5% aqueous sodium hydroxide (2 × 10 ml.), then water (3 × 10 ml.). Dried with magnesium sulfate, the solution was evaporated *in vacuo*. Recrystallization of the residue from isopropyl alcohol gave 4.13 Gm. (64%) of product, m.p. 94–95°. $\nu_{max.}$ 1605, 1600, 1580, 1500 (C=C); 1520, 1350 (NO_2); 1250, 1060 (C—O—C); 805, 800 (*m*-C_6H_4); 762, 742, 700 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data and for other compounds prepared by this method.)

m-Aminophenyl β-Phenethyl Ether Hydrochloride (XXIVb)—*Method C*—A solution of 1.83 Gm. (7.5 mmoles) of XXII*b* in 100 ml. of ethanol containing 0.75 ml. of 12 *N* aqueous hydrochloric acid (9 mmoles) was shaken with hydrogen at 2–3 Atm. in the presence of 0.15 Gm. of 5% palladium-charcoal; reduction was complete in about 20 min. The filtered solution was evaporated *in vacuo*. Recrystallization of the residue from isopropyl alcohol–petroleum ether (b.p. 40–60°) gave 1.05 Gm. (56%) of white crystals, m.p. 126–128°. $\nu_{max.}$ 2870 (broad, NH); 2600, 1950 (NH_3^+); 1620, 1600, 1530, 1498 (NH, C=C); 1275, 1028 (C—O—C); 795, 785 (*m*-C_6H_5); 755, 702, 690 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data for other compounds prepared by this method.)

4-(m-Aminophenyl)butyl Phenyl Ether Hydrochloride (XXXIII)—*Method D*—To a magnetically stirred mixture of 1.51 Gm. (10 mmoles) of *m*-nitrobenzaldehyde, 5.25 Gm. (11 mmoles) of XXX (24), and 15 ml. of reagent tetrahydrofuran, cooled in an ice bath and protected from moisture, was added 1.12 Gm. (10 mmoles) of potassium *tert*-butoxide. After being stirred in an ice bath for 1 hr. and at ambient temperature for 18 hr., the mixture was filtered and the insolubles washed with tetrahydrofuran. The combined filtrate and washings were evaporated *in vacuo*. The residual oil was dissolved in benzene and passed through a 3-cm. diameter column containing 64 Gm. of silica gel to remove triphenylphosphine oxide. The first fraction of yellow colored solution was collected and evaporated *in vacuo;* yield, 1.83 Gm. (68%) of XXXI as a yellow oil. $\nu_{max.}$ (film) 1600, 1595, 1500 (C=C); 1525, 1350 (NO_2); 1245, 1040 (C—O—C); 800 (*m*-C_6H_4); 735, 690 cm.$^{-1}$ (C_6H_5).

A solution of 1.83 Gm. of XXXI in 100 ml. of ethanol containing 0.75 ml. of 12 *N* aqueous hydrochloric acid was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide catalyst; reduction was complete in 15 min. The reaction mixture was processed as described under *Method C;* yield, 1.10 Gm. (69%), m.p. 147–149°. A second recrystallization from isopropyl alcohol–petroleum ether (b.p. 40–60°) gave the analytical sample as white crystals, m.p. 150–152°. $\nu_{max.}$ 2850, 2600, 1950 (NH, NH_3^+); 1600, 1570, 1510, 1490 (NH, C=C); 1255, 1035 (C—O—C); 790 (*m*-C_6H_4); 758, 690 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data.)

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[m-(2-phenoxyethoxy)phenyl]-s-triazine Hydrochloride (XV)—*Method E*—A mixture of 664 mg. (2.5 mmoles) of XXIV*d*, 217 mg. (2.7 mmoles) of cyanoguanidine, and 4 ml. of reagent acetone was refluxed for 20 hr. with magnetic stirring. The cooled mixture was filtered and the product washed with acetone; yield, 631 mg. (65%), m.p. 168–170°. Recrystallization from ethanol–petroleum ether (b.p. 40–60°) gave 559 mg. (57%) of white crystals, m.p. 173–174°. $\lambda_{max.}$ 246, 268–280 (plateau) mμ; $\nu_{max.}$ 3300, 3150 (broad, NH); 1660 (C=NH^+); 1630, 1600, 1545, 1520 (NH, C=C, C=N); 1250, 1070 (C—O—C); 800, 785 (*m*-C_6H_4); 750, 705, 690 cm.$^{-1}$ (C_6H_5). (See Table III for analytical data and additional compounds made by this method).

REFERENCES

(1) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967.
(2) *Ibid.*, chap. IX.
(3) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 470(1966).
(4) Baker, B. R., *J. Med. Chem.*, to be published.
(5) Hitchings, G. H., and Burchall, J. J., *Advan. Enzymol.*, **27**, 417(1965).
(6) Baker, B. R., and Shapiro, H. S., *J. Pharm. Sci.*, **55**, 308(1966).
(7) *Reference 1*, chap. X.
(8) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **4**, 31(1967).
(9) Baker, B. R., and Meyer, R. B., Jr., *J. Pharm. Sci.*, **56**, 570(1967).
(10) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417 (1966).
(11) Baker, B. R., and Ho, B.-T., *ibid.*, **54**, 1261(1965).
(12) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *ibid.*, **55**, 295(1966).
(13) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(14) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(15) *Reference 1*, chap. VIII.
(16) Baker, B. R., Lourens, G. J., and Jordaan, J. H., *J. Heterocyclic Chem.*, **4**, 39(1967).
(17) Baker, B. R., Ho, B.-T., and Lourens, G. J., *J. Pharm. Sci.*, **56**, 737(1967).
(18) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 72(1965).
(19) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **53**, 1137(1964).
(20) Modest, E. J., *J. Org. Chem.*, **21**, 1(1956).
(21) Russell, P. B., *J. Chem. Soc.*, **1954**, 2951.
(22) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(23) Baker, B. R., Ho, B.-T., and Chheda, G. B., *ibid.*, **1**, 88(1964).
(24) Mel'nikov, N. N., and Kraft, V. A., *Zh. Obshchei Khim.*, **30**, 1918(1960); through *Chem. Abstr.*, **55**, 6417*c* (1961).
(25) Sova, J., Sehera, A., and Vrba, C., *Chem. Listy*, **51**, 2339(1957).
(26) Cavill, G. W. K., Dean, P. M., McGookin, A., Marshall, B. M., and Robertson, A., *J. Chem. Soc.*, **1954**, 4573.
(27) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).

Action of Angiotensin II Upon Renal Vascular Resistance

By WILLIAM E. HAGEMAN* and JOSEPH P. BUCKLEY

The effects of angiotensin II, vasopressin, *l*-norepinephrine, and elevated perfusion pressure were investigated on the peripheral resistance of the vascular beds in isolated rabbit kidneys in which the rate of flow was controlled by a Sigmamotor pump. Angiotensin II produced marked rhythmic fluctuations in perfusion pressure, having a mean amplitude of 52 mm. H_2O (n = 124); and when flow was decreased so that perfusion pressure was equal to control levels, the mean amplitude of rhythmic fluctuations was still 40 mm. H_2O. This effect of angiotensin II appeared to be affected by both age and sex with peak activity occurring at 2 to 4 months of age and the kidneys from female rabbits being significantly more responsive than those of male rabbits. Vasopressin also significantly increased the amplitude of the rhythmic fluctuations in perfusion pressure but to a much lesser degree than angiotensin II. The effect of *l*-norepinephrine was not significantly different from that induced by simple elevation of perfusion pressure. These data suggest that angiotensin II may exert a direct effect upon renal vascular smooth muscle of the "single-unit" type by increasing the number of spontaneous, local depolarizations through an increased influx of Ca^{2+} into the muscle cells. Such an action might also involve angiotensin II in the autoregulation of renal blood flow.

THE INVOLVEMENT of the kidney as a possible cause of, or contributor to, arterial hypertension was suspected in the latter part of the 19th century. Tigerstedt and Bergman (1) demonstrated that crude saline kidney extracts produced a pressor response when injected into anesthetized rabbits. The active principle(s) of the extract was postulated to have a direct vasoconstrictor action; and although it was impure and unidentified, they called it renin. This work lay dormant for over a quarter of a century before interest was renewed when Goldblatt *et al.* (2) demonstrated a reproducible hypertension of renal origin in dogs by the production of renal ischemia, and Volhard (3) introduced his theory of "pale hypertension" and its relationship to nephrosclerosis.

Since Goldblatt's discovery, the vasoactive principles, renin and angiotensin II, have been isolated, identified, and extensively investigated. These findings have stimulated profuse pharmacological research in the area of renal hypertension. However, the true physiological mechanism of the hypertensive state and the relationship of the kidney, angiotensin, and renin in arterial hypertension are not yet clearly defined. Considerable controversy and conflicting experimental evidence exist, and angiotensin is a focal point of these controversies.

Studies involving the role of the kidney in cardiovascular changes or disorders must take into consideration all of the factors involved in the complex relationships of the renal and cardiovascular systems. One such factor is the kidney's remarkable capacity to rapidly readjust in the wake of changing systemic arterial pressure so that renal blood flow remains fairly constant as the arterial pressure is altered. Waugh (4) reported that, within a normal range of arterial blood pressure, a 50% elevation of arterial pressure resulted in only a 5% increase in renal blood flow. Investigators have been cognizant of this phenomenon for many years, and the mechanisms of such "autoregulation" have been the subject of extensive research. However, there is still much discussion over the role of autoregulation and the factors involved in its mechanisms.

Regardless of investigators' disagreement on the mechanisms involved in the initiation of this phenomenon, it is almost universally accepted that vascular components play a major role in autoregulatory processes. In light of this fact, it might well be pertinent to ask what happens in a pathological state such as that produced by hypertension. The classic experiments of Goldblatt *et al.* (2) indicated that pathological changes of the renal arterial vasculature in dogs can produce a sustained arterial hypertension which is hemodynamically similar to essential hypertension in man. Byrom (5) reported uniform constriction interrupted by sharply localized dilatations in intestinal and cerebral arteries of renal hypertensive rats. Giese (6) infused synthetic angiotensin into nephrectomized rats to produce acute hypertensive vascular disease. He was able to not only demonstrate a very characteristic pattern of "alternating constrictions and dilatations" along the course of intestinal arteries, but,

Received March 20, 1967, from the Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213

Accepted for publication May 5, 1967.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by research grant HE-03475 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

* American Foundation for Pharmaceutical Education Fellow, 1965–1967.

TABLE I—EFFECT OF PRESSURE AND ANGIOTENSIN INFUSION UPON THE PERFUSION PRESSURE OF ISOLATED PERFUSED[a] KIDNEYS OF RABBITS

Conditions	N	Mean Perfusion Pressure, mm. Hg ± S.E.[b]	Rhythmic Activity Amplitude, mm. H_2O ± S.E.[b]	Rhythmic Activity Rate/min. ± S.E.[b]
Pre-Angiotensin II Infusion				
Perfusion pressure	151	75.31 ± 1.76	9.74 ± 0.89	2.79 ± 0.16
Elevated perfusion pressure[c]	144	108.80 ± 2.05	19.60 ± 1.46	3.68 ± 1.25
Angiotensin II Infusion				
Perfusion pressure	124	109.43 ± 2.32	52.42 ± 3.60	3.27 ± 0.11
Decreased perfusion pressure[d]	44	64.23 ± 6.38	40.14 ± 3.99	2.58 ± 0.22
Post-Angiotensin II Infusion				
Perfusion pressure	123	76.87 ± 2.40	14.41 ± 1.18	2.89 ± 0.20
Elevated perfusion pressure[c]	89	111.24 ± 2.94	29.72 ± 3.07	3.49 ± 0.15

[a] Perfused with Tyrode's solution. [b] S.E. = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$. [c] Perfusion pressure increased by adjusting Sigmamotor pump to approximate pressure induced by angiotensin II. [d] Perfusion pressure decreased by adjusting Sigmamotor pump.

using vascular labeling with colloidal carbon particles, he was able to show increased vascular permeability which was restricted to the dilated segments of the vessels.

Several investigators have reported similar changes in vascular elements subsequent to the infusion of angiotensin. Benelli *et al.* (7) reported a spontaneous rhythmic motility of the parenchymal as well as the vascular smooth muscle apparatus of the isolated cat spleen during perfusion with an angiotensin solution. Buckley (8) reported an induced rhythmicity in isolated rabbit kidneys upon perfusion with angiotensin, which seemed to be diminished with the increasing age of the animal.

Spontaneous rhythmic changes in renal vascular resistance have also been noted. Waugh (9) perfused isolated kidneys of dogs with PVP-Locke solution and observed "an unusually high degree of brief rhythmic changes in renal flow and prevenous resistance," concomitant with autoregulation of flow and subsequent to elevation of perfusion pressure, which were not affected by procaine anesthetization of intrarenal neural elements, but which were abolished by higher doses of procaine which were sufficient to depress the reactivity of the renal vascular elements to barium chloride or epinephrine injections. In view of these aforementioned results, the intent of the present investigation was to investigate further the nature of the rhythmic alterations in the renal vasculature and to determine its relation to angiotensin and other factors.

METHODS

Effect of Angiotensin II Upon Isolated Perfused Rabbit Kidneys and the Production of Rhythmic Alterations in Perfusion Pressure—New Zealand albino rabbits were selected at random and anesthetized with 30 mg./Kg. of sodium pentobarbital *via* a marginal ear vein. Each kidney was carefully isolated, the renal artery and vein were distally ligated, and the renal artery cannulated. A polyethylene cannula, made by building polyethylene tubing up to a $^3/_{16}$-in. bore starting with size PE 60 tubing, was used. Each layer of tubing was treated with epoxy cement to assure a tight, leak-proof seal. The ureter and renal vein were severed and the kidney removed from the animal and quickly suspended in a perfusion system which maintained the temperature of the internal and external environment of the kidney at 37°. The cannula was connected to one orifice of a small T tube which was also connected to a transducer, recording the perfusion pressure onto a Grass polygraph calibrated at two different sensitivities to allow the perfusion pressure to be monitored in both mm. Hg and mm. H_2O.

The kidney was perfused with Tyrode's solution mol. wt. 73,000, containing 1% dextran[1] (Pharmachem Corp., Bethlehem, Pa.) in such a manner the perfusion pressure ranged approximately between 75–100 mm. Hg and the flow between 12 and 20 ml./min. A Sigmamotor pump was used to maintain a constant flow from two 32-oz. reservoir bottles, one containing Tyrode's solution and the other containing Tyrode's solution plus angiotensin II, 0.01 mcg./ml. The reservoirs were kept in a constant-temperature bath at 37° and aerated with 95% oxygen and 5% carbon dioxide *via* fritted-glass gas dispersion tubes in order to assure adequate and balanced aeration of the solutions. The right kidney was prepared first, followed immediately by the left; and both were suspended in similar perfusion systems. Approximately 15–30 min. were allowed for the venous flow to stabilize to between 12–20 ml./min., after which time each kidney was treated according to the following procedure.

(*a*) The perfusion pressure was increased by adjusting the flow of the Sigmamotor pump to a level of pressure similar to that expected to be achieved by the infusion of angiotensin II, as

[1] Stock solution *1:* NaCl, 200 Gm.; KCl, 5 Gm.; $CaCl_2$ (anhydrous), 5 Gm.; $MgCl_2 \cdot H_2O$, 5 Gm.; glass-distilled water *q.s. ad.* 1000 ml. Stock solution *2:* $NaHCO_3$, 50 Gm.; $NaH_2PO_4 \cdot H_2O$, 2.5 Gm.; glass-distilled water *q.s. ad.* 1000 ml. Stock solution *3:* Dextran (mol. wt. 73,000), 100 Gm.; dextrose (anhydrous), 25 Gm.; glass-distilled water *q.s. ad.* 1000 ml. Mix 80 ml. of stock solution *1*, 40 ml. of stock solution *2*, add 200 ml. of stock solution *3*, and make up to a total volume of 2 L. with glass-distilled water.

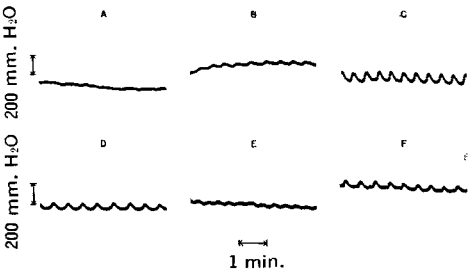


Fig. 1—Typical responses due to angiotensin II infusion and perfusion pressure changes in isolated perfused rabbit kidney. Key: A, *pre-angiotensin II infusion at normal perfusion pressure;* B, *pre-angiotensin II infusion at elevated perfusion pressure;* C, *angiotensin II infusion;* D, *angiotensin II infusion at decreased perfusion pressure level;* E, *post-angiotensin II infusion at normal perfusion pressure;* F, *post-angiotensin II infusion at elevated perfusion pressure.*

determined from preliminary experiments. (*b*) The perfusion pressure was returned to original levels and allowed to stabilize for 5–10 min. (*c*) Angiotensin II was infused until the maximum effect was obtained. If rhythmic fluctuations in perfusion pressure occurred, the perfusion pressure was readjusted to pre-angiotensin II infusion pressure levels by reducing flow *via* the Sigmamotor pump. (*d*) Flow was returned to control levels *via* the Sigmamotor pump, and angiotensin II infusion stopped. (*e*) Steps (*a*), (*b*), and (*c*) were repeated several times until maximal rhythmic alterations were observed. (*f*) Perfusion pressure levels were monitored in mm. Hg, and amplitude changes of the rhythmic alterations were measured in mm. of water. Records were made at a chart speed of 0.25 mm./sec.

Effect of Other Vasopressor Agents on the Rhythmic Fluctuations in the Perfusion Pressure of Isolated Perfused Rabbit Kidneys—The procedure described above was followed utilizing either *l*-norepinephrine,[2] 0.25 mcg./ml., or vasopressin,[3] 0.01 units/ml., in place of angiotensin II in one of the perfusion reservoirs.

Effect of Age and Sex upon the Rhythmic Alterations in Perfusion Pressure Induced by Angiotensin II Infusion into Isolated Perfused Rabbit Kidneys—New Zealand albino rabbits of both sexes were obtained at certain selected ages. Females were used at ages of 1, 2, 4, 8, and 12 months; and males were used up to the age of 8 months since 12-month-old male rabbits were not available during the course of the experiment. The previously described procedure for isolation, removal, perfusion, and treatment was followed.

Analysis of Data—All data were punched onto IBM cards and analyzed by either the BMD 02V, BMD 05V, or BMD 07V analysis of variance programs on the IBM 7090 computer.

RESULTS

Effect of Angiotensin II Infusion Upon Rhythmic Fluctuations in Perfusion Pressure of Isolated Perfused Rabbit Kidneys—The data obtained in these experiments are summarized in Table I. Angiotensin II,[4] when infused at a concentration of 0.01 mcg./ml. and at a rate of 12–20 ml./min., produced rhythmic alterations in perfusion pressure up to 250 mm. H_2O (Fig. 1) with a mean amplitude of 52.4 ± 3.6 mm. H_2O. Even when the perfusion pressure was decreased to pre-angiotensin II levels by decreasing the rate of perfusion, there was only a 15–20% decrease in the amplitude of rhythmic fluctuations to 40.1 ± 4 mm. H_2O. Although simply increasing the perfusion pressure did produce rhythmic pressure alterations, the response to angiotensin II was over 2.5 times that achieved by pressure alone. These data indicated that pressure *per se* cannot fully account for the rhythmic changes in perfusion pressure produced by the infusion of minute doses of angiotensin II, and that this polypeptide plays a direct role in inducing these rhythmic alterations in perfusion pressure. Although the rhythmicity produced by angiotensin II is significantly greater ($p = <0.01$) than that present either prior to or following the infusion of angiotensin II, the post-angiotensin II responsiveness is also significantly greater both at normal and increased perfusion pressure ($p = <0.01$). Angiotensin II did not significantly alter the rate of the rhythmic changes.

Effect of Other Vasopressor Agents on the Rhythmic Fluctuations in the Perfusion Pressure of Isolated Perfused Rabbit Kidneys—The data obtained in these experiments are summarized in Tables II and III. *l*-Norepinephrine at a concentration of 0.25 mcg./ml. and at a rate of 12–20 ml./min. produced rhythmic activity approximately equivalent to that induced by the elevation of perfusion pressure alone. When compared with the effects of angiotensin II infusion, *l*-norepinephrine produced approximately 1/4 the increase in amplitude of rhythmic activity induced by angiotensin II ($p = <0.01$), the mean amplitude of the rhythmic alterations in perfusion pressure induced by *l*-norepinephrine being 12.1 ± 2.9 mm. H_2O compared to a mean amplitude of 52.4 ± 3.6 mm. H_2O produced by angiotensin II infusion.

Vasopressin, 0.01 units/ml., 12–20 ml./min., significantly greater rhythmic fluctuations in perfusion pressure than that produced by simple elevation of perfusion pressure ($p = < 0.05$). However, the 24.6 ± 1.5 mm. H_2O amplitude of rhythmic alterations in perfusion pressure produced by vasopressin was less than 50% of that produced by angiotensin II infusion. Vasopressin did not seem to produce a sensitization as did angiotensin II since there was no significant difference between the pre- and post-infusion period responses at either normal or elevated perfusion pressure. The effect of vasopressin upon the amplitude of the perfusion pressure alterations was 133% of the effect produced by simple elevation of perfusion pressure, but the response to vasopressin infusion was over 200% greater than the response to *l*-norepinephrine infusion ($p = <0.05$).

Effect of Sex Upon Rhythmic Alterations in Perfusion Pressure of Isolated Perfused Rabbit Kidneys—In order to analyze these data for the over-all effect of sex, the kidneys from female rabbits were grouped together, ignoring age, and compared with the kidneys from male rabbits at

[2] Levophed Bitartrate, Winthrop Laboratories, New York, N. Y.
[3] Pitressin, Parke, Davis & Co., Detroit, Mich.
[4] Hypertensin, Ciba Pharmaceutical Co., Summit, N. J.

TABLE II—EFFECT OF PRESSURE AND VASOPRESSIN INFUSION UPON THE PERFUSION PRESSURE OF ISOLATED PERFUSED[a] KIDNEYS OF RABBITS

Conditions	N	Mean Perfusion Pressure, mm. Hg ± S.E.[b]	Rhythmic Activity Amplitude, mm. H_2O ± S.E.[b]	Rhythmic Activity Rate/ min. ± S.E.[b]
Pre-Vasopressin Infusion				
Perfusion pressure	151	75.31 ± 1.76	9.74 ± 0.98	2.79 ± 0.16
Elevated perfusion pressure[c]	144	108.80 ± 2.05	19.60 ± 1.46	3.68 ± 1.25
Vasopressin Infusion				
Perfusion pressure	15	102.47 ± 8.50	24.60 ± 5.18	3.00 ± 0.22
Decreased perfusion pressure[d]	11	69.36 ± 6.50	15.45 ± 4.01	2.18 ± 0.42
Post-Vasopressin Infusion				
Perfusion pressure	15	65.00 ± 6.69	11.67 ± 2.43	2.83 ± 0.33
Elevated perfusion pressure[c]	12	100.83 ± 7.17	21.00 ± 4.27	3.15 ± 0.49

[a] Perfused with Tyrode's solution. [b] S.E. = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$. [c] Perfusion pressure elevated by adjusting Sigmamotor pump to approximate the pressure induced by vasopressin. [d] Perfusion pressure decreased by adjusting the Sigmamotor pump.

TABLE III—EFFECT OF PRESSURE AND *l*-NOREPINEPHRINE INFUSION UPON THE PERFUSION PRESSURE OF ISOLATED PERFUSED[a] KIDNEYS OF RABBITS

Conditions	N	Mean Perfusion Pressure, mm. Hg ± S.E.[b]	Rhythmic Activity Amplitude, mm. H_2O ± S.E.[b]	Rhythmic Activity Rate/ min. ± S.E.[b]
Pre-*l*-Norepinephrine Infusion				
Perfusion pressure	151	75.31 ± 1.76	9.74 ± 0.98	2.79 ± 0.16
Elevated perfusion pressure[c]	144	108.80 ± 2.05	19.60 ± 1.46	3.68 ± 1.25
***l*-Norepinephrine Infusion**				
Perfusion pressure	14	108.57 ± 7.23	12.14 ± 2.90	3.90 ± 0.73
Decreased perfusion pressure[d]	5	81.00 ± 5.79	7.80 ± 3.38	5.40 ± 1.16
Post-*l*-Norepinephrine Infusion				
Perfusion pressure	14	77.50 ± 4.82	4.68 ± 2.15	2.86 ± 0.75
Elevated perfusion pressure[c]	8	112.50 ± 6.94	9.00 ± 3.42	3.75 ± 1.03

[a] Perfused with Tyrode's solution. [b] S.E. = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$. [c] Perfusion pressure elevated by adjusting Sigmamotor pump to approximate pressure induced by *l*-norepinephrine. [d] Perfusion pressure decreased by adjusting Sigmamotor pump.

each of the five parameters studied: pre-angiotensin II infusion at both normal and elevated perfusion pressures, during angiotensin II infusion, and post-angiotensin II infusion at normal and elevated perfusion pressures.

The kidneys from female rabbits were statistically more responsive to angiotensin II than those kidneys obtained from male rabbits ($p = <0.05$), the mean amplitude of rhythmic fluctuations of perfusion pressure of kidneys from females being 56.9 ± 5.4 mm. H_2O compared to a mean amplitude of 42.2 ± 4.1 mm. H_2O in the males' kidneys.

Effect of Age Upon Rhythmic Alterations in Perfusion Pressure of Isolated Perfused Rabbit Kidneys—The contribution of age to the induction of rhythmic changes in perfusion pressure is graphically presented in Figs. 2 and 3. The amplitude of the rhythmic alterations in perfusion pressure was compared at different age levels in kidneys from male and female rabbits separately. The amplitude of the rhythmic changes in perfusion pressure in kidneys from female rabbits was significantly affected by age at all five parameters studied ($p = <0.05$). In kidneys from male rabbits, there was only a significant effect of age during perfusion at normal perfusion pressures prior to and after angiotensin II infusion ($p = <0.05$). The rhythmic fluctuations were greatest between the ages of 1 to 4 months in all five parameters studied in both male and female rabbits' kidneys. In both sexes at normal perfusion pressure prior to angiotensin II infusion, the most sensitive age was 1 month. Under all other parameters the peak response was at 4 months in both sexes except during angiotensin II infusion, where the peak sensitivity was at 2 months of age in kidneys from female rabbits where the mean amplitude of rhythmic changes was 77.5 ± 8.2 mm. H_2O. The peak response produced by angiotensin II in the kidneys of male rabbits was at 4 months of age where the mean amplitude was 53.3 ± 11.2 mm. H_2O.

There was a marked decrease in the amplitude of rhythmic alterations in perfusion pressure in kidneys of 8-month-old rabbits of both sexes, the mean amplitude in the kidneys of females being 45.0 ± 11.3 mm. H_2O and that of kidneys from males being 29.5 ± 6.11 mm. H_2O. Angiotensin II produced rhythmic alterations in perfusion pressure having a mean of 36.3 ± 12.8 mm. H_2O in the kidneys of 12-month-old female rabbits.

DISCUSSION

The relatively slight changes in renal blood flow from normal levels under various physiological conditions have become a well accepted fact. Sympathetic vasoconstrictor control of the renal vessel under normal conditions has been ruled out

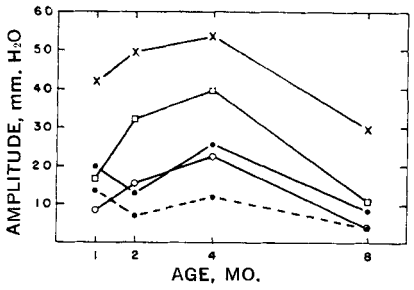


Fig. 2—The effect of age on rhythmic fluctuations in perfusion pressure of isolated kidneys from male rabbits. Key: ×, angiotensin II infusion; □, post-angiotensin II ↑ pressure; ○, post-angiotensin II normal pressure; ●——●, pre-angiotensin II ↑ pressure; ●- - - - -●, pre-angiotensin II normal pressure.

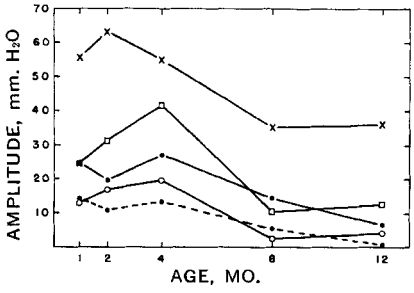


Fig. 3—The effect of age on rhythmic fluctuations in perfusion pressure of isolated kidneys from female rabbits. Key: ×, angiotensin II infusion; □, post-angiotensin II ↑ pressure; ○, post-angiotensin II normal pressure; ●——●, pre-angiotensin II ↑ pressure; ●- - - - -●, pre-angiotensin II normal pressure.

because surgical and pharmacological denervation do not elevate the renal blood flow. Such changes in renal resistance to blood flow as a consequence of changes in blood pressure have inherited the term autoregulation of renal blood flow, because no extrarenal, nervous, or humoral factors are believed to be involved. There is experimental evidence that an elevated tension in the arteriolar wall stimulates the vascular smooth muscle to contract. Little doubt exists that during normal kidney function, resistance changes are caused by smooth muscle reactions. There does exist, however, a basic question of whether smooth muscle contraction during autoregulation is initiated only by the tension change in the arteriolar wall or whether it is a muscle response to either a change in renal metabolism or an intrinsic humoral factor, both of which might be related to arterial perfusion pressure.

There has been a considerable amount of evidence supporting the proponents of the myogenic theory for autoregulation by vascular smooth muscle. Many conclusions have been drawn based upon the electrophysiological studies performed by Bozler (10) and Bulbring (11) on other types of smooth muscles. Funaki (12) confirmed this work on vascular smooth muscle. Evidence from these experiments indicated that there are two types of smooth muscle cells, some with a higher, more stable membrane potential (multi-unit) and some with lower, less stable membrane potentials (single-unit). The multi-unit type smooth muscle is dominant in the larger vessels, and the single-unit type dominates in the smaller vessels. A slight continuous distension of the wall of a blood vessel due to elevated blood pressure might then affect the smooth muscle cell by increasing the rate of the spontaneously generated local depolarizations in proportion to the extent of distension which, in turn, initiates depolarization followed by excitation–contraction.

There is also experimental evidence in dogs suggesting that renin release occurs subsequent to pressure changes within the kidney (13). Anatomically, the individual nephron is ideally arranged so that changes in the dynamic or chemical pattern of the intratubular fluid can be sensed by the vasa afferens at the initial portion of the distal convoluted tubule, the area in which the juxtaglomerular apparatus is located. It has been suggested that the juxtaglomerular apparatus is involved in a mechanism to prevent a further increase in renal blood flow, glomerular filtration rate (GFR), and intrarenal pressure when the renal arterial blood pressure increases above 90 mm. Hg (14). Such a mechanism would narrow autoregulation down to a problem of adjusting the physical factors, which are responsible for the tubular load, to the tubular reabsorption capacity, which has to handle the filtered load. Both physical and metabolic factors are involved in the formation of urine; and since the physical factors occur at the initial phase of urine formation, the most likely candidate for achieving a glomerular–tubular balance might be maintenance of a stable physical component (effective filtration pressure). Also it is possible to regulate the effective filtration pressure by a single mechanism, *i.e.*, tonus of afferent glomerular vessels, whereas regulation of tubular activity would be far more difficult and complex. The possibility then exists that the renin–angiotensin system, which seems to have its origin at the juxtaglomerular apparatus, being sensitive to both changes in electrolytes (15) and effective in altering the vascular resistance in the kidney, may be involved in renal autoregulation of blood flow as a result of the maintenance of glomerular tubular balance. A sudden elevation of renal arterial blood pressure initially increases renal blood flow and thereby increases GFR which causes increased tubular load and urine flow velocity. Such changes could change the dynamics of flow of the tubular fluid and increase the osmolarity at the macula densa within seconds. The increased osmolarity could then stimulate the juxtaglomerular apparatus to release a vasoactive substance which would thereby decrease the GFR *via* afferent vasoconstriction.

The results of the present study indicate that angiotensin II may have an effect upon the vasculature of the kidney other than that of direct vasoconstriction. This effect expressed itself as a rhythmic fluctuation in the perfusion pressure of the isolated perfused rabbit kidney. Pressure elevation was also capable of inducing a similar type of fluctuation, as would be expected according to the myogenic theory; but angiotensin II was significantly more effective in inducing such alterations. Such a reaction could possibly be interpreted on an electrophysiological basis using the results of

Funaki's experiments as guide lines. It is possible that angiotensin II exerts its effect upon vascular smooth muscle, at least in part by decreasing the resting membrane potential by increasing the influx of Ca^{2+} ions. This theory is supported by recent evidence that isolated renal arteries constrict in response to angiotensin II only in the presence of the calcium ion (16). Tobian and Chesley (17) have also demonstrated that small arteries and arterioles from renal hypertensive rats have a significantly greater Ca^{2+} content than those of normotensive control animals. The decreased membrane potential increases the number of spontaneous local depolarizations, thereby reaching more muscle fibers and exaggerating the vasomotion of the small vessels. The frequency of firing, however, did not seem to be influenced in that there was no significant difference in the rate of occurrence of the rhythmic fluctuations between control conditions and during angiotensin II infusion. In other words, the effect of elevated pressure initiates rhythmic fluctuations probably by the myogenic mechanism described by Funaki (12); however, the influence of a hormone closely related to the kidney may be capable of greatly accentuating the effect of pressure upon the activity of vascular smooth muscle and might possibly be the direct-acting intrinsic hormonal factor concerned with renal autoregulation of blood flow.

The effect of angiotensin II in producing the rhythmic changes in perfusion pressure cannot be fully explained by the myogenic theory as described by Bayliss (18), since the rhythmic fluctuations were only slightly decreased when the perfusion pressure was reduced to control levels during angiotensin II infusion. Also, the effect of angiotensin II is significantly greater than the effects produced by elevation of perfusion pressure or the effect produced by the two other vasoconstrictor agents, *l*-norepinephrine and vasopressin. The effect of *l*-norepinephrine was only slightly greater than that of pressure elevation alone, whereas vasopressin, another polypeptide, did have some effect other than the influence of pressure; but it was not nearly so pronounced as that of angiotensin II.

This study also indicated that these effects of angiotensin II upon the vasculature of the kidney are both age and sex dependent. The pattern which is seen when the age of the animal is varied is interesting in that there seemed to be an increasing reactivity up to the age of 4 months, and then a drastic decline in responsiveness of the rabbit kidney occurred. It might be speculated that when the animal is very young, the kidney's vasculature is not completely developed; and the majority of the vessels are still relatively small. It is also possible that during this time these small vessels are dominated by the single-unit type of muscle cell described by Bozler and therefore possess an inherently lower resting membrane potential, thereby being less stable and more prone to depolarization. As the animal matures, the vessels become larger; and the dominance shifts to that of the multi-unit muscle cell which possesses a more stable membrane potential and lacks automaticity. The occurrence of such events seems logical in the light of Funaki's report that the vasculature of the larger vessels is dominated by multi-unit smooth muscle cells and the smaller vessels by single-unit smooth muscle cells, even though both types of cells can be found in both large and small vessels. This is by no means a suggestion that an older animal has lost the ability of autoregulation. The mature animal still possesses an adequate supply of small preglomerular arterioles which are dominated by the single-unit type of muscle cells, but the major vessels are almost completely composed of the multi-unit smooth muscle cells. It would perhaps be more correct to say that the young animal is better able to regulate renal blood flow in the face of changing cardiovascular patterns, so that during its young formative period the rabbit is better able to assure the constancy of its internal environment.

There is also the question of the effects of sex upon the responsiveness of the renal vasculature, since the female rabbit's kidneys were more responsive at almost every age tested. The explanation for this phenomenon probably lies in the difference in the hormonal nature of internal environment of the two sexes; and perhaps the female endocrine system, particularly estrogens, potentiates the responsiveness of the vascular smooth muscle.

REFERENCES

(1) Tigerstedt, R., and Bergman, T. G., *Arch. Physiol.*, **8**, 598(1898).
(2) Goldblatt, H., Lynch, C., Hanzal, R. F., and Summerville, W., *J. Exptl. Med.*, **59**, 347(1938).
(3) Volhard, F., *Schweiz. Med. Wochschr.*, **78**, 1189(1948).
(4) Waugh, W. H., *Circulation Res.*, **6**, 363(1958).
(5) Byrom, F. B., and Dodson, L. F., *J. Pathol. Bacteriol.*, **60**, 357(1948).
(6) Giese, J., *Acta Pathol. Microbiol. Scand.*, **62**, 497 (1964).
(7) Benelli, G., Della Bella, D., and Gandini, A., *Brit. J. Pharmacol.*, **22**, 211(1964).
(8) Buckley, J. P., *Acta Physiol. Scand.*, **65**, 273(1965).
(9) Waugh, W. H., *Circulation Res.*, **15**, 156(1964).
(10) Bozler, E., *Experientia*, **4**, 213(1948).
(11) Bulbring, E., *Pflug. Arch. Physiol.*, **273**, 1(1961).
(12) Funaki, S., *Nature*, **191**, 1102(1961).
(13) Scott, J. B., Daughterty, R. M., Jr., Dabney, J. M., and Haddy, F. J., *Am. J. Physiol.*, **208**, 813(1965).
(14) McManus, J. F. A., *Lancet*, **2**, 374(1942).
(15) Guyton, A. C., Langston, J. B., and Navar, G., *Circulation Res.*, **15**, 187(1964).
(16) Kovalcik, M. V., quoted by Gillespie, J. S., *Pharmacol. Rev.*, **18**, 538(1966).
(17) Tobian, L. J., Jr., and Chesley, G., Proceedings of the Council for High Blood Pressure Research: Hypertension, 13, American Heart Association, Inc., Cleveland, Ohio, 1964, p. 187.
(18) Bayliss, W. M., *J. Physiol.*, **28**, 220(1902).

Laboratory Studies with Nitrofurantoin

Relationship Between Crystal Size, Urinary Excretion in the Rat and Man, and Emesis in Dogs

By HENRY E. PAUL, KENYON J. HAYES, MARY F. PAUL, and A. RUSSELL BORGMANN

Studies were conducted relating the effect of varying the crystal size of orally administered nitrofurantoin on absorption and urinary excretion in the rat and man and emesis in dogs. It was discovered that there is an optimal average crystal size of about 150 mesh, or its equivalent surface area falling between 80 to 200 mesh limits, which reduces emesis while still permitting ample urinary excretion for efficacy.

THE USE OF nitrofurantoin[1] as a urinary tract antibacterial agent has become firmly established since initial reports of the first laboratory (1, 2) and clinical studies (3–5). During the years of successful use of this drug a limited number of patients have exhibited nausea or emesis to the extent that they were deprived of an effective medication. Little or no success attended attempts to overcome this defect by combining other substances with nitrofurantoin, although experience indicates administration of the drug with meals or with milk is helpful (6). A recent review article (7) provides general information on nitrofurantoin.

Since obvious means of controlling or reducing nausea and/or emesis associated with drug administration have not proved completely satisfactory in the case of nitrofurantoin, a thorough examination of the problem was undertaken. While studies in these laboratories have not clearly delineated the mechanism of nitrofurantoin-induced nausea or emesis, there is some evidence to indicate this undesirable side effect may be of neural origin. Rate of drug absorption could possibly be a factor. Accordingly, modification of the absorption pattern of individual nitrofurantoin doses by controlling release into the gastrointestinal tract might reduce this undesirable side effect.

Although there are several means whereby the absorption pattern of a drug may be modified or controlled (8, 9), one effective way with compounds having limited water solubility is the control of the surface area available for solution in the gut by varying the crystal size. Numerous reports (10–13) demonstrate the fact that the rate and amount of absorption of slightly soluble drugs can be markedly increased by reducing crystal size thereby providing increased surface area for a given dosage. Unfortunately, increased toxicity often accompanies this change (14, 15). Conversely, the use of larger crystals of drug having less surface area per dose, toxicity (as evidenced by nausea and vomiting) might be eliminated or greatly reduced. Such an approach, however, is beset with the likelihood of diminution of efficacy. Since nitrofurantoin has a water solubility of only about 200 to 400 mg./L. at 37° at the physiological pH values expected in application, larger crystals having considerably less surface area for a fixed dosage would dissolve much more slowly, thereby slowing the rate of absorption.

EXPERIMENTAL

Nitrofurantoin of different crystal sizes was made by recrystallization from nitromethane. These crystals of rhombic anhydrous nitrofurantoin were sieved into appropriate size ranges and some micronized in a Sturdevant mill for finest material. To explore emetic effect, very coarse (50–60 mesh) crystals, fine crystals (200–325 mesh), and micronized material (<10 μ) were orally administered in gelatin capsules to 20 fasted dogs in two independent comparative studies in single doses ranging from 10 to 20 mg./Kg. in the first and at a higher 25 mg./Kg. level in the second trial. An ample clearance time of 3 days between doses was established. Data from these studies are summarized in Table I. At these dosages emesis occurred in 40 to 75% of the dogs receiving either the fine 200–325 mesh or finer (micronized) material, but only in 5% of those receiving the coarse 50–60 mesh crystals—a great improvement. The crystal size ranges studied here actually measure from less than 10 μ up to about 300 μ in diameter, presenting almost a *thirtyfold* difference in available surface area for the *same weight* of drug.

While this control of emesis might be a result of the slower absorption rate produced by the decreased surface area of the larger crystals, it could

Received November 4, 1966, from the Research Laboratories, The Norwich Pharmacal Co., Norwich, NY 13815

Accepted for publication April 4, 1967.

This study was a cooperative effort between the Research Divisions of Biology, Chemistry, and Pharmacy.

Appreciation is especially due to Donald T. Humphrey and Esther G. Nohle for numerous nitrofurantoin assays, to Paul H. Seehausen for extending and refining crystal preparation methods, and to Dr. Julian G. Michels for establishing crystal size control procedures. The authors wish particularly to thank Dr. Paul V. Newland for arranging and supervising the human volunteer studies and Dr. William B. Stillman for over-all direction.

[1] The registered trademark of the Norwich Pharmacal Co. for nitrofurantoin is Furadantin.

TABLE I—EFFECT OF NITROFURANTOIN CRYSTAL SIZE ON EMESIS IN DOGS

Study and Single Dosage	Crystal Size *vs.* No. of Dogs Vomiting Out of 20: 50–60 Mesh	200–325 Mesh	Micronized
First Study			
150 mg./dog (10–20 mg./Kg.)	1	8	9
Second Study			
25 mg./Kg./dog	1	14	15

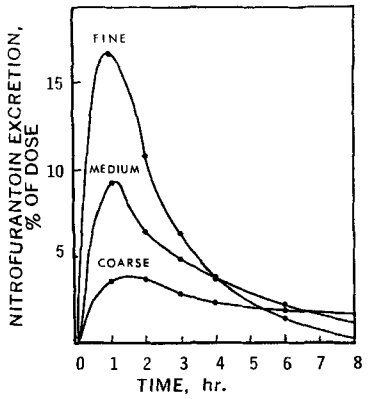


Fig. 1—Effect of crystal size of orally administered nitrofurantoin on urinary recovery rate in the rat. Key: coarse, 50–80 mesh (300–180 μ); medium, 80–200 mesh (180–75 μ); fine, 200 mesh to micronized (75–10 μ or less).

also have been the result of reduced total absorption, a possibility that must be considered when too large a crystal size of a slightly soluble drug is employed (16, 17). Since the effectiveness of nitrofurantoin is associated with its urinary concentration (18, 19), a reduction in toxicity (emesis) would have no medical practicality if excretion were also *proportionately* reduced.

Quantitative urinary excretion studies were initiated in rats, a physiologically suitable animal, to determine the effect of crystal size on both rate and total excretion of orally administered nitrofurantoin. This information would also provide an indication of the rate and amount of nitrofurantoin absorbed from the gut (13, 17, 20). Good correlation exists in the urinary excretion of nitrofurantoin by the rat, dog, and man (7).

In studying the effect of variation of nitrofurantoin crystal size on absorption, as measured by both urinary excretion rate and total excretion, several exploratory studies were conducted to establish size of oral dose, collection periods, crystal size ranges to study, *etc.* Urines were assayed by a spectrophotometric method (21). The nitrofurantoin crystals of selected size were administered as an aqueous suspension by gavage to adult rats—usually a group of four for each crystal size test. Following single dosage at the 10 mg./Kg. level, collection periods were established at 0–1, 1–2, 2–3, 3–4, 4–8, and 8–24 hr. to supply the required information. Of particular interest would be a substantial reduction of the absorption rate immediately following dosage. That this can be accomplished is clearly shown in the excretion curves covering the 0 to 8 hr. period as presented in Fig. 1. Each curve is based on data from 8 to 12 rats (2 or 3 groups of 4 animals each) receiving, respectively, relatively coarse (50–80 mesh), medium (80–200 mesh), and fine (200 mesh to micronized material). It is apparent that the particle size of nitrofurantoin alone can greatly influence the rate of intestinal absorption which is reflected in the measured urinary excretion (20). The rapidity with which urinary concentration rises and falls following administration of the finer crystal range shows that solution and absorption must have been rapid indeed. In fact, it has been shown that for micronized (<10 μ) material absorption can be so rapid that half of the total amount excreted can be recovered in the urine by the end of the first hour! Conversely, it will be noted that in the case of the medium and coarser crystals appreciable nitrofurantoin was still being excreted at 8 hr. In this study the 0 to 24 hr. total recovery of the dose given was 45% and 42% for the fine and medium crystals, but this had dropped to 33% for the coarser crystals. Not shown in this figure is an important further finding: in the last 8–24 hr. collection period the remaining portion excreted related to the total amount excreted (not to the total dose) was 3% for the fine, 20% for the medium, and 40% for the coarser material. The coarsest material alone resulted in prolonged excretion coupled with appreciably reduced total excretion.

Further studies with rats were conducted with particular emphasis on the relation of crystal size to total urinary excretion or drug recovery. Some variation in total recoveries was encountered between studies, but the over-all crystal size relationship to rate and total urinary excretion remained clear. It was found that sodium carboxymethylcellulose should not be used without proper precaution as a thickener for an aqueous suspension of nitrofurantoin. Unless this suspension is used *immediately*, needle-like nitrofurantoin hydrate crystals form on prolonged mixing or standing which appreciably affect nitrofurantoin-crystal size relationship studies. Methylcellulose or some other inert thickening agent could be used.

More refined and extensive comparative tests were conducted with humans and dogs to study the relationship of crystal size to total urinary excretion in humans and nitrofurantoin-induced emesis in dogs. In these tests 32 purebred beagles and a population of 15 humans were used. Wherever possible, each test animal or human received every crystal size for comparison. A previously established sensitive dose of 10 mg./Kg./dog was used. Humans were standardized at 100 mg./subject. Ample clearance time was allowed—3 days for dogs and 7 days for humans. Crystals were admixed with lactose and administered in capsules. To demonstrate the relationship between crystal size, drug excretion, and emesis, data from representative studies are plotted in Fig. 2. Contrary to expectation there is a crystal size range in which there is a marked reduction of nausea and minimal reduction of total nitrofurantoin excretion.

A crystal size of about 150 mesh nitrofurantoin (about 100 μ), or its surface area equivalent, was selected as an optimal desired size from a potentially

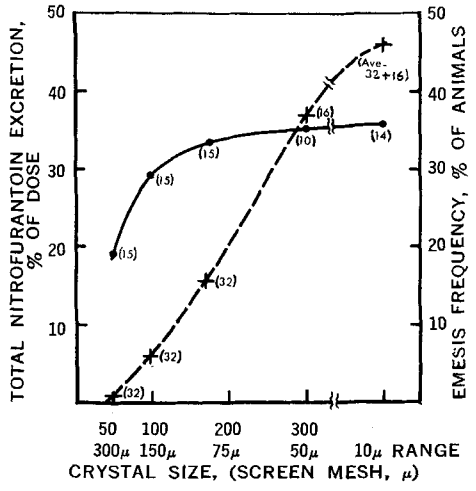


Fig. 2—Relation between total urinary recovery in humans and emesis frequency in dogs following oral administration of nitrofurantoin of different crystal sizes. Values in parentheses are numbers of subjects used. Points are plotted essentially at median values for nonoverlapping crystal size ranges used. Key: ●, urinary excretion (humans); ×, emesis (dogs).

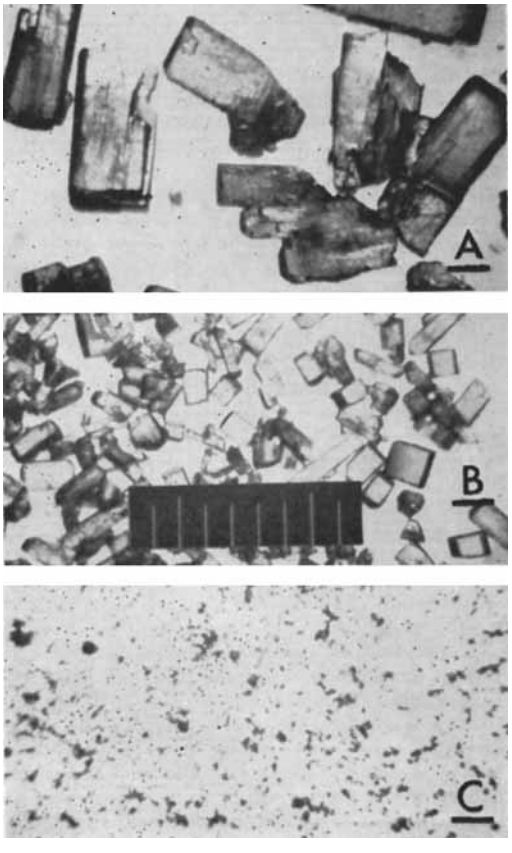


Fig. 3—Nitrofurantoin crystals. Key. A, coarse, 50 to 80 mesh or 300 to 180 μ; B, medium, 80 to 200 mesh or 180 to 75 μ; reference scale 100 μ/division; C, fine, micronized crystals. 25× magnification.

useful range of 50 to 400 mesh. For practical reasons a narrower working range was chosen of 80–200 mesh (180–75 μ size), or its surface area equivalent of about 200–500 cm.²/Gm. Figure 3 illustrates crystals of this median size range compared to coarse (50–60 mesh) and fine material (micronized or marketed tablet—as providing crystals in the 10 μ range, equivalent to a theoretical 1500 mesh).

These findings were amplified by conducting appropriate studies in humans to obtain information on the effect of crystal size (surface area) on the urinary excretion rate, urinary concentration, and total excretion of nitrofurantoin following single oral doses to couple maintenance of efficacy with reduced nausea potential. Fifteen volunteer subjects were used in comparative studies, each serving as his own control. Subjects ate a standard breakfast immediately prior to administration of a selected crystal size in a single test dose of 100 mg. nitrofurantoin in a capsule. Marketed tablets[2] (100 mg.) were included in the study for comparison. Fluid intake was controlled and caffeine and alcohol were prohibited. Total urine collections were made at specified time intervals and nitrofurantoin concentrations determined on each collection from each subject by a recently developed method (22) or modification of an earlier one (23).

Table II presents a summary of the results. From the five columns it will be noted that: (*a*) the maximum per cent of nitrofurantoin excreted in *any* 2-hr. collection period decreased as the crystal size was increased, being definitely reduced in the case of the coarsest material, demonstrating decreased absorption rate with larger crystal sizes. (*b*) The average time at which maximum percentage excretion occurred increased with particle size. (*c*) The maximum urinary concentration attained was lower with the larger crystals, partially a result of the extended excretion period. (*d*) The average time at which maximum urinary concentration was reached was later for larger crystals, again reflecting slower absorption. (*e*) The total amount of nitrofurantoin excreted (*i.e.*, per cent of the dose given) in the urine decreases with increased particle size, but only in a degree to cause serious concern in crystal sizes larger than 50 mesh.

DISCUSSION

It appears that these human studies basically agree with those conducted in rats. From a potentially useful range of 50 to 400 mesh a more practical range of nitrofurantoin crystals of the 80–200 mesh size has been selected as providing ample urinary concentration with satisfactory total urinary excretion. Accordingly, such nitrofurantoin crystals, termed "macrocrystals," have now been introduced into clinical trial for critical testing of efficacy and emetic action in patients having urinary tract infections who, in addition, suffer nausea and emesis from commercial nitrofurantoin tablets. In the crossover studies planned, each patient will serve as his own control, making such tests more meaningful with reasonable numbers, rather than to test the macrocrystals on all patients using nitrofurantoin for urinary tract infections where nausea and emesis percentages are relatively low.

[2] Furadantin Tablets, Norwich Pharmacal Co.

TABLE II—SUMMARY OF CRITERIA MEASURED IN HUMAN VOLUNTEERS RECEIVING VARIOUS SIZES OF NITROFURANTOIN CRYSTALS[a]

Crystal Size[b] of Nitrofurantoin	(1) Max. % Excreted in Any 2-hr. Period	(2) Av. Time of Max. % Excretion, hr.	(3) Max. Urinary Concn. Attained, mg./L.	(4) Av. Time Max. Urinary Concn. Attained, hr.	(5) Total % of Initial Dose Excreted
50–60 mesh	8.3	4.9	83	5.5	19.6
80–120 mesh	12.9	4.6	124	5.0	29.8
140–200 mesh	16.6	3.8	159	4.1	32.3
200–400 mesh	17.8	3.6	156	3.4	35.4
Marketed nitrofurantoin tablets (Provide fine crystals in the 10 μ range)	20.0	3.6	151	3.0	36.1

[a] Each value based on 15 complete individual studies—no pooling of samples. Exceptions: 10 individuals only were available from same population for 200–400 mesh and 14 for tablets. [b] Size checked by microscopic measurement.

SUMMARY

A study of the effect of crystal size of orally administered nitrofurantoin on its urinary excretion in the rat and man demonstrates a slower rate of absorption from larger crystal size than from fine crystals with ample total urinary excretion from a suitably selected size or size range.

Studies with dogs reveal that the larger crystals are much less provocative of emesis than the smaller crystal size.

Based on quite favorable relationships between nitrofurantoin crystal size, total urinary excretion, and reduction of emesis established by these studies, nitrofurantoin crystals ranging from 80 to 200 mesh and of about 150 mesh average size, or its equivalent in surface area as determined by solubility rate, have been selected for clinical study.

REFERENCES

(1) Paul, H. E., Hayes, K., and Bender, R. C., Program 120th Annual Meeting of the American Association for the Advancement of Science, Boston, Mass., 1953, p. 122.

(2) Hayes, K. J., U. S. pat. 2,610,181 (to the Norwich Pharmacal Co., Norwich, N. Y.)

(3) Norfleet, C. M., Jr., Beamer, P. R., and Carpenter, H. M., *J. Urol.*, **70**, 113(1953).

(4) Beutner, E. H., Petronio, J. J., Lind, H. E., Trafton, H. M., and Correia-Branco, M., "Antibiotics Annual," Medical Encyclopedia, Inc., New York, N. Y., 1954–1955, p. 988.

(5) Carroll, G., and Brennan, R. V., *J. Urol.*, **71**, 650 (1954).

(6) MacLeod, P. F., Rogers, G. S., and Anzlowar, B. R., *Intern. Record Med. Gen. Pract. Clin.*, **169**, 561(1956).

(7) Paul, H. E., and Paul, M. F., "Experimental Chemotherapy," vol. II, Academic Press Inc., New York, N. Y., 1964, pp. 307–370; *ibid.*, vol. IV, 1966, pp. 521–536.

(8) Delgado, J. N., and Cosgrove, F. P., *Tex. State J. Med.*, **59**, 1008(1963).

(9) *Ibid.*, **59**, 1106(1963).

(10) Levy, G., *Am. J. Pharm.*, **135**, 78(1963).

(11) Nelson, E., *Clin. Pharmacol. Therap.*, **3**, 673(1962).

(12) Dare, J. G., *Australasian J. Pharm.*, **45**, 558(1964).

(13) Fincher, J. H., Adams, J. G., and Beal, H. M., *J. Pharm. Sci.*, **54**, 704(1965).

(14) Kraml, M., Dubuc, J., and Dvornik, D., *Arch. Dermatol.*, **87**, 179(1963).

(15) Kelsey, F. O., *Am. J. Hosp. Pharm.*, **21**, 320(1964).

(16) Kraml, M., Dubuc, J., and Gaudry, R., *Antibiot. Chemotherapy*, **12**, 239(1962).

(17) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).

(18) McCabe, W. R., and Jackson, G. G., *New Engl. J. Med.*, **272**, 1037(1965).

(19) Stamey, T. A., Govan, D. E., and Palmer, J. M., *Medicine*, **44**, 1(1965).

(20) Nelson, E., *J. Pharm. Sci.*, **50**, 181(1961).

(21) Paul, H. E., Austin, F. L., Paul, M. F., and Ells, V. R., *J. Biol. Chem.*, **180**, 345(1949).

(22) Conklin, J. D., and Hollifield, R. D., *Clin. Chem.*, **11**, 925(1965).

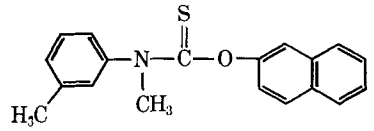
(23) Reckendorf, H. K., Castringius, R., and Spingler, H., *Med. Welt*, (No. 15), 816(1963).

Qualitative and Quantitative Tests for Tolnaftate

By EDWARD F. SALIM* and WILBUR S. FELKER†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

2-NAPHTHYL-N-METHYL-N-(3-TOLYL)THIONOCARBAMATE; $C_{19}H_{17}NOS$; mol. wt. 307.42. The structural formula of tolnaftate may be represented as:



Physical Properties—Tolnaftate occurs as a white to creamy white fine powder, m.p. 110–113° (U.S.P., class I). It is freely soluble in chloroform and in acetone, slightly soluble in alcohol, sparingly soluble in ether, and practically insoluble in water.

Identity Tests—A 1 in 100,000 solution of tolnaftate in methanol exhibits an ultraviolet absorbance maximum at about 257 mμ [absorptivity (*a*) about 71] and a minimum at about 243 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of tolnaftate in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of tolnaftate, accurately weighed, in vacuum at 65° for 3 hr.: it loses not more than 0.5% of its weight.

Char about 1 Gm. of tolnaftate, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the heavy metals content of tolnaftate by the U.S.P. heavy metals test, method II: the heavy metals limit for tolnaftate is 20 p.p.m.

Assay—Dissolve 50 mg. of tolnaftate, accurately weighed, in sufficient methanol to make 250.0 ml. Dilute 5.0 ml. of this solution with methanol to 100.0 ml., and mix. Dissolve an accurately weighed quantity of tolnaftate reference standard in methanol, and dilute quantitatively and stepwise with methanol to obtain a standard solution having a concentration of about 10 mcg./ml. Concomitantly determine the absorbance of each solution in 1-cm. cells, at the wavelength of maximum absorbance at about 257 mμ, with a suitable spectrophotometer, using methanol as the blank. Calculate the quantity, in mg., of $C_{19}H_{17}NOS$ in the portion of tolnaftate taken by the formula $5\ C\ (A_u/A_s)$, in which C is the concentration, in mcg./ml., of tolnaftate in the standard solution, A_u is the absorbance of the sample solution, and A_s is the absorbance of the standard solution. The amount of tolnaftate found is not less than 98.0% and not more than 102.0% of the weight of the sample taken.

DOSAGE FORMS OF TOLNAFTATE

Tolnaftate Solution

Identity Test—The ultraviolet absorption spectrum of the chloroform solution obtained in the *Assay* exhibits an absorbance maximum and minimum at the same wavelengths as that of the standard solution.

Assay—Transfer an accurately measured volume of tolnaftate solution equivalent to about 10 mg. of tolnaftate, to a separator, add 50 ml. of chloroform, and extract with 50 ml. of 0.1 *N* sodium hydroxide. Filter the chloroform phase through chloroform-washed cotton into a 250-ml. volumetric flask and extract the aqueous phase with four 45-ml. portions of chloroform, filtering each portion into the flask. Make to volume with chloroform, and mix. Dilute 25.0 ml. of this solution to 100.0 ml. with chloroform, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of tolnaftate reference standard, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 260 mμ, with a suitable spectrophotometer, using chloroform as the blank. Calculate the quantity, in mg., of $C_{19}H_{17}NOS$ in each ml. of solution taken by the formula $(C/V) \times (A_u/A_s)$, in which C is the concentration, in mcg./ml., of tolnaftate in the standard solution, V is the volume, in ml., of solution taken, A_u is the absorbance of the sample solution, and A_s

Received February 28, 1967, from the * Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication April 21, 1967.

† Schering Corp., Bloomfield, NJ 07003. Schering Corp. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The authors express their appreciation to John J. Callahan and Emanuel Zenno, Schering Corp., and Hannah Klein, Drug Standards Laboratory, for valuable contributions to the monograph.

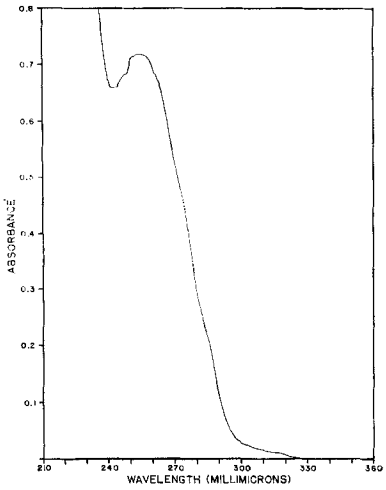


Fig. 1—Ultraviolet absorption spectrum of tolnaftate in methanol (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

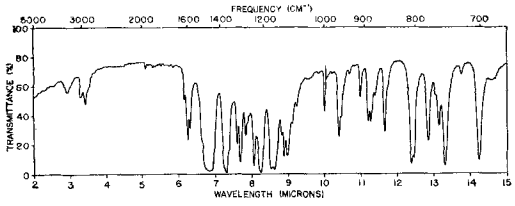


Fig. 2—Infrared spectrum of tolnaftate in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

is the absorbance of the standard solution. The amount of tolnaftate found is not less than 90.0% and not more than 115.0% of the labeled amount.

Tolnaftate Cream

Identity Test—The ultraviolet absorption spectrum of the chloroform solution obtained in the *Assay* exhibits an absorbance maximum and minimum at the same wavelengths as that of the standard solution.

Assay—Weigh accurately an amount of tolnaftate cream equivalent to about 10 mg. of tolnaftate and transfer to a 250-ml. separator. Add 100.0 ml. of chloroform and shake the chloroform solution successively with two 25-ml. portions of 0.1 *N* sodium hydroxide, two 25-ml. portions of 0.1 *N* hydrochloric acid, and 25 ml. of water, transferring the bulk of the chloroform phase to a second separator after each extraction. Dilute 5.0 ml. of the chloroform solution to 50.0 ml. with chloroform, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of tolnaftate reference standard, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 260 mμ, with a suitable spectrophotometer, using chloroform as the blank. Calculate the quantity, in mg., of $C_{19}H_{17}NOS$ in the portion of cream taken by the formula $C\ (A_u/A_s)$, in which C is the concentration, in mcg./ml., of tolnaftate in the standard solution, A_u is the absorbance of the sample solution, and A_s is the absorbance of the standard solution. The amount of tolnaftate found is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Tolnaftate[1] is an antifungal agent which is useful in the topical treatment of fungal infection caused by various species of *Trichophyton* and *Microsporum* (1–3). Tolnaftate, applied in a polyethylene glycol solution or cream, is particularly effective in the treatment of tinea pedis, tinea cruris, tinea corporis, and tinea manuum.

Identity Tests—Thin-layer chromatography may be included for testing the purity of bulk tolnaftate as well as an additional identity test for the drug and dosage forms. Benzene–alcohol (1:1) solutions of tolnaftate drug and reference standard are prepared at a concentration of about 1 mg./ml. The remainder of the *Assay* solutions obtained from tolnaftate solution and cream are evaporated to dryness and the residue dissolved in 1 ml. of benzene–alcohol (1:1). A 10-μl. portion of test and standard solutions is applied to a thin-layer plate prepared with Silica Gel G and the chromatogram developed using toluene as the mobile phase. The spots are visualized using ultraviolet light (254 mμ) and/or a 1 in 100 solution of iodine in carbon tetrachloride sprayed on the chromatogram and air dried.

Quantitative Tests—Analyses of bulk tolnaftate and tolnaftate solution and cream are based on ultraviolet spectrophotometric measurements. A solution of the basic drug in methanol compared to a similarly prepared reference standard solution gave an average recovery value of 100.5 ± 0.4%.[2] Absorbance measurements of tolnaftate in the commercial preparations are conveniently determined in chloroform solution. The ultraviolet absorption spectrum of tolnaftate in chloroform is similar to the spectrum shown in Fig. 1 with a slight shift in maximum absorbance to 260 mμ. Spectrophotometric analyses of tolnaftate solution and cream gave average values of 107.6 ± 1.3%[2] and 108.4 ± 0.7%[2] of the labeled amounts, respectively. The suitability of each procedure was verified by quantitative recovery of a standard tolnaftate solution carried through the extractive steps as included for the solution and cream.

REFERENCES

(1) Noguchi, T., Kaji, A., Igarashi, Y., Shigematsu, A., and Taniguchi, K., *Antimicrobial Agents Chemotherapy*, **1962**, 259.

(2) Weinstein, J., Oden, E. M., and Moss, E., *ibid.*, **1964**, 595.

(3) Robinson, H. M., Jr., and Raskin, J., *J. Invest. Dermatol.*, **42**, 185(1964).

[1] Marketed as Tinactin by the Schering Corp., Bloomfield, N. J.

[2] Maximum deviation from the mean value.

Compression Characteristics of Some Pharmaceutical Materials

By S. LEIGH, J. E. CARLESS, and B. W. BURT

A commercial single-punch tablet machine (Manesty E 2) fitted with 1/2-in. punches was instrumented with strain gauges to monitor the forces simultaneously on the upper punch and the die-wall. The complete pressure cycles of a number of pharmaceutical materials at various levels of compression have been studied. The materials were sucrose granules and crystals, acetaminophen granules, aspirin crystals, and sodium chloride crystals. It has been found that these materials conform to three different types of compression pattern. The results indicate that materials which laminate on compression behave like a Mohr's body, *e.g.*, acetaminophen granules without binder. Granulation of acetaminophen with polyvinylpyrrolidone altered the compression pattern to that of a body with a constant yield stress. Aspirin compacts were found to exhibit elastic properties. Compression patterns are given for 20/40 mesh and 40/60 mesh crystals and granules of sucrose. No significant differences due to particle size were found, but differences were noted between the crystals and granules. The poisson ratios of these materials are given.

MEASUREMENTS of radial pressures exerted on the die-wall during uniaxial compression of materials to form compacts have been described by Nelson (1), Long (2), Windheuser (3), and Higuchi (4).

Nelson (1) and Higuchi (4), using load cells and strain gauges, respectively, mainly studied the transmission of forces to the die-wall, during the application of axial pressure, *i.e.*, on axial loading. The investigations failed to cover the relaxation/dissipation of pressure during removal of the compressional forces, *i.e.*, during unloading. Long (2) used a split die to measure the radial pressures. He studied the complete pressure cycles during loading and unloading of materials used in powder metallurgy and a theory was put forward regarding the nature of the residual radial pressure. No attention was paid to the effect of particle size, moisture content, and different physical forms.

The object of the present investigation was to find out the type and nature of the pressure cycles involved during compression of different pharmaceutical materials in granular and crystalline form. It was of particular interest to find out whether the ability of a material to form a "good" or "bad" tablet (particularly with respect to capping) was manifested in its pressure cycles.

APPARATUS

A Manesty E2 single-punch tableting machine, fitted with 1/2-in. plain punches was instrumented with strain gauges to monitor the forces involved during a compression cycle. The die-plate was suitably modified to house the die and the wires from the transducers without interfering with the movement of the upper punch and the hopper. To monitor the upper punch force and lower punch force, 1/4-in. linear foil gauges (Saunders Roe, resistance 120Ω, gauge factor 2.2) were used. Three foil gauges were bonded to the three slots around the lower punch holder, and connected in series, to form one active arm of a Wheatstone bridge. Three dummy gauges were used for the compensating arm as described by Shotton (5). In both cases, the transducers were connected as a half-bridge circuit with the resistors in the carrier amplifiers completing the circuit.

Silicone gauges (type 2A-1A-250 P)[1] were used to monitor the die-wall pressure. A 1/2-in. die was cut away on opposite sides, leaving a wall thickness of 1/8 in. on the die. One silicone gauge was bonded on either side of the cut-away surface. A silicone rubber solution was used to fill in the cut-away portions. With the solution hardened, the circumference of the die conformed to its original shape and size. The two silicone gauges formed opposite arms of a Wheatstone bridge. A full bridge was completed by using two resistors.

Type 2506 Carrier amplifiers[2] were employed to activate the transducers on the upper punch and lower punch holder. The oscillator frequency was set at 5 Kc./sec. and the output voltage was 3 v. A low gain amplifier (CA31)[2] of frequency 3 Kc./sec. and output adjusted at 6 v., powered by a power unit PU21,[2] was employed to energize the semiconductors.

The signals from the transducers were received in a 4 channel U.V. recorder (type 1706 Visicorder),[2] using suitably damped galvanometers (BB 250A)[2] and recorded at a chart speed of 6 mm./sec.

Received January 16, 1967, from the Department of Pharmacy, Chelsea College of Science and Technology, University of London, London, England.
Accepted for publication March 23, 1967.

[1] Ether Langham Thompson Ltd.
[2] Honeywell Controls Ltd.

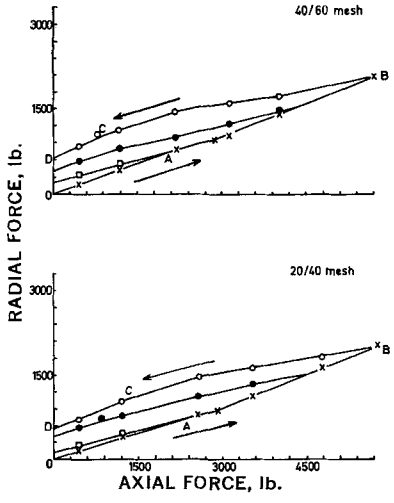


Fig. 1—Compressional cycle of sucrose granules.

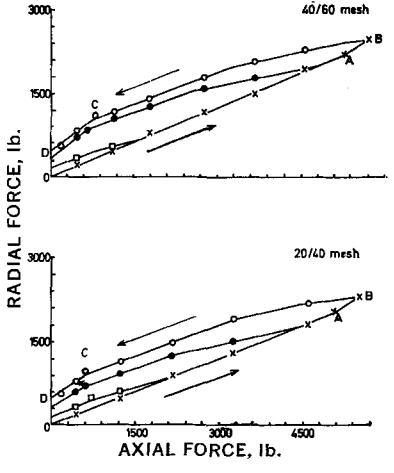


Fig. 2—Compressional cycle of sucrose crystals.

CALIBRATIONS

The upper punch and lower punch were calibrated by the method of Shotton and Ganderton (5). The die was calibrated by two different methods. First, it was clamped between two plates. Water, under pressure, was forced into the die. The height of the water column within the die was 0.16 in. This was of the same height as the tablets to be compressed. A linear relationship was obtained between the hydraulic pressures and the galvanometer deflections. A second calibration was carried out using a rubber plug as a confirmation. The die-wall response was of the same order as before.

EXPERIMENTAL

To investigate the type and nature of pressure cycles exhibited by different forms of the same material, sucrose granules and sucrose crystals were chosen. Two different sizes of each (20/40 mesh; 40/60 mesh) were compressed to investigate the effect of particle size.

The materials used were: sucrose granules (20/40 mesh, 40/60 mesh); sucrose crystals (20/40 mesh, 40/60 mesh); aspirin crystals (20/40 mesh); sodium chloride crystals (20/40 mesh); acetaminophen granules (20/40 mesh); and acetaminophen granules (97% acetaminophen, 3% polyvinylpyrrolidone (20/40 mesh).

The sucrose granules were prepared using distilled water. Acetaminophen granules were prepared using acetone. Granules of 97% acetaminophen and 3% polyvinylpyrrolidone (PVP) were prepared by moist granulation with the PVP in acetone. The granules were dried at 85° overnight. The

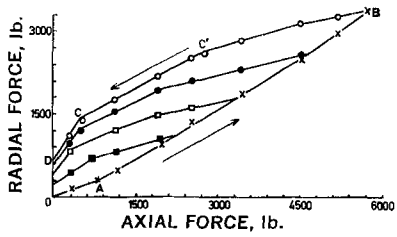


Fig. 3—Compressional cycle of sodium chloride (20/40 mesh).

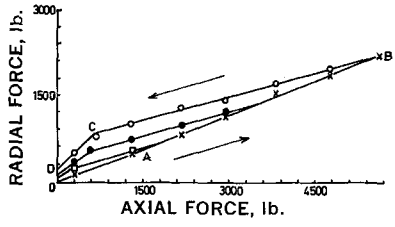


Fig. 4—Compressional cycle of acetaminophen granules (20/40 mesh).

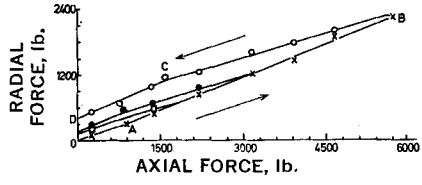


Fig. 5—Compressional cycle of acetaminophen granules and 3% PVP (20/40 mesh).

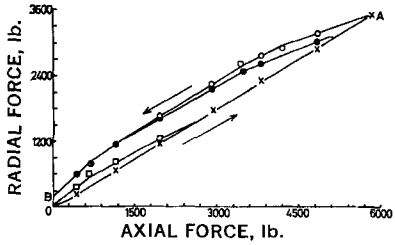


Fig. 6—Compressional cycle of aspirin crystals (20/40 mesh).

TABLE I—SLOPES

Material	Mesh Size	Slope of OA	Slope of AB	Slope of BC	Slope of CD	Max. Axial Load, lb.
Sucrose crystals	40/60	0.45	0.82	0.43	0.84	5600
Sucrose crystals	20/40	0.45	0.81	0.43	0.84	5600
Sucrose granules	40/60	0.36	0.42	0.36	0.42	5600
Sucrose granules	20/40	0.36	0.42	0.36	0.40	5600
Aspirin crystals	20/40	0.53	0.62			
Acetaminophen granules + 3% PVP	20/40	0.33	0.43	0.36	0.43	5600
Acetaminophen granules	20/40	0.38	0.42	0.38	1.00	5600
Sodium chloride crystals	20/40	0.40	0.70	0.48	1.41	5600
					0.70	2200

TABLE II—POISSON RATIOS (ν)

Material	Mesh Size	ν
Sucrose crystals	40/60	0.31
Sucrose crystals	20/40	0.31
Sucrose granules	40/60	0.27
Sucrose granules	20/40	0.27
Aspirin crystals	20/40	0.38
Acetaminophen + PVP granules	20/40	0.24
Acetaminophen granules	20/40	0.29
Sodium chloride crystals	20/40	0.30

two fractions, 20/40 mesh and 40/60 mesh, were obtained by sieving, using a test sieve shaker[3].

All materials were further dried over silica gel in an oven at 50° and stored over P_2O_5 in a desiccator prior to compression. The moisture content of each sample was determined by loss of weight after drying at 95° for 45 min. using infrared heat. Weight loss was found to be below 0.3% in each case.

All samples were lubricated with 0.5% magnesium stearate (100 mesh) and tumbled in a jar for 5 min. (A lubricant was included, because the object of this investigation was to determine the nature of the pressure cycles rather than die-wall friction.) The weight of material compressed in each case was such that a compact of 0.16 in. (height to diameter ratio 0.32) was obtained at the maximum loading. Each sample was weighed to the nearest mg. The compression cycle extended over 30 sec. for the loading and 30 sec. for the unloading. Loading and unloading were carried out evenly over the entire cycle. Five samples of each material were taken up in stages to load levels of approximately 2000, 3000, 4000, 5000, and 5600 lb. on separate occasions before unloading was commenced from these levels.

RESULTS

Figures 1–6 show the relationship between the radial forces and axial forces of the materials during a complete pressure cycle, *i.e.*, during loading and unloading. The slopes of OA, AB, BC, and CD are given in Table I.

Figure 1 shows the nature of the pressure cycles between two different sizes (20/40 and 40/60 mesh) of sucrose granules. Figure 2 shows the nature of the pressure cycles between different sizes (20/40 mesh and 40/60 mesh) of the sucrose crystals.

Table II gives the poisson ratio of the materials. Figure 4 shows the pressure cycle of acetaminophen granules which cap and laminate on compression, while Fig. 5 is the pressure cycle for the same material, granulated with 3% polyvinylpyrrolidone where capping and lamination were absent.

[3] Pascall Inclyno.

DISCUSSION

In general, a force acting on one part of a body will be transmitted across the section to the other part. This force may be resolved into two components, one normal to the body and the other in the tangential direction. The former is a normal stress and the latter a shearing stress (6). The normal component is consistent with volume changes, whereas the shearing forces (couples) are sources of, or response to, deformation of a body.

When granules or crystals are compressed within a die by means of two opposing punches, the axial load that is applied through the upper punch is transmitted to the die as a shearing force. In addition, force is transmitted radially to the die-wall. The pattern and magnitude of this latter force is governed by the elastic or plastic behavior of the compact. In studying the pressure transmitting characteristics of materials during tableting, using a single punch machine, it must be noted that although the forces involved in the sequence starting from loose material to close packing to compact formation are being monitored from the moment the upper punch enters the die, these forces cannot be recorded until the material forms a sufficiently firm compact to offer resistance to the downward movement of the upper punch. The moment that the compact is sufficiently firm, the upper punch force and the die-wall force can be recorded simultaneously. From this stage onward, the compact behaves more or less like a solid body and follows certain characteristics of solid bodies under uniaxial compression.

Consider the three following examples of solid bodies under uniaxial compression within a die. It is assumed that the die is perfectly rigid and that die-wall friction is absent. In the first case, it is assumed that the body is perfectly elastic. When an axial loading is applied, the transmitted radial force is of the same magnitude and the relationship is of the order:

$$\sigma = \nu(\tau) \qquad \text{(Eq. 1)}$$

where σ = axial force, τ = radial force, and ν = the poisson ratio.

Figure 7 shows this relationship in graphical form. This will be valid up to the elastic limit of the material. If the elastic limit has not been

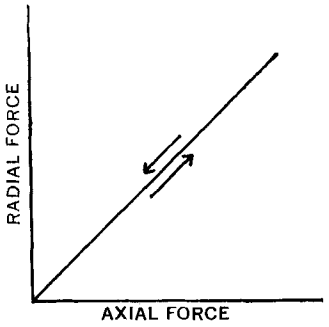


Fig. 7—Compressional cycle of an ideal elastic body.

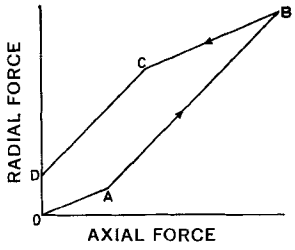


Fig. 8—Compressional cycle of a body with constant yield stress in shear.

exceeded, on unloading, the radial force will dissipate at the rate of ν times the axial force. When the axial force returns to zero, there will be no residual radial force exerted on the die-wall and the body will be free to move out from the die.

In the second case, the axial force is increased above the yield point (A) of the material, when the body starts to deform. The relationship between the axial and radial forces will now depend on the conditions of yield of the material. In this example it is assumed that the body has a constant yield stress in shear, independent of the magnitudes of the principal stresses to which it is subjected. Friction is assumed absent. The principal stresses are σ and τ. After yield has taken place, the following equation applies (2)

$$\sigma - \tau = 2S \qquad \text{(Eq. 2)}$$

S = yield stress in shear, and graphically it follows the course AB (Fig. 8). After a point B has been reached, if the axial force is allowed to fall, the body is no longer forced to yield. The radial force will fall at ν times the rate of fall of the axial force, following the course BC. The slope of the line BC = OA. Provided that the maximum axial loading has been sufficiently large, the point C will be reached at which the radial force is greater than the axial force by an amount equal to $2S$. Yield will again occur. The difference between the radial force and axial force ($\tau - \sigma$) will again be constant, and the relationship will follow the course CD. Taking this example, it is evident that after the upper punch has been withdrawn, the body will exert a residual radial force of $2S$ on the die-wall. Because of this force, the body will be constrained at the die-wall and will not be free to move out from the die. Whether this value is reached will depend on the maximum axial loading on the body. If this load is not large enough, the difference ($\tau - \sigma$) will not attain a value as high as $2S$. The change of the slope at C, the yield point on unloading, will not be evident from the graph of the pressure cycle.

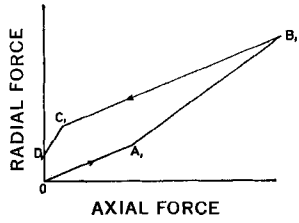


Fig. 9—Compressional cycle of a Mohr's body.

Consider the third case, where the body is also rigid, but after yield has taken place, the shearing stress (Mohr's theory) (7) in a plane of slip depends on the value of the normal stress σ_n acting in the same plane. In other words, the yield stress in shear is a function of the normal stress on the plane of shear. In the simple case where the body yields when:

$$\text{shearing stress} = S + \mu\sigma_n \qquad \text{(Eq. 3)}$$

the term μ is similar to a friction force, and the proportionality factor μ may be interpreted as a coefficient of internal solid (Coulomb type) friction. In this case, the normal stress on the plane of shear $= (\sigma + \tau)/2$ and the shearing stress $= (\sigma - \tau)/2$ (computed from Mohr's circle for principal stresses).

At yield from Eq. 3:

$$\sigma - \tau = 2S + \mu(\sigma + \tau) \qquad \text{(Eq. 4)}$$

$$\therefore \tau = [(1 - \mu)\sigma - 2S]/(1 + \mu) \qquad \text{(Eq. 5)}$$

On unloading, providing the axial loading has been sufficiently large (B'), the radial force will diminish according to the equation $\tau = \nu\sigma$, and then when the material yields again at the point C',

$$\tau = [(1 + \mu)\sigma + 2S]/(1 - \mu) \qquad \text{(Eq. 6)}$$

The slope $A'B' = (1 - \mu)/(1 + \mu)$. The final slope $C'D' = (1 + \mu)/(1 - \mu)$. The typical pressure cycle for this case takes the form shown in Fig. 9.

It must be realized that the pressure cycles described above are for models of solid, isotropic bodies under uniaxial compression, assuming that die-wall friction is absent. Compressing materials within a die to form a compact would obviously be somewhat different, as in this case, die-wall friction cannot be overlooked. In addition, the shape and size of the material (granules/crystals) could modify the pressure cycles.

Comparing the graphs of the pressure cycles of sucrose granules, sucrose crystals, aspirin crystals, sodium chloride crystals, acetaminophen granules, and acetaminophen granules with 3% polyvinylpyrrolidone (PVP), with the pressure cycles described, it can be seen that the cycles have certain features in common and certain analogies may be drawn. The pressure cycles of sucrose crystals, sucrose granules, sodium chloride crystals, and acetaminophen granules with 3% PVP are similar to the behavior of a solid body with a constant yield stress in shear, under uniaxial compression. Acetaminophen granules alone share a striking resemblance to that of a Mohr's body. It is interesting to note that the Mohr's body type pressure cycle of acetaminophen changes to that of a body with a constant yield stress in shear when granulated with 3% PVP. It is well known that acetaminophen on compression tends to cap and laminate. However, when granulated with 3% PVP no capping or lami-

nation is evident. The aspirin compact appears to have a very low yield stress. It behaves more like an elastic body. The hysteresis loss is comparable to that of an elastic body. On completely removing the axial load, the radial force dissipates almost completely. The small residual radial force that is recorded may be due to some die-wall friction.

With all the materials investigated, the axial loading was taken up to various levels from 1000–5600 lb. on separate occasions before unloading was commenced. However, in the plot of the axial-radial pressure cycles shown, only three loading levels (about 2200, 4000, and 5600 lb.) for each material are illustrated. Except in the case of aspirin crystals, it appears that after yield has started, the pressure cycles conform to the same pattern. However, the residual radial force increases with the increase in the axial loading. With aspirin crystals, the residual radial force appears to have reached a limiting value at an axial loading of about 4000 lb. This illustrates the behavior of the compact at different levels of axial loading and seems to support the assumption that the solid aspirin compact behaves like an elastic body. The direct relationship between the residual radial force and axial force of the materials (except for aspirin) may be attributed to the permanent deformation of the compacts after yield has started. It is probably significant that the ratio of height to diameter (0.32) of the aspirin compact remained constant after an axial loading of about 4000 lb. Since all the materials were lubricated with the same amount of lubricant, the difference in behavior would indicate a fundamental difference in the materials.

Table I shows the slopes of the lines corresponding to OA, AB, BC, and CD of Figs. 1–6. The slopes of the lines OA and BC are similar in the case of sucrose granules, sucrose crystals, sodium chloride crystals, and acetaminophen granules granulated with 3% PVP, having values ranging from 0.36 to 0.57. These slopes are directly related to poisson's ratio where the proportionality constant is the same for tablets of the same dimensions. The slopes of the lines BC and CD for sucrose granules, sucrose crystals, and acetaminophen granules with 3% PVP are more or less the same in each case as expected, for materials with a constant yield stress in shear.

The behavior of sodium chloride crystals is rather interesting. At an axial loading of about 2000 lb. it behaves almost like a perfect model of a body with a constant yield stress in shear. At the maximum axial loading of about 5600 lb., although the values of OA and BC are still the same, there is a break in the unloading curve at C′. After the yield point C has been exceeded in the unloading cycle, the radial force exerted is larger than the axial force.

In the three models described (Figs. 7–9) the yield points in each half of the cycle (representing loading and unloading) are clearly defined. With the materials under investigation this is not always the case. For instance, with sodium chloride it is seen that the slope of the line in the first half of the cycle is not constant. The initial change of the line may be taken as the yield point for the material. The subsequent change may be due to either (*a*) closer packing of the compacts, or (*b*) crystal slip or crystal twinning (*i.e.*, a shifting of the position of the lattice in a part of a crystal into a second position, so that the second part is in a symmetrical position relative to the first part and some plane of symmetry). It is likely that both phenomena contribute. On unloading the compact of sodium chloride, the slope of the line changes gradually down to the point C′ before it corresponds to the initial slope in the first half of the cycle (corresponding to poisson's ratio). This may be due to an "after flow" effect.

Comparing the behavior of sucrose granules with the crystals, it is seen that in the case of the crystals, the yield point A is higher and it has just been reached before unloading was commenced at B. The poisson ratio of the crystals is also higher than that of the granules. The residual radial pressure of the granules is, however, higher. After the yield point has been exceeded, the crystals appear to transmit more radial force. The slope of the line changes from 0.42 initially to 0.82, as compared with 0.36 to 0.42 in the case of the granules. The pressure cycles of the two sizes of sucrose granules are almost identical. However, the smaller of the sucrose crystals seem to have a higher yield point (A) at 5000 lb. compared to 4700 lb. for the larger crystals. This is to be expected as the yield point would depend more on the particle size of the material, but once a compact is formed the original particle size would not play a great role in its behavior, as the body now becomes a homogeneous isotropic mass.

Acetaminophen granules appear to behave like a Mohr's body. In this case, the final slope of the line C′D′ is 1. From the slope of A′B′ the value μ (coefficient of internal friction) is found to be 0.40.

The present investigation reveals significant differences between different granular and crystalline materials. Taking the case of acetaminophen it is seen that the change from a freely capping and laminating compact to a solid one is associated with the transformation from a Mohr's body type of pressure cycle to one with a constant yield stress in shear. It is perhaps more than coincidence that the materials which form firm and solid compacts exhibit the pressure cycle of a body with a constant yield stress in shear. At this stage, although no definite conclusion may be drawn, reasonable evidence exists that the classification of materials that form "good" or "bad" tablets may be carried out by an analysis of their pressure cycles, rather than from surmise. Admittedly, a wider range of materials will have to be investigated before any definite form of behavior can be predicted. No account has been taken in this investigation of the crystal shape. Indeed the crystal shape (anisotropy or isotropy) would modify the pressure cycles to a great extent. Further work is being conducted on the effect of lubrication on the pressure cycles.

REFERENCES

(1) Nelson, E. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 494(1955).
(2) Long, W. M., *Powder Met.*, **6**, 173(1960).
(3) Windheuser, J. J., Misra, J., Eriksen, S. P., and Higuchi, T., *J. Pharm. Sci.*, **52**, 767(1963).
(4) Higuchi, T., Shimamoto, T., Eriksen, S. P., and Yashiki, T., *ibid.*, **54**, 111(1965).
(5) Shotton, E., and Ganderton, D., *J. Pharm. Pharmacol.*, **12**, 87T(1960).
(6) Reiner, M., "Deformation, Strain and Flow," 2nd ed., H. K. Lewis, London, England, 1960.
(7) Nadai, A., "Theory of Flow and Fracture of Solids," 2nd ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1950, chap. 15.

Investigation of Hepatic Drug Metabolism by Isolated Perfused Liver *In Situ*

By G. BENZI, F. BERTÉ, and A. CREMA

The metabolizing activity of the hepatic tissues in an isolated *in situ* liver preparation has been examined. In this experiment the organ remained connected to the other parts of the body, but its blood circulation was supplied by a pump–oxygenator system. By this method some hepatic metabolic activities, such as demethylation, acetylation, and glucuronide-conjugation, have been evaluated.

THE TECHNIQUE of local perfusion of organs was developed with the specific purpose of minimizing the toxicity and increasing the chemotherapeutic activity of carcinolytic agents. Klopp *et al.* (1) maintained in 1950 that the therapeutic effect of drugs could be enhanced and the toxicity greatly reduced by intravascular injections into regions of the body containing tumors. In 1958 Creech *et al.* (2) and Ryan *et al.* (3) began the use of local therapy by isolating the tumor-bearing area from the remainder of the circulation and perfusing it with the aid of a pump–oxygenator system. According to these authors, the technique of regional perfusion is based on the concept that various anatomic regions can be isolated from the general circulation, supplied with a separate extraneous system for pumping and oxygenating the blood, and maintained for varying periods of time under these conditions without significant systemic effects.

Subsequently, these or related techniques have been utilized by many investigators, and Stehlin *et al.* (4), Creech *et al.* (5), Shingleton *et al.* (6), Ausman and Aust (7), Clarkson and Lawrence (8), and others have published excellent descriptions.

In the course of systematic investigations of drug metabolism (9–11), we developed a method to evaluate the liver metabolic activity *in situ*, utilizing the concepts of the authors quoted above. To test the method itself, we have also examined some metabolic transformations of aminopyrine and oxazepam.

EXPERIMENTAL

The abdominal technique of liver perfusion was carried out on 16 dogs (14.5 to 29.0 Kg.) and on 4 monkeys (*C. hamadryas;* 11.4 to 16.2 Kg.) preanesthetized with urethan (0.4 Gm./Kg. i.p.). Anesthesia was induced and maintained in closed circuit by nitrous oxide, cyclopropane, or ethyl ether. The animals were given artificial ventilation after tracheal intubation by Warne tube during succinylcholine chloride action (1 mg./Kg. i.v.). The operative procedures are indicated in Fig. 1 and are briefly summarized here.

Before laparotomy, a large outflow tube is inserted into the right external iliac vein (*via* femoral vein, 10) and connected with a large inflow tube inserted into the right external jugular vein, 9, in the dog or into the right internal jugular vein in the monkey; the connection is made by a large silicone coated para-rubber cannula joined to a Sigmamotor pump, 12.

After laparotomy, the inferior cava vein, 6, is ligated above the renal veins, 17. The phrenico-abdominal veins are also ligated or excluded. Into the inferior cava vein, clamped below the liver, a special three-way outflow cannula is inserted immediately above the ligature; this tube, 5, contains a movable rubber balloon (connected with a syringe, 14), which after clamp removal, is passed into the cava, 7, over the diaphragmatic foramen. The rubber balloon, 18, remains deflated, and the outflow tube, 5′, is clamped. It is necessary to tie the accessory portal veins, and particularly the gastro-hepatic, paraumbilical, diaphragmatic, and cardial groups.

A T-shaped outflow tube, 13, inserted into the porta or the mesenteric vein, is connected, by a silicone coated para-rubber cannula, with the Sigmamotor apparatus, 12.

An inflow cannula, 3, is inserted into the portal vein and ligated below the liver but immediately before the portal vein divides into two or more branches. Both the portal inflow cannula, 3, and the cava outflow cannula, 5, are connected with the pump–oxygenator system, 4.

The rubber balloon, 18, is inflated by the syringe, 14, and drawn back (under X-ray examination) to

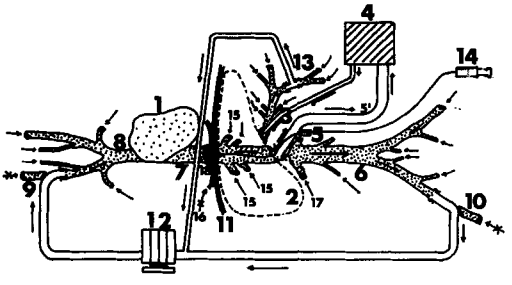


Fig. 1—Diagram of the experimental arrangement in dog. Key: 1, *heart;* 2, *liver;* 3, *portal inflow cannula;* 4, *pump–oxygenator system;* 5, *three-way cannula with outflow tube,* 5′; 6, *under-diaphragmatic inferior cava vein;* 7, *over-diaphragmatic inferior cava vein;* 8, *superior cava vein;* 9, *right external jugular vein;* 10, *right femoral vein;* 11, *diaphragm;* 12, *Sigmamotor pump;* 13, *T outflow cannula;* 14, *syringe connected with the rubber balloon,* 18; 15, *hepatic veins;* 16, *phrenic veins;* 17, *renal veins;* 18, *movable rubber balloon.*

Received January 16, 1967, from the Departments of Pharmacology, Pavia and Pisa Universities, Italy.
Accepted for publication March 9, 1967.

block, in the diaphragmatic foramen, both the cava vein and the blood flow from the phrenic tributaries, 16. At the end, the hepatic artery is ligated and the perfusion begins. It is necessary to make several ligatures to block the lymphatic ducts of the hepatic hilum and the pancreas.

The pump–oxygenator system consists of: a venous reservoir, an oxygenator with gasmeter, a roller-type pump with flowmeter, a blood filter, an apparatus to eliminate the blood foam, a perfusion pressure regulator, and a blood heat exchanger with telethermometer. Before the extracorporeal liver perfusion, the pump–oxygenator system is filled with 300–400 ml. of heparinized blood previously obtained from the animal itself that was infused with 300–400 ml. of blood from donor animals. The priming blood is circulated through the extracorporeal system, fully oxygenated, and warmed. A flow of oxygen into the oxygenator is maintained at the rate of 4–8 L./min. During the extracorporeal liver perfusion the blood flow rate was 4–10 ml./min./Kg.

The evaluation of any leakage from the extracorporeal circuit can be determined: (*a*) during the metabolic research, by taking samples from the general circulation of the blood and the lymph to verify the absence of the studied substance or its metabolites; (*b*) at the end of the metabolic research, by adding to the blood of the extracorporeal circuit either a dye (which can be examined, for its eventual leakage, by autopsy) or a radiopaque substance (which can be traced by radiography).

To confirm the proper function of the liver, the concentration of serum glutamate-oxalacetate transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT), sorbit dehydrogenase (SDH); leucine aminopeptidase (LAP) and seric hemoglobin was determined in blood samples from the extracorporeal circuit. From bile samples, the time of bromosulphonthalein (BSP) elimination from the blood of the extracorporeal circuit was determined.

To evaluate the metabolizing activity of the liver, the following were examined: (*a*) the demethylation by studying the transformation of aminopyrine to 4-aminoantipyrine, using the method of Brodie and Axelrod (12), after the addition of 150–300 mcg./ml. of aminopyrine into the extracorporeal circuit; (*b*) the acetylation, by studying the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine under the above-mentioned conditions; (*c*) the glucurono-conjugation, by studying the transformation of oxazepam to glucuronide, using the method of Walkenstein *et al.* (13), after addition of 25–50 mcg./ml. of oxazepam (7-chloro-1,3-dihydro - 3 - hydroxy - 5 - phenyl - 2*H* - 1,4 - benzodiazepine-2-one) into the extracorporeal circuit.

RESULTS

Following these preparations, the venous blood from the hepatic veins (Fig. 1, 15) passes through the inferior cava into the pump–oxygenator system which is connected with the portal inflow tube. The abdominal venous blood by-passes through an artificial cava into the right side of the heart.

During the extracorporeal liver perfusion (from 2 to 3 hr.) no difference was observed between initial and terminal values of the following parameters: SGPT, SDH, LAP, and the time of BSP elimination. In contrast, there was a slight linear increase of SGOT during the perfusion depending on the increase of serum hemoglobin content. In fact, it has been shown that even a low-grade hemolysis leads to false positive values for SGOT (14).

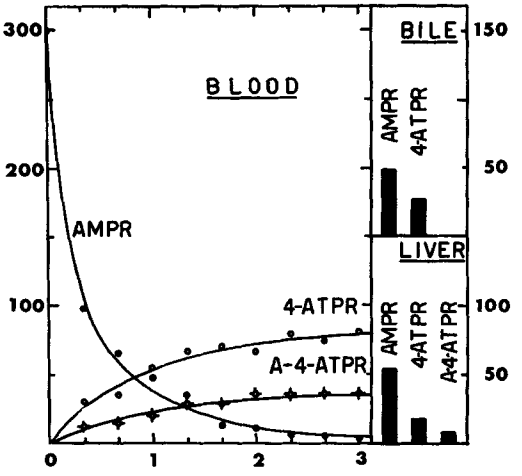


Fig. 2—Aminopyrine metabolism studied by the isolated liver perfusion in situ *in a dog weighing 25.8 Kg. Blood in extracorporeal circuit = 600 ml.; blood flow rate of the liver extracorporeal circuit = 130 ml./min.; initial concentration of aminopyrine in the plasma of the extracorporeal circuit = 300 mcg./ml. At left, the plasmatic concentration curves (mcg./ml., in ordinate) of aminopyrine (AMPR), 4-aminoantipyrine (4-ATPR), and* N*-acetyl-4-aminoantipyrine (A-4-ATPR) during 3 hr. (in abscissae) after aminopyrine addition. At right, bile and liver concentrations (mcg./ml. or mcg./Gm., in ordinate) of aminopyrine and its two metabolites, 3 hr. after adding the aminopyrine into the extracorporeal liver perfusion.*

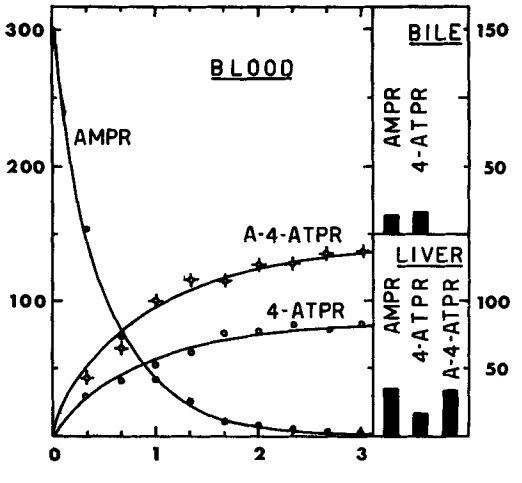


Fig. 3—Aminopyrine metabolism studied by the isolated liver perfusion in situ *in a monkey* (C. hamadryas) *weighing 13.8 Kg. Blood in liver extracorporeal circuit = 480 ml.; blood flow rate of the extracorporeal circuit = 70 ml./min.; initial concentration of aminopyrine in the plasma of extracorporeal circuit = 300 mcg./ml. Left and right same as Fig. 2.*

Concerning the metabolizing activity of *in situ* isolated liver preparation, aminopyrine and oxazepam disappear from the extracorporeal blood of dog and monkey at a very rapid rate: the half-life for aminopyrine ranges from 10 to 14 min. in dog and from 16 to 21 min. in monkey, and for oxazepam from 7 to 10 min. in both animals.

Figures 2 and 3 show two typical examples of the rate of transformation of aminopyrine in two metabolities: the demethylation is quite similar in dog and monkey, whereas the acetylation is higher in monkey than in dog. Likewise, at the end of the liver perfusion, the hepatic concentration of 4-aminoantipyrine is similar in both species, while the hepatic concentration of *N*-acetyl-4-aminoantipyrine is higher in the monkey than in the dog.

Figure 4 shows a typical example of the high glucurono-conjugation produced by the liver which eliminates the glucuronide into the bile.

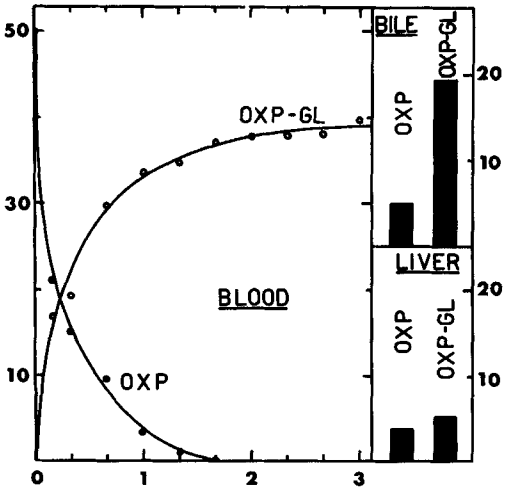


Fig. 4—Oxazepam metabolism studied by the isolated liver perfusion in situ *in a dog weighing 23.5 Kg. Blood in liver extracorporeal circuit = 600 ml.; blood flow rate of extracorporeal circuit = 120 ml./min.; initial concentration of oxazepam in the plasma of the extracorporeal circuit = 50 mcg./ml. At left, the plasmatic concentration curves (mcg./ml., in ordinate) of oxazepam (OXP) and its glucuronide (OXP-GL), assayed for 3 hr. (in abscissae) after oxazepam addition. At right, the bile and liver concentrations (mcg./ml. or mcg./Gm., in ordinate) of oxazepam and its glucuronide, assayed after 3 hr. of the extracorporeal liver perfusion with the addition of oxazepam.*

CONCLUSIONS

The described method permits us to investigate *in situ* the metabolizing activity of the liver normally connected with the body except that its blood circulation is supplied by a pump–oxygenator system. The liver functional tests remained within normal limits during the experiment (2 to 3 hr.). In such a way, the hepatic metabolic activity can be examined under experimental arrangements more similar to the normal conditions than traditional *in vitro* methods.

SUMMARY

The technique of the local perfusion of the organs *in situ* has been applied to evaluate the metabolizing hepatic activity. The liver can be isolated from general circulation, supplied with a separate extraneous system for pumping and oxygenating the blood, and maintained from 2 to 3 hr. under these conditions without significant local damage.

To test the method, the authors studied certain hepatic metabolic activities such as demethylation, acetylation, and glucurono-conjugation. The results suggest that the method could be useful in evaluating the metabolizing activity of the liver *in situ* without interference from other tissues.

REFERENCES

(1) Klopp, C. T., Alford, T. C., Bateman, J., Berry, G. N., and Winship, T., *Ann. Surg.*, **132**, 811(1950).
(2) Creech, O., Jr., Krementz, E. T., Ryan, R. F., and Winblad, J. N., *ibid.*, **148**, 616(1958).
(3) Ryan, R. F., Krementz, E. T., Creech, O., Jr., Winblad, J. N., Chamblee, W., and Cheek, H., *Surg. Forum*, **8**, 158(1958).
(4) Stehlin, J. S., Clark, R. L., White, E. C., Smith, J. L., Jr., Griffin, A. C., Jesse, R. H., Jr., and Healey, J. E., Jr., *Ann. Surg.*, **151**, 605(1960).
(5) Creech, O., Jr., Krementz, E. T., Ryan, R. F., Reemtsma, K., Elliot, J. L., and Winblad, J. N., *J. Am. Med. Assoc.*, **171**, 2069(1959).
(6) Shingleton, W. W., Reeves, J. W., Jr., Keppel, R. A., Mahaley, S., and Taylor, H. M., *Ann. Surg.*, **151**, 741(1960).
(7) Ausman, R. K., and Aust, J. B., *Surg. Forum*, **12**, 123(1961).
(8) Clarkson, B., and Lawrence, W., Jr., *Med. Clin. N. Am.*, **45**, 689(1961).
(9) Mascherpa, P., *Actualités Pharmacol.*, **16**, 121(1963).
(10) Mascherpa, P., and Berté, F., *Biochem. Pharmacol.*, **5**, 200(1966).
(11) Crema, A., and Berté, F., *Arch. Expil. Pathol. Pharmakol.*, **239**, 475(1960).
(12) Brodie, B. B., and Axelrod, J., *J. Pharmacol. Expil. Therap.*, **99**, 171(1950).
(13) Walkenstein, S. S., Wiser, R., Gudmundsen, C. H., Kimmel, H. B., and Corradino, R. A., *J. Pharm. Sci.*, **53**, 1181(1964).
(14) Schmidt, F. H., *Proc. European Soc. Drug Toxicity*, **1965**, 97.

Relationship Between Lesion Production, Absorption, and Distribution of ^{14}C-Labeled Acetylsalicylic Acid

By ERDIE MORRIS*, ANTOINE A. NOUJAIM†, TOM S. MIYA, and JOHN E. CHRISTIAN

Carbon-14-labeled acetylsalicylic acid in conjunction with liquid scintillation counting and autoradiography was used to study the absorption and localization of the drug in the rat stomach. Lesions were produced in rats following oral administration of from 50 to 600 mg./Kg. The concentration of acetylsalicylic acid in the rumen and corpus portions of the stomach and in other tissues was determined for various time intervals after administration. The localization of the drug was studied in both the mucosal and muscularis surfaces of the stomach.

ALTHOUGH MANY divergent reports have been made on the blood loss and the gastric lesions caused by acetylsalicylic acid, it is clear that gastric bleeding does occur in humans and in experimental animals administered acetylsalicylic acid. As early as 1938, Douthwaite and Lintott (1) found severe mucosal reactions in 13 of 16 subjects following oral administration of acetylsalicylic acid.

These observations were extended by Hurst in 1943 (2). However, Wolf and Wolff in 1943 (3) were unable to produce gastric lesions by direct application of acetylsalicylic acid to the mucosa of a gastrostomy subject. Paul (4) observed no lesions in 107 subjects after acetylsalicylic acid administration. Caravati and Cosgrove in 1946 (5) repeated this work with 12 subjects with similar results. Since 1955, however, many investigations (6–10) have been reported agreeing, in general, with the earlier findings of Douthwaite and Lintott (1) and Hurst (2).

Davenport (7), in extensive studies of the production of mucosal damage by acetylsalicylic acid in vagally denervated dogs, suggested that the damage may be the result of the exchange of H^+ and Na^+ with a consequent efflux of Na^+, K^+, and Cl^- ions, eventually leading to damage of the capillary walls and subsequent hemorrhage.

Based on studies of gastric damage in guinea pigs, Anderson (8) concluded that the acetylsalicylic acid-induced gastric damage depended upon gastric absorption probably originating at the cellular level when the rate of entry into the mucosal cells exceeded some critical value. Initially the surface epithelial cells are shed, and this is followed by necrosis of the underlying glandular but not muscular tissue. If this is the case, the mechanism would therefore appear to involve an effect on the mucosal cells and is not directly related to gastric hyperacidity or peptic activity.

Martin (9) has suggested that the gastric mucosal injury is brought about by an appreciable accumulation of the drug anion in gastric mucosal cells because of the high pH gradient (stomach *versus* inside of cell; pH 1.4–6.8).

Croft (10) concluded that the gastric bleeding following repeated ingestion of acetylsalicylic acid appeared to be a result of gastric erosions brought about by the loss of surface cells. Davison (11) demonstrated that undissolved particles of aspirin are necessary for bleeding to occur in the dog.

From these and other reports, it is evident that the mechanism by which acetylsalicylic acid causes gastric lesions and subsequent blood loss is not well understood. The present study was carried out to uncover additional information about the concentration, localization, and penetration of the drug.

The terminology applied to the gross anatomy of the stomach in this paper was adapted from Shay (12).

EXPERIMENTAL

Materials and Methods—Carbon-14-carboxyl-labeled acetylsalicylic acid (Tracerlab, Waltham, Mass.), was used for all radiometric phases of this study. Radiocompound purity was confirmed by means of thin-layer chromatographic assay using a solvent system composed of benzene–ether–acetic acid–methanol (120:60:18:1). Polyvinylpyrrolidone (Antara Chemicals, Chicago, Ill.) was used as the suspending agent for oral administration. Kodak No-Screen medical X-ray film was used for the gross autoradiography. Liquid scintillation counting was accomplished using a model 3003 TriCarb liquid scintillation spectrometer (Packard Instrument Co., LaGrange, Ill.), with external standard attachment for quench correction. Tissue samples were prepared for counting by dissolving them in 2 ml. of hyamine–hydroxide solution (1 *M* methanolic hyamine HCl, 3 parts; 30% potassium hydroxide, 1 part). Excessive color was removed with 0.5 ml. of 30% hydrogen peroxide. Each sample was then acidified with 1.0 ml. of glacial acetic acid after which 15 ml. of XDC scintillation fluid consisting of xylene–cellusolve–dioxane (1:3:3), PPO (2,5-diphenyloxazole) 10 Gm./L., dimethyl POPOP (dimethyl 1,1-4-bis-2-(5-phenyloxazolyl) benzene, 0.5 Gm./L., and naphthalene, 80 Gm./L.

Production of Lesions—Preliminary experiments were carried out to confirm the production of lesions by oral administration of acetylsalicylic acid. U.S.P. grade acetylsalicylic acid was suspended in 5.0% polyvinylpyrrolidone and administered to 24 male rats of the Holtzman strain, weighing 200 to 260 Gm., in doses of 10, 50, 100, and 600 mg./Kg. The animals were fasted for 36 hr. prior to administration of the drug and were allowed no food or water during the experiment. Lesions were produced in the corpus in every case with doses of from 50 to 600 mg./Kg. No lesions were observed in the control animals which were

Received December 15, 1966, from the Bionucleonics and Pharmacology Departments, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication March 22, 1967.

This investigation was supported in part by a grant from Menley and James Laboratories, Philadelphia, Pa., and in part by the Division of Radiological Health, U. S. Public Health Service, Bethesda, Md.

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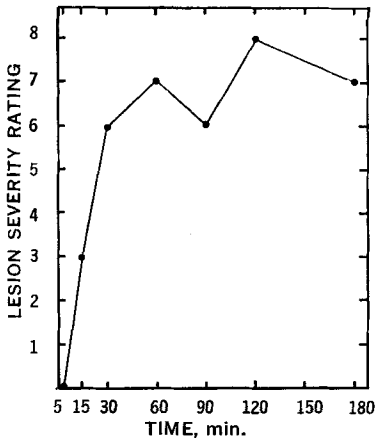


Fig. 1—Average gastric lesion ratings produced by oral 50 mg./Kg. acetylsalicylic acid. The condition of the stomachs was rated according to the following scale: No. 1, normal, no lesions, erythema or blood; No. 2, erythema only, no lesion or blood evident; No. 3, lesions present, less than 5, all of which are smaller than 1 mm. in diameter; No. 4, lesions present, less than 5 with one or more larger than 1 mm. in diameter; No. 5, lesions present, more than 5 but less than 10, all of which are less than 1 mm. in diameter; No. 6, lesions present, more than 5 but less than 10 with one or more larger than 1 mm. in diameter; No. 7, lesions present, more than 10, all of which are smaller than 1 mm. in diameter; No. 8, lesions present, more than 10 with one or more larger than 1 mm. in diameter.

given similar doses of polyvinylpyrrolidone alone in like volumes. The localization of lesions produced by aspirin to the corpus region has been previously noted by Gray (13).

Using time intervals between dose and sacrifice of 5, 15, 30, 60, 90, and 120 min., the time required to produce lesions and the severity of the lesions produced were studied. Three animals were used in each time group and a 50 mg./Kg. dose contained in 0.7 ml. of suspension was used in this and all subsequent experiments. The results are summarized in Fig. 1.

Surgical Techniques—The stomachs were removed, freed from all adhering tissue, and opened along the lesser curvature. Each stomach was pinned to a No. 14 rubber stopper such that the rugal folds were flattened out. All stomachs were washed with exactly 100 ml. of 0.9% saline solution by gently spraying from a plastic wash bottle. A previous experiment indicated that a 100-ml. wash was sufficient to remove surface contamination from the mucosa.

Determination of Drug Concentration in Selected Tissues—Twenty-one rats were administered a 50-mg./Kg. dose of ^{14}C-carboxyl-labeled acetylsalicylic acid having a specific activity of 0.625 μc./mg. Three control animals were given a similar dose of nonlabeled drug. The animals were sacrificed in groups of 3 at time intervals of 5, 15, 30, 60, 90, and 120 min. after dosing. After removal, the stomachs were washed with saline and random samples (50 to 100 mg.) were taken from the rumen and corpus portions, placed in a previously weighed Wheaton vial, and the wet weight of the tissue accurately determined. The samples were taken by cutting

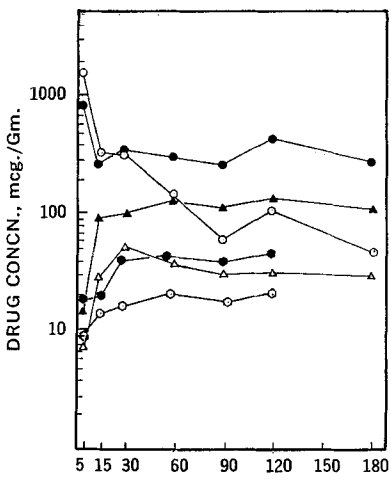


Fig. 2—Concentration of acetylsalicylic acid in the rumen and corpus portions of the stomach, skeletal muscle, kidney, blood, and adrenal gland of the rat. Key: ●, rumen portion; ○, corpus portion; △, skeletal muscle; ▲, kidney; ⬢, blood; ⬡, adrenal gland.

through the entire stomach wall with a hand cork borer. Samples were also taken from the liver, lung, kidney, adrenal gland, skeletal muscle, and blood (1 ml.). All samples were counted as previously described, and from the specific activity of the original dose, the amount of drug present per Gm. of tissue was determined for each sample (see Fig. 2).

Gross Autoradiography—Eighteen rats were dosed and sacrificed as previously described; 3 rats served as controls. After the stomachs had been removed, pinned, and washed, they were quick frozen with dry ice and removed from the rubber stoppers. In the dark room, the stomachs were covered on both sides with Saran wrap and arranged in groups according to the time intervals of 5, 30, and 120 min., respectively. Each group was then placed between 2 sheets of No-Screen medical X-ray film such that one film was in apposition to the mucosal surface and the other in apposition to the muscularis surface. The films with the stomachs in position were then placed in light tight cassets and stored at −20° for 60 days. The films were developed and photographic prints were made so that the dark areas corresponded to the areas of radioactivity where the drug was concentrated. Respresentative examples of these prints are shown in Figs. 3 through 8.

Intravenous Administration of Acetylsalicylic Acid—Eighteen animals were given *via* the caudal vein 50 mg./Kg. labeled drug dissolved in propylene glycol. Three control rats were used. Gross autoradiographs were prepared in the same manner as previously described (see Figs. 9 and 10). After exposure of the gross autoradiographs, samples were taken from the stomachs and counted to determine the drug concentrations. Drug concentration was found to be greatest 15 min. after intravenous administration in both the rumen and corpus portions.

DISCUSSION AND COMMENTS ON RESULTS

From the results of the drug concentration studies in selected tissues, it is apparent that very rapid

absorption occurs through the mucosa of the corpus portion of the stomach (Figs. 3–5). Five minutes after oral administration, the drug concentration in the corpus and rumen mucosa is greater by a factor of 10 than those concentrations found in any other tissue at any time. Furthermore, it was shown that the drug passes rapidly through the corpus mucosa into the muscularis where it is absorbed. Consequently, within 30 min. after administration, the concentration of drug

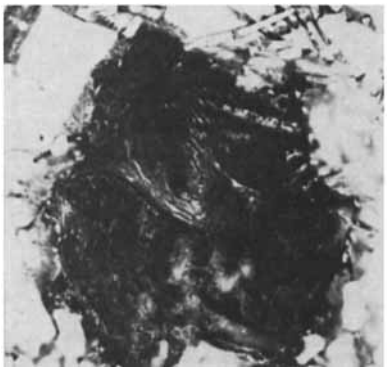


Fig. 3—Mucosal surface autoradiograph of a rat stomach 5 min. after oral administration. Note the high concentration of drug (acetylsalicylic acid and/or metabolites) in rumen and corpus portions.

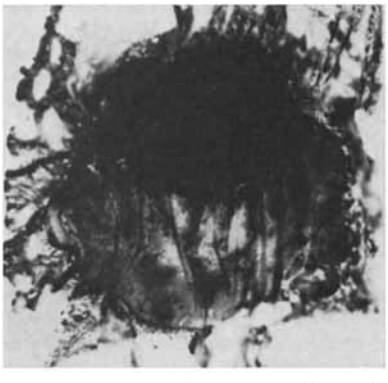


Fig. 4—Mucosal surface autoradiograph of a rat stomach 30 min. after oral administration. Note the decreasing concentration in the corpus portion with the rumen concentration remaining the same.

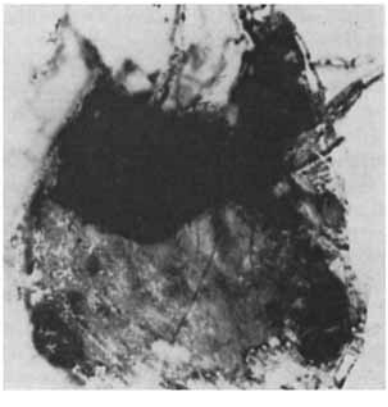


Fig. 5—Mucosal surface autoradiograph of a rat stomach 120 min. after oral administration. Note the lack of drug in the corpus portion.

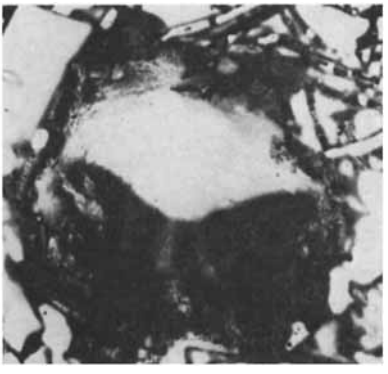


Fig. 6—Muscularis surface autoradiograph of a rat stomach 5 min. after administration. Note the high concentration of drug in the corpus portion and little or no concentration in the rumen portion.

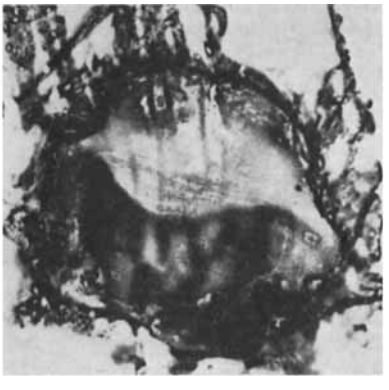


Fig. 7—Muscularis surface autoradiograph of a rat stomach 30 min. after administration. Note the drug in the corpus portion. The rumen concentration remains low.

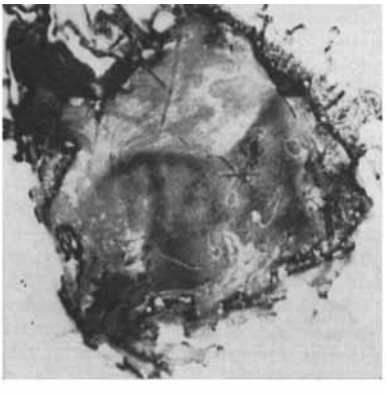


Fig. 8—Muscularis surface autoradiograph of a rat stomach 120 min. after administration. Note the greatly decreased drug in the corpus portion as compared to the 30-min. preparation. There is still little or no concentration of drug in the rumen portion.

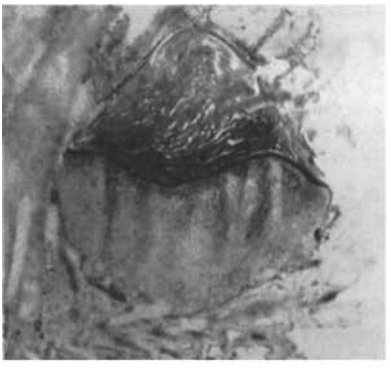


Fig. 9—Mucosal surface autoradiograph of a rat stomach 120 min. after intravenous administration.

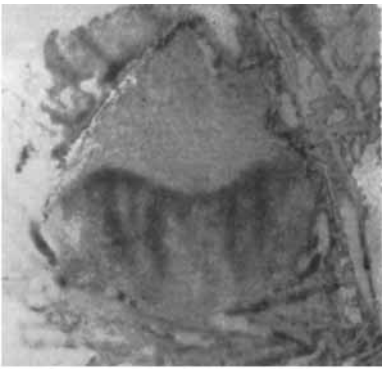


Fig. 10—Muscularis surface autoradiograph of a rat stomach 120 min. after intravenous administration.

remaining in the corpus mucosa is less than that found in the rumen mucosa. After 60 min. the drug levels in the corpus portion are essentially the same as those found in most other tissues. On the other hand, although the original absorption in the rumen portion is not nearly so great as in the corpus, there appears to be very little loss of drug from the rumen, so that 30 min. after administration until at least 3 hr., the concentration levels in the rumen are much greater than those found in any other tissue.

The gross autoradiographs clearly show the greater concentration of drug (acetylsalicylic acid and/or metabolites) in the corpus mucosa at 5 min. after administration and greater in the rumen mucosa after 120 min. The muscularis surface autoradiograms show increasing concentration in the corpus muscularis, followed by gradual decrease indicating absorption through this portion. No significant concentration is seen in the rumen portion of the muscularis indicating a lack of absorption through this portion.

SUMMARY AND CONCLUSIONS

The results may be summarized as follows. (*a*) Acetylsalicylic acid in doses of from 50 to 600 mg./Kg. produced gastric lesions in the corpus portion of the rat stomach. (*b*) Acetylsalicylic acid is absorbed through the mucosa of the corpus portion at a rapid rate with maximum concentration being reached within 5 min. after drug administration. (*c*) The drug concentration in the mucosa of the rumen portion remains relatively constant over a 3-hr. period following administration while the concentration in the corpus is initially high with steady decrease beginning 5 min. after oral administration. The drug concentration decreases in the corpus portion to a level comparable to that found in most other tissues after about 1 hr., while in the rumen it remains at a significantly higher level.

Although more work must be done before the entire mechanism of lesion production can be understood, the data presented here tend to refute the hypothesis of focal necrosis at least as the sole mechanism of gastric erosion. The data tend to support the concepts submitted by Davenport (7) as to the importance of tissue permeability and ion flux across the mucosa in the production of capillary wall damage eventually resulting in blood loss and erosion of the mucosa.

REFERENCES

(1) Douthwaite, A. H., and Lintott, G. M., *Lancet*, **235**, 1222(1938).
(2) Hurst, A., *Brit. Med. J.*, **I**, 768(1943).
(3) Wolf, S., and Wolff, H. G., "Human Gastric Function," Oxford University Press, London, England, 1943, pp. 165, 167.
(4) Paul, W. D., *J. Iowa Med. Soc.*, **33**, 155(1943).
(5) Caravati, C. M., and Cosgrove, E. F., *Ann. Internal Med.*, **24**, 638(1946).
(6) Muir, A., and Cossar, I. A., *Brit. Med. J.*, **II**, 7(1955).
(7) Davenport, H. W., *Gastroenterology*, **46**, 245(1964).
(8) Anderson, K. W., *Arch. Intern. Pharmacodyn.*, **152**, 379(1964).
(9) Martin, B. K., *Nature*, **198**, 896(1963).
(10) Croft, D. N., *J. Pharm. Pharmacol.*, **18**, 354(1966).
(11) Davison, C., Hertig, D. H., and Devine, R., *J. Clin. Pharmacol. Therap.*, **7**, 239(1965).
(12) Shay, H., Komarov, S. A., Fels, S. S., Merance, D., Grüenstein, M., and Siplet, H., *Gastroenterology*, **5**, 43(1945).
(13) Gray, J. E., Jones, P. M., and Feenstra, E. S., *Toxicol. Appl. Pharmacol.*, **2**, 514(1960).

Synthesis of Pyrrolidine-2-^{14}C

By JOHN M. WALTS*, WAYNE V. KESSLER, and JOHN E. CHRISTIAN

The preparation of pyrrolidine-2-^{14}C picrate in satisfactory yields from potassium cyanide-^{14}C and from barium carbonate-^{14}C is reported. Reaction of pyrrolidine picrate with lithium hydroxide followed by distillation separated the free base.

THE SYNTHESIS of ^{14}C-labeled pyrrolidine was of interest since pyrrolidine is often found in a variety of biologically active compounds. The routes of synthesis were evaluated, and two were selected. The criteria used in selecting these routes were that relatively inexpensive starting materials were available, special equipment such as hydrogenators and autoclaves were not needed, vapors and gases were easily controlled, and an excessive number of reactions were not required. Pyrrolidine was labeled with ^{14}C at position 2 by both routes. It was isolated as its picrate salt for ease in handling and for convenience in purification and radioactivity determinations.

In method *A* (Scheme I), potassium cyanide-^{14}C and 3-methoxy-1-iodopropane (I) were refluxed in methyl alcohol to give 4-methoxybutyronitrile-1-^{14}C (II) (1). The latter compound was reduced with lithium aluminum hydride in ether to form the corresponding amine, 4-methoxybutylamine-1-^{14}C (III) (2). Heating this amine with hydrobromic acid produced 4-bromobutylamine-1-^{14}C hydrobromide (IV). Neutralization of IV with aqueous alkali followed by fractionation yielded pyrrolidine-2-^{14}C (V) (3). Addition of an ethereal solution of picric acid precipitated pyrrolidine-2-^{14}C picrate (VI) (3).

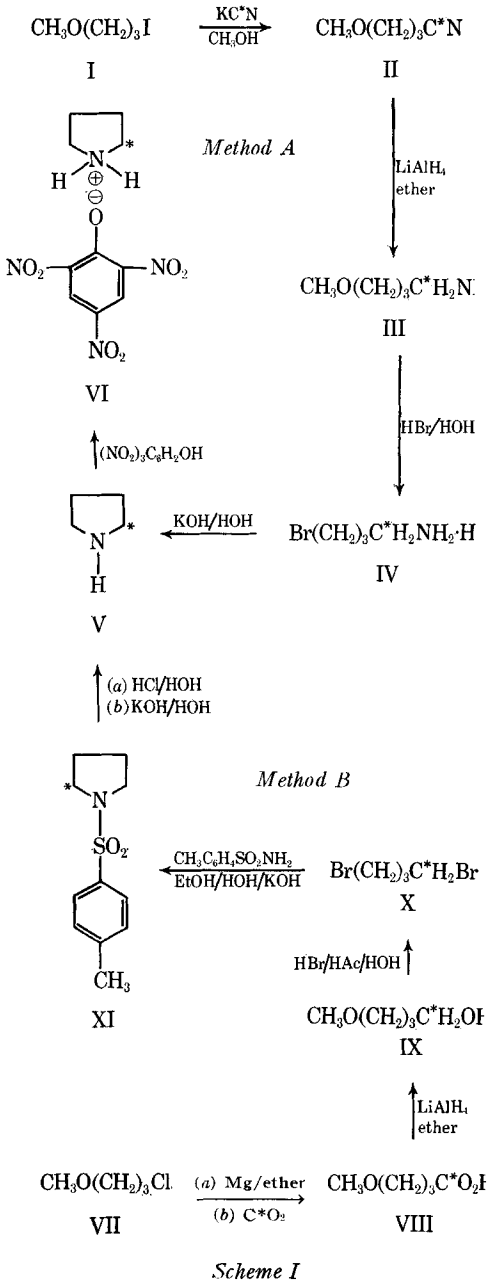
In method *B* (Scheme I), carbonation of the Grignard reagent of 3-methoxy-1-chloropropane (VII) in ether with carbon dioxide-^{14}C gave 4-methoxybutanoic-1-^{14}C acid (VIII) (4). Reduction of this acid with lithium aluminum hydride in ether produced the corresponding alcohol, 4-methoxy-1-butanol-1-^{14}C (IX) (2). Heating this alcohol with hydrobromic acid and acetic acid formed 1, 4-dibromobutane-1-^{14}C (X) (5). Reaction of the latter compound with *p*-toluenesulfonamide in ethyl alcohol and aqueous alkali yielded *N*-*p*-toluenesulfonylpyrrolidine-2-^{14}C (XI) (6). Acid hydrolysis of this amide, followed by neutralization and distillation, gave pyrrolidine-2-^{14}C (V) (6) in aqueous solution. Extraction with a solution of ether and petroleum ether followed by addition of an ether–petroleum ether solution of picric acid to the extractions precipitated pyrrolidine-2-^{14}C picrate.

The synthetic procedures reported in the literature were modified for preparing the labeled compounds. These modifications were necessary for ease in handling the small amounts of labeled materials and for obtaining maximum yields. Intermediate products were not isolated in certain reaction steps.

Pyrrolidine picrate was cleaved by aqueous lithium hydroxide (7). Distillation gave the free base in an ether–petroleum ether solution. The cleavage was proved by addition of an ethe solution of picric acid to the ether–petroleum et solution to obtain the starting material.

EXPERIMENTAL

Melting and boiling points are uncorrected. duced pressure refers to the use of a water aspira (8–20 mm.). Petroleum ether refers to the fract



Scheme I

Received April 6, 1964, from the Bionucleonics Department, Purdue University, Lafayette, IN 47907

Accepted for publication April 5, 1967.

The authors thank Rufus T. Dixon, Purdue University Chemistry Department, for his assistance in constructing certain glass apparatus.

This work was supported by the Purdue Research Foundation.

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boiling between 35–65° Fractional distillations were performed with a vacuum jacketed Vigreaux column.

Method *A*

Pyrrolidine-2-^{14}C picrate was prepared starting with potassium cyanide-^{14}C and I. The latter compound was prepared from the corresponding chloride (VII) in 62.7% yield (8). The chloride in turn was synthesized from 3-methoxy-1-propanol in 83.9% yield (9), and the alcohol was prepared from 1,3-propanediol in 64.8% yield (10).

4 - Methoxybutyronitrile - 1 - ^{14}C (II)—A solution containing 16.2 mg. (0.25 mmole, 0.123 mCi./mg.) of potassium cyanide-^{14}C (New England Nuclear Corp., Boston, Mass.), 1.61 Gm. (24.8 mmoles) of unlabeled potassium cyanide, and 4.75 Gm. (23.8 mmoles) of freshly distilled I in 50 ml. of methyl alcohol was refluxed for 30 hr. and cooled. The solvent was removed under reduced pressure, and the residue was washed portionwise with 100 ml. of ether. The washings were filtered by suction, combined, allowed to stand over anhydrous sodium thiosulfate for 12 hr., and decanted. The solvent was removed under reduced pressure, and the residue was distilled yielding 1.42 Gm. (57.4% based on potassium cyanide) of a colorless liquid boiling at 63–64° (10 mm.), n_D^{20} 1.4083; reported (11) b.p. 172–175° (Atm.).

Pyrrolidine-2-^{14}C Picrate (VI)—To 1.36 Gm. (35.9 mmoles) of lithium aluminum hydride in 50 ml. of anhydrous ether at 0–10° was added with stirring a solution containing 1.42 Gm. (14.3 mmoles) of II in 5 ml. of anhydrous ether. When the addition was complete (about 30 min.), two 2-ml. portions of anhydrous ether were added and stirring was continued for 2 hr. at 0–10°. To the mixture at 0–10° were added slowly with rapid stirring 30 drops of water, 23 drops of 20% sodium hydroxide, 5.0 ml. of water, and 20 ml. of ether. Stirring was continued for 30 min., the granular precipitate was filtered by suction, and washed portionwise with 100 ml. of ether, and the washings were combined with the filtrate.

The combined ethereal solution was saturated with hydrogen bromide and extracted with five 10-ml. portions of 10% hydrobromic acid. The extractions were combined and placed in a flask fitted with a fractionating column and adapter set for distillation. The solution was distilled under reduced pressure until about 30 ml. of liquid was removed. To the remaining volume was added 30 ml. of hydrobromic acid (d. 1.49), and the solution was distilled at atmospheric pressure until about 20 ml. of liquid was removed. This procedure was repeated using four 10-ml. portions of hydrobromic acid. The residual solution was then refluxed gently for 15 hr., and 20 ml. of liquid was removed by distillation under reduced pressure. The remaining volume was cooled to 0–10° and 15 ml. of 50% sodium hydroxide was added dropwise with stirring. When the addition was complete, the solution was heated slowly, and the fraction distilling below 95° was collected in a 25-ml. flask immersed in an acetone–dry ice bath and containing 1 Gm. of anhydrous sodium carbonate and 10 ml. of an ether–petroleum ether solution (4:1).

This solution was filtered by suction into a 500-ml. flask. The drying agent was washed portionwise with 100 ml. of an ether–petroleum ether solution (4:1), and the washings were combined with the filtrate. To the combined solution was added, through a filter and with stirring, a solution containing 3.29 Gm. (14.4 mmoles) of picric acid (N.F. grade) in 250 ml. of anhydrous ether. When the addition was complete, the mixture was stirred for 15 min., and the precipitate was filtered by suction. Recrystallization of the pale yellow colored, light solid two times with ethyl propionate (b.p. 98–100°) and *n*-heptane (b.p. 97–98°) yielded 1.41 Gm. (18.8% based on potassium cyanide) of fine, yellow colored needles melting at 113–114°. [Reported (6) m.p. 113°.] The melting point did not change upon further recrystallization of a small sample.

Method *B*

Pyrrolidine-2-^{14}C picrate was prepared starting with barium carbonate-^{14}C and the Grignard reagent of VII.

4 - Methoxy - 1 - butanol - 1 - ^{14}C (IX)—The procedure of Dauben *et al.* (4) for preparing carboxylic acids was followed for preparing 4-methoxybutanoic-1-^{14}C acid (VIII).

Carbonation of 25.8 mmoles of the Grignard reagent of VII, prepared from 4.13 Gm. (0.17 mole) of magnesium and 16.3 Gm. (0.15 mole) of VII, was effected using 123.0 mg. (0.62 mmole, 0.129 mCi./mg.) of barium carbonate-^{14}C (Oak Ridge National Laboratories, Oak Ridge, Tenn.) and 4.50 Gm. (22.8 mmoles) of unlabeled barium carbonate. The reaction mixture was worked up in the usual way; however, the product was not purified.

The crude material was dissolved in 20 ml. of anhydrous ether and added with stirring to 1.03 Gm. (27.1 mmoles) of pulverized lithium aluminum hydride in 40 ml. of anhydrous ether. When the addition was complete (about 15 min.), two 5-ml. portions of anhydrous ether were added, and stirring was continued for 4 hr. at room temperature. The mixture was cooled to 0–10° and decomposed by adding, slowly with stirring, 23 drops of water, 18 drops of 20% sodium hydroxide, and 3.8 ml. of water. Stirring was continued for 15 min., and the granular precipitate was filtered and washed portionwise with 100 ml. of ether. The washings and filtrate were combined, dried over anhydrous potassium carbonate, and decanted. The solvent was removed under reduced pressure, and the residue was fractionated giving 2.04 Gm. (83.7% based on barium carbonate) of a colorless liquid boiling at 77–79° (10 mm.). [Reported (12) b.p. 63–64° (7 mm.).]

1,4-Dibromobutane-1-^{14}C (X)—Hydrogen bromide was bubbled through a solution consisting of 2.04 Gm. (19.6 mmoles) of IX, 7.5 ml. of glacial acetic acid, and 7.5 ml. of water while the temperature was increased slowly to 95°. When the solution was saturated (about 1–1.5 hr.), it was heated for 15 hr. between 90–100°, cooled, and poured into 20 ml. of water. The organic layer was separated and the aqueous layer was extracted exhaustively with petroleum ether. The extractions and organic layer were combined and washed with 5 ml. of 20% sodium hydroxide followed by 10 ml. of water. The aqueous washings were extracted with two 10-ml. portions of petroleum ether. The extractions and organic solutions were combined, dried over anhydrous sodium sulfate, and

decanted. The solvent was removed under reduced pressure, and the residue was distilled. There was obtained 3.47 Gm. (68.6% based on barium carbonate) of a colorless liquid boiling at 83–85° (14 mm.). [Reported (5) b.p. 85° (15 mm.).]

N-p-Toluenesulfonylpyrrolidine-2-^{14}C (XI)—A mixture consisting of 3.47 Gm. (16.1 mmoles) of X, 2.75 Gm. (16.1 mmoles) of *p*-toluenesulfonamide, and 9 ml. of ethyl alcohol was heated with stirring until a clear solution resulted. A solution containing 3.3 ml. of 58% potassium hydroxide in 6.8 ml. of ethyl alcohol (95%) was then added portionwise with stirring over a 9–10-hr. period. When the addition was complete, the mixture was refluxed for 20 hr. and cooled. The alcohol was removed by distillation at atmospheric pressure, and 10 ml. of water was added to the residue. The mixture was poured while still hot into a 250-ml. flask, cooled, and extracted exhaustively with ether. The extractions were combined, and the solvent was allowed to evaporate. The residue was warmed for 20 min. with 15 ml. of 15% sodium hydroxide, filtered by suction, and washed with water. This process was repeated using 10 ml. of 15% sodium hydroxide. The residue was then allowed to dry, washed with 25 ml. of ice-cold ligroin (b.p. 100–115°), allowed to dry, and sublimed under reduced pressure (0.4 mm.). There was obtained 2.78 Gm. (52.7% based on barium carbonate) of a white crystalline powder melting at 120–121°. [Reported (6) m.p. 123°.]

Pyrrolidine-2-^{14}C Picrate (VI)—A mixture consisting of 2.78 Gm. (12.4 mmoles) of XI and 15 ml. of hydrochloric acid (d. 1.19) was heated in a Pyrex bomb tube (19 × 25 × 400 mm.) for 15 hr. between 170–180°. The mixture was then cooled slowly and frozen in an acetone–dry ice bath. The tube was opened, the contents were allowed to liquefy, and 10 ml. of water was added. The organic layer was separated, and the aqueous layer was extracted with three 10-ml. portions of petroleum ether. The extractions and organic layer were combined and washed with two 5-ml. portions of water, and the washings were added to the aqueous layer. Approximately 15–20 ml. of the combined aqueous solution was removed by distillation at atmospheric pressure. The remaining volume was cooled to 0–10°, and 10 ml. of 40% sodium hydroxide was added with stirring. The mixture was distilled at atmospheric pressure until crystals began to form in the distilling flask. The distillate was then collected in a 100-ml. flask immersed in an acetone–dry ice bath and containing 5 Gm. of potassium hydroxide and 5 Gm. of sodium chloride. The distillate was allowed to liquefy and was extracted exhaustively with an ether–petroleum ether solution (4:1). The extractions were combined, dried over anhydrous potassium carbonate, and filtered by suction into a 500-ml. flask containing a magnetic stirring bar. The drying agent was washed portionwise with 100 ml. of an ether–petroleum ether solution (4:1). The washings were combined with the filtrate.

To the ether–petroleum ether extractions was added, through a filter and with stirring, a solution containing 3.27 Gm. (14.3 mmoles) of picric acid in 230 ml. of an ether–petroleum ether solution (9:1). When the addition was complete the mixture was stirred for 15 min., and the precipitate was filtered by suction. Recrystallization of the pale yellow-colored, light solid once with ethyl propionate and *n*-heptane yielded 2.69 Gm. (38.3% based on barium carbonate) of fine, yellow colored needles melting at 113–114°. [Reported (6) m.p. 113°.] The melting point did not change upon further recrystallization of a small sample.

Cleavage of Pyrrolidine Picrate—Pyrrolidine picrate, 1.50 Gm. (5.0 mmoles), was cleaved by the method of Burger (7). The free base was recovered in 68.7% yield as the picrate salt by the procedure described above for preparing the picrate salt.

Radioactivity Determinations—The specific activity of pyrrolidine-2-^{14}C picrate obtained by methods *A* and *B* was determined using a Tri-Carb liquid scintillation spectrometer. The XDC liquid scintillator (13) was used. Counting efficiency was determined using a benzoic-^{14}C acid standard. The specific activities of the products from methods *A* and *B* were 0.203 μCi./mg. and 1.75 μCi./mg., respectively. These specific activities did not change upon further recrystallization.

SUMMARY

Pyrrolidine-2-^{14}C was prepared by two different methods for the purpose of evaluating a suitable isotopic synthesis. Both methods were satisfactory. Method *B* is better than method *A* with respect to chemical yield (38.3% *versus* 18.8%). In addition, method *B* is more economical. Method *A*, however, is less time consuming.

REFERENCES

(1) Hass, H. B., and Marshall, J. R., *Ind. Eng. Chem.*, **23**, 352(1931).
(2) Amundsen, L. H., and Nelson, L. S., *J. Am. Chem. Soc.*, **73**, 242(1951).
(3) Gabriel, S., *Chem. Ber.*, **24**, 3231(1891).
(4) Dauben, W. G., Reid, J. C., and Yankwich, P. E., *Anal. Chem.*, **19**, 828(1947).
(5) Hamonet, J., *Bull. Soc. Chim. France*, **33**, 513(1905).
(6) Muller, A., and Sauerwald, A., *Monatsh. Chem.*, **48**, 155(1927).
(7) Burger, A., *J. Am. Chem. Soc.*, **67**, 1615(1945).
(8) Finkelstein, H., *Chem. Ber.*, **43**, 1528(1910).
(9) Letsinger, R. L., and Schnizer, A. W., *J. Org. Chem.*, **16**, 704(1951).
(10) Smith, L. I., and Sprung, J. A., *J. Am. Chem. Soc.*, **65**, 1276(1943).
(11) Schlinck, J., *Chem. Ber.*, **32**, 947(1899).
(12) Palomaa, M. H., and Jansson, R., *ibid.*, **64**, 1606 (1931).
(13) Bruno, G. A., and Christian, J. E., *Anal. Chem.*, **33**, 1216(1961).

Hydrogenation of 1,1-Diphenyl-4-dimethylamino-2-butyn-1-ol to 1,1-Diphenyl-4-dimethylamino-2-buten-1-ol with a Modified Lindlar Catalyst

By MORRIS FREIFELDER

The selective reduction of 1,1,-diphenyl-4-dimethylamino-2-butyn-1-ol to 1,1-diphenyl-4-dimethylamino-2-buten-1-ol is described using a modified Lindlar catalyst.

THERE ARE many reports in the literature which claim that selective reduction of acetylenic compounds to the corresponding olefins can be achieved by the use of palladium catalyst. Ott and Schroter (1), for example, obtained selectivity with palladium on carbon catalyst. A better method of accomplishing this is to carry out the hydrogenation in the presence of a lead-poisoned palladium catalyst such as the one described by Lindlar (2). However, such a catalyst at times may be inhibited too strongly by the amount of lead present. This point was well illustrated in an attempt in this laboratory to convert 1,1-diphenyl-4-dimethylamino-2-butyn-1-ol (I) to the corresponding 2-buten-1-ol (II).

The hydrogenation of I with commercial palladium on carbon yielded a mixture of products which consisted of over 50% of recovered I, 34% of II, and about 12% of the saturated compound III. When the author used Lindlar catalyst prepared according to the described directions (2), reduction was slow and incomplete. However, when half the amount of lead acetate was added to poison palladium on calcium carbonate during its preparation successful reduction took place.

EXPERIMENTAL[1]

Compound III was prepared as a standard for comparison with some of the reduction products of I.

α-(3-Dimethylaminopropyl)benzhydrol (III)—A solution of 2.65 Gm. (0.01 mole) of 1,1-diphenyl-4-dimethylamino-2-butyn-1-ol (I)[2,3] in 75 ml. of absolute ethyl alcohol was hydrogenated in the presence of 0.03 Gm. of platinum oxide under 2 Atm. pressure. Uptake was complete in about 30 min. After filtration from the catalyst the solution was concentrated to dryness and 2.3 Gm. (85%) of III was obtained, m.p. 118–119°, after recrystallization from hexane; hydrochloride salt, m.p. 197°.[3,4]

Anal.—Calcd. for $C_{18}H_{23}NO$: C, 80.25; H, 8.61; N, 5.20. Found: C, 80.08; H, 8.76; N, 5.14.

1,1-Diphenyl-4-dimethylamino-2-buten-1-ol (II)—A solution of 2.65 Gm. (0.01 mole) of I in 50 ml. of absolute alcohol was hydrogenated in the presence of 0.27 Gm. of Lindlar catalyst prepared as described (2) but modified by the addition of 50% of the amount of lead acetate used in its preparation. The reaction, conducted at 1.25 Atm. pressure, was complete in about 1 hr. After filtration from the catalyst, concentration of the solution, and recrystallization of the residue from hexane, 1.6 Gm. (60%) was obtained, m.p. 93–94° (the crude product melted at 88–91°; yield, 79%).

Anal.—Calcd. for $C_{18}H_{21}NO$: C, 80.83; H, 7.91; N, 5.24; O, 6.02. Found: C, 81.01; H, 7.86; N, 5.31; O, 6.03.

A hydrochloride salt of II was prepared. It melted at 177–178°. Mixed melting points with samples of the hydrochloride of III were greatly depressed.

Hydrogenation of I with Lindlar Catalyst—When the reduction of 2.65 Gm. of I in ethyl alcohol was carried out in the presence of 0.27 Gm. of Lindlar catalyst, prepared in the exact manner as described (2), hydrogen uptake was 12% in 3 hr. Warming produced little additional uptake. Filtration and addition of 0.54 Gm. of Lindlar catalyst and warming induced further reaction in 3 hr. However, uptake stopped of its own accord at about 35% of 1 mole equivalent.

Hydrogenation of I with Palladium on Carbon—The hydrogenation of 0.01 mole of I in alcohol was carried out in the presence of 0.05 Gm. of 5% palladium on carbon and under slight hydrogen pressure, about 0.7 Atm. The low catalyst ratio was resorted to in an effort to keep hydrogen uptake from proceeding beyond 1 mole equivalent. The amount of hydrogen present was only 0.0125 mole, also to prevent overhydrogenation. Uptake was complete in 1 hr. After removal of catalyst, the filtrate was concentrated to dryness. The residual solid was treated with anhydrous ether. The insoluble portion melted between 130–140°. It was shown by infrared examination to be identical to I. The residue, obtained by concentration of the ether filtrate, was recrystallized from pentane. The first crop, 0.3 Gm., melted at 116–120°. It appeared to be a mixture of II and III. Further concentration of the filtrate gave 0.9 Gm. of material melting at 90°. On conversion to the hydrochloride salt, it melted at 166–170°. On recrystallization from alcohol or dry ether the melting point rose to 171–172°. It was odd that when mixed with the hydrochloride salt of III the melting point dropped only to 170°. Nevertheless, on treatment of the salt with aqueous ammonia the base melted at 93–94°, as did the analytical sample of II obtained from the reduction of I with the modified Lindlar catalyst. Elemental analysis of the base, however, showed low carbon, but higher hydrogen values to suggest that it was contaminated with III.

Received October 27, 1966, from the Organic Chemical Research Department, Research Division, Abbott Laboratories, North Chicago, IL 60064

Accepted for publication March 22, 1967.

[1] The author is indebted to Mr. Orville Kolsto and his group for the microanalyses and to Mr. A. Kammer and Mr. W. Washburn for infrared examination of some of the compounds.

[2] Preparation described by Libman, N. M., and Kuznetsov, S. G., *Zh. Obshch. Khim.*, **30**, 1197(1960); through *Chem. Abstr.*, **55**, 452(1961).

[3] *Footnote 2* by Croxall, W. J., and Dawson, J. W., U. S. pat. 2,584,429(1952).

[4] Ose, M., and Kaneko, H., Japanese pat. 8316-7(1959); through *Chem. Abstr.*, **54**, 17335(1960) give 118° for base and 201.5–202° for hydrochloride salt. Croxall and Dawson (see *Footnote 3*) give 122–123.5°.

REFERENCES

(1) Ott, E., and Schroter, R., *Ber.*, **60**, 624(1927).
(2) Lindlar, H., *Helv. Chim. Acta*, **35**, 446(1952).

Examination of Structural Effect on Thin-Layer Chromatographic Multiple Spot Formation of Sympathomimetic Amines in the Presence of Hydrochloric Acid

By N. H. CHOULIS

From a number of solutions of sympathomimetic amines in (*a*) water or (*b*) 10 *N* hydrochloric acid, chromatographed on thin layer of alumina, cellulose, or silica gel using a water–hydrochloric acid–phenol solvent system, only two produced multiple spots when the solvent system and an amine solution in excess hydrochloric acid, on cellulose layers, were used. The appearance of multiple spots does not depend upon the chemical structure of the compounds used, since the phenomenon was not reproduced when alumina or silica gel layers were used. The combination of adsorption and partition forces, the presence of the carboxyl groups in prepared cellulose, and the continuity of the thin layer are the main factors leading to the formation of multiple spots.

THE PHENOMENON of multiple spot formation of sympathomimetic amines when paper or cellulose thin layers are used has often been reported. Shepherd and West (1) observed that adrenaline, liberated from tissues using trichloroacetic acid, gave two spots when chromatographed in various solvent systems; they suggested that the multiple spot formation is due to the presence of trichloroacetic acid.

West (2) also observed double spots with trichloroacetic acid and tryptamine derivatives of sympathomimetic amines, and later Beckett *et al.* (3, 4) showed that the spot deformation and multiplication occurs with many bases in the presence of many diverse acids. Robinson and Shepherd (5) considered that the formation of the additional amine spot which was obtained in the presence of trichloroacetic acid is assisted by the formation of loose complexes between the bases, acting as electron donors, and the trichloroacetic acid, acting as electron acceptor by virtue of the marked electronegativity of the three chlorine atoms.

Roberts (6) also observed the phenomenon of multiple spot formation in an attempt to examine some factors which were affecting the R_f values of sympathomimetic amines, but on paper chromatography only. It was found that hydrochloric acid present in the developing solvent converted some of the norepinephrine acid tartrate to norepinephrine hydrochloride. The presence or absence of multiple spots, according to Roberts (7), depends upon the chemical structure of each amine.

The application of thin-layer chromatography for the examination of these phenomena (8–10) indicated that, just as in paper chromatography, it is possible to obtain two amine spots of an amine salt (or pure amine in the presence of one or more equivalents of acid) when a neutral or acidic solvent system is used in thin-layer chromatography involving cellulose as the thin layer.

EXPERIMENTAL

Materials—The amines used were: amphetamine hydrochloride, norepinephrine hydrochloride, normetanephrine hydrochloride, metanephrine hydrochloride, dopamine, ephedrine hydrochloride, isopropylarterenol hydrochloride, nordefrin hydrochloride, dopa, epinephrine, and phenylephrine hydrochloride.

Developing Systems—(*a*) Phenol containing 15% v/v of 0.1 *N* hydrochloric acid in water. (*b*) *n*-Butanol–acetic acid–water (4:1:5 v/v); the liquids were mixed together, left aside overnight, separated, and the organic layer was used.

Detection Methods—The spots were detected using either (*a*) a solution of 0.6 Gm. of potassium ferrocyanide and 0.5 Gm. of sodium hydroxide in 100 ml. of water or (*b*) ninhydrin in butanol (0.2% w/v) and heating the chromatograms at 100° for 3 min.

Thin-Layer Plates—All plates used were 20 × 20 cm.

Using MN 300 G cellulose powder (Machery, Nagel and Co.), Silica Gel G (Merck), and aluminum oxide G (Merck), plates were prepared according to Stahl (11).

The plates were kept in a desiccator using calcium chloride and reactivated at 100° for 5 min. before use.

General Method—The amines, 0.1-μl. quantities, were chromatographed on alumina, cellulose, or silica gel plates from freshly prepared solutions (10 mg./ml.) in distilled water or 10 *N* hydrochloric acid. The solutions were subjected to further chromatographic development at intervals of 1 day, 2 days, 1 week, 3, 6, and 8 weeks.

All chromatograms were carried out at room temperature (22–24°) and until the solvent front

TABLE I—RESULTS USING CELLULOSE THIN-LAYER PLATES

Amines	R_f Values[a] Freshly Prepared Soln.	Aged Soln.
Amphetamine HCl	84 ± 0.05	85 ± 0.05
Normetanephrine HCl	67 ± 0.05	66 ± 0.05
Metanephrine HCl	80 ± 0.03	79 ± 0.05
Dopamine	40 ± 0.02	44 ± 0.03
Arterenol HCl	35 ± 0.05– 12 ± 0.03	36 ± 0.03– 12 ± 0.03
Ephedrine HCl	93 ± 0.02	93 ± 0.02
Isopropylarterenol	76 ± 0.04	76 ± 0.04
Nordefrin HCl	42 ± 0.01	45 ± 0.02
Dopa	43 ± 0.01	36 ± 0.02
Epinephrine	62 ± 0.05– 35 ± 0.02	64 ± 0.01– 36 ± 0.01
Phenylephrine HCl	79 ± 0.02	81 ± 0.01

[a] R_f values and number of spots of various sympathomimetic amines in 10 *N* hydrochloric acid solution, when chromatographed in water–hydrochloric acid–phenol solvent system.

Received January 6, 1967, from the School of Pharmacy, Texas Southern University, Houston, TX 77004
Accepted for publication March 5, 1967.

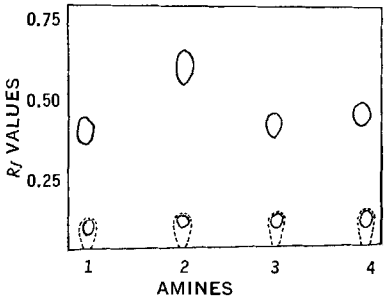

Fig. 1—TLC of certain amines freshly prepared in 10 N *HCl, on cellulose, when* n*-butanol–acetic acid–water solvent system is used. Continuous line indicates amines. Dotted line indicates acid. Key: 1, arterenol HCl; 2, isopropylarterenol HCl; 3, dopa; 4, epinephrine.*

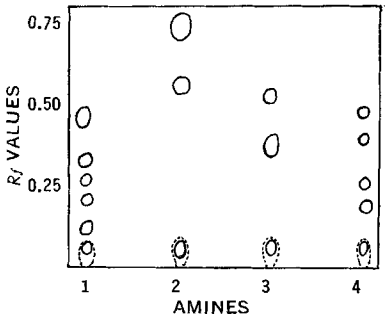

Fig. 2—TLC of certain amines as aged solutions in 10 N *HCl, on cellulose, when* n*-butanol–acetic acid–water solvent system is used. Continuous line indicates amines. Dotted line indicates acid. Key: 1, arterenol HCl; 2, isopropylarterenol HCl; 3, dopa; 4, epinephrine.*

was advanced 10 cm. (65 to 75 min.). The chromatoplates were removed from the solvent, air dried, and the detecting reagents were used to locate the spots.

The R_f values were measured from the center of the spots.

RESULTS

Using silica gel thin-layer plates, freshly prepared aqueous solutions of all amines used produced one spot. The spots, with the exception of arterenol (R_f value 0.75) and dopa (R_f value 0.81), were moved with the solvent front. A similar pattern was observed for the same solutions when chromatographed in intervals of up to an 8-week period, with the exception of dopa which gave an elongated spot, when an 8-week-old solution was chromatographed.

In the presence of 10 *N* hydrochloric acid, the amine spots were completely separated from the acid spots and moved with the solvent front, with the exception of arterenol and dopa, where the spots were moved faster (about 0.8 cm.) than those of the free amines. Hydrochloric acid, in all cases, gave elongated spots, having an R_f value of 0.53 ± 0.03.

However, when an 8-week-old solution was chromatographed, only dopa gave an elongated amine spot having the same R_f value as that of the free base.

In all cases where alumina thin-layer plates were employed, no movement of the amine spots was observed.

The results when cellulose thin-layer plates were used are summarized in Table I.

These results indicated that only arterenol and epinephrine appeared to produce a second spot when the 10 *N* hydrochloric acid solution was used. The second spot was observed in a freshly prepared as well as in an aged solution.

Using *n*-butanol–acetic acid–water solvent system and the cellulose thin layer, arterenol, isopropylarterenol, dopa, and epinephrine gave two discrete spots, when a fresh solution of the amines in 10 *N* hydrochloric acid was chromatographed (Fig. 1). However, when an 8-week-old solution of the amines in 10 *N* hydrochloric acid was used, isopropylarterenol and dopa gave three spots, while epinephrine gave five; six spots were observed during the development of arterenol (Fig. 2).

On alumina or silica gel thin layers and the above solvent system, only elongated spots were detected.

DISCUSSION

From the results obtained, it is noted that when the water–hydrochloric acid–phenol solvent system and alumina thin layers were used, only one spot of the utilized amines was always produced in the presence of 10 *N* hydrochloric acid. The amine spots remained at the starting line, which indicates that the spots were unaffected by the presence of even an excess of acid.

In thin-layer chromatograms with alumina, adsorption forces are operating exclusively.

The results, using silica gel thin layers and the same solvent system as above, showed that the amine spots moved faster than when alumina layers were used, and that most of them followed the solvent front. However, single spots of the amines are obtained, with the exception of dopa, which in an 8-week-old solution, either in water or 10 *N* hydrochloric acid, gave an elongated spot.

With silica gel thin layers, partition effect will in general be more important than adsorption forces.

In a thin layer of cellulose, both partition and adsorption forces exist and their relative role depends upon the conditions (8–10).

Since the added acid has a negligible effect on the movement of the amines on alumina, the effect of single adsorption forces does not account for the multiple spots of amines in cellulose (arterenol and epinephrine). On silica gel plates, the added acid has an effect on the movement of the amines and it seems probable that the added acid, as it moves along the plate, has a substantial effect on the partition of the amines between the mobile solvent and the stationary phase, but two spots were not produced on silica gel layers.

However, using the *n*-butanol–acetic acid–water solvent system and cellulose thin layers, most of the amines chromatographed developed a second spot when in a freshly prepared 10 *N* hydrochloric acid solution; moreover, the number of the spots was increased with the age of the acid solution, presumably due to the oxidation products of the amines which were developed during the prolonged presence of the acid of the solution, leading to the formation of the additional spots.

It is suggested that the formation of double spots does not depend upon the structure of the amine,

since amines giving two spots in one acidic solvent (*i.e.*, *n*-butanol–acetic acid–water) give only one spot in another (*i.e.*, water–hydrochloric acid–phenol). Moreover, amines which appeared to give six or more spots when chromatographed from freshly prepared solutions in 10 *N* hydrochloric acid on cellulose paper (7) gave only two spots when chromatographed from the same freshly prepared solution on cellulose thin layer. In the former, a gradual decrease, while in the latter, a gradual increase in the number of the spots with the age of the solution was observed.

It is therefore concluded that the formation of two amine spots, when cellulose thin layers are used, results from the following factors: (*a*) a continuity of adsorption forces along the direction of the mobile solvent on cellulose thin layers (9, 10), (*b*) a combination of adsorption and partition forces, and (*c*) the presence of the carboxyl groups in the prepared cellulose. That the presence of the carboxyl groups in the prepared cellulose is leading to the formation of double spots, has been previously discussed (10) and therefore further discussion here is unnecessary.

This investigation shows that the presence of more than one spot in cellulose chromatograms, using extracted biological material, does not necessarily indicate that two or more amines are present.

REFERENCES

(1) Shepherd, D. M., and West, G. B., *Nature*, **169**, 797 (1952).
(2) West, G. B., *J. Pharm. Pharmacol.*, **11**, 595(1959).
(3) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *Nature*, **186**, 775(1960).
(4) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *J. Pharm. Pharmacol., Suppl.*, **12**, 203(1961).
(5) Robinson, H., and Shepherd, D. M., *ibid.*, **13**, 374 (1961).
(6) Roberts, D. J., *ibid.*, **16**, 549(1964).
(7) *Ibid.*, **18**, 182(1966).
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By JOSEPH SAM and A. J. BEJ

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THE POTENTIAL of *dl*-1-(4-hydroxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (V*a*) as an intermediate to medicinal agents prompted an investigation of its synthesis. The levorotatory base (V*a*) was first isolated by Kupchan and coworkers (1, 2) from *Nelumbo lutea* and named (−)-*N*-norarmepavine. The alkaloid subsequently was isolated by Tomita, Yang, and Lu (3), and by Yang (4).

A total synthesis of V*a* *via* the Bischler-Napieralski reaction has been reported by Yamaguchi and Nakano (5). The benzyl group was utilized for the protection of the phenolic hydroxyl group. This report describes the use of the carboethoxy protecting group in the preparation of V*a* and V*b*.

The requisite amide (III*b*) for the Bischler-Napieralski reaction was prepared either from III*a* or by the condensation of I with the acid chloride (6) of II*b*. The cyclization of III*b* to IV*a* was accomplished with phosphorus oxychloride. The low pressure reduction of IV*a* followed by hydrolysis gave V*a*. Alternately, V*a* was prepared by the hydrolysis of IV*a* to IV*b*, followed by sodium borohydride reduction. It was observed also that sodium borohydride converts IV*a* directly to V*a*. Treatment of V*a* with formic acid and formaldehyde provided *dl*-armepavine (V*b*). Since V*a* was converted to *dl*-armepavine (V*b*), as described above, this sequence also comprises a total synthesis of the alkaloid (Scheme I.)

The nuclear magnetic resonance (NMR) spectrum of V*a* in deuteriochloroform showed six aromatic protons (τ = 3.0–3.4), two labile protons (τ = 5.4, by D_2O exchange), one tertiary proton (quadruplet centered at τ 5.85), and six methoxyl protons (broad doublet centered at τ 7.05), which is in good agreement with the reported NMR spectrum of (−)-*N*-norarmepavine (2).

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EXPERIMENTAL[1]

N-β-(3,4-Dimethoxyphenethyl)-4-hydroxyphenylacetamide (IIIa)—A mixture of 3.6 Gm. (0.02 mole) of homoveratrylamine, 3.0 Gm. (0.02 mole) of *p*-hydroxyphenylacetic acid, 10 ml. of dry decalin, and 10 ml. of tetralin was refluxed at 180–185° for 8 hr. The solvent was decanted from the semisolid; the last traces of solvent were removed by steam distillation. The residual solid was washed successively with 10% sodium bicarbonate, water,

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since amines giving two spots in one acidic solvent (*i.e.*, *n*-butanol–acetic acid–water) give only one spot in another (*i.e.*, water–hydrochloric acid–phenol). Moreover, amines which appeared to give six or more spots when chromatographed from freshly prepared solutions in 10 *N* hydrochloric acid on cellulose paper (7) gave only two spots when chromatographed from the same freshly prepared solution on cellulose thin layer. In the former, a gradual decrease, while in the latter, a gradual increase in the number of the spots with the age of the solution was observed.

It is therefore concluded that the formation of two amine spots, when cellulose thin layers are used, results from the following factors: (*a*) a continuity of adsorption forces along the direction of the mobile solvent on cellulose thin layers (9, 10), (*b*) a combination of adsorption and partition forces, and (*c*) the presence of the carboxyl groups in the prepared cellulose. That the presence of the carboxyl groups in the prepared cellulose is leading to the formation of double spots, has been previously discussed (10) and therefore further discussion here is unnecessary.

This investigation shows that the presence of more than one spot in cellulose chromatograms, using extracted biological material, does not necessarily indicate that two or more amines are present.

REFERENCES

(1) Shepherd, D. M., and West, G. B., *Nature*, **169**, 797 (1952).
(2) West, G. B., *J. Pharm. Pharmacol.*, **11**, 595(1959).
(3) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *Nature*, **186**, 775(1960).
(4) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *J. Pharm. Pharmacol., Suppl.*, **12**, 203(1961).
(5) Robinson, H., and Shepherd, D. M., *ibid.*, **13**, 374 (1961).
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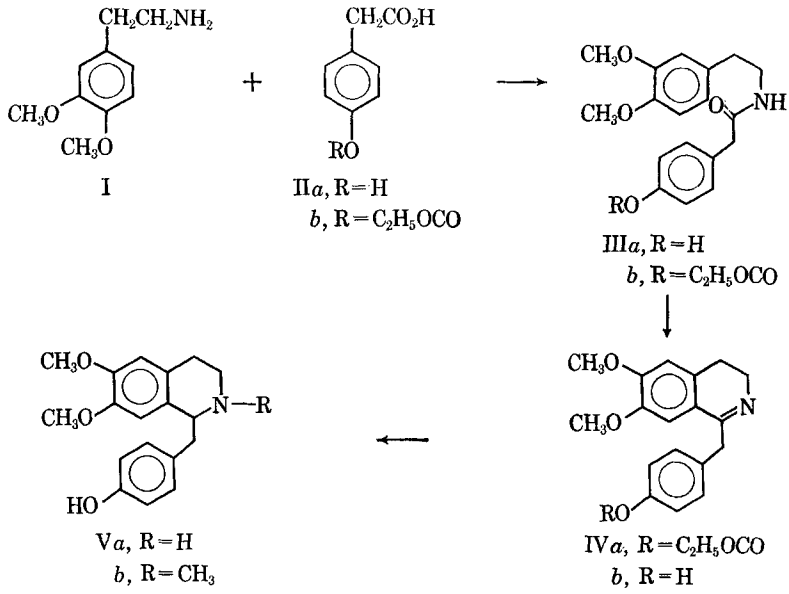

Scheme I

10% hydrochloric acid, and water. The recrystallization of the residual material from ethanol gave 5.0 Gm. (81%) of an amorphous solid, m.p. 153–155°. The infrared spectrum possessed an absorption peak at 6.1 μ characteristic of an amide function. The methoxy analog was prepared in the usual manner and recrystallized from ethanol, m.p. 123–124°. [Lit. (7) m.p. 123–125°).]

N-β-(3,4-Dimethoxyphenethyl)-4-ethoxycarbonyloxyphenylacetamide (IIIb)—*Method A*—A solution of 3.1 Gm. (0.01 mole) of *N*-β-(3,4-dimethoxyphenethyl)-4-hydroxyphenylacetamide (III*a*) in 45 ml. of 5% potassium hydroxide was treated slowly, with shaking, with 1.1 Gm. (0.01 mole) of ethyl chloroformate. The mixture was shaken for 15 min. and then filtered to remove the solid product. The filtrate again was treated with 1.1 Gm. (0.01 mole) of ethyl chloroformate as above. The combined solid product was washed with a copious amount of water and then recrystallized from a mixture of benzene and petroleum ether (30–60°). There was obtained 3 Gm. (81%) of feathery needles, m.p. 95–96°. The infrared spectrum possessed two carbonyl absorption peaks at 5.8 μ and 6.1 μ, characteristic of esters and amides, respectively.

Anal.—Calcd. for $C_{21}H_{25}NO_5$: C, 65.1; H, 6.5; N, 3.6. Found: C, 65.3; H, 6.5; N, 3.8.

Method B—A solution of 4-ethoxycarbonyloxyphenylacetyl chloride (6) [from 5 Gm. (0.022 mole) of the acid] in 15 ml. of tetraethylene glycol dimethyl ether was added dropwise at approximately 16° to a stirred mixture of 3.6 Gm. (0.02 mole) of homoveratrylamine, a solution of 2 Gm. of sodium hydroxide in 6 ml. of water, and 15 ml. of tetraethylene glycol dimethyl ether. During the mixing the temperature rose to about 30°. After the addition was complete (5 min.), the reaction mixture was poured into 100 ml. of cold water and adjusted to pH 10. The crude product was removed by filtration and washed with water. The recrystallization of the crude material from a mixture of benzene and petroleum ether (30–60°) gave 2.5 Gm. (31%) of product, m.p. 95–96°. A mixed melting point with the product obtained from method *A* showed no depression.

1-(4-Ethoxycarbonyloxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline Hydrochloride (IVa)—The method described by Finkelstein (6) was followed using 2.6 Gm. (0.007 mole) of *N*-β-(3,4-dimethoxyphenethyl)-4-ethoxycarbonyloxyphenylacetamide (III*b*), 30 ml. of dry toluene, and 1.5 Gm. (0.01 mole) of phosphorus oxychloride. The toluene was decanted from the reaction mixture and the unreacted phosphorus oxychloride was distilled under reduced pressure. The residual solid was recrystallized from ethanol to give 2.2 Gm. (76%) of product, m.p. 210–211°.

Anal.—Calcd. for $C_{21}H_{24}ClNO_5$: C, 62.1; H, 6.0; Cl, 8.7; N, 3.5. Found: C, 62.1; H, 6.1; Cl, 8.9 N, 3.4.

1-(4-Hydroxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline Hydrochloride (IVb)—Six grams (0.015 mole) of 1-(4-ethoxycarbonyloxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline hydrochloride (IV*a*) was heated on a steam bath with 70 ml. of 20% hydrochloric acid until evolution of carbon dioxide ceased (90 min.). The solvent was distilled under reduced pressure. The residual semisolid was treated with 200 ml. of dry benzene and distilled to remove the water. The residual solid (m.p. 195–200°, 4.7 Gm., 94%) was triturated with 30 ml. of acetone and recrystallized from methanol, m.p. 202.5–204°.

Anal.—Calcd. for $C_{18}H_{20}ClNO_3$: Cl, 10.6; N, 4.2. Found: Cl, 10.1; N, 3.9.

dl-1-(4-Hydroxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (dl-N-Norarmepavine) (Va)—*Method A*—A solution of 2.0 Gm. (0.005 mole) of 1-(4-ethoxycarbonyloxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline hydrochloride (IV*a*) in 50 ml. methanol was treated with 50 mg. of PtO_2 and hydrogenated at 25° for 1.7 hr. The product was isolated in the usual manner. The crude hydrogenation product was treated with 5 ml. of 15% hydrochloric acid and heated on a steam bath for 20 min. The acid solution was cooled to room

temperature and refrigerated for 10 hr. The hydrochloride (0.8 Gm., 50%, m.p. 194–196°) was removed by filtration and recrystallized twice from a mixture of methanol and ethyl acetate, m.p. 194–196°.

Anal.—Calcd. for $C_{18}H_{22}ClNO_3$: C, 64.4; H, 6.6; Cl, 10.5; N, 4.1. Found: C, 64.2; H, 6.6; Cl, 10.5; N, 4.3.

The free base was prepared in the usual manner and recrystallized from a mixture of ethyl acetate and *n*-hexane, m.p. 99–101°, softens around 94°. [Lit. (5) m.p. 95–96°.] The oxalate was prepared in the usual manner and recrystallized from methanol, m.p. 214–215°. [Lit. (5) m.p. 202–204°.]

Method B—A mixture of 1.6 Gm. (0.005 mole of 1 - (4 - hydroxybenzyl) - 6,7 - dimethoxy - 3,4 - dihydroisoquinoline hydrochloride (IV*b*), 50 ml. of methanol, and 3.5 Gm. of sodium borohydride was stirred for 30 min. The reaction mixture was treated with 2 ml. of water and refluxed with stirring on a steam bath for 1.7 hr. Most of the methanol was distilled under reduced pressure. The residue was poured onto a mixture of crushed ice and water (250 Gm.). The mixture was adjusted to pH 8 and extracted with three 50-ml. portions of ether. The ether extract was dried over anhydrous sodium sulfate. The evaporation of the ether left 1 Gm. (62%) of an oil. The hydrochoride was prepared in the usual manner and recrystallized from a mixture of methanol and ethyl acetate, m.p. 194–196° A mixed melting point with a sample prepared by method *A* showed no depression. The oil was crystallized from a mixture of ethyl acetate and hexane or from acetone to give 0.5 Gm. (31%) of an amorphous solid, m.p. 99–101°, softens around 94°. The infrared spectrum in chloroform of this sample was identical with the infrared spectrum of the sample obtained by method *A*. The oxalate was prepared in the usual manner and recrystallized from methanol, m.p. 214–215°.

Method C—A solution of 4.0 Gm. (0.01 mole) of 1 - (4 - ethoxycarbonyloxybenzyl) - 6,7 - dimethoxy-3,4-dihydroisoquinoline hydrochloride (IV*a*) in 100 ml. of methanol was reduced with 10 Gm. of sodium borohydride and worked up as described in method *B*. The free base (1 Gm., 34%), the hydrochloride, and the oxalate were identical (mixed melting point showed no depression) with the samples obtained by methods *A* and *B*.

dl-Armepavine (Vb)—A mixture of 0.5 Gm. (0.0017 mole) of *dl*-1-(4-hydroxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (V*a*), 6 ml. of 98% formic acid, and 6 ml. of 40% formalin was heated under reflux for 6 hr. The mixture was diluted with water, adjusted to pH 9, and extracted three times with 50-ml. portions of chloroform. The chloroform extract was washed with water and dried over anhydrous sodium sulfate. The distillation of the chloroform under reduced pressure left an oil which was chromatographed on a column of Woelm neutral alumina (activity I) with acetone as the eluent. From the acetone eluent was obtained an oil which was crystallized from a mixture of ethyl acetate and hexane to yield 0.15 Gm. (29%) of *dl*-armepavine, m.p. 158.5–160.5°. [Lit. (8, 9) m.p. 159–161°.]

REFERENCES

(1) Kupchan, S. M., DasGupta, B., Fujita, E., and King, M. L., *J. Pharm. Sci.*, **51**, 599(1962).
(2) Kupchan, S. M., DasGupta, B., Fujita, E., and King, M. L., *Tetrahedron*, **19**, 227(1963).
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(5) Yamaguchi, H., and Nakano, K., *Yakugaku Zasshi*, **79**, 1106(1959); through *Chem. Abstr.*, **54**, 4637(1960).
(6) Pinkelstein, J., *J. Am. Chem. Soc.*, **73**, 550(1951).
(7) Kondo, H., and Kondo, T., *J. Pharm. Soc. Japan*, **48**, 324(1928); through *Chem. Abstr.*, **22**, 3414(1928).
(8) Marion, L., Lemay, L., and Portelance, V., *J. Org. Chem.*, **15**, 216(1950).
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[1] Melting points (uncorrected) were determined on a Kofler block. Rotations wereme asured at 20°. The ultraviolet spectrum was recorded in solution in absolute ethanol on a SP 700 spectrophotometer. Infrared spectra were recorded on a Perkin Elmer 137 spectrophotometer.

(Maule) were dried at 80–90°. A 1550-Gm. quantity of dried and ground plant was extracted in a Soxhlet extractor with petroleum ether (b.p. 65–75°) to exhaustion. This dark solution was concentrated to yield 50 Gm. of a dark green product. This product was refluxed for 1 hr. in a 5% sodium hydroxide ethanolic solution. The neutral product was found to be a polymethylenic alcohol of low melting point. The acidic fraction yielded, after several recrystallizations, 100 mg. of a compound, m.p. 273–275°; $[\alpha]_D^{20°}$ + 61° (pyridine c 0.45).

Alcoholic Extract—The defatted plant material was dried and the ethanol-soluble constituents were extracted. This dark solution was concentrated to yield 415 Gm. of a water-soluble product. This product was treated with 2.5 *N* ethanolic hydrochloric acid. The mixture was then refluxed for 5 hr. and poured into ice water. After 48 hr. the precipitate was collected on a filter and washed with water, yielding 215 Gm. of a light green mass. The crude ursolic acid was partially purified by continuous extraction, first with petroleum ether (b.p. 65–75°), then with a mixture of petroleum ether–benzene and, finally, with benzene. After recrystallizations of the product extracted with the mixture petroleum ether–benzene and benzene, 450 mg. of an acid, m.p. 267–279°, was obtained.

Ursolic Acid—Several recrystallizations of the previously obtained compound yielded ursolic acid, m.p. 281°; $[\alpha]_D^{20°}$ +61° (pyridine c 0.6), $\nu_{max.}^{KCl}$ 3350 and 1695 cm.$^{-1}$.

Ursolic Acid Acetate—The pure compound was obtained as needles after crystallization from methanol–chloroform, m.p. 281°.

Ursolic Acid Methyl Ester—This ester was prepared by diazomethane treatment of the acid in ethereal solution, crystallized from methanol–chloroform as colorless crystals, m.p. 170°; $\lambda_{max.}^{EtOH}$ 206 mμ ε 5000, $\nu_{max.}^{CHCl_3}$ 3410, 1754, and 1642 cm.$^{-1}$.

The melting point of this compound was undepressed upon admixture with an authentic specimen, m.p. 171°, and was found to be identical with the methyl ester of the acidic compound, m.p. 273–275°, previously isolated from the petroleum ether extract.

Ursolic Acid Methyl Ester Acetate—It was obtained as needles after crystallization from methanol–chloroform, m.p. 243–244°; $[\alpha]_D^{20°}$ +60° (chloroform c 0.48).

REFERENCES

(1) Giral, F., and Silva, M., *Ciencia Mex.*, **18**, 30(1958).
(2) Silva, M., and Mancinelli, P., *Bol. Soc. Chilena Quim.*, **9**, 49(1959).
(3) Silva, M., and Dujisin, K., *ibid.*, **10**, 23(1960).
(4) Silva, M., and Mancinelli, P., *J. Pharm. Sci.*, **50**, 975 (1961).
(5) Silva, M., Mancinelli, P., and Cheul, M., *ibid.*, **51**, 289 (1962).
(6) Silva, M., and Stück, R., *Arch. Pharm.*, **295**, 1, 5 (1962).
(7) Silva, M., Stück, R., and Chavez, P., *ibid.*, **295**, 7, 515(1962).
(8) Silva, M., Stück, R., and Mancinelli, P., *Bol. Soc. Chilena Quim.*, **12**, 29(1962).
(9) Moyano, I., Tésis de la Escuela de Educación, Universidad de Chile, 1962.

Synthesis and Basic Pharmacology of *N*-Substituted and *N,N'*-Disubstituted Allyl Barbiturates

By M. H. WEINSWIG, A. M. BURKMAN*, W. D. JONES, and E. J. ROWE

***N*-Substituted and *N,N'*-disubstituted allyl derivatives of barbital, phenobarbital, hexobarbital, and diallylbarbital have been synthesized by the use of a strongly basic anion-exchange resin. The barbiturate is first absorbed on the resin and the resin then agitated mechanically with an ethanol solution of benzyl chloride. None of the allyl barbiturates approached phenobarbital in terms of pharmacological potency.**

IN PREVIOUS reports (1, 2) a method was described for the synthesis of benzyl ethers of a variety of phenols and of 5,5-disubstituted barbiturates by the use of a strongly basic anion-exchange resin. The method involved the reaction of the phenolate or the barbiturate form of the resin with benzyl chloride. The synthesis of the *N*-allyl and the *N,N'*-diallyl barbiturate derivatives seemed feasible, using this method, since the allyl and the benzyl halides are believed equally reactive chemically. This report presents an analogous method for the synthesis of *N*-allyl and *N,N'*-diallyl derivatives of four 5,5-disubstituted barbituric acids (phenobarbital, barbital, hexobarbital, and diallylbarbital) and the synthesis of 5,5-diallylbarbituric acid.

SYNTHESIS

The synthesis of these derivatives involved the reaction of the barbiturate form of a strongly basic anion-exchange resin with allyl bromide. The resin employed was a polystyrene polymer containing reactive quaternary ammonium groups. Proceeding at room temperature, a mixture of *N*-allyl and *N,N'*-diallyl derivatives was obtained from the reaction. Separation of the derivatives was effected by taking advantage of the solubility of the *N*-allyl derivative in dilute alkali. Table I presents the analytical data for allyl derivatives of barbituric acid, phenobarbital (5-ethyl-5-phenyl barbituric acid), barbital (5,5-diethyl barbituric acid), and hexobarbital (*N*-methyl-5-methyl-5-cyclohexenyl barbituric acid) prepared by the use of a strongly basic anion-exchange resin. The *N,N'*-diallyl derivative is the main product when an equivalency of the resin in the OH form is used with the barbiturate form and the quantity of the allyl bromide is doubled plus 10% excess.

Resin Preparation—Synthetic ion-exchange resin made from styrene-divinylbenzene copolymer[1] supplied commercially in the chloride form (20–50

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Accepted for publication April 20, 1967.
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(Maule) were dried at 80–90°. A 1550-Gm. quantity of dried and ground plant was extracted in a Soxhlet extractor with petroleum ether (b.p. 65–75°) to exhaustion. This dark solution was concentrated to yield 50 Gm. of a dark green product. This product was refluxed for 1 hr. in a 5% sodium hydroxide ethanolic solution. The neutral product was found to be a polymethylenic alcohol of low melting point. The acidic fraction yielded, after several recrystallizations, 100 mg. of a compound, m.p. 273–275°; $[\alpha]_D^{20°}$ + 61° (pyridine c 0.45).

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REFERENCES

(1) Giral, F., and Silva, M., *Ciencia Mex.*, **18**, 30(1958).
(2) Silva, M., and Mancinelli, P., *Bol. Soc. Chilena Quim.*, **9**, 49(1959).
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(4) Silva, M., and Mancinelli, P., *J. Pharm. Sci.*, **50**, 975 (1961).
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By M. H. WEINSWIG, A. M. BURKMAN*, W. D. JONES, and E. J. ROWE

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SYNTHESIS

The synthesis of these derivatives involved the reaction of the barbiturate form of a strongly basic anion-exchange resin with allyl bromide. The resin employed was a polystyrene polymer containing reactive quaternary ammonium groups. Proceeding at room temperature, a mixture of *N*-allyl and *N,N'*-diallyl derivatives was obtained from the reaction. Separation of the derivatives was effected by taking advantage of the solubility of the *N*-allyl derivative in dilute alkali. Table I presents the analytical data for allyl derivatives of barbituric acid, phenobarbital (5-ethyl-5-phenyl barbituric acid), barbital (5,5-diethyl barbituric acid), and hexobarbital (*N*-methyl-5-methyl-5-cyclohexenyl barbituric acid) prepared by the use of a strongly basic anion-exchange resin. The *N,N'*-diallyl derivative is the main product when an equivalency of the resin in the OH form is used with the barbiturate form and the quantity of the allyl bromide is doubled plus 10% excess.

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TABLE I—ANALYSIS OF THE *N*-ALLYL AND *N,N'*-DIALLYL BARBITURATES

Compd.	Yield, %	M.p., °C.[b]	Empirical Formula	Anal.,[a] % Calcd.	Found
N-Allylbarbital	47	80–81[c]	$C_{11}H_{10}N_2O_3$	C, 58.93 H, 7.14 N, 12.50	59.13 7.40 12.46
N,N'-Diallylbarbital	30	120.5–121.5[d]	$C_{14}H_{20}N_2O_3$	C, 63.64 H, 7.57 N, 10.60	63.71 7.77 10.95
N-Allylphenobarbital	37	73.0–73.5[e]	$C_{15}H_{16}N_2O_3$	C, 66.14 H, 5.87 N, 10.29	65.92 5.96 10.24
N,N'-Diallylphenobarbital	35	40[f]	$C_{18}H_{20}N_2O_3$	C, 69.23 H, 6.41 N, 8.94	69.23 6.46 8.75
N-Allylhexobarbital	70	75–76[g]	$C_{15}H_{20}N_2O_3$	C, 65.19 H, 7.30 N, 10.14	65.43 7.33 9.94
5,5-Diallylbarbituric acid	45	169–170[h]	$C_{10}H_{12}N_2O_3$	C, 57.69 H, 5.77 N, 13.46	57.63 5.84 13.70
5,5,*N,N'*-Tetraallylbarbituric acid	25	148.5–149[i]	$C_{16}H_{20}N_2O_3$	C, 66.67 H, 6.94 N, 10.00	66.96 7.09 10.25

[a] Analyses were performed by Midwest Microlabs, Indianapolis, Ind., and Weiler and Strauss, Oxford, England. [b] Melting points were determined on a Fisher-Johns melting point apparatus. [c] Literature (4) 80°. [d] Literature (4) b.p. 156–157°. [e] Literature (4) 68–69°. [f] Literature (4) 40°. [g] Literature (4) 72°. [h] Literature (5) 169°. [i] Not reported in literature.

mesh), was converted to its hydroxyl form by the usual column technique with 5% aqueous sodium hydroxide (2, 3).

Absorption of the Barbiturate on the Resin—A general procedure was followed. The barbiturate as the sodium salt (0.05 mole) was dissolved in 300 ml. of distilled water and passed through a resin column 25 mm. in diameter. The resin was first rinsed with distilled water until the washings were neutral to phenolphthalein T.S., then with two 100-ml. portions of ethanol.

Synthesis of Allyl Barbiturates—A general procedure was followed for the synthesis of all allyl barbiturates. A description of the preparation of *N*-allyl and *N,N'*-diallylbarbital illustrates the procedure used.

N-Allylbarbital—A 4.1-ml. (0.041 mole) quantity of allyl bromide dissolved in 200 ml. of ethanol was added to the column containing the resin[1] (25 Gm.) upon which 7.0 Gm. (0.038 mole) of sodium barbital had been adsorbed. The mixture was then agitated for 48 hr. by rotating the column horizontally with an electric motor at 78 r.p.m. At the end of this period, the column was drained and the resin washed with three 75-ml. portions of ethanol. Evaporation of the eluate and washings left a yellow oily residue. The combined residue was dissolved in 30 ml. of ether and then extracted with 1 *N* sodium hydroxide solution. The alkaline aqueous portion, from which any *N,N'*-diallylbarbital was removed, was acidified with diluted hydrochloric acid and extracted with several portions of ether. The combined ether extractives were washed free of acid and evaporated to dryness. A crystalline residue was obtained which, when recrystallized from 50% methanol in water, melted at 80–81°. [Lit. (4) m.p. 80°.] The yield was 3.75 Gm. (47%).

Anal.—Calcd. for $C_{11}H_{20}N_2O_3$: C, 58.93; H, 7.14; N, 12.50. Found: C, 59.13; H, 7.40; N, 12.46.

N,N'-Diallylbarbital—A 6.6-ml. (0.066 mole) quantity of allyl bromide dissolved in 200 ml. of ethanol was added to the column containing the resin[1] (25 Gm.) upon which 6.18 Gm. (0.03 mole) of sodium barbital had been adsorbed. The mixture was agitated as described previously. At the end of this period, the column was drained and the resin washed with ethanol. The ethanolic solution was evaporated down to a yellow oily residue. The residue was washed with 1 *N* sodium hydroxide solution and extracted with several portions of ether. Evaporation of the ether extractives, first washed free of alkali, yielded *N,N'*-diallylbarbital which, when crystallized from 50% methanol in water, melted at 121°. [Lit. (4) b.p. 156–157°.] The yield was 2.65 Gm. (30%).

Anal.—Calcd. for $C_{14}H_{20}N_2O_3$: C, 63.64; H, 7.57; N, 10.60. Found: C, 63.71; H, 7.77; N, 10.95.

PHARMACOLOGY

Preparation—Each of the barbiturates was suspended, with the aid of a motorized Ten Broeck tissue homogenizer, in 25 ml. of distilled water containing 1% w/v of methylcellulose (1500 cps.). The resulting suspensions were diluted to 50 ml. with water yielding stock preparations containing 0.5% methylcellulose and 10^{-3} moles of barbiturate per ml. of suspension. Further dilutions were made, when necessary, with a 0.5% methylcellulose vehicle.

Animals—Male albino mice, 18–28 Gm., were employed as test subjects. They were selected from stock colonies of 200–300 animals and isolated 1–2 hr. prior to drug administration. Tests were conducted in rooms having an air temperature of 24–26°.

Standard—Phenobarbital was adopted as the reference standard against which the allyl barbiturates were compared.

Protocol—Mice were weighed, injected intra-

TABLE II—INFLUENCE OF ALLYL BARBITURATES ON THE RIGHTING REFLEX OF MALE MICE

Drug	Nominal Mol. Wt.	Dose mole/Kg.	Dose mg./Kg.	Incidence of R.R. Loss	Mean Onset Time, min.	Mean Duration, min.
Phenobarbital	232	3×10^{-4}	70	1/8	39[a]	107[a]
		5×10^{-4}	116	8/8	25	134
		7×10^{-4}	162	8/8	17	532
N-Allylphenobarbital	272	3×10^{-4}	91	0/8	...	...
		5×10^{-4}	136	2/8	13	15
		7×10^{-4}	194	8/8	11	40
N,N'-Diallylphenobarbital	312	5×10^{-4}	156	0/8	...	...
		7×10^{-4}	218	0/8	...	...
		1×10^{-3}	312	0/8	...	...
		2×10^{-3}	624	0/8	...	...
N-Allylbarbital	224	5×10^{-4}	112	0/8	...	...
		7×10^{-4}	157	1/8	20[a]	36[a]
		1×10^{-3}	224	8/8	4	334
N,N'-Diallylbarbital	264	5×10^{-4}	132	0/8	...	...
		7×10^{-4}	184	0/8	...	...
		1×10^{-3}	264	5/16	6	18
N-Allylhexobarbital	276	5×10	138	0/8	...	...
		7×10	193	0/8	...	...
		1×10	276	0/8	...	...
		2×10	552	0/8	...	...
Tetraallylbarbituric acid	288	5×10^{-4}	144	0/8	...	...
		7×10^{-4}	201	0/8	...	...
		1×10^{-3}	288	0/8	...	...
		2×10^{-3}	576	0/8	...	...

[a] One animal.

peritoneally with the appropriate doses of barbiturate (maximum volume injected was 0.6 ml.), and placed in individual 4-L. glass observation chambers. The animals were observed continuously during the first 4-hr. postinjection and periodically during the next 20-hr. period for the appearance of behavioral symptoms characteristic of barbiturate medication, *e.g.*, prodromal excitation, sedation, loss of righting reflex.

Results—A summary of the results is presented in Table II. In terms of both subtle and gross evidence of CNS depression, none of the allyl barbiturates approached phenobarbital in potency. *N*-Allylphenobarbital, *N*-allylbarbital, and *N,N'*-diallylbarbital exhibited mild hypnotic action at doses below those necessary to influence the righting reflex. The first instances of righting reflex loss occurred at 3×10^{-4} mole/Kg. (phenobarbital), 5×10^{-4} mole/Kg. (*N*-allylphenobarbital), 7×10^{-4} mole/Kg. (*N*-allylbarbital), and 10^{-3} mole/Kg. (*N,N'*-diallylbarbital). The most active depressant among the allyl barbiturates was *N*-allylphenobarbital. *N,N'*-Diallylphenobarbital evoked only the mildest degree of sedation and doses up to 2×10^{-3} mole/Kg. failed to influence the righting reflex. *N*-Allylhexobarbital and tetraallylbarbituric acid were devoid of all obvious CNS actions at doses up to 2×10^{-3} mole/Kg.

The appearance of mild prodromal excitability characterized the initial response to several of the compounds. Phenobarbital induced the characteristic locomotor excitability in mice at doses of 5×10^{-4} mole/Kg. and higher. Similar but less intense effects were observed when 7×10^{-4} mole/Kg. of *N*-allylphenobarbital was administered. *N*-Allylbarbital induced a severe clonus of short duration in animals receiving doses above 7×10^{-4} mole/Kg. The remaining compounds evoked no evidence of CNS stimulation.

The ranking of depressant potencies of the allylbarbiturates is in good agreement with the ranking of acute LD_{50}'s reported by Kaku *et al.* (4). Gylys and Proctor (6) examined the anticonvulsant properties of *N*-allylbarbital and *N*-allylphenobarbital. They reported that *N*-allyl substitution on barbital increased anticonvulsant activity but decreased duration of action while *N*-allyl substitution on phenobarbital decreased anticonvulsant activity both in terms of intensity and duration. Doses of the two drugs at which activity was observed were in the range of 50–100 mg., concentrations too low to alter the righting reflex.

REFERENCES

(1) Rowe, E. J., Kaufman, K. L., and Piantadosi, C. *J. Org. Chem.*, **23**, 1622(1958).
(2) Rowe, E. J., and Weinswig, M. H., *J. Pharm. Sci.*, 53, 226(1964).
(3) Kunin, R., and Myers, R. J., "Ion Exchange Resins," John Wiley & Sons, Inc., New York, N. Y., 1950, p. 150.
(4) Kaku, T., Kase, Y., and Nagai, M., *J. Pharm. Soc. Japan*, **74**, 122(1954).
(5) "Merck Index," Merck & Co., Rahway, N. J., 1960, p. 333.
(6) Gylys, J. A., and Proctor, C. D., *Illinois Acad. Sci. Trans.*, **48**, 97(1956).

Diazoxide Blood Levels in Man

By S. SYMCHOWICZ, L. WINSTON, J. BLACK*, M. SMITH, B. CALESNICK, and I. I. A. TABACHNICK

Blood diazoxide levels were measured in six adult human males at various times after i.v. drug administration. On the basis of these results, the diazoxide half-life in blood was determined as 28.0 ± 8.3 hr. The presence of diazoxide in blood was verified by thin-layer chromatography (TLC) and by its U.V. spectrum. This communication describes a simple spectrophotometric quantitative method and a useful TLC method for determination of diazoxide in biological fluids.

DIAZOXIDE is a nondiuretic benzothiadiazine which was originally developed for the treatment of hypertension. Early observations showed that diazoxide produced hyperglycemia in animals and man (1–5) which limited its use as an antihypertensive agent in man (6–8). However, the hyperglycemic effect of diazoxide is being increasingly utilized for treatment of patients with severe hypoglycemia (9–16).

A relationship was shown (17) between diazoxide dosage forms, dissolution rates, and blood levels. This report presents data on blood half-life of diazoxide in man and describes the methods for diazoxide analysis in biological fluids.

MATERIALS AND METHODS

Diazoxide was given intravenously at 5 mg./Kg. to six young adult males (weighing between 66 and 88 Kg.) in good physical health with no known cardiovascular or renal disease, and no abnormal glucose tolerance curve or family history of diabetes. The sterile diazoxide solution contained the equivalent of 300 mg. of the drug in 20 ml. of water adjusted to pH 11.4 with NaOH. Blood samples were drawn at 0, 5, 15, 30, and 60 min. and 2, 4, 6, 12, 24, 48, and 72 hr. after injection of diazoxide which lasted 2 min. All morning samples were taken before breakfast. Strenuous physical activity was avoided and normal diet was maintained.

The diazoxide blood levels were determined quantitatively as follows: 1 ml. of distilled water and 2 ml. of whole blood were mixed vigorously in a 15-ml. glass-stoppered centrifuge tube for 1 min. with 4 ml. of ethyl acetate. After brief centrifugation (2–3 min. at 1,300 r.p.m., International centrifuge PR-2), 2.5 ml. of the organic phase was transferred to another centrifuge tube containing 2.5 ml. of 4% sodium carbonate solution. The contents of the tube were mixed thoroughly (1–2 min.) and after centrifugation, the clear, aqueous phase was transferred to a quartz cell (1 cm. light path). The absorbance was read at 280 mμ in a Beckman DU spectrophotometer. A 4% sodium carbonate solution was used to set the instrument to zero absorbance. All samples were corrected for individual "blank" readings (zero hour sample).

The diazoxide blood concentration was determined from a standard curve. A linear relationship was obtained with diazoxide standards over a wide range of drug concentration (1–25 mcg./ml.). Studies with blood, plasma, and water, by our procedure, yielded on the average about 80% recovery of the drug.

Thin-layer chromatography was employed as an additional tool for identification of diazoxide and for its semiquantitative estimation. One to two milliliters of plasma was extracted with 4 ml. of ethyl acetate. The organic solvent was concentrated to a small volume (under nitrogen or on a rotating evaporator) and then applied quantitatively to Silica Gel F 254 precoated plates (Brinkmann Co.) which were developed with benzene–methanol (1:4) for 30–45 min. Diazoxide was visualized as a purple spot on the chromatographic plates with a short-wave U.V. lamp.

The half-life of diazoxide was estimated from the mathematical function $\log \hat{y} = a + bt$, where $\hat{y}$ = drug concentration (mcg./ml.), t = time in hours, a = y intercept (value of y when $t = 0$), and b = slope.

The function was fitted by the method of "least squares" (18).

RESULTS AND DISCUSSION

Table I summarizes the results obtained by spectrophotometric analysis of diazoxide blood levels at various time intervals. The half-lives were estimated from the function $\log y = a + bt$ (see *Methods*) and they range from 20.9 hr. (subject 1) to 34.7 hr. (subject 2). All slopes (b) are significantly different from 0 ($p < 0.01$, Student t test).

The mean blood half-life in man was estimated after testing the homogeneity of the regression lines (derived from Table I) by analysis of covariance. A weighted regression estimate for diazoxide is given by the function; $\log \hat{y} = 1.0483 - 0.0110t$ (Fig. 1) from which the half-life was derived as 28.0 + 8.3 hr. (95% confidence interval). The individual drug blood concentrations at various time intervals are shown in Fig. 1.

TABLE I—HALF-LIFE OF DIAZOXIDE IN HUMAN BLOOD

Subject	a	b	Estimated[a] Half-Life, hr.
1	0.9928	0.0142	20.9
2	0.9701	0.0087	34.7
3	1.1083	0.0136	22.2
4	1.0584	0.0094	32.1
5	1.0989	0.0096	31.4
6	1.1087	0.0107	28.2

[a] Derived from function $\log \hat{y} = a + bt$, where a = log mcg./ml. at time 0 and b = (rate of change per unit change in time), log mcg./ml.

Received February 6, 1967, from the Research Division, Schering Corp., Bloomfield, NJ 07003, and the Department of Pharmacology, Hahnemann Medical College and Hospital, Philadelphia, PA 19102

Accepted for publication April 21, 1967.

The authors thank Mr. M. Miller for statistical analysis of diazoxide half-life and Dr. B. Katchen for helpful suggestions in TLC chromatography. Dr. W. D. Peckham is credited with developing the spectrophotometric method for blood diazoxide.

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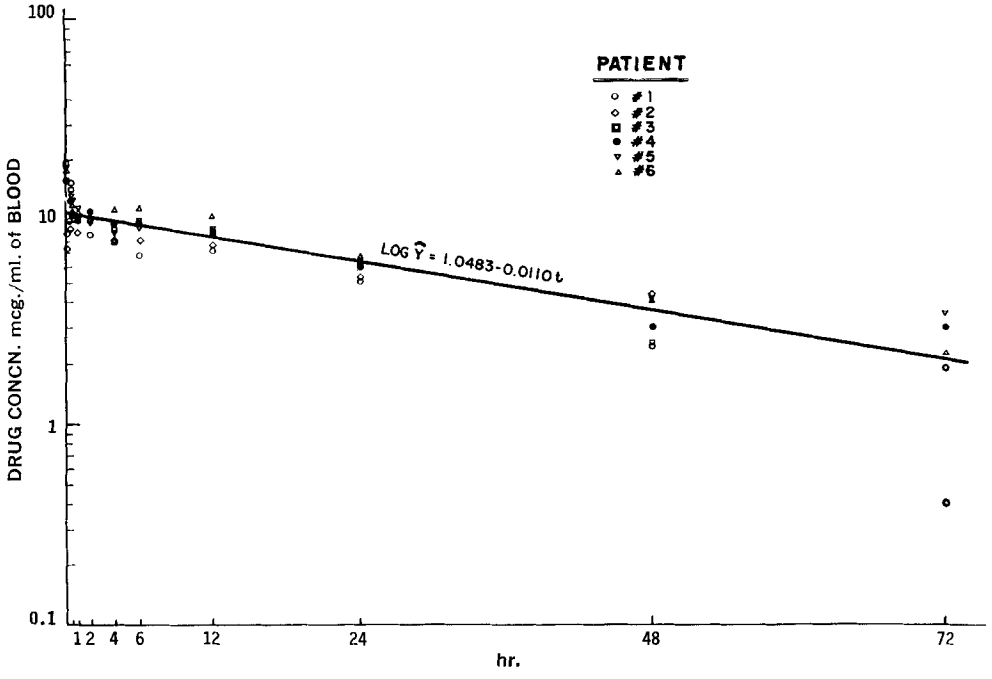

Fig. 1—Least square regression line for blood half-life evaluation in man.

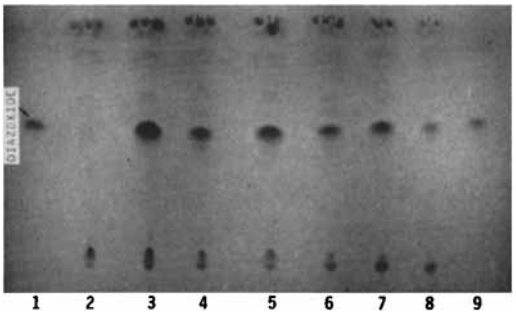

Fig. 2—TLC pattern of diazoxide extracted from plasma (photographed under U.V. light). Samples 1 and 9 show the position of diazoxide standards (5 mcg.). Sample 2 represents the pattern of untreated (0 hr.) plasma. Samples 3 and 4 are from subject 1, at 30 min. and 24 hr., respectively. Samples 5 and 6 are corresponding time intervals for subject 2 and samples 7 and 8 for subject 6.

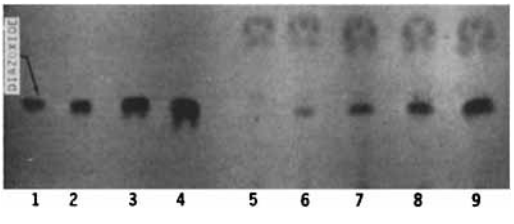

Fig. 3—TLC analysis of diazoxide recovered from plasma (photographed under U. V. light). Samples 1, 2, 3, and 4 are diazoxide standards spotted directly at concentrations of 5, 10, 20, and 40 mcg., respectively. Samples 6, 7, 8, and 9 represent the same respective diazoxide concentrations recovered from plasma. Sample 5 is plasma control (0 hr.).

To identify the material read at 280 mμ, particularly at the later time intervals, thin-layer chromatography was employed. Thirty-minute and 24-hr. plasma samples from subjects 1, 2, and 6 were extracted with ethyl acetate (see *Methods*) and chromatographed. The results in Fig. 2 clearly show that diazoxide is the only major component visible in plasma when compared with control (0 hr., spot 2). Samples from each subject show, as expected, more intense diazoxide spots at the earlier time interval. Other spots visible under U.V. light (Fig. 2) correspond to unidentified constituents of plasma (compare with control plasma, spot 2). Samples 1 and 9 are the diazoxide standards (5 mcg.).

Further identification of the diazoxide spot visible upon chromatography of plasma samples was obtained as follows. The areas on the glass plate (samples 2, 3, and 4, Fig. 2) were scraped out, and extracted with ethyl acetate, which was then extracted with 4% sodium carbonate. The U.V. spectrum of the sodium carbonate solution was recorded on a Perkin-Elmer recording spectrometer and compared with 5 mcg. of a chromatographed standard diazoxide solution (sample 1, Fig. 2) subjected to the same procedure. Both the standard and plasma samples showed identical U.V. characteristics, thus verifying the identity of the diazoxide spot on TLC plates.

The TLC procedure in this investigation is quite sensitive for diazoxide and gives good plasma recoveries. Figure 3 shows that the intensities of spots are proportional to the amounts (5, 10, 20, and 40 mcg.) of diazoxide standard spotted. Similar results were obtained by adding the same amounts of diazoxide standard to 1-ml. plasma samples and subjecting them to the described extraction procedure (samples 6–9, Fig. 3). Sample 5 is plasma control (no drug added).

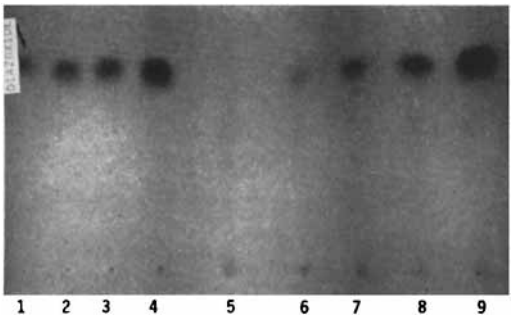

Fig. 4—TLC analysis of diazoxide recovered from urine (photographed under U. V. light). Samples 1, 2, 3, and 4 represent diazoxide standards 5, 10, 20, and 40 mcg., respectively. Samples 5, 6, 7, 8, and 9 represent diazoxide recovered from urine to which 0,5, 10, 20, and 40 mcg. of the drug was added, respectively.

Although no attempt was made to analyze diazoxide levels in urine, the TLC procedure is applicable to urine as well. It is recommended, however, that after extraction of urine with ethyl acetate, the organic solvent be washed with 2.5 ml. of 4% sodium bicarbonate solution before it is concentrated for chromatography. This will remove many interfering materials present in urine and give good diazoxide recoveries. Samples 6–9 (Fig. 4) present results obtained on recovery from urine of 5, 10, 20, and 40 mcg. of diazoxide, respectively. The size and intensity of diazoxide spots compare favorably (assuming some losses due to extraction procedure) with that obtained by direct chromatography of respective diazoxide standards (samples 1 4). Sample 5 represents control urine with no drug added.

REFERENCES

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The plant is an odoriferous, glandular shrub 2.3 M. tall, collected near Greaterville, Ariz., where it has become naturalized in a pasture.

EXPERIMENTAL

Ten Kg. of the dry pods of *C. gilliesii* was extracted with approximately 15 L. of water at room temperature. The aqueous extract was lyophilized in a Repp Industries model 15 sublimator and then washed with MeOH and EtOH. The yield was 760 Gm. One-hundred grams of this crude compound was added to 1 L. of water at room temperature. The insoluble part was separated by centrifuge and discarded.

The aqueous solution was extracted with 5 × 200 ml. of ether to remove fatty materials, and increased in volume threefold by the addition of 95% ethanol. A light-colored precipitate appeared and was separated by centrifuge, dissolved in water, and dialyzed against running water at room tem-

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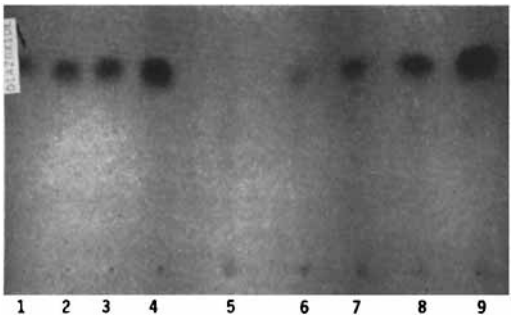

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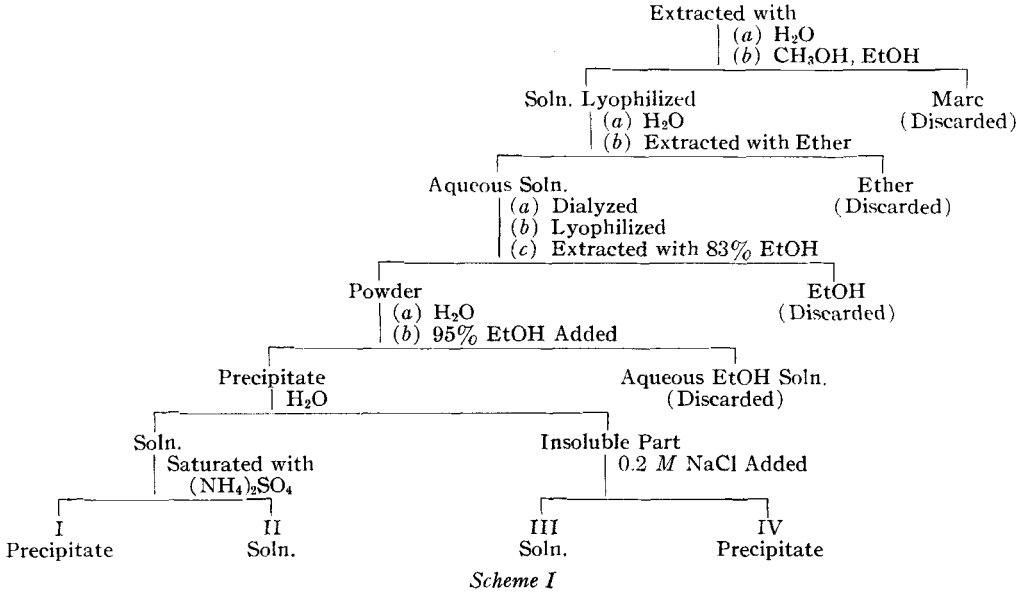
Schematic Presentation of Extraction
C. gilleisii



Scheme I

perature using a dialyzing tube of 5/8 in. diam. (No. 4465-A2, Arthur H. Thomas Co., Philadelphia, Pa.). After a period of 7 days, the solution in the tubes was lyophilized. The material thus obtained was extracted with 83% boiling ethanol. A red-colored solution was obtained. The remaining powder was dissolved in water and lyophilized. Twenty-five grams of compound was obtained. This crude material showed a marked activity against the sarcoma 180 test system and showed a reduction in tumor size of 82% and 89% at a dose of 4.4 mg./Kg. This corresponds to a T/C[3] value of 18 and 11%, respectively. The elementary analysis showed 17.3% inorganic material which consists mostly of calcium and phosphate. Sulfur and nitrogen were also present.

Four grams of the crude material was added to 50 ml. of water. The insoluble part was separated by centrifuge. To the soluble portion, $(NH_4)_2SO_4$ was added until saturation. A precipitate was then separated by centrifuge. The precipitate (I) and the solution (II) after being dissolved in water were dialyzed and lyophilized. The insoluble part was dissolved in 0.2 *M* NaCl (pH 8), and was separated by centrifuge. The soluble part (III) and the insoluble part (IV) were both dialyzed and lyophilized (Scheme I).

Column chromatography was employed to further separate these fractions (I, II, III, and IV). Substrates used included ion-exchange resins[4] and synthetic dextran derivatives.[5] The columns, upon treatment with the following buffer systems, gave the following test results: I when washed with 0.02 *M* phosphate buffer solution pH 5.6 yielded one fraction (a) and with a barbital buffer solution pH 8.6 a second fraction (b). II yielded two fractions (c, d) with the buffer solution pH 5.6; III gave one fraction (e) with the same buffer solution. No other fraction was obtained. IV yielded only one fraction (f) with a buffer solution pH 8.6. The four fractions indicated above were tested against the 5WM system. I exhibited a T/C[6] value of 3% at 33 mg./Kg and III a value of 28% at 100 mg./Kg.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF PROTEIN FRACTIONS

Paper electrophoresis of the six fractions was run utilizing a barbital buffer solution of pH 8.6, 0.05 ionic strength in a Spinco model Beckman instrument, and after 14 hr of electrophoresis using a 2 ma. current, the papers were developed using periodic acid and Schiff reagent. Mobilities of the six different fractions are shown in Table I.

The fractions were hydrolyzed with 6 *N* hydrochloric acid in a sealed and evacuated glass tube at 105°. The resulting solution was applied on Whatman No. 1 paper using the same solvent systems previously reported (2, 3).

One hundred milligrams of the six fractions were hydrolyzed separately with 3 *N* HCl in a boiling water bath for 24 hr. in preparation for characterization of the sugars. Three different solvent systems previously reported (3) were employed to detect the sugars using paper chromatography. Aniline phthalate and silver nitrate solutions were used to develop the chromatograms. The results are described in Table I. Results of nitrogen determination of six fractions using micro-Kjeldahl method and U. V. absorption are also shown in Table I.

Infrared curves of the six fractions showed characteristic protein peaks at 3400, 2950, 1650, and

[3] The criteria for activity is defined as being a per cent T/C (test/control) value of less than 42 in a satisfactory dose response test (1).

[4] Amberlite IR-120, IR-4B, IRC-50, and IRA-400, Rohm and Haas Co., Philadelphia, Pa.

[5] Sephadex, Pharmacia Fine Chemicals, Inc., New Market, N. J. The most effective column was G-200 Sephadex.

[6] Activity in this system is defined as a per cent T/C value of less than 60 in a satisfactory dose response test (1).

TABLE I—PHYSICAL AND CHEMICAL CHARACTERISTICS OF SIX PROTEIN FRACTIONS

Fraction	Mobilities, cm.²/sec. v.	Sugars	U.V. Abs. Maxima mμ	Nitrogen, %	Ash Content, %
a	1.07×10^{-6}	Mannose, fructose	204, 208	12.8	1.5
b	6.4×10^{-7}	Mannose, rhamnose	204, 265	11.6	1.5
c	2.2×10^{-6}	Mannose, glucosamine	204, 280	5.7	3.9
d	1.2×10^{-6}	Mannose, glucosamine	204, 280	6.2	0
e	1.2×10^{-6}	Mannose, glucosamine	204, 265	13.6	6.0
f	1.6×10^{-6}	Mannose, glucosamine rhamnose	204, 240, 280	14.08	6.0

TABLE II—PRELIMINARY AMINO ACID ANALYSIS OF *Caesalpinia* PROTEINS

Amino Acids	% μm. of Amino Acids I	II	III	IV
Aspartic acid	10.5	6.01	10.7	10.5
Threonine	6.04	7.66	5.81	5.08
Serine	6.66	13.00	5.88	6.01
Glutamic acid	16.20	7.18	13.60	15.80
Proline	5.02	3.32	4.90	4.95
Glycine	11.30	7.62	10.1	9.73
Alanine	8.89	13.3	10.9	8.90
Cystine	2.82	0.522	0.783	1.02
Valine	5.07	3.72	6.57	6.05
Methionine	1.04	0.522	1.52	1.27
Isoleucine	3.22	1.62	4.28	4.23
Leucine	6.02	3.53	7.75	7.87
Tyrosine	2.23	0.731	2.01	2.25
Phenylalanine	3.19	1.25	3.75	3.64
Lysine	3.99	2.41	3.21	3.57
Histidine	1.51	0.731	1.59	1.78
Arginine	6.24	1.51	5.95	6.73
Glucosamine	...	1.50[a]	0.644	0.454
Hydroxyproline	...	23.8[a]	...	...

[a] Estimated values.

1540 cm.$^{-1}$. Table II represents the results of an amino acid analysis using a Beckman analyzer model 120B.

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Synthesis of Some Substituted Amides of Terephthalic and Isophthalic Acids

By JOSEPH P. LAROCCA* and MOHAMED A. Y. SHARKAWI†

The syntheses of 14 diamide derivatives of terephthalic acid and six diamide derivatives of isophthalic acid are described. Evaluation of reaction conditions and solvent systems led to the use of anhydrous ether at 0° as the preferred method of preparing these amides from the corresponding acid chloride and amine. Preliminary pharmacological screening is reported.

A LARGE NUMBER of drugs are available which depress the central nervous system. These drugs elicit sedative, hypnotic, anticonvulsant, and psychotherapeutic effects. Many of these drugs contain a carbonyl-nitrogen-carbonyl group-

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TABLE I—SELECTION OF SOLVENT FOR DIAMIDE SYNTHESIS: AMINE WITH TEREPHTHALOYL CHLORIDE

Method	Solvent	Amine	% Yield[a]	Reaction Temp.
A	Aq. NaOH	Propyl	52.4	...
B	CCl_4	Propyl	58.4	24°
C	Diethyl ether	Propyl	76.6	20°
	Diethyl ether	Di-*n*-butyl	74.7	20°
D	No solvent	Di-*n*-butyl	82.4	[b]

[a] Based on crude yield. [b] Vigorous exothermic reaction with discoloration. Difficulties in purification made this procedure less desirable.

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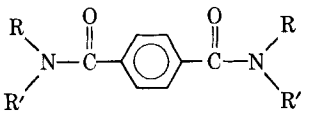
A	Aq. NaOH	Propyl	52.4	...
B	CCl_4	Propyl	58.4	24°
C	Diethyl ether	Propyl	76.6	20°
	Diethyl ether	Di-*n*-butyl	74.7	20°
D	No solvent	Di-*n*-butyl	82.4	[b]

[a] Based on crude yield. [b] Vigorous exothermic reaction with discoloration. Difficulties in purification made this procedure less desirable.

ing; however, similar effects have been noted in other compounds such as simple amides, *N*-substituted amides, dehydes, alalcohols, and glycols. The anticonvulsant *N*-benzyl-2-chloropropionamide is an example of such a compound (1). Numerous *N*-substituted amides have been synthesized in this laboratory and reported in the literature (2–6). Since these compounds have shown various degrees

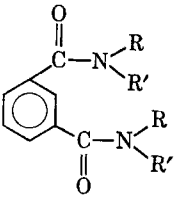
TABLE II—AMIDES OF TEREPHTHALIC ACID



No.	R	R′	% Yield	M.p., °C.[a]	%N Calcd.	%N Found
1	Hydrogen	*n*-Propyl	75.7	243–244	11.29	10.85 11.12
2	Hydrogen	*i*-Propyl	65.3	285–286	11.29	11.18 11.25
3	*n*-Propyl	*n*-Propyl	74.1	103.5–104	8.43	8.31 8.33
4	*i*-Propyl	*i*-Propyl	73.4	274–275	8.43	8.46 8.46
5	Hydrogen	*n*-Butyl	68.1	225–226	10.14	10.03 10.07
6	*n*-Butyl	*n*-Butyl	74.7	81.5–82.5	7.21	8.14 7.20
7	*i*-Butyl	*i*-Butyl	83.2	133–134	7.21	7.25 7.28
8	Hydrogen	Allyl	57.2	208–210	11.47	11.63 11.39
9	Hydrogen	Phenyl	94.3	340–345	8.86	8.69 8.54
10	Hydrogen	α-Methylbenzyl	65.3	275–278	7.53	7.66 7.36
11	Phenyl	Phenyl	99.1	340–341	5.98	6.29 6.34
12	Hydrogen	Cyclohexyl	34.7	273–275	8.54	8.40 8.34
13	Piperidino[b]		38.3	208–209	9.33	9.05 9.16
14	Morpholino[b]		7.89	212–215	9.21	9.85

[a] All melting points are uncorrected and were determined on a Thomas-Hoover capillary melting point apparatus. [b] For compounds *13* and *14*, R and R′ together represent piperidine and morpholine, respectively.

TABLE III—AMIDES OF ISOPHTHALIC ACID



No.	R	R′	% Yield	M.p., °C.[a]	% N Calcd.	% N Found
1	Hydrogen	*n*-Propyl	58.4	131–132	11.29	11.33 11.46
2	Hydrogen	*i*-Propyl	56.4	210–211	11.29	11.27 11.32
3	*i*-Propyl	*i*-Propyl	43.9	132–133	8.43	8.37 8.39
4	Hydrogen	*n*-Butyl	71.3	131–132.5	10.14	10.19 10.12
5	*i*-Butyl	*i*-Butyl	88.6	104–105	7.21	7.20 7.19
6	Hydrogen	Allyl	20.5	119–120	11.47	11.30 11.35

[a] Footnote same as Table I.

of activity on the central nervous system, the synthesis of the diamides of terephthalic and isophthalic acids was undertaken.

EXPERIMENTAL

Methods—The best and most general reaction for the preparation of amides has been reported to be the acylation of amines by acyl halides (7). A study of the reaction medium best suited for the synthesis of the desired compounds was undertaken. For this purpose the reaction of terephthaloyl chloride with alkyl amines (propylamine and *N*-di-*n*-butyl amine) was selected for detailed investigation. The results are summarized in Table I.

As a result of these studies anhydrous ether was chosen as the reaction medium. The following procedure was employed for the synthesis of the compounds reported.

A fourfold excess of the amine was dissolved in 100 ml. of anhydrous ether and cooled to 4°. A solution of the chloride (0.05 mole in 150 ml. anhydrous ether) was added slowly with vigorous stirring. The reaction temperature was not allowed to exceed 20° during the addition process. After the addition was complete, the stirring was continued for 15 min. The reaction mixture was stored overnight at 4° during which time the desired product crystallized (if the product failed to crystallize, the ether was removed by evaporation). The crystals or residue obtained were washed with distilled water to remove the amine hydrochloride and recrystallized from 50% ethanol. The melting points, yields, and analytical data for the amides of terephthalic acid are given in Table II, and for the amides of isophthalic acid in Table III.

Pharmacological Survey[1]—The preliminary mouse toxicity study showed that, in general, these compounds are nontoxic and free of any major CNS activity. The *N,N'*-di-*n*-butylterephthalamide and the *N,N,N',N'*-tetra-isopropylisophthalamide were also screened as antidepressants. Although both showed some activity, the activity was not sufficient to warrant further study.

REFERENCES

(1) Harned, B. K., Cunningham, R. W., Clark, M. C., Hine, C. H., Kane, M. M., Smith, F. H., Jr., Vessley, R. E., Yuda, N. N., and Zabransky, F. W., *J. Pharmacol. Exptl. Therap.*, **107**, 402(1935).
(2) LaRocca, J. P., Leonard, J. M., and Weaver, W. E., *J. Org. Chem.*, **16**, 47(1951).
(3) Byrum, W. R., and LaRocca, J. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 100(1952).
(4) Easterly, W. D., and LaRocca, J. P., *ibid.*, **43**, 59 (1954).
(5) LaRocca, J. P., *J. Pharm. Sci.*, **50**, 448(1961).
(6) LaRocca, J. P., and Culpepper, W. C., *ibid.*, **54**, 489 (1965).
(7) Wagner, R. B., and Zook, H. D., "Synthetic Organic Chemistry," John Wiley & Sons, Inc., New York, N. Y., 1953, p. 556.

[1] The authors are indebted to Dr. Warren J. Close, Abbott Laboratories, North Chicago, Ill., for the pharmacological screening.

Multi-Chamber System for Toxicity Studies in Mice at Simulated High Altitude

By I. P. BAUMEL, S. M. ROBINSON, and W. F. BLATT

Plastic desiccators have been adapted to serve as chambers in a simulated high altitude system. The multi-chambered system is convenient for observations of grouped mice or other small animals subjected to decompression. Toxicity data are given for representative drugs at sea level and a simulated altitude of 19,000 ft.

In examining the effects of simulated high altitude on drug toxicity in mice, it was necessary to utilize chambers which could accommodate a group of animals and permit a clear, unobstructed view of them. Accordingly, the authors devised a system incorporating relatively inexpensive plastic desiccators designed for routine laboratory use. The simulated altitude afforded by this system was used to evaluate the lethality to mice of agents representative of various drug classes.

EXPERIMENTAL

Apparatus—The modified spherical desiccators[1] shown in the lower portion of Fig. 1 can be evacuated to below 0.01 μ, a value considerably below that normally required for altitude studies. In single chamber operation, the air valve at the top portion of the chamber was attached directly to a vacuum pump. For air entry, a hole was drilled 2 in. above the level of the porcelain platform in the lower portion of the desiccator, and a suitable tubing adapter was inserted. If desired, additional openings can be made to accommodate separate manometers and/or monitoring equipment.

The operation of several chambers through a multi-port manifold requires the exact duplication of size and length for the air evacuation and entry lines. The $^3/_4$-in. tubing assembly, shown in the top portion of Fig. 1, was devised to serve four chambers. The upper manifold was attached to the pump; the lower section provided for the constant fresh air intake regulated by the control valve. Uniform pieces of thick-walled rubber tubing ($^1/_2$ in.) were used to attach the chambers to the manifold. A $^1/_{12}$-h.p. pump[2] was adequate for establishing a simulated altitude of 19,000 ft. at an air flow of 2.5 L./min. The barometric pressure within the system was monitored by a mercury manometer attached to the end chamber. The unit, during normal operation, is shown in the lower portion of Fig. 1. The speed of ascent was controlled by the air intake valve—more than adequate air flow was maintained at the 1,000

Received February 24, 1967, from the Biochemistry-Pharmacology Division, U. S. Army Research Institute of Environmental Medicine, Natick, MA 01760
Accepted for publication May 5, 1967.
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(2) LaRocca, J. P., Leonard, J. M., and Weaver, W. E., *J. Org. Chem.*, **16**, 47(1951).
(3) Byrum, W. R., and LaRocca, J. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 100(1952).
(4) Easterly, W. D., and LaRocca, J. P., *ibid.*, **43**, 59 (1954).
(5) LaRocca, J. P., *J. Pharm. Sci.*, **50**, 448(1961).
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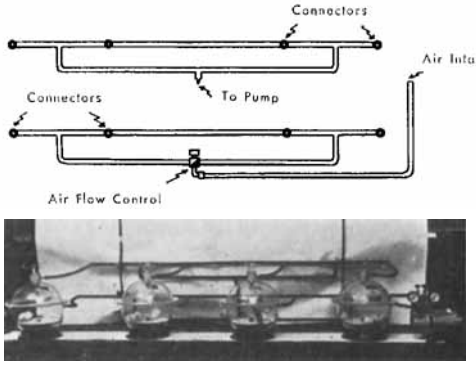

Fig. 1—Four-unit simulated altitude system. Key: top, manifold assembly for chambers showing tubing connections to pump and air entry lines; bottom, assembled system attached to $^1/_{12}$-h.p. pump.

ft./min. ascent rate utilized in this system. The sufficiency of the air flow obviated a possible problem; the turnover was such that normal room air conditioning could maintain the chambers within one degree of the external ambient temperature.

Drugs and Procedure—Male albino mice (CD_1, Charles River Mouse Farms), approximately 20.0 Gm., were placed in the chambers in groups of four, with 16 mice per dosage level. As part of ongoing studies on the interaction of altitude and drugs affecting the central nervous system (1), the following representative agents were selected for preliminary evaluation: sodium phenobarbital, reserpine, and tranylcypromine sulfate. Drugs were administered intraperitoneally at a constant dosage volume of 0.1 ml./10.0 Gm. of body weight. For the altitude determinations, the mice were then brought to 19,000 ft. at a rate of 1,000 ft./min. The number dead were noted for the 4-hr. period following arrival at the specified altitude. Occasionally an animal succumbed during ascent and as such was discarded from group computation. LD_{50} values and 95% confidence limits were determined by the method of Litchfield and Wilcoxon (2).

RESULTS AND DISCUSSION

Representative data for altitude and comparable sea level determinations are shown in Table I. An increased toxicity was obtained with reserpine in altitude-exposed mice; in contrast, no differences between sea level and simulated altitude could be demonstrated with phenobarbital or tranylcypromine. The observations thus far, while attesting to the feasibility of the system, are too limited to permit speculation on the mechanisms operative in the increased toxicity.

TABLE I—INTRAPERITONEAL LD_{50} DETERMINATIONS OF DRUGS AT SEA LEVEL AND ALTITUDE (19,000 ft.)

Dose, mg./Kg.	Observed/Tested		LD_{50}, mg./Kg.	
	Sea Level	Altitude	Sea Level	Altitude
Na Phenobarbital				
200	0/16	.....	290	273
250	2/16	2/16	(277–303)[a]	(256–286)
275	5/16	8/16		
300	10/16	15/16		
375	16/16	.....		
Reserpine				
75	.....	3/15	188	101
100	1/16	9/16	(138–256)	(80–127)
125	.....	8/16		
200	9/16	.....		
300	14/16	.....		
Tranylcypromine SO_4				
40	1/16	.....	107	122
80	2/16	1/16	(86–134)	(105–142)
100	2/16	3/16		
125	.....	7/12		
160	13/16	.....		
200	16/16	.....		

[a] Figures in parentheses represent values for 95% confidence limits. [b] Solubilized in 20% ascorbic acid.

Although the authors have used this system mainly for acute studies, the apparatus can be used for more protracted periods of exposure. For the latter application, cages containing standard feed and watering devices can be placed within the chamber and, following descent, rapid cleaning made by complete change of cage unit. During prolonged stay at altitude, it is suggested that a safety release valve be inserted within the system to protect against sudden unforeseen increases in altitude.

The apparatus can be expanded to accommodate an increased number of chambers by modification and expansion of the pipe assemblies, holding to the same general principle of equal connecting lengths. It also lends itself, with no major changes, to studies that require varied gas mixtures.

REFERENCES

(1) Robinson, S. M., Baumel, I. P., and Bass, D. E., *Federation Proc.*, **26**, 720(1967).
(2) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).

Trichloroethyl Carbonate I. Synthesis, Physical Properties, and Pharmacology

By H. C. CALDWELL, H. J. ADAMS, DONALD E. RIVARD, and JOSEPH V. SWINTOSKY*

Trichloroethyl carbonate was prepared from trichloroethanol and phosgene. It is a tasteless, crystalline solid with limited solubility in water. Pharmacological comparisons of trichloroethyl carbonate and trichloroethanol reveal that these compounds are about equitoxic and equipotent in decreasing spontaneous motor activity in mice after oral administration. These observations suggest that this carbonate is a prodrug of trichloroethanol.

CHLORAL HYDRATE which was used as a sedative by Liebreich (1) as early as 1869 is still one of the least toxic and most beneficial sedatives and hypnotics available (2). A hypnotic dose produces drowsiness within 15 to 30 min. and physiological sleep, which lasts for 6 to 8 hr., soon follows, and no hangover reaction is observed. However, the usefulness of the drug has been restricted by certain undesirable properties; it has a penetrating odor, and it possesses a bitter taste. Furthermore, it frequently causes stomach irritation, and it may produce nausea and vomiting at high doses. These factors, in addition to the popularity of the barbiturates, may account for the neglect of this drug. Chloral hydrate is rapidly converted in the body to trichloroethanol (3–5), which is also a potent sedative and hypnotic. Mackay and Cooper (3) studied this conversion in detail and showed chloral hydrate to be a potent hypnotic agent of short duration, and that the long duration of action observed was due to the metabolite trichloroethanol. Thus, the pharmacological effects noted after administration of chloral hydrate are chiefly due to its metabolite trichloroethanol.

While trichloroethanol has long been known to possess these pharmacological properties and has been studied at length in humans (6, 7), it has nonetheless been neglected as a therapeutic agent. No doubt its physical properties had much to do with this neglect because it is a bad tasting, hygroscopic liquid that often produces gastric irritation.

Recognizing that chloral hydrate and trichloroethanol are useful drugs, the authors felt it would be desirable to convert one of them to a more acceptable chemical form that would, after oral ingestion, be reconverted to the parent drug. Ideally this form should be a solid that can be conveniently formulated as tablets, capsules, or liquid forms, and it was thought that the carbonate derivative of trichloroethanol bis-(2,2,2-trichloroethyl) carbonate (or trichloroethyl carbonate[1]) might meet these criteria. This compound was reported in recent communications (8, 9).

EXPERIMENTAL

Chemistry—*Synthesis*—To a solution of 20 ml. of phosgene in 100 ml. of toluene was slowly added with stirring and cooling a mixture of 74.7 Gm. (0.5 mole) of trichloroethanol and 39.6 Gm. (0.5 mole) of pyridine. The mixture was stirred for 3 hr. at room temperature and the excess phosgene was removed *in vacuo*. Pyridine hydrochloride was filtered off and the filtrate was washed with 10% sodium hydroxide, dried, and concentrated. Dilution with hexane gave 58.4 Gm. of product, m.p. 86–87°.

Anal.—Calcd. for $C_5H_4Cl_6O_3$: C, 18.49; H, 1.24; Cl, 65.49. Found: C, 18.59; H, 1.28; Cl, 65.30.

Pharmacology—Initial testing of the trichloroethyl carbonate was aimed primarily at comparing its (*a*) acute oral toxicity and (*b*) CNS depressant activity with the parent drug trichloroethanol. For the latter, the effects of the two drugs on spontaneous motor activity in mice was measured, since it is well known that agents causing CNS depression in rodents will cause a concomitant decrease in spontaneous motor activity (10).

Acute Oral Toxicity in Mice—Male Carworth Farms mice (18–23 Gm.) were randomly divided into groups of 10 and dosed orally with suspensions of drug in 1% tragacanth. Suspensions were prepared with the aid of a Thomas glass-Teflon tissue grinder. Grinding was just sufficient (1–3 min.) to ensure a uniform suspension. Concentrations of drug were calculated so that each animal received a dose-volume of 10 ml./Kg. and a control group of 10 mice received 10 ml./Kg. of vehicle. After dosing, the animals were housed 10 per cage. They were observed for several hours immediately after dosing and then checked daily until no deaths occurred for 2 successive days following the fifth day post-drug. All deaths occurred within 48 hr. after dosing. The LD_{50}'s and 95% confidence limits were calculated by the logit χ^2 method of Berkson (11).

CNS Depressant Activity—Spontaneous Motor Activity (SMA) in Mice—Male Carworth Farms mice (15–25 Gm.) were randomly divided into groups of 10 and dosed with suspensions of drug in 1% tragacanth. Suspensions were prepared with the aid of a Thomas glass-Teflon tissue grinder as before and concentrations were calculated so that each animal received a dose volume of 10 ml./Kg. A control group received 10 ml./Kg. of vehicle. A 30-min. optimal pretreatment time for the drugs was determined in exploratory trials. Spontaneous motor activity was measured by means of an actophotometer. Exactly 30 min. after being dosed, an animal was placed into the activity chamber and its SMA, measured by the number of times the animal interrupted the light beams in the chamber, was determined for a 10-min. period. All counts were made on individual animals, and the reduction in SMA for each group was calculated by means of the following equation:

$$\% \text{ reduction in SMA} = \frac{\left(\begin{matrix}\text{mean count of}\\ \text{control group}\end{matrix}\right) - \left(\begin{matrix}\text{mean count of}\\ \text{treated group}\end{matrix}\right)}{\text{mean count of control group}} \times 100$$

Received February 27, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication April 5, 1967.

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[1] Chlorethate.

TABLE I—ACUTE ORAL TOXICITIES OF TRICHLOROETHYL CARBONATE AND TRICHLOROETHANOL IN MICE (MALE CF_1)

Dose, mg./Kg.	% Mortality	LD_{50} and 95% Fiellер Limits
	Trichloroethyl Carbonate	
800	0	
900	30	
1000	30	
1100	80	1003(939–1089)mg./Kg.[a]
1200	100	
	Trichloroethanol	
400	0	
600	10	
800	40	930(769–1264)mg./Kg.
1000	50	
1600	100	

[a] Not statistically significantly different from trichloroethanol.

TABLE II—DEPRESSION OF SPONTANEOUS MOTOR ACTIVITY (SMA) BY TRICHLOROETHYL CARBONATE OR TRICHLOROETHANOL ORALLY IN MICE

Dose, mg./Kg.	% Reduction in SMA	ED_{50} and 95% Confidence Limits
	Trichloroethyl Carbonate	
150	14	288(175–483)mg./Kg.[a]
250	38	
350	61	
450	63	
	Trichloroethanol	
200	13	212(130–290)mg./Kg.
300	77	
400	84	
500	83	

[a] Not statistically significantly different from trichloroethanol.

The ED_{50}'s and 95% confidence limits were calculated by the method described by Tedeschi *et al.* (12).

RESULTS AND DISCUSSION

Synthesis and Properties of Trichloroethyl Carbonate—No particular difficulties were encountered in the synthesis of this new compound, though cognizance must be taken of its latent capability for undergoing hydrolytic cleavage. By the laboratory method employed, yield of compound was 72%.

Conversion of trichloroethanol to its carbonate results in a profound change in physical-chemical properties. Whereas trichloroethanol is a dense, hygroscopic liquid of ethereal odor, the carbonate is an odorless, friable, white, crystalline, solid, m.p. 86–87°. Trichloroethanol has a disagreeable taste, but the carbonate is virtually tasteless. Trichloroethanol is soluble in water to about 1 in 12, but the carbonate dissolves in water only to about 4 mcg./ml. Trichloroethyl carbonate is essentially a lipophilic compound showing the following approximate solubilities at room temperature in several solvents: ethanol (2.6%), methanol (4.9%), ethyl ether (43%), chloroform (49%), carbon tetrachloride (37%).

Trichloroethanol has not lent itself to convenient use in therapeutics, but trichloroethyl carbonate possesses pharmaceutical characteristics that permit it to be encapsulated or tableted. In conjunction with appropriate suspending agents, it can be dispersed in water for convenient oral administration.

Acute Oral Toxicity in Mice—The oral LD_{50}'s of the carbonate and the alcohol are 1003 and 930 mg./Kg., respectively (Table I). This indicates the two compounds are equitoxic in mice.

CNS Depressant Activity—Spontaneous Motor Activity in Mice—The oral ED_{50}'s of the carbonate and the alcohol are 288 and 212 mg./Kg., respectively. This indicates the two compounds are equipotent in depressing spontaneous motor activity in mice.

These studies show that, mg. for mg., trichloroethyl carbonate and trichloroethanol are approximately equipotent in depressing spontaneous motor activity and also about equitoxic orally in mice. Studies with other carbonate derivatives, which will be reported in subsequent papers, show that the carbonate linkage is quite labile and subject to enzymatic cleavage by enzymes present in dilute human plasma. The authors infer from these observations that the pharmacological activities manifested after oral administration of trichloroethyl carbonate are the result of *in vivo* regeneration of the parent drug, trichloroethanol. Hydrolytic cleavage of trichloroethyl carbonate yields two molecules of trichloroethanol and a molecule of carbon dioxide for each molecule of parent drug:

$$CCl_3CH_2OCOCH_2CCl_3 + H_2O \rightarrow 2\ CCl_3CH_2OH + CO_2$$

Subsequent pharmacological studies have been aimed at qualitative as well as quantitative comparisons between trichloroethyl carbonate, trichloroethanol, and chloral hydrate. The results of these studies and of pharmaceutical studies will be reported in future publications. Pharmacology, safety, and utility of this compound in humans have been reported elsewhere (13).

REFERENCES

(1) Liebreich, O., "Das Chloralhydrate, Ein Neues Hypnoticum und Anaestheticum," Otto Müller's Verlag, Berlin, Germany, 1869; through Mackay, F. J., and Cooper, J. R., *J. Pharmacol. Exptl. Therap.*, **135**, 271(1962).
(2) Friend, D. G., "Molecular Modification in Drug Design," American Chemical Society, Washington, D. C., 1964.
(3) Mackay, F. J., and Cooper, J. R., *J. Pharmacol. Exptl. Therap.*, **135**, 271(1962).
(4) Butler, T. C., *ibid.*, **92**, 49(1948).
(5) Friedman, P. J., and Cooper, J. R., *ibid.*, **129**, 373 (1960).
(6) Imboden, J., and Lasagna, L., *Bull. Johns Hopkins Hosp.*, **99**, 91(1956).
(7) Owens, A. H., Jr., Marshall, E. K., Jr., Broun, G. O., Jr., Zubrod, C. G., and Lasagna, L., *ibid.*, **96**, 71(1955).
(8) Swintosky, J. V., Adams, H. J., Caldwell, H. C., Dittert, L. W., Ellison, T., and Rivard, D. E., *J. Pharm. Sci.*, **55**, 992(1966).
(9) Caldwell, H. C., U. S. pat. 3,193,573 (July 6, 1965).
(10) Lim, R. K. S., Pindell, M. H., Glass, H. G., and Rink, K., *Ann. N. Y. Acad. Sci.*, **64**, 667(1956).
(11) Berkson, J., *J. Am. Stat. Assoc.*, **48**, 565(1953).
(12) Tedeschi, D. H., Fowler, P. J., Cromley, W. H., Pauls, J. F., Eby, R. Z., and Fellows, E. J., *J. Pharm. Sci.*, **53**, 1046(1964).
(13) Beard, W. J., and Free, S. M., Jr., *J. Clin. Pharmacol.*, **7**, 41(1967).

Constituents of *Helenium plantagineum*

By MARIO SILVA

From flowers of *Helenium plantagineum* four compounds were isolated—a polymethylenic alcohol, β-sitosterol, linifolin A, and deacetoxylinifolin A.

In recent years there has been considerable interest in the study of the constituents of the *Helenium* genus (1–5).

During a current project dealing with Chilean flora (6) the authors had occasion to re-examine *Helenium plantagineum* (D.C.) Macbride in order to study the compounds isolated by Taito (7).

The petroleum ether soluble fraction yielded four compounds characterized as: a high molecular weight aliphatic alcohol, which was not further studied; β-sitosterol; linifolin A (8); and deacetoxylinifolin A. Deacetoxylinifolin A was also isolated through crystallizations from the benzene soluble fraction.

EXPERIMENTAL[1]

Petroleum Ether Extract—Flowers of *Helenium plantagineum*, collected in January 1962 near Florida (Concepción), were dried at 80–90°. A 2064-Gm. quantity of dried powder was extracted to exhaustion in a Soxhlet extractor with petroleum ether (b.p. 65–75°). This solution was concentrated to yield 461 Gm. of a dark product. This product was chromatographed many times on neutral alumina to give four compounds. The first compound is probably a polymethylenic alcohol, m.p. 78°, $[\alpha]_D$ 0° (chloroform, c 1), molecular weight 432 (Rast), which was not further studied.

β-Sitosterol—After several crystallizations of the second compound from ethanol, β-sitosterol was obtained, m.p. 137–138°, $[\alpha]_D$ −33.5° (chloroform, c 0.39), $\nu_{max.}^{KCl}$ 3380 cm.$^{-1}$.

The mixed melting point of this compound with authentic β-sitosterol showed no depression.

β-Sitosterol Acetate—The pure compound was obtained as needles after crystallizations from ethanol, m.p. 128–129°, $[\alpha]_D$ −49° (chloroform, c 0.43), $\nu_{max.}^{Nujol}$ 1736, 1250 cm.$^{-1}$.

Linifolin A—From the third product, through crystallizations from ethanol, 167 mg. of linifolin A was obtained, m.p. 202–203°, $[\alpha]_D$ +28.1° (chloroform, c 0.39), $\lambda_{max.}^{EtOH}$ 217 mμ, ϵ 13000, $\nu_{max.}^{Nujol}$ 1745, 1706, 1658, 1590, and 1235 cm.$^{-1}$.

Anal.—Calcd. for $C_{17}H_{20}O_5$: C, 67.09; H, 6.62. Found: C, 67.35; H, 6.36.

This compound did not show depression with an authentic sample of linifolin A on mixed melting point determination.

Deacetoxylinifolin A—The fourth compound, after recrystallizations from ethanol, gave 250 mg. of deacetoxylinifolin A, m.p. 251°, $[\alpha]_D$ +58.4° (pyridine c 0.38), $\lambda_{max.}^{EtOH}$ 217 mμ, ϵ 15150, $\nu_{max.}^{Nujol}$ 3717, 1745, 1698, and 1582 cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{18}O_4$: C, 68.68; H, 6.92. Found: C, 68.48; H, 7.00.

Deacetoxylinifolin A Acetate—Deacetoxylinifolin A on acetylation with pyridine–acetic anhydride at room temperature gave, after crystallization from methanol–chloroform, the acetate, m.p. 194°, $[\alpha]_D$ +28.8° (chloroform, c 0.52), $\lambda_{max.}^{EtOH}$ 217 mμ, ϵ 14400, $\nu_{max.}^{Nujol}$ 1745, 1706, 1658, 1590, and 1236 cm.$^{-1}$.

This acetate did not show depression with an authentic sample of linifolin A on mixed melting point determination.

Benzene Extract—The defatted plant material was dried and the benzene soluble constituents were extracted. This solution was concentrated to yield 80 Gm. of a dark product. This product, after several recrystallizations from ethanol, yielded 2.5 Gm. of deacetoxylinofolin A, m.p. 253–254°, $[\alpha]_D$ +59° (pyridine, c 0.7).

The mixed melting point of this compound with deacetoxylinifolin A, previously isolated, showed no depression.

Received January 9, 1967, from the Laboratorio de Fitoquímica, Departamento de Botánica, Instituto Central de Biología, Universidad de Concepción, Chile.

Accepted for publication March 27, 1967.

This work was generously supported by the Scientific Research Council of the University of Concepción.

The author acknowledges his indebtedness to Professor D. H. R. Barton for making available an authentic sample, and for his constant help and encouragement. The authentic sample of linifolin A was obtained from Professor Werner Herz through Professor Barton.

[1] Melting points (uncorrected) were performed on a Kofler block. Rotations were measured at 20°. The microanalyses were performed at Dr. Alfred Bernhardt's Institute, Ruhr, Germany. Ultraviolet spectra were recorded in solution in absolute ethanol on a SP 700 spectrophotometer. Infrared spectra were recorded on a Perkin Elmer 137 spectrophotometer.

REFERENCES

(1) Herz, W., and Laksmikantham, M. V., *Tetrahedron*, **21**, 1711(1965).

(2) Romo, J., Joseph-Nathan, P., and Díaz, A. F., *ibid.*, **20**, 79(1964).

(3) Parker, B. A., and Geissman, T. A., *J. Org. Chem.*, **27**, 4127(1962).

(4) Lucas, R. A., Rovinski, S., Kiesel, R. J., Dorfman, L., and Macphillamy, H. B., *ibid.*, **29**, 1549(1964).

(5) Lucas, R. A., Smith, R. G., and Dorfman, L., *ibid.*, **29**, 2101(1964).

(6) Silva, M., *J. Pharm. Sci.*, **56**, 908(1967).

(7) Taito, R., *Anales Fac. Quím. Farm., Concepción, Chile*, **13–14**, 176(1962–1963).

(8) Herz, W., *J. Org. Chem.*, **27**, 4043(1962).

N-Bromohydantoins in Pharmaceutical Analysis I. Determination of Sulfonamides and Some Local Anesthetics with 1,3-Dibromo-5,5-dimethylhydantoin

By MARÍA MARTÍNEZ DE BERTORELLO

This communication reports the use of 1,3-dibromo-5,5-dimethylhydantoin in the quantitative determination of sulfanilamide, 3,4-dimethyl-5-sulfanilamidoisoxazole, 5-methoxy-2-sulfanilamidopyrimidine, ethyl aminobenzoate, and *p*-aminobenzoyldiethylaminoethanol hydrochloride in acid aqueous medium.

THE *N*-BROMOHYDANTOINS occupy a very important position in preparative organic chemistry because of their efficient bromating and oxidizing action. They are simply and economically prepared, stable, and the dibromated derivatives have a high positive bromine content. They have been successfully tested with a wide variety of organic compounds as shown in numerous publications (1).

Reactions with *N*-bromohydantoins take place under mild conditions and are highly selective. Halogenating experiments are usually carried out under the conditions recommended by Ziegler *et al.* (2) for other similar reagents, using nonpolar solvents, preferably carbon tetrachloride. The few examples studied with these reagents using polar solvents, such as acetic acid (3), indicate an exceptional reaction rate even at low temperatures (15–20°), nuclear halogenation being particularly favored (4).

N-Bromohydantoin has not yet been used for analytical purposes. In the present paper the application of a derivative, 1,3-dibromo-5,5-dimethylhydantoin (I) has been studied for the evaluation of some organic substances of pharmaceutical interest.

This stable halogenating reagent of high bromine content (55.9%), when tested in an aqueous acid medium under conditions similar to those used by Barakat *et al.* (5) for *N*-bromosuccinimide (II), proved to be an excellent brominating agent for several sulfonamides and some local anesthetics. This property made it useful for the volumetric determination of these drugs.

Under these conditions it reacts quantitatively with sulfanilamide (III), 3,4-dimethyl-5-sulfanilamidoiso xazole (sulfisoxazole) (IV), 5-methoxy-2-sulfanilamidopyrimidine (V), ethyl aminobenzoate (benzocaine) (VI), and *p*-aminobenzoyldiethylaminoethanol hydrochloride (procaine hydrochloride) (VII), with the reagent/substrate molar relationships of 1:1, 3:2, 1:1, 1:1, 1:1, respectively. The end point is directly visualized using methyl red as an indicator.

1,3-Dibromo-5,5-dimethylhydantoin decolorizes the indicator in an aqueous acidic medium, but halogenates preferentially the substrates; only when the halogenation has been total does a small excess of reactant decolorize the indicator. To achieve further accuracy a blank experiment is recommended with the indicator under the same conditions.

EXPERIMENTAL

Reagents—(*a*) 1,3-Dibromo-5,5-dimethylhydantoin[1] was prepared according to the method described by Orazi *et al.* (6) by means of bromine acting on an alkaline solution of 5,5-dimethylhydantoin. By means of repeated crystallizations in hot water (70–80°), the pure product is obtained with a 55.9% bromine content.

The positive quality of bromine in 1,3-dibromo-5,5-dimethylhydantoin permits the liberation of iodine from an acid solution of potassium iodide which makes it possible to carry out its quantitative determination by the iodometric method (7).

(*b*) Solution of 1,3-dibromo-5,5-dimethylhydantoin[2] at 0.1% w/v in distilled water: it is prepared by dissolving the pure substance in hot distilled water (70°), cooling it at once after the solution is obtained. Once the environmental temperature is reached, distilled water is added up to a total of 100 ml. The titer of the solution is iodometrically determined. The solution must be kept in a cool place and must be protected from light, controlling its factor every 3 days. Solutions prepared for more than 7 days should not be used.

(*c*) Solution 0.1%, of III, IV, V, VI, and VII in 10% hydrochloric acid.

(*d*) Hydrochloric acid solution, 10% w/v in distilled water.

(*e*) Methyl red solution (0.02%) in 50% ethanol.

(*f*) Acetic acid, glacial.

(*g*) *N*-Bromosuccinimide solution at 0.1% w/v in distilled water.

Procedure—A convenient volume of the substrate solution, accurately measured, *i.e.*, 5 ml. is poured into a conical flask, then 5 ml. of acetic acid and 2 drops of the indicator are added and titrated with the 0.1% titrant, shaking frequently until the indicator is decolorized. The volume required for the blank assay is deduced from the number of milliliters used.

Blank Experiment—The same procedure is followed using, instead of the substrate solution, an equivalent volume of 10% hydrochloric acid solution (in the given sample, 5 ml.).

The results obtained in the tested samples are indicated in Tables I, II, and III. Each value represents the average of three parallel determinations.

The range of applicability of I and other similar titrants is currently under study in this laboratory.

CONCLUSIONS

The results in Tables I and II indicate that 1,3-dibromo-5,5-dimethylhydantoin is a suitable analytical reagent for the quantitative determination of some sulfonamides and local anesthetics; the reaction takes place rapidly and quantitatively at room temperature, experimental errors range about 1% and, moreover, quantities as small as 1 mg. can be determined.

Received January 9, 1967, from the Instituto de Ciencias Químicas, Universidad Nacional de Cordoba, Córdoba, Argentina.

Accepted for publication March 30, 1967.

[1] Commercial products are supplied by the K E K Laboratories, Inc., New York, and Fluka A. G. Chemische Fabrik, Schweiz.

[2] Solubility in water at 75°, 8.5 mg./ml., and solubility at 25°, 1.75 mg./ml.

TABLE I—DETERMINATION OF SULFONAMIDES WITH 1,3-DIBROMO-5,5-DIMETHYLHYDANTOIN (I)

Substrate	Soln. Used, ml.	Soln. I,[a] ml.	Content, mg.	Found, mg.	Error, %
Sulfanilamide (III)	1	1.68	1.00	1.01	1
	2	3.36	2.00	2.02	1
	3	5.02	3.00	3.02	0.6
	4	6.63	4.00	3.99	0.25
	5	8.29	5.00	4.99	0.20
3,4-Dimethyl-5-sulfanilamidoisoxazole (IV)	5	8.00	5.00	4.98	0.4
	6	9.58	6.00	5.97	0.6
	7	11.30	7.00	7.04	0.57
	8	12.79	8.00	7.97	0.37
	9	14.59	9.00	9.09	1
	10	16.09	10.00	10.02	0.2
5-Methoxy-2-sulfanilamidopyrimidine (V)	1	1.02	1.00	1.00	0
	2	2.06	2.00	2.02	1
	3	3.04	3.00	2.98	0.67
	4	4.02	4.00	3.99	0.25
	5	5.05	5.00	4.95	1

[a] One milliliter of a 0.1% solution of I = 0.602 mg. of III, 0.623 mg. of IV, and 0.980 mg. of V.

TABLE II—DETERMINATION OF LOCAL ANESTHETICS WITH 1,3-DIBROMO-5,5-DIMETHYLHYDANTOIN (I)

Substrate	Soln. Used, ml.	Soln. I,[a] ml.	Content, mg.	Found, mg.	Error, %
Ethyl aminobenzoate (VI)	1	1.74	1.00	1.01	1
	2	3.49	2.00	2.02	1
	3	5.24	3.00	3.03	1
	4	6.95	4.00	4.02	0.50
	5	8.74	5.00	5.05	1
p-Aminobenzoyl diethylaminoethanol hydrochloride (VII)	1	1.06	1.00	1.01	1
	2	2.11	2.00	2.01	0.50
	3	3.18	3.00	3.03	1
	4	4.24	4.00	4.04	1
	5	5.23	5.00	4.99	0.2

[a] One milliliter of solution 0.1% of I = 0.578 mg. of VI and 0.954 mg. of VII.

TABLE III—DETERMINATION OF SULFONAMIDES WITH 1,3-DIBROMO-5,5-DIMETHYLHYDANTOIN (I) AND *N*-BROMOSUCCINIMIDE (II)

Substrate	Soln. Used, ml.	Soln., ml. I[a]	Soln., ml. II[b]	Content, mg.	Found, mg. I	Found, mg. II	Error, % I	Error, % II
Sulfanilamide (III)	5	8.29	10.42	5.00	4.99	5.03	0.20	0.60
3,4-Dimethyl-5-sulfanilamidoisoxazole	5	8.00	10.05	5.00	4.98	5.03	0.40	0.60

[a] One milliliter of solution 0.1% = 0.602 mg. of III and 0.623 mg. of IV. [b] One milliliter of solution 0.1% of II = 0.483 of III and 0.501 mg. of IV.

Comparative studies with *N*-bromosuccinimide (11) against sulfanilamide and 3,4-dimethyl-5-sulfanilamidoisoxazole show that the results obtained with both reactants are similar; however, the hydantoin derivatives offer the advantage of their better stability (8).

REFERENCES

(1) Corral, R. A., and Orazi, O. O., *J. Org. Chem.*, **28**, 1100 (1963).

(2) Ziegler, K., Spat, A., Schaf, E., Schuman, W., and Winkelmann, E., *Ann.*, **551**, 80(1942).

(3) Corral, R. A., Orazi, O. O., and de Bertorello, M. M., *Anales Asoc. Quim. Arg.*, **52**, 251(1964).

(4) de Bertorello, M. M., "Estudio Sobre Nuevos Agentes Halogenantes," Ph.D. Thesis, Instituto de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, 1962.

(5) Barakat, M. Z., and Shaker, M., *Analyst*, **89**, 216 (1964).

(6) Orazi, O. O., and Orio, O. A., *Anales Asoc. Quim. Arg.*, **41**, 153(1953).

(7) Scott, W. W.," Standard Methods of Chemical Analysis," vol. 1, 5th ed., D. Van Nostrand Co., New York, N. Y., 1939, p. 452.

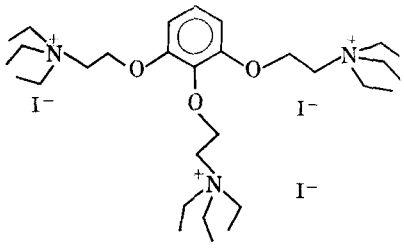
(8) Reed, R. A., *Chem. Prod.*, **1960**, 299.

Quaternary Salts of 2,4-Bis(dialkylaminoalkoxy)quinazolines. Potential Curaremimetics

By RONALD E. ORTH

Bis-quaternary salts of 2,4-bis(dialkylaminoalkoxy)quinazolines were prepared from the corresponding bis-tertiary amines as potential curaremimetic agents.

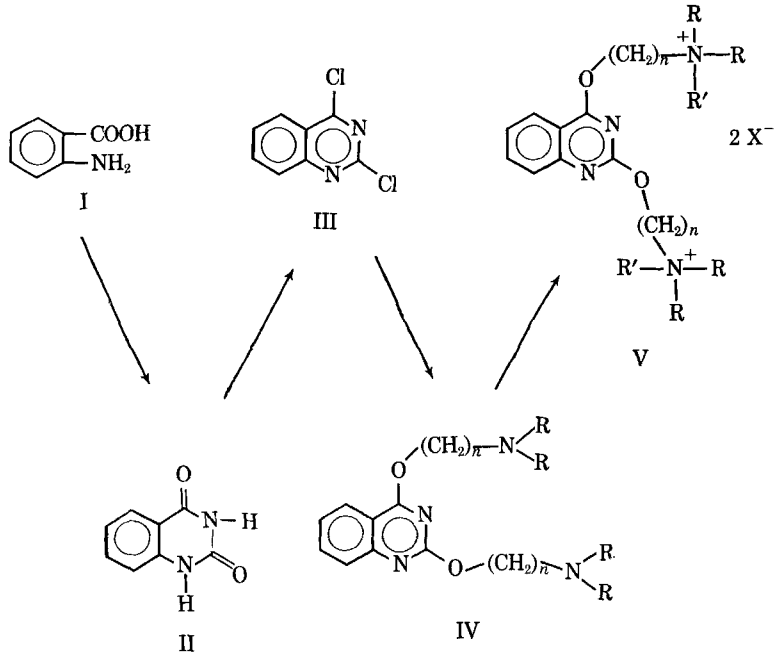
TUBOCURARINE CHLORIDE and drugs which act in a similar manner are utilized to induce muscle relaxation in neuromuscular disorders and during abdominal surgery. These applications were made only after it was demonstrated that the drug acts at the myoneural junction. However, tubocurarine chloride exerts undesirable autonomic ganglionic effects which have led investigators to continue the quest for a more specific myoneural blocking agent. Numerous investigations have ensued. Brown and Fraser suggested the pharmacological activity of various quaternary salts (1) and Craig found that all "onium" containing compounds may be expected to exhibit curare-like activity (2). Two or more quaternized moieties within a single substance were shown to produce higher activity by virtue of a more firm attachment at the site of action (3, 4). A distance of approximately 15 Å. between "onium" heads in a homologous series was suggested for maximum curariform activity. Stenlake *et al.* studied linear chemicals with three, four, and five "onium" heads and found that the number of these centers influenced potency, reversibility, and the duration of action



Gallamine Triethiodide N.F. XII

(5, 6). In 1949 Bovet *et al.* attempted to reproduce synthetically a simplified version of the tubocurarine molecule (7). They achieved the synthesis of a series of bis-quinoline derivatives wherein the aromatic structures were connected through a methylene chain by ether linkages. The same group examined some simple mono- and polyphenolic ethers of certain aminoalcohols and found that these substances also possessed a striking curariform activity. The best-known of this latter series is the triethiodide of tris(β-diethylaminoethoxy)-1,2,3-benzene (gallamine triethiodide).

Hohmann and Jones demonstrated high curare-like activity in several quaternary salts of 2,4,6-tris(dialkylaminoalkoxy)-1,3,5-triazines and 2,4-bis(dialkylaminoalkoxy)quinazolines (8). The paralyzing doses required in the mouse sloping-screen and rabbit head drop tests were calculated and sum-

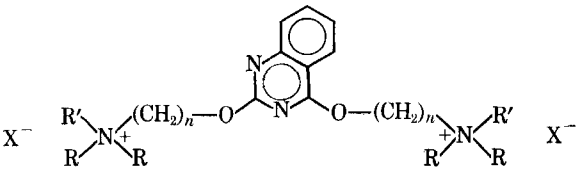


I, anthranilic acid; II, 2,4-(1*H*,3*H*)quinazolinedione (9); III, 2,4-dichloroquinazoline (10); IV, 2,4-bis(dialkylaminoalkoxy)quinazoline (8); V, title compounds. *n* is 2 or 3; R is methyl or ethyl; R′ is ethyl or benzyl; X is chloride, iodide.

Scheme I

Received January 3, 1967 from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506
Accepted for publication March 22, 1967.

TABLE I—BIS-QUATERNARY SALTS OF 2,4-BIS(DIALKYLAMINOALKOXY)QUINAZOLINES



	Quaternary Salt				% N		% Halide			
	n	R	R′	X⁻	Calcd.	Found[b]	Calcd.	Found[b]	Dec. Pt., °C.	% Yield
I	2	Me	Et	I⁻	9.09	8.77	41.2	40.1	261–263	71
II	2	Me	Bzyl[a]	Cl⁻	10.0	10.0	12.7	12.0	257–258	85
III	2	Et	Et	I⁻	8.33	8.31	36.3	36.3	232–233	68
IV	2	Et	Bzyl[a]	Cl⁻	9.07	9.02	11.6	11.4	244	81
V	3	Et	Et	I⁻	8.00	7.83	36.3	36.3	254–256	77
VI	3	Et	Bzyl[a]	Cl⁻	8.73	8.67	11.1	11.2	271–273	91

[a] Benzyl. [b] Elemental analysis by Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England.

marized using gallamine as a standard. In both tests 2,4,6-tris(β-diethylaminoethoxy)-1,3,5-triazine tribenzchloride proved superior to gallamine and other salts of the triazine and quinazoline series. Unfortunately, the LD_{50} for this substance was 5.05 mg./Kg. The quinazolines, however, were potent and much less toxic than the corresponding triazines. No obvious increase in potency was attributed to the trimethylene side chain moiety over dimethylene in the quinazoline series. It was noted in both series that *N,N*-diethyl substitution produced more potent activity than *N,N*-dimethyl. Since only the methiodides of the quinazolines were prepared, several of the potentially more active ethiodides and benzchlorides are synthesized. (Scheme I.)

EXPERIMENTAL

2, 4 - Bis (dialkylaminoalkoxy) quinazolines—Twelve grams of 2,4-dichloroquinazoline, prepared by the method of Lange *et al.* (10), was dissolved in 50 ml. of dry benzene and added to a cooled suspension of sodium aminoalkoxide prepared by reacting 2.8 Gm. (0.12 *M*) of sodium with 0.18 *M* of the dialkylaminoalkanol. The mixture is refluxed for 2 hr., cooled, washed with water, and dried over Na_2SO_4. Most of the benzene is then removed at atmospheric pressure. The remaining mixture is distilled twice to obtain the pure amine (8). 2,4-Bis(diethylaminoethoxy)quinazoline: 225–226°/4 mm. (54% yield). 2,4-Bis(diethylaminopropoxy)quinazoline: 230–233°/2 mm. (40% yield). 2,4-Bis(dimethylaminoethoxy)quinazoline: 234–236°/9 mm. (35% yield).

Diethiodide and Dibenzchloride Salts—Reflux 0.1 *M* of the amine and 0.5 *M* of the halide in 50 ml. of absolute ethanol for 2 hr. Absolute ether is then added until the quaternary salt precipitates. The solid is collected on a filter, washed with dry ether, and dried in a vacuum oven at 60°. The yields, melting points, and analytical data are given in Table I.

REFERENCES

(1) Brown, A. C., and Fraser, T., *Tr. Roy. Soc. Edinburgh*, **25**, 151, 693(1868–1869).
(2) Craig, L. E., *Chem. Rev.*, **42**, 285(1948).
(3) Phillips, A. P., and Castillo, J. C., *J. Am. Chem. Soc.*, **73**, 3949(1951).
(4) Cavallito, C. J., Soria, A. E., and Hoppe, J. O., *ibid.*, **72**, 2661(1950).
(5) Edwards, D., Stenlake, J. B., Lewis, J. J., and Stothers, F., *J. Med. Pharm. Chem.*, **3**, 369(1961).
(6) Carey, F. M., Furst, C. I., Lewis, J. J., and Stenlake, J. B., *J. Pharm. Pharmacol.*, **16**, Suppl. 89T(1964).
(7) Bovet, D., Depierre, F., Courvoisier, S., and deLestrange, Y., *Arch. Intern. Pharmacodyn.*, **80**, 172(1949).
(8) Hohmann, J. R., and Jones, J. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 453(1954).
(9) Lange, N. A., and Sheibley, F. E., "Organic Synthesis," coll. vol. II, Blatt, A. H. ed., John Wiley & Sons, Inc., New York, N. Y., 1943, pp. 79–80.
(10) Lange, N. A., Roush, W. E., and Asbeck, H. J., *J. Am. Chem. Soc.*, **52**, 3696(1930).

Isolation of Ergosterol from *Gibberella zea* Mycelium

Sir:

Gibberella zea Schwabe is the perfect stage of *Fusarium graminearum*, a plant pathogen causing stalk, ear, and root rot of corn and scab diseases on barley. This organism has been implicated in many reports of animal toxicities. Two different types of effects have been reported from studies of animals having ingested infested grains. Christensen *et al.* (1) and Stob *et al.* (2) have isolated a fungal product having uterotrophic activity. Curtin and Tuite (3) have reported on the emetic principle contained in *G. zea* infested corn. Christensen *et al.* (1) have also given evidence that ergosterol is produced by the fungus when cultured on cracked corn.

The authors wish to report the isolation of ergosterol as the major sterol product of *G. zea* when grown in an agitated, submerged, completely defined fermentation medium.

A growth medium composed of 3% glucose and 0.5% succinic acid plus salts [a modification of a synthetic medium described by Pacifici *et al.* (4) for the production of *Claviceps purpurea*] was inoculated with a liquid culture of *G. zea*, incubated at 26° for 5 days, and the mycelium removed by filtration. The mycelium was frozen immediately, freeze-dried, and powdered. This was extracted in a Soxhlet apparatus for 36 hr. with Skelly B.

The solvent was removed from the extract by evaporation *in vacuo*. The extract (0.69 Gm.), representing 34 Gm. freeze-dried mycelium, was extracted twice with 5 ml. of 90% ethanol each time. To the combined extracts was added 4 ml. of 1% digitonin solution. The resultant precipitated digitonide was recovered by filtration and dissolved in 6 ml. dimethylsulfoxide and treated according to the procedure of Issidorides *et al.* (5). The solution was heated for 15 min. on a steam bath, then cooled to room temperature and extracted once with 15 ml. and then 4 times with 6 ml. of *n*-hexane. The hexane solution was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. The sterol isolated (0.11 Gm.) was recrystallized twice from ethanol.

This sterol was chromatographed on Silica Gel G (250 μ layer) buffered (pH 9.3) thin-layer plates using a solvent composed of methylene chloride–carbon tetrachloride–methanol (64:32:5). The hR_f values of the *G. zea* sterol and a recrystallized sample of authentic ergosterol were identical. The same results were obtained when both substances were chromatographed on Silica Gel G thin-layer nonbuffered plates with a solvent composed of cyclohexane–chloroform (1:1). The melting point was not depressed with mixtures of authentic ergosterol and the *G. zea* sterol. The ultraviolet spectrum of the *G. zea* sterol was identical in all respects to the spectrum of an authentic sample of ergosterol (maxima at 262, 271, 282, and 293.5 mμ). Infrared spectra of the *G. zea* sterol and ergosterol were also identical in every respect.

Although earlier reports of ergosterol as a product of *G. zea* were based upon extraction of not only the mycelium of this fungus but also the substrate upon which it was produced, the authors have conclusively proven that ergosterol is in fact the major sterol of *G. zea*, and that this organism can produce ergosterol when grown in a simple medium containing glucose and succinic acid as the sole source of carbon.

(1) Christensen, C. M., Nelson, G. H., and Mirocha, C. J., *Appl. Microbiol.*, **13**, 653(1965).
(2) Stob, M., Baldwin, R. S., Tuite, J., Andrews, F. N., and Gillette, K. G., *Nature*, **196**, 1318(1962).
(3) Curtin, T. M., and Tuite, J., *Life Sciences*, **5**, 1937 (1966).
(4) Pacifici, L. R., Kelleher, W. J., and Schwarting, A. E., *Lloydia*, **25**, 37(1962).
(5) Issidorides, C. H., Kitigawa, I., and Mosettig, E., *J. Org. Chem.*, **27**, 4693(1962).

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Received April 14, 1967.
Accepted for publication May 22, 1967.

This investigation was supported by grant EF-00483 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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Effect of Bed Rest on Distribution and Elimination of Drugs

Sir:

Most pharmacokinetic studies are conducted on healthy ambulatory subjects. It is recognized, however, that many of these investigations should be carried out also on sick patients in order to assess the effect of various pathological conditions on the kinetics of drug absorption, distribution, and elimination. Such comparative studies will ordinarily involve bedridden patients and a control group of healthy ambulatory subjects (or the same patients upon complete restoration of health). It is the purpose of this communication to point out that bed rest *per se* can have an appreciable effect on drug distribution and elimination. This effect, if unrecognized, can cause considerable errors in the interpretation of pharmacokinetic data.

Schmidt and Roholt (1) have recently determined serum levels and urinary excretion of benzylpenicillin after intramuscular injection of 5 million units of benzylpenicillin sodium in six subjects while these were in bed and, subsequently, while they were ambulatory. For the purpose of this report, data for 5 of these subjects have been subjected to pharmacokinetic analysis. (Data for subject *3* were incomplete and had to be excluded.) It was found that penicillin concentrations in the plasma declined exponentially with time over a concentration range of three orders of magnitude. Half-life values ($t_{1/2}$) were determined graphically and were used to calculate apparent first-order rate constants for elimination (k). Apparent first-order rate constants for excretion of unchanged drug (k_e) were obtained from fk, where f is the fraction of the dose excreted unchanged. Rate constants for metabolism (k_m) were determined by subtracting k_e from k. The apparent volume of distribution (V_d) was calculated on the basis of the relationship A = dose $k^{-1}V_d^{-1}$, with the area (A) under the serum level *versus* time curve obtained by planimetry. Renal clearance was calculated as the product of k_e and V_d. Mathematical derivations for these equations may be found in the review by Nelson (2).

The pharmacokinetic constants for five subjects while ambulatory and during bed rest are listed in Table I. The fraction of the administered dose excreted unchanged and the renal clearance of benzylpenicillin were significantly increased and k_m was significantly decreased during bed rest ($p < 0.05$ by t-test, paired comparisons). The average V_d was increased during bed rest; this effect was not quite statistically significant, but it occurred in all five subjects.

The most interesting effect of bed rest is the apparent decrease in the first-order rate constant for metabolism (and possibly extra-renal excretion) of benzylpenicillin (k_m) to one-half the value observed while the subjects were ambulatory. It is conceivable that similar results, if obtained in the course of a comparative study of ambulatory normal subjects and bedridden patients suffering from certain disease(s), would be interpreted as being indicative of impaired drug metabolizing capacity due to the disease. In fact, it appears that bed rest *per se* can bring about pronounced changes in the distribution, metabolism, and excretion of drugs relative to values obtained in ambulatory subjects. This may be due to the higher metabolic rate of ambulatory subjects (1), or to the marked circulatory and plasma volume differences found in upright and recumbent subjects, respectively. These latter differences are due to the increased hydrostatic pressure of plasma associated with an upright as opposed to a recumbent posture (3). The increased hydrostatic pressure in the lower parts of the upright body causes plasma water to enter the interstitial space and leads to an increase in the concentration of plasma proteins (4). Similar effects are observed following physical exercise (5). This in turn results in higher plasma concentrations of plasma protein-bound drugs, although the total amount of drug in the plasma may be decreased due to the smaller plasma volume. A remarkable example of this effect and the type of artifact it can produce have been described recently by Dettli and Spring (4). These investigators determined plasma levels of sulfasymazine in hospitalized patients at 12-hr. intervals; apparent $t_{1/2}$ values calculated for any given 12-hr. period were much higher if the patient left the bed prior to removal of the second blood sample. In some instances the plasma obtained from a patient who had left the bed before the blood sample was drawn had a higher sulfonamide concentration than the plasma obtained 12 hr. earlier while the patient was in bed!

Pharmacokinetic models in current use do not provide for variations in plasma volume, variable blood flow rates through organs, and other region- or organ-specific circulatory changes. The effects of such variations will appear in the V_d and rate constants of presently used pharma-

TABLE I—PHARMACOKINETIC CONSTANTS FOR BENZYLPENICILLIN IN ADULT HUMAN MALES WHILE AMBULATORY AND DURING BED REST[a]

Subject	$t_{1/2}$, hr.	k, hr $^{-1}$	f	k_e, hr.$^{-1}$	k_m, hr.$^{-1}$	V_d, L.	V_dk_e, ml. min.$^{-1}$
			Ambulatory				
1	.85	.82	.54	.44	.38	45	330
2	.90	.77	.74	.57	.20	47	440
4	.85	.82	.78	.64	.18	35	370
5	.70	.99	.58	.57	.42	28	270
6	.90	.77	.70	.54	.23	33	290
Mean[b]	.84	.83	.67	.55	.27	37	340
			Bed Rest				
1	.75	.92	.76	.70	.22	47	550
2	1.1	.63	.88	.55	.076	51	470
4	1.1	.63	.98	.62	.013	53	550
5	.70	.99	.84	.83	.16	29	410
6	1.0	.69	.64	.44	.25	55	410
Mean[c]	.93	.75	.82[c]	.61	.13[c]	47	480[c]

[a] Based on data from *Reference 1*. All data are expressed in two significant figures based on the results of calculations carried out to three significant figures. [b] All mean values are based on the arithmetic mean half-life. [c] Significantly different ($p < 0.05$) from values obtained while the subjects were ambulatory.

cokinetic models. Thus, the pronounced difference in the k_m values for benzylpenicillin during the ambulatory state and during bed rest, respectively, is probably only an apparent one which is not necessarily indicative of an intrinsically impaired capacity for drug biotransformation during bed rest.

A review of general physiology (3) and particularly of renal physiology (6) suggests to this author that the circulatory and distributive changes in man due to changes in posture [or due to physical exercise (5, 7)] may be too complex to incorporate in pharmacokinetic models. It appears therefore that comparative pharmacokinetic studies on sick, bedridden patients and healthy controls require that the latter be in bed also. An additional complication arises from the fact that prolonged bed rest causes a gradual deterioration of cardiovascular function which is associated with a *decrease* of plasma volume (8). Such selective effects of bed rest give rise to potentially formidable problems in the pharmacokinetic interpretation of drug disposition during disease.

(1) Schmidt, H., and Roholt, K., *Acta Pathol. Microbiol. Scand.*, **68**, 396(1966).
(2) Nelson, E., *J. Pharm. Sci.*, **50**, 181(1961)
(3) Best, C. H., and Taylor, N. B., "The Physiological Basis of Medical Practice," 7th ed., Williams and Wilkins, Baltimore, Md., 1961.
(4) Dettli, L., and Spring, P., *Helv. Med. Acta*, **33**, 291 (1967).
(5) König, E., and Lemp, A., *Klin. Wocshr.*, **44**, 862 (1966).
(6) Smith, H. W., "Principles of Renal Physiology," Oxford University Press, New York, N. Y., 1957.
(7) Aurell, M., Fritjofsson, Å., and Grimby, G., *Clin. Sci.*, **31**, 461(1966).
(8) Taylor, H. L., Erikson, L., Henschal, A., and Keys, A., *Am. J. Physiol.*, **144**, 227(1945).

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Received April 3, 1967.
Accepted for publication May 17, 1967.

REVIEWS

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF **Pharmaceutical Sciences**

August 1967 volume 56, number 8

Review Article

Isergonic Relations and Their Significance for Catalysis

By RICHARD L. SCHOWEN

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Received from the Department of Chemistry, University of Kansas, Lawrence, KA 66044

All errors of fact, judgment, and concept in this article are my own, but the same cannot be said for any truths which may be present. I wish especially to thank Professor E. E. Smissman for his encouragement and aid. Some of these ideas were presented in a lecture at the Fourth Annual Medicinal Chemistry Meeting-in-Miniature (MIKI Meeting) in Lawrence, Kan., last year, and I was greatly influenced by discussions with that group. Professor Bernard Belleau, who visited Lawrence in the Chemical Biology Seminar, and Dr. R. J. Thorn, whose visit was sponsored by the Chemical Physics Seminar, both contributed a number of insights. I would like to thank my colleagues and coworkers, especially Professors Earl S. Huyser and John A. *Landgrebe and Stephen* C. Hoops, H. Jayaraman, Larry Kershner, and C. G. Mitton, for many ideas and criticisms. I am grateful also for financial support by the National Science Foundation and National Institutes of Health of work discussed herein.

AN INTEREST in the phenomenon of catalysis, the acceleration of chemical reactions by substances having little or no effect on the gross stoichiometry, is shared by physical organic chemists and by medicinal chemists whose desire is to understand the character of enzyme action for purposes of drug design. The use of the concepts and theories of physical organic chemistry in the solution of problems of medicinal chemistry, such as the analysis of receptor sites and the synthesis of active compounds, is already well known to readers of the *Journal of Pharmaceutical Sciences*. In this article, the author would like to call attention to some current ideas in physical organic chemistry which are believed to have special relevance to the problems faced by pharmaceutical scientists, and to present some elementary applications of them to questions of catalysis.

Transition-State Theory—It is a nearly universal practice of solution kineticists today to present their results in terms of the transition-state theory (1–5), a custom justified by the enormous success enjoyed by this theory in producing a detailed understanding, in molecular language, of the mechanisms of a wide variety of organic and inorganic reactions. According to this theory, the reactants are viewed as participating in a quasi-equilibrium with the species (or configuration) at the maximum of energy separating the reactants from the next minimum of the Gibbs

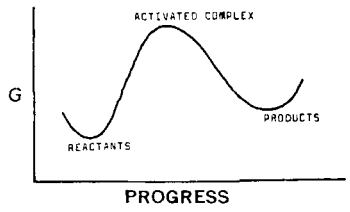


Fig. 1—Free energy profile for a simple reaction.

free energy surface[1] (Fig. 1). The maximum energy configuration (the *activated complex* or, colloquially, the *transition state*) is treated as a molecule for which "thermodynamic" functions (the free energy of activation, ΔG^*, the enthalpy of activation, ΔH^*, and the entropy of activation, ΔS^*) may be defined using a quasi-equilibrium constant, K^*, for its formation from reactants[2] (Eqs. 1 and 2):

$$\Delta G^* = -RT \ln K^* \qquad \text{(Eq. 1)}$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad \text{(Eq. 2)}$$

The rate constant k is then given by Eq. 3:

$$k = (\mathbf{k}T/h)K^* \qquad \text{(Eq. 3)}$$

where $\mathbf{k}$ is the Boltzmann constant, h the Planck constant, and T the absolute temperature. In addition to the free energy, enthalpy, and entropy of activation, other quasi-thermodynamic functions of activation may be defined and measured, notably the heat capacity and volume of activation, but these will not concern us here, although the volume of activation, in particular, may be of importance in catalytic processes (7).

The relevance of these considerations to problems of catalysis lies in the accuracy, economy, and elegance with which the activation parameters can be used to describe the molecular origins of reaction-rate variation with conditions (1–5, 7, 8). The understanding of the function of a catalyst must consist in the specification of the way in which it affects the activation parameters of the reaction of interest by interaction with the substrate and the activated complex.

Any mechanistic model, if formulated in sufficient detail, includes the relevant interactions. If the effect of each of these on the enthalpy and entropy of activation, and thus on the free energy of activation, is estimated, then the sum over all interactions gives the rate of the catalyzed reaction, relative to the rate of the uncatalyzed reaction or to the reaction catalyzed by another substance, *i.e.*, the *catalytic efficiency*. A similar estimation of the effect of changing the substrate structure on these quantities can yield the relative rates of the catalyzed reaction for various substrates, or the *catalytic specificity*.

The estimation process can be aided, and some general statements can be made, by use of relations which have been observed between the enthalpy and entropy changes associated with certain interactions, so that the two variables are not independent. In fact, it will be shown that the existence of such relations permits the formulation of a simple quantity which expresses numerically the catalytic specificity associated with a given kind of interaction in terms of its biological value.

Table I—Some Important Factors Which Contribute to the Enthalpy and Entropy Changes for Solution Reactions

Enthalpy: Potential and Kinetic Energies; Work	*Entropy:* Statistical Probability
Covalent bonding	Number of particles (translational freedom)
Electrostatic attractions and repulsions, including those of dipoles	Freedom of external and internal rotations
Nonbonded attractions and repulsions (van der Waals and London forces)	Low-frequency vibrations
Hydrogen bonding	Freedom of translation and rotation of solvent molecules
Charge-transfer bonding	Symmetry
Strain and distortion of bond lengths and angles	
Solvation energies	
Resonance energies	
Zero-point energies	
Pressure-volume work	

Free Energy, Enthalpy, and Entropy—For the convenience of the reader, Table I recalls the major factors contributing to the magnitudes of enthalpy and entropy changes for reactions in solution. From the standpoint of transition-state theory, it is immaterial whether these quantities refer to the conversion of reactants into products in an equilibrium process or to the conversion of reactants into activated complex in a rate process. No comment is really required about these factors, which are familiar to most people from discussions of reaction mechanisms. Wiberg (3) has discussed the subject in great detail in a very clear fashion. It should be mentioned that the form of the transition-state theory given here (unit transmission coefficient) is inapplicable to reactions involving changes in electron-spin multiplicity (9).

[1] This is the appropriate variable for the vertical axis of such plots (see the discussion in *Reference 6*). Johnston's objection (*Reference 5*, chap. 16) can be met by use of an appropriate standard state for the stoichiometric assembly for each set of experimental conditions. The step functions shown by Johnston are correct, rather than the curves shown here (and usually).

[2] K^* is a quasi-equilibrium constant and the associated 'thermodynamic" functions are given quotation marks (or are designated quasi-thermodynamic) because the degree of freedom for decomposition of the activated complex has been removed from consideration. (See *Reference 4* for a recent, lucid discussion.)

ISERGONIC RELATIONS

Should there exist a relation between the enthalpy change and the entropy change which occur during a chemical process? A number of models suggest that the answer is yes. Consider the formation of a bond between two polyatomic species. As the two particles combine, enthalpy (bond energy) is liberated and the translational and rotational entropy is decreased by the conversion of two particles into one. If a series of such association processes, of progressively stronger binding, were studied, the enthalpy liberated would increase with the stronger binding, but the translational entropy loss would be the same in all cases and the rotational entropy loss little different. However, stronger binding usually means shorter bonds, and for polyatomic species, the smaller separation of the more strongly bound moieties could easily lead to greater losses of entropy due to restriction of bending vibrations and internal rotations through mutual interaction. Thus stronger and more exothermic binding might well be accompanied by a progressively more negative entropy of combination.

Consider also the ionization of a neutral molecule in a solvent which interacts readily with ions. Suppose a series of dissociation rates is studied, such that the degree of conversion of the reactant into its ionic fragments at the activated complex steadily increases. As the ions become more fully formed, the solvent will interact more strongly with the activated complex with liberation of a correspondingly greater amount of solvation energy. At the same time, the tighter bonding of the solvent molecules will lead to an increasing reduction in entropy, as more solvent molecules become involved (translational and rotational entropy) and as the rotational and vibrational motions of those already bound are further restricted. Thus the solvation process should make increasingly more negative enthalpy and entropy contributions to the free energy of activation as the degree of charge separation at the activated complex increases.

Both of these models predict a *compensatory* relation of enthalpy and entropy, *i.e.*, a correspondence such that the effect of one quantity on the free energy tends to be cancelled by the effect of the other, but neither model (in the form above at least) is detailed enough to determine the functional form of the relation. For this we turn to experiment.

Linear Compensatory Relations—Alder and Leffler (10) observed that the enthalpies and entropies of activation for the decomposition of phenylazotriphenylmethane into free radicals in a series of solvents were related *linearly*, according to Eq. 4:

$$\delta_M \Delta H^* = \beta \delta_M \Delta S^* \qquad \text{(Eq. 4)}$$

with $\beta = 310°$ K.; the positive value of β expresses the fact that the behavior is compensatory. The symbol δ_M occurring in Eq. 4 is the very useful Leffler-Grunwald operator (11) for medium variation. $\delta_M \Delta G^* = \Delta G_M^* - \Delta G_0^*$, where the subscript M refers to some arbitrary solvent and the subscript 0 to a standard solvent. The expression $\delta_M \Delta G^*$ can conveniently be read, "the effect of medium on ΔG^*." Other operators can be defined for any variable, such as structure (δ_R), catalyst (δ_C), *etc.* The net effect on the free energy of activation in the Alder-Leffler system is obtained by substitution of Eq. 4 into Eq. 2, after application of δ_M to the latter, yielding Eq. 5.

$$\delta_M \Delta G^* = (\beta - T)\delta_M \Delta S^* \qquad \text{(Eq. 5)}$$

This study illustrated that lack of a solvent effect on rate at a given temperature ($\delta_M \Delta G^* \sim 0$) could not be taken as evidence that no solvent-interaction change was arising on activation. In this case, at temperatures near 310° K., the solvation enthalpies and entropies cancel each other and no rate effect is found. The molecular interactions responsible for these observations for free radical reactions are still under investigation (12).

Since, in principle, no variation in rate at all would be observed if the reaction were studied at a temperature equal to β, this quantity was denoted the *isokinetic temperature* (13, 14). If enthalpy–entropy compensation is observed for an equilibrium process, this terminology is etymologically incorrect. Perhaps a more desirable general term is *isergonic temperature* (the slope of an *isergonic relation*, such as Eq. 4), which we adopt here.

Leffler and Grunwald (11) have collected a large number of isergonic relations for both rate and equilibrium processes, all approximately linear with positive slope, *i.e.*, compensatory in nature. The values of β ranged from about 100° K. to about 1300° K.

The same authors have further shown that some data sets which did not yield isergonic reactions (in which enthalpy *versus* entropy plots either showed no correlation or a nonlinear one) could be analyzed in terms of a superposition of more than one isergonic relation (Eqs. 6–8):

$$\delta\Delta H = \sum_i \delta\Delta H_i; \quad \delta\Delta S = \sum_i \delta\Delta S_i \qquad \text{(Eq. 6)}$$

$$\delta\Delta H_i = \beta_i \delta\Delta S_i \qquad \text{(Eq. 7)}$$

$$\delta\Delta H = \sum_i \beta_i \delta\Delta S_i \qquad \text{(Eq. 8)}$$

They reason that each of these terms in the summations corresponds to a particular interaction mechanism and that the observed enthalpies and entropies are merely the resultant. The plots may be analyzed by vector addition of the individual relations to obtain the locus of a given point. As shown in Eq. 7, each interaction mechanism is presumed to have its own characteristic isergonic temperature β_i. The reader will agree that such cases as this are likely to be of the greatest value in analyzing the most interesting cases of catalysis, in which manifold interactions between catalyst and substrate are expected.

The Role of Experimental Error—Two important criticisms of the use of isergonic relations have appeared. Petersen, Markgraf, and Ross (15) criticized most of the relations which had appeared before 1961 on the grounds that (*a*) the errors in ΔH and ΔS are related—if the temperature dependence of a rate or equilibrium constant is used to evaluate them, ΔH is obtained from the slope and ΔS from the intercept (effectively) of the same line—so that spurious relations may arise from experimental error, and (*b*) that a number of reactions showing apparent isergonic relations in fact had total ranges of variation of the enthalpy smaller than the maximum error in ΔH, as estimated from the errors of the extreme temperature points of the Arrhenius or Eyring plots.

Leffler and Grunwald (11, 14) have shown, however, that this method of error analysis is incorrect, since if the maximum error of ΔH is placed on the enthalpy–entropy plot, it will include regions of impossibly high or low ΔG (in which the error is always much less than in ΔH). The true error contour is thus an ellipse tilted with its major axis along a line of slope equal to the mean experimental temperature. It would appear that most of the originally described isergonic relations do in fact represent actual correspondences of enthalpy and entropy, although it emerges clearly from this controversy that larger temperature ranges than have been usual ought to be employed in the measurement of activation and thermodynamic parameters, inasmuch as the maximum error in ΔH increases as the inverse of the absolute temperature range studied.

Petersen (16) has raised a further objection to the use of isergonic reactions. If an isergonic temperature actually exists, he points out, then it is clear that the Arrhenius or Eyring plots for all the individual systems correlated must intersect at that point. No variation in rate at the isergonic temperature will then be observed. Petersen (*a*) constructed a set of artificial data which displayed a satisfactorily linear isergonic relation (correlation coefficient 0.998), but for which the individual Eyring plots did not intersect at a common point, and (*b*) found that in no case of an isergonic system from the literature did the Eyring plots have the requisite intersection property.

This demonstrates very convincingly that isergonic relations are approximate, a result which on reflection is more satisfying than the existence of exact relations. It would be amazing if the shift in enthalpy change produced by a structural or environmental variation were always accompanied by an exactly proportional entropy change; we are unaccustomed to such rigor in chemistry. On the other hand, the fact that structurally induced variations in ΔH and ΔS are in the same direction and approximately proportional is a very informative observation from a mechanistic point of view. Indeed, the isergonic temperature (or approximate change in ΔH per unit change in ΔS) is directly useful inasmuch as it must be quantitatively consistent with an acceptable mechanistic model. Herein is the utility of the concept, rather than in the existence of an experimental temperature at which no rate variation is observed.

It is certainly true that Petersen's work shows the term isergonic temperature (or isokinetic temperature) to be a misnomer. Nevertheless, as Leffler has said (14), such a term is conveniently mnemonic as a name for the slopes of linear enthalpy–entropy relations.

Solvation Phenomena and Electrostatic Interactions—As we noted in the initial arguments above, an isergonic relation due to solvation effects might be expected for a series of reactants in an ionization rate process. The same arguments indicate that solvation energy and solvation entropy ought in general to be related in a compensatory way and that processes in which solvation changes contribute in a significant way to the energetics may exhibit the effects of this compensation.

In the case of solvation phenomena involving electrically charged species, however, we must consider the values of the enthalpy and entropy changes associated with the electrostatic work of separating the charges (in ionization) or the work done by their combination. The free energy change, enthalpy change, and entropy change [as obtained by Frost and Pearson (17) in a simple way] are given by Eqs. 9, 10, and 11:

$$\Delta G = (-Z_A Z_B e^2/Dr) \qquad \text{(Eq. 9)}$$

$$\Delta S = (-Z_A Z_B e^2/Dr)\,(\partial \ln D/\partial T)_P \qquad \text{(Eq. 10)}$$

$$\Delta H = (-Z_A Z_B e^2/Dr) \times (1 - T[\partial \ln D/\partial T]_P) \quad \text{(Eq. 11)}$$

where the Z's are the charges (in units of the electronic charge) on the ions, e the charge on the electron, D the dielectric constant of the medium, and r the charge separation in the initial state; the values are for separation of the ions to infinity. These and related expressions have been used and discussed critically by Frost and Pearson, by Lumry (7), and by Linderstrøm-Lang and Schellman (18) as well as many others. Their limitations are rather severe inasmuch as they ignore the difference between macroscopic and microscopic dielectric constant, treat the solvent as a continuum, and ignore particulate interactions of the solvent with the ions. As is evident from inspection, they do not predict any dependence on the structure of the ions; for example, ΔS of combination for univalent ions in water should be about -10 e.u. A number of cases do not depart seriously from this rule (17).

However, the heat and entropy of combination of all ions of the same charge type are not equal, as is implied by this treatment. Duncan and Kepert (19) collected the data of a number of workers on the thermodynamics of ion-pair formation for many different types of salts in several protic solvents. These data are correlated isergonically with a slope of 200° K. The explanation probably lies in the interaction of solvation energies and entropies in the hydration shells, as discussed above.

Brown (20), in a wide ranging study of isergonic relations, found that the data for ionization of a variety of organic acids were correlated by lines with an isergonic temperature of 280° K. This number is in striking agreement with an estimate by Belleau (21) of the isergonic temperature associated with the "melting" of the "icebergs" which, according to the Frank-Evans theory of the structure of aqueous solutions,[3] surround nonpolar solutes in water. The term "iceberg" being taken literally, the heat of fusion of ice (1440 cal./mole) was divided by the entropy of fusion of ice (5.6 e.u.) to obtain $\beta = 273°$ K. This agreement with Brown's finding supports an *ad hoc* hypothesis that these correlations are arising from formation and destruction of solvation shells surrounding the reactants and products, and that these shells bear some resemblance to ice.

Hepler,[4] in a landmark contribution to the understanding of chemistry in protic solvents, applied the isergonic temperature of 280° K. suggested by Brown's observations for solvation phenomena in water to the data for phenol ionization and arrived at a completely meaningful interpretation of the substituent effect on this reaction. This theory will be discussed in more detail under *Linear Free Energy Relations*, but at this point a particular property, noted by Hepler, conferred on solvation phenomena in water by the isergonic temperature of 280° K. should be brought to light. According to Eq. 5 above, the total contribution of solvation phenomena in any process to its free energy change is given by the change in solvation entropy times $(\beta - T)$; but since β is very close to room temperature, the very important conclusion emerges from Hepler's theory that *solvation changes will have little effect on the rate or site of equilibrium of a reaction studied in aqueous solution near room temperature.*

Linear Free Energy Relations[5]—In his original discussion of the limitations of the Hammett equation (Eq. 12):

$$\log k/k_0 = \rho\sigma = \frac{\delta_R \Delta G^*}{-2.3\ RT} \quad \text{(Eq. 12)}$$

Hammett (24) noted that if the entropy change for a series of reactions were constant, then the free energy change would be equal to the enthalpy change, which might reasonably be expected to vary according to the potential energy changes which are usually considered to lie at the basis of such linear correlations. At a later time, Taft (25) showed this criterion to be too restrictive and demonstrated that linear free energy relations could result from data sets in which the entropy changes were not constant along the series but instead varied linearly (isergonically) with the enthalpy changes (Eqs. 4 and 13).

$$\delta_R \Delta G^* = \left(\frac{\beta - T}{\beta}\right) \delta_R \Delta H^* \quad \text{(Eq. 13)}$$

Studies of the ionization of substituted phenols in water by Hepler (23), already mentioned, produced data which were correlated by the Hammett equation but which fulfilled neither of the criteria just mentioned: the changes in entropy were far from constant (in fact, the major part of the substituent effect appeared in the entropy term) and they were not linearly related to the corresponding enthalpy changes. Hepler proposed to explain the data in a way which can be formulated as a two-interaction–mechanism isergonic relation. He assumed the enthalpy effects to be a sum of internal contributions ($\delta\Delta H_i$, essentially the potential energy effects customarily invoked for substituents) and external contributions due to solvation ($\delta\Delta H_e$) as expressed by Eq. 14:

[3] See Kavanau (22) for a summary of this and other ideas.
[4] See Hepler (23) and other papers by the same author.
[5] Readers unfamiliar with free energy relations should consult Wiberg (3) and then Leffler and Grunwald (8).

$$\delta\Delta H = \delta\Delta H_i + \delta\Delta H_e \quad \text{(Eq. 14)}$$

He assumed the observed entropies to comprise a similar sum but hypothesized the internal entropy changes (in the rigid aromatic system) to be negligible compared to those derived from solvation effects (an infinite isergonic temperature was thus assumed for internal changes since $\delta\Delta S_i = 0$). Thus, the observed $\delta_R\Delta S$ were taken equal to $\delta_R\Delta S_e$. Furthermore, on the basis of the observations of Brown cited above, he assumed the solvation energies and entropies to be isergonically related with an isergonic temperature of 280° K. (Eq. 15):

$$\delta_R\Delta H_e = (280)\delta_R\Delta S_e = (280)\delta_R\Delta S \quad \text{(Eq. 15)}$$

The observed enthalpies of ionization are then related to the corresponding entropies by Eq. 16:

$$\delta_R\Delta H = \delta_R\Delta H_i + (280)\delta_R\Delta S \quad \text{(Eq. 16)}$$

Notice that this relation could be represented graphically by an isergonic plot with $\beta_e = 280°$ with each experimental point displaced vertically (parallel to the $\delta\Delta H$ axis since $\beta_i = \infty$) from the line by an amount $\delta\Delta H_i$. Now if the substituent effect on the free energy is examined, Eq. 17 is obtained:

$$\delta_R\Delta G = \delta_R\Delta H_i + (280 - T)\delta_R\Delta S \quad \text{(Eq. 17)}$$

Since the experimental temperature for which the observed Hammett correlation of these data was made is near 280° (the data were for 298° as usual), the contribution of the entire solvation effect (contained in the last term of Eq. 17) is almost completely lost from the free energy. In fact, as Hepler points out, if the 280° isergonic temperature is generally applicable to solvation phenomena in water, then solvation effects are nearly always negligible and the Hammett equation "sees" only the potential energy contributions to the free energy. This explains why the Hammett equation in aqueous solution (or, as we shall see below, in other protic solvents) near room temperature is so much more successful than it "should be" in providing data explicable in simple potential energy terms.

It has been found (26) that Hepler's theory is not limited to aqueous solution, to ionization processes, or to equilibrium data. The enthalpies of activation and the entropies of activation for the methoxide-catalyzed methanolysis of aryl methyl carbonates in methanol (to give dimethyl carbonate and the phenol) appear to be unrelated. Rates of methoxyl exchange from tritium-labeled esters were found to be much slower than solvolysis so that addition to the carbonyl group of the ester by methoxide ion is the rate-determining step. The rate process under study then is essentially addition to an aryloxy-substituted carbonyl group. According to the Hepler theory, the vertical displacements of the individual points from an isergonic line for solvation interactions should give $\delta\Delta H_i$* for each substituent, the effect of the substituent on the internal enthalpy (roughly the internal potential energy) of activation. If 320° K. is adopted as the solvation isergonic temperature for methanol, these deviations in fact give an excellent correlation with the Hammett σ. The slope is consistent with inductive stabilization of the negative charge being introduced in the activated complex and with resonance stabilization of the electron density being released from the carbonyl group onto the aryl oxygen by addition of the methoxide ion. As predicted by the Hepler theory, the free energies are correlated (roughly) by the Hammett σ and very well by the free energies of ionization of the phenols, determined under the same conditions.

Under the discussion of catalytic interactions, we shall see that Hepler's theory probably explains the great success of the Brønsted equation in correlating catalytic constants and basicities or acidities. It also seems entirely possible that the new and extremely successful applications of linear free energy relations to biological phenomena such as drug action, by Hansch (27) and others, will be found to rest in part on the existence of isergonic relations for certain perturbing interactions which cause their cancellation or near-cancellation from the observed free energy data.

Arnett and Burke (28) have recently shown as well that Hepler's theory explains the correlation they found between heats of ionization of acids in nonaqueous or water-poor media and free energies of ionization in water-rich media. They cite other data to support their opinion, which agrees with mine, that this theory underlies a "vast array of free-energy–structure correlations."

Nonbonded Interactions—This term is probably used most often to refer to the repulsive interactions between groups forced to exist closely enough to experience mutual repulsion of their outer electron clouds. No entropy change would be expected to result from this kind of interaction among structureless groups. However, if the groups contain a sufficient array of substructure that close steric interaction causes the restriction of internal rotations, or increases the frequency of vibrations (usually bends) which have a low enough initial frequency to have

some population of molecules in the excited vibrational states at ordinary temperature (and thus initially some vibrational entropy), then a reduction in entropy is expected to arise coincidentally with the onset of steric repulsion. If both of these processes came about concurrently and gradually, an enthalpy–entropy relation would be expected, with an increase in enthalpy (incursion of repulsion) being accompanied by a reduction in entropy (restriction of motions). This model thus predicts a negative isergonic temperature, which has not been observed for cases of this kind.

The reason perhaps lies in the very steep distance dependence of steric interactions: they arise only at very short distances and the associated energies then increase very rapidly as the distance is further shortened between the interacting groups (3). Furthermore, rotational motion can probably be restricted by mere development of a potential well within which the former rotor now sits stationary, but not experiencing any increase in potential energy; in fact the energy will have decreased due to the loss of the kinetic energy of the rotor. Thus as the two interacting groups approach, the internal rotations of the substructure probably are lost at distances too great for severe steric repulsion. Later, when the repulsion begins to increase the energy, the groups are already frozen and no further entropy can be lost.

The latter model is in agreement with some data collected and tabulated by Taft (25). The steric strain energies of activation for esterification and hydrolysis reactions of esters were (in effect) taken as equal to the deviations (in energy units) from the Taft equation plot of the experimental enthalpies. These are plotted *versus* the entropies of activation in Fig. 2. The model predicts effectively a sharp change from an isergonic temperature of zero (entropy loss with no accompanying energy change) to an isergonic temperature of infinity (energy increase with no further entropy change), which Fig. 2 shows to be the case.

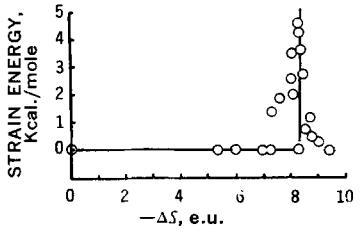


Fig. 2—Strain energies of activation versus *entropies of activation for hydrolysis and esterification of esters under various conditions, from the tabulation by Taft (25).*

Charge–Transfer Interactions—We have just seen that the restriction of internal rotations by steric interaction is unlikely to produce an observed relation due to contributions from the entropy loss of the former and the enthalpy increase of the latter; if some longer range force is simultaneously coming into operation, however, it might be possible to observe a relation between the enthalpy change from this source and the entropy loss from the restriction of internal rotations mentioned above. A possible system is offered by charge–transfer complexation of structured molecules.

A number of cases have been collected and examined for such correlations by Andrews and Keefer (29) An isergonic relation (β = 690° K.) is found for the complexation in carbon tetrachloride solution of iodine and iodine monochloride with alkylbenzenes, *tert*-butyl alcohol, and dioxane (although most oxygen bases do not fit the same equation). A similar correlation is obtained for iodine with aliphatic amines in *n*-heptane solution (β = 637° K.) and with nitriles, and for iodine, iodine monochloride, and iodine monobromide in carbon tetrachloride solution (β = 540° K.). The absolute magnitudes of the entropy losses in the first cited series are from 3 to 9 e.u. The range (6 e.u.) is not inconsistent with the ascription of the variation to losses of internal rotations, for which values of 2–3 e.u. per rotation are probably appropriate.

The model fits the data in all respects, as noted by Andrews and Keefer. For example, the data for complexation of iodine with hexamethylbenzene (ΔH = −4 Kcal./mole, ΔS = −7 e.u.) and hexaethylbenzene (ΔH = −2 Kcal./mole, ΔS = −3 e.u.) indicate that the greater steric bulk of the ethyl groups restricts the complexing agent to a greater distance from the π-cloud, thus making the bonding less exothermic, but also requiring the restriction of fewer rotations, thus making the entropy change less negative. The values in general of β about 600° appear to show that closer approach of the complexing agent, such that entropy equivalent to that of one internal rotation is lost ($-\Delta S \sim$ 2–3 e.u.), produces a bond about 1.2–1.8 Kcal./mole tighter than before.

Hydrogen Bonding—Hydrogen bonding is a long range force because the proton bridge, which *per se* has practically no steric requirement, serves to keep the two bonding moieties at a sufficient removal from one another that little or no steric interaction (an exceedingly short-range force) between the bonding groups is ex-

pected.[6] Thus, if a single hydrogen bond binds two species together, only the loss of translational entropy is expected, together with some entropy loss from restriction of rotation about the bonds forming the hydrogen bridge itself. Neither of these should vary much with the strength of the hydrogen bond, so that enthalpy–entropy relations would not be expected from this source. In aqueous solution, some changes in solvation on formation of the hydrogen bond might occur, leading to an isergonic relation with $\beta \sim 280$–$300°$, as discussed above.

The situation differs if more than one hydrogen bond forms between the same two bodies, or if the hydrogen bond is one of several forces binding the two moieties together. In this case the formation of the hydrogen bond should nearly always be accompanied by a loss of entropy, from restriction of internal rotational motions on conversion of linear into cyclic conformations and from conversion of low-frequency chain-deformation vibrational modes into higher frequency ring-deformation modes. Furthermore, ring formation of this type will promote closer steric interaction between the sides of the new ring, thereby restricting internal rotational motion to a still greater degree and probably increasing the frequency of some bending vibrations. In general, a stronger hydrogen bond, formed with greater liberation of enthalpy, ought to lead to a more rigidly restricted product and thus to a greater loss in entropy.

Although the data are not ideal for a test of this hypothesis, an isergonic plot has been constructed in Fig. 3 from a variety of measurements reported by Davies (31). The scatter is not surprising in view of the variety of types and sources of information included, but the correlation is satisfactory, with an isergonic temperature of about 270°. An entropy loss equivalent to freezing of one internal rotation (2–3 e.u.) thus results from strengthening of the hydrogen bonding system by about 0.5–0.8 Kcal./mole.

Covalent Bonding—Covalent bonding between structured species might be expected to generate isergonic relations in the same fashion described in the cases above for other types of bonding. Some of the many isergonic relations tabulated by Leffler and Grunwald (11) for association and displacement reactions may fall in this category, but the possibility of other origins remains open because of the universal complexity of the systems studied. Covalent bonds in organic compounds are generally formed with exothermicities of 30 to more than 100

[6] Covitz and Westheimer (30) have shown that such steric effects are not entirely negligible.

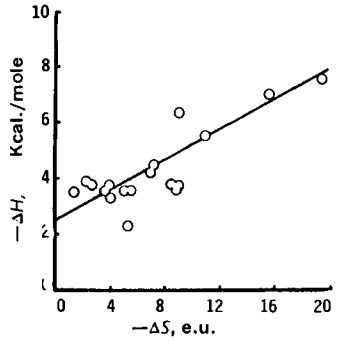


Fig. 3—Compensation of energy and entropy in the formation for hydrogen bonds. The data are taken from Davies (31) and are for dimerization and polymerization in benzene solution of amides, measured by Davies and Thomas (32), and association of diphenylamine, phenol, and benzyl alcohol with dimethylformamide, dioxane, acetonitrile, benzyl acetate, and azobenzene in carbon tetrachloride solution, determined by Flett (33).

Kcal./mole; if the formation is accompanied by the combination of two species into one, the entropy may decrease by as much as 50–60 e.u., while if the bond is formed intramolecularly, only 2–3 e.u. may be lost in the process. The simple ratios of $\Delta H/\Delta S$ thus range from 500 to 50,000° K., although this model cannot generate a continuously varying sequence of enthalpies and entropies.

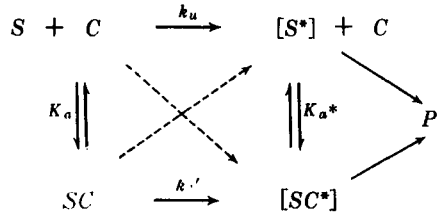
Thorn and his co-workers (34) have observed energy–entropy correlations in high temperature systems and have attributed the results to a fundamental relation between the energy of formation of a substance at the absolute zero and the entropy of the substance at higher temperatures, since structural information is present in both quantities. Thorn's efforts to develop the underlying theory promise to provide a complete statistical-mechanical basis for both isergonic and linear free-energy relations.

QUESTIONS IN CATALYSIS

A Simple Model of Catalysis—To facilitate the consideration of catalytic processes, we will develop a simple model, based on the concept of virtual equilibria among activated complexes, recently applied by Kurz (35) to several mechanistic problems.

Scheme I portrays a system of reactions in which a substrate S may be converted to a product P by (a) a route catalyzed by C, involving formation of a substrate–catalyst complex SC^*, or (b) an uncatalyzed route through activated complex S^*. The rate law is given by Eq. 18:

$$v = k_c'[SC] + k_U[S] = [S](K_a k_c'[C] + k_U) \quad \text{(Eq. 18)}$$



Scheme I

It is readily shown that introduction of the extra, dashed routes makes no change. Scheme I also includes the equilibrium constant, K_a^*, for virtual interconversion of the activated complexes S^* and SC^*, according to Eq. 19:

$$S^* + C \rightleftarrows SC^* \quad K_a^* \qquad \text{(Eq. 19)}$$

It does not matter that the actual interconversion does not occur directly; the concentrations of the two are the same as if it did. Then, since $k_c'K_a = k_UK_a^*$, the rate law may also be written as in Eq. 20:

$$V = [S](k_U + k_UK_a^*[C]) \qquad \text{(Eq. 20)}$$

This can be written in terms of the analytical concentration of S ($[S]_{tot}^{\circ}$), for conditions of excess catalyst as Eq. 21, or in terms of the analytical concentration of C ($[C]_{tot}$) for conditions of excess substrate (as in many enzyme experiments) as in Eq. 22:

$$V = k_U[S]_{tot}\left\{\frac{1 + K_a^*[C]}{1 + K_a[C]}\right\} \qquad \text{(Eq. 21)}$$

$$V = k_U[S]\left\{1 + \frac{K_a^*[C]_{tot}}{1 + K_a[S]}\right\} \qquad \text{(Eq. 22)}$$

The point is clear from either of these expressions that the larger K_a^* is relative to K_a, the more efficient C is as a catalyst. In other words, the stronger the association of catalyst with S^* to form SC^*, the more rapidly the catalyzed reaction will proceed. Quasi-thermodynamic functions are of course associated with K_a^* (Eq. 23):

$$\Delta G_a^* = -RT\ln K_a^* = \Delta H_a^* - T\Delta S_a^* \qquad \text{(Eq. 23)}$$

Let us divide up the free energy (and enthalpy and entropy) change on association of S^* and C into contributions from each of the interactions which occurs (Eq. 24):

$$\Delta G_a^* = \sum_i g_i = \sum_i h_i - T\sum_i s_i \qquad \text{(Eq. 24)}$$

Assume that h_i and s_i for each of these interactions are related, at least roughly, isergonically (Eq. 25):

$$h_i = \beta_i s_i \qquad \text{(Eq. 25)}$$

Then ΔG_a^* can be written as a sum in either enthalpies or entropies or both, for individual interactions (Eq. 26):

$$\Delta G_a^* = \sum_i (\beta_i - T)s_i = \sum_i (1 - T/\beta_i)h_i = \sum_j (\beta_j - T)s_j + \sum_k (1 - T/\beta_k)h_k \qquad \text{(Eq. 26)}$$

Notice that if the catalyzed reaction is to proceed faster than the uncatalyzed reaction, ΔG_a^* must be negative. Thus any interaction which contributes effectively to catalysis must have $g_i = (\beta_i - T)s_i < 0$. For interactions which involve binding of some kind, s_i will usually be negative. Thus only if $\beta_i > T$ (the isergonic temperature exceeds the experimental temperature) is the interaction likely to enhance catalytic efficiency. Of the interactions considered in the last section, covalent bonding (probable $\beta \sim$ 500–50,000° K.) and charge-transfer bonding ($\beta \sim$ 500–700° K.) are clearly catalytic near room temperature, while hydrogen bonding ($\beta \sim$ 270° K.) and ion-pairing ($\beta \sim$ 200° K.) are noncatalytic.

Use of the average values for β_i's obtained in the last section may give spurious results, however. All those values were obtained by use of systems where the forces were involved as primary (or very important) binding forces. In a catalytic activated complex, other forces may already hold a functional group in such a position that the interaction can occur with an abnormally small loss in entropy; the effective value of $\beta(\delta\Delta H/\delta\Delta S)$ will then be increased and the interaction becomes more catalytically effective. Thus, if a hydrogen bond with an enthalpic strength of 2 Kcal./mole were able to form with only a restriction of a single internal rotation (say, loss of 3 e.u.) in a preformed environment, $\beta_{eff} = 2000/3 \sim 700°$ K., and the interaction is nicely catalytic.

Conceivable interactions which come under scrutiny in the formulation of a mechanistic model can be designated (in the context of the activated-complex model) as *obligatory* (the model requires that they occur) or *elective* (the model makes no statement). Models will sometimes include obligatory interactions with positive g_i, *i.e.*, anticatalytic interactions. Steric repulsions which necessarily must be included to allow other, more favorable, interactions are examples. Of the elective interactions, those with $g_i > 0$ will not occur and may be omitted from the summation (that is, it is unreasonable to postulate that they occur). Those with $g_i < 0$ will occur and are catalytic. The best model is of course the one in which the obligatory and elec-

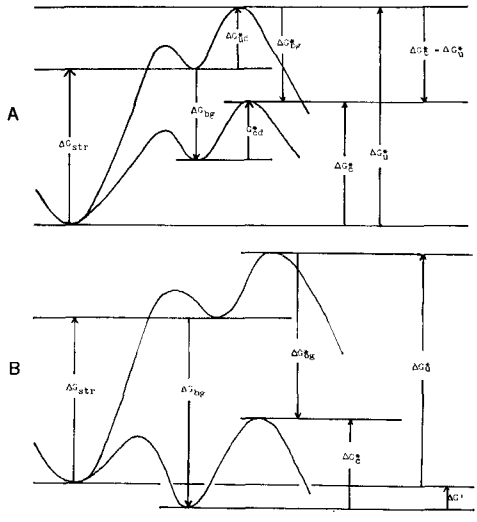


Fig. 4—Key: A, *distortion-catalysis in a system where the substrate is chiefly free;* B, *distortion-catalysis in a system where the substrate is chiefly complexed.*

tive interactions have been so balanced as to make ΔG_a^* as negative as possible.

Is the present model sufficiently sophisticated to apply to the complex kinetic systems frequently encountered in practice, particularly with enzymes? With some qualification, an affirmative answer is possible. Equation 22 is readily shown to be equivalent to the Michaelis-Menton equation. Lumry (7) has discussed in detail how experimental activation parameters from such a treatment can be related to mechanisms for more complex reactions. For our purposes, we need to regard ΔG_a^* as referring not to formation of a single activated complex SC^* but rather an abstract weighted-average entity for all activated complexes along the reaction path. The weighting factor for the ith activated complex is exp (G_i^*/RT).

Catalysis by Strain and Distortion—The view seems widely current[7] that many enzymes are catalytically active by virtue of the ability to induce in the substrate molecule some sort of strain or distortion, increasing its energy and thereby decreasing the energy required to transform it into a (presumably no longer strained) activated complex. Notable examples of such ideas are the "rack" hypothesis of Eyring and his co-workers (38) according to which the enzyme stretches bonds of the substrate, rendering their fission more facile; the proposal of Westheimer and his co-workers (39) that ribonuclease functions by intermediate formation of strained cyclic five-membered phosphate esters which, demonstrably in model systems, hydrolyze enormously more rapidly than their open-chain analogs; and the proposal of Bruice and Pandit (40) that esterases may bind esters in the unfavorable *trans* rotameric form, facilitating attack at the carbonyl group by nucleophilic reagents.

These hypotheses must be considered in two different ways, depending upon whether the major form of the reactant in limiting concentration under the conditions of interest is the free substance or the substance bound in the enzyme–substrate complex. Both circumstances are shown in Fig. 4. Figure 4A, refers to the former situation (free reactants). We compare the catalyzed reaction (lower curve) with the (perhaps hypothetical) uncatalyzed reaction passing through the strained or distorted form (pictured as the high minimum) along the upper curve. Shown on the diagram are ΔG_{str}, the increase in free energy of the substrate on conversion to the strained form; ΔG_{bg}, the free energy released on binding of the strained form of the substrate to the catalyst; ΔG_{ud}^*, the free energy of activation for conversion of the strained substrate to the uncatalyzed activated complex; ΔG_{cd}^*, the similar quantity for conversion of the strained-substrate–catalyst complex to the catalyzed activated complex; ΔG_U, the over-all free energy of activation for the uncatalyzed reaction; and ΔG_C, the over-all free energy of activation for the catalyzed reaction. It is graphically obvious that the entire catalytic efficiency, $\Delta G_U - \Delta G_C$, is given by the free energy of binding of the catalyst to the activated complex, the distortion energy cancelling out of the observable quantities. Thus the distortion, while surely an interesting characteristic of the catalytic mechanism, does not directly enter into the catalytic efficiency. This model applies to enzymatic catalysis at such reactant concentrations that the velocity is considerably less than maximum.

Figure 4B, shows the other possibility, the same quantities being displayed on the diagram. Here the complex is more stable than the free reactants. Inspection now shows that the catalytic efficiency is given by $-\Delta G_{bg} + \Delta G'$, where $\Delta G'$ is the excess of binding free energy over strain free energy for the complex (expressed as a positive number). The larger $\Delta G'$, the less effective the catalysis; the more negative ΔG_{bg}, the more effective the catalysis. Once again, the important point is that the catalyst bind strongly to the activated complex relative to the reactant.

Thus the methods of reasoning given above apply to these cases equally well.

[7] For a discussion of this and the enthalpy–entropy question in intramolecular catalysis, omitted here for lack of space, see *References 36* and *37*.

TABLE II—ACTIVATION PARAMETERS FOR THE MUTAROTATION OF GLUCOSE ANALYZED BY HEPLER'S ISERGONIC THEORY[a]

Catalyst	$\Delta H^*)$ [b]	$\Delta S^*)$ [c]	$\delta_C \Delta H^*)$ [d]	$\delta_C \Delta S^*)$ [d]	$\delta_C \Delta H$	$\delta_C \Delta H_i$
H_2O	17.2	−24.9	(0)	(0)	(0)	(0)
HCO_2^-	17.1	−15.2	−0.1	+9.7	+2.7	−2.8
$CH_3CO_2^-$	18.2	−10.4	+1.0	+14.5	+4.1	−3.1
NH_3	13.3	−17.6	−3.9	+7.3	+2.0	−5.9
Glucosate ion	17.3	−0.4	+0.1	+24.5	+6.9	−6.8
HO^-	18.3	+11.8	+1.1	+36.7	+10.0	−8.9

[a] Data are from Schmid, H., and Bauer, G., *Monatsh Chem.*, **96**, 1503(1965) All enthalpy data are in Kcal./mole; entropies in e.u. [b] Errors are about ±0.4 Kcal./mole. [c] Errors are about ±1.5 e.u. All figures are given, even if not significant.

Acid–Base Catalysis and Catalytic Efficiency—Catalytic efficiencies for acid-base catalysis are most frequently described by the Brønsted relation (Eq. 27), one of the oldest linear free energy relations (41–43):

$$\delta_C \Delta G^* = \alpha \delta_C \Delta G^\circ \qquad \text{(Eq. 27)}$$

Here ΔG^* is the free energy of activation, ΔG° the free energy of neutralization or ionization of the catalyst, and δ_C the Leffler-Grunwald operator for catalyst variation. It was early rationalized (41) in terms of intersecting Morse curves, implying that potential energy variations were at its basis, while Leffler (44) later provided a noncommittal free-energy argument. In any case, examination of activation parameters for various catalysts correlated by the equation frequently reveals startling irregularities (45). Exemplary are the data of Schmid and Bauer (46) for the mutarotation of glucose, shown in Table II.

The validity of the Brønsted relation under these circumstances is understandable in terms of Hepler's isergonic theory, outlined above. Following Hepler, we assume $\delta_C \Delta S^* = \delta_C \Delta S_e$, entirely due to solvation. Furthermore, we assume $\delta_C \Delta H_e = 280 \delta_C \Delta S_e$ and that $\delta_C \Delta H^* = \delta_C \Delta H_i + \delta_C \Delta H_e$. These quantities are all listed in Table II. Note that the $\delta_C \Delta H_i$ are all negative and decrease with increasing basicity of the catalyst. These quantities are the enthalpies for the process shown in Scheme II.

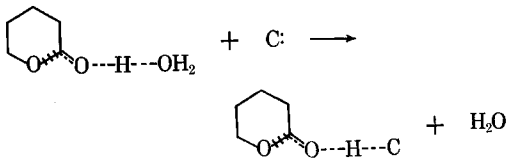
They are therefore effectively the strengths of the hydrogen bonds by which each catalyst is bound to the activated complex. The values are unreasonably large for ordinary hydrogen bonds, in agreement with a recent suggestion that unusually strong hydrogen-bonding capabilities are displayed by polarizable activated complexes.[8]

The Brønsted relation holds because, as shown in Eq. 28,

$$\delta_C \Delta G^* = \delta_C \Delta H_i + (\beta - T)\delta_C \Delta S \approx \delta_C \Delta H_i \qquad \text{(Eq. 28)}$$

$\delta_C \Delta G^* \approx \delta_C \Delta H_i$, because $\beta = 280^\circ \approx 298^\circ$

[8] See *Reference 47*. For another point of view, see *References 48* and *49*.



Scheme II

Furthermore, this makes the Morse curve argument and the Leffler rationale essentially equivalent.

The Hydrophobic Bond—Many discussions of this phenomenon have emphasized the "entropic" driving force of the liberated water molecules in stabilizing the combined form of nonpolar solutes or side chains in water, while others (7) have postulated an increase in the strength of hydrogen bonding by these liberated molecules.

If the solvation changes involved here are also described by Hepler's isergonic theory, then we can write for the free energy of combination of two nonpolar solutes:

$$\Delta G = \Delta G_i + \Delta H_e - T\Delta S_e = \Delta G_i + (\beta - T)\Delta S_e \qquad \text{(Eq. 29)}$$

and regardless of which effect is postulated above, $\beta \approx 280^\circ \approx T$ and $\Delta G \approx \Delta G_i$. This suggests that the actual driving forces are simply dispersion forces, *etc.* Of course the driving force may experimentally *appear* to be entropic if $\Delta G_i \approx -\Delta H_e$ by coincidence.

Catalytic Specificity and Free Energy—By a simple extension of the above model for catalysis we can examine the relative rates of reaction of two different substrates (catalytic specificity) by using the virtual equilibrium constant of Eq. 30 and its associated functions (Eqs. 31 and 32):

$$S_1 + S_2C^* \rightleftarrows S_2 + S_1C^* \; K_{12}^* \qquad \text{(Eq. 30)}$$

$$\Delta G_{12}^* = -RT \ln K_{12}^* = \sum_i g_i \qquad \text{(Eq. 31)}$$

$$g_i = h_i - Ts_i = (\beta_i - T)s_i \qquad \text{(Eq. 32)}$$

which, as before, are divided into contributions from individual interactions. g_i is then the change in free energy of the ith interaction of the

catalyst on interchange of S_2 for S_1 at the binding site. A negative value of ΔG_{12}^* means a faster reaction for S_1; a negative value of g_i means that the ith interaction favors a faster reaction with S_1.

A large negative value of ΔG_{12}^* means that the reaction is much faster for S_1 than for S_2 and the catalyst is said to be "specific" for S_1.

Assume the catalyst is reasonably specific for S_1. The interactions which confer this specificity are likely to be binding interactions for which the s_i (Eq. 32) is negative. In order for the interaction actually to contribute to specificity, $(\beta_i - T)$ must also then be positive; the more positive it is, the more effective the interaction in aiding the catalyst to discriminate between S_1 and S_2, for a given amount of binding.

Just as was found with efficiency, above, a large value of β_i permits an interaction to contribute strongly to catalytic specificity. The phenomena of specificity and efficiency, as Lumry (7) predicted, would appear strongly linked.

The Biological Value of Specificity: Information and Entropy (50–54)—The present treatment permits an interesting look at catalytic specificity in a living system in terms of its value to the system. Why are biological catalysts generally unusually capable of selecting one substrate over another? The probable answer is that any other course would lead to chaos in the cell—to a general breakdown of the intricate metabolic scheme which underlies continued life. This intricate metabolic scheme is a pattern of information and the value of enzymatic specificity is that it preserves this information.

Many authors have discussed the relation of information to entropy and the fact that living systems, because of their high information content, are reservoirs of negative entropy. As was seen above, the interactions which lend specificity to catalysts are likely to be ones of negative entropy—in order to "see" that it has the right substrate, the enzyme must bind it intimately (the *total* entropy of binding or catalysis may, of course, have any sign or magnitude).

Consider the free energy change when a "wrong" substrate is bound in preference to a "right" one (in Eq. 30 let S_1 now be "wrong" and S_2 "right"); ΔG_{12}^* must be positive (in order for the "wrong" reaction to be slower). For specificity-conferring interactions, as was just argued, s_i will be positive. The more "wrong" the substrate is, the more positive s_i will be. This is reasonable since if the "wrong" reaction occurs, information will be lost to the system (and its entropy will increase). In fact, if one bit (binary unit) of information (the amount required to answer one yes-or-no question) is lost, the associated increase in entropy is $R \ln 2$, or 1.4 e.u.[9] By what factor will the rate decrease if this much information is to be lost? The answer is given by g_i: for each increment of 2.3 RT (1.42 Kcal./mole of 37° C.), the rate will decrease by a factor of 10. The ratio g_i/s_i for any interaction then gives the ratio of decrease in rate of the specific reaction (x powers of 10) which will occur if the structure of the substrate is sufficiently "wrong" that y bits of information are lost (Eq. 33):

$$\frac{g_i}{s_i} = \frac{(2.3\ RT)x}{(R \ln 2)y} \cong 1000\ \frac{x}{y} = (\beta_i - T) \quad \text{(Eq. 33)}$$

This is just $\beta_i - T$ for the interaction in question. In fact x/y (in units of orders/bit) $= (\beta_i - T)/1000$ can be called the *informational specificity* of an interaction. For reasonable ranges of β_i, estimated above, these are listed for a few interactions in Table III.

TABLE III—INFORMATIONAL SPECIFICITY AT 37° C. FOR SOME CATALYTIC INTERACTIONS

Interaction	$[(\beta_i - T)/1000]$ orders/bit
Covalent bonding	0.2 to 50
Hydrogen bonding	0 to 2.2
Electrostatic bonding	0 to 1
Charge-transfer bonding	0.5 to 0.8

Modification of the model should be possible to include specificity hypotheses such as "wrong-way binding" (56).

REFERENCES

(1) Glasstone, S., Laidler, K. J., and Eyring, H., "The Theory of Rate Processes," McGraw-Hill Book Co., New York, N. Y., 1941.
(2) Eyring, H., and Eyring, E. M., "Modern Chemical Kinetics," Reinhold Publishing Corp., New York, N. Y., 1963.
(3) Wiberg, K. B., "Physical Organic Chemistry," John Wiley & Sons, Inc., New York, N. Y., 1964.
(4) Thornton, E. R., "Solvolysis Mechanisms," Ronald Press Co., New York, N. Y., 1965.
(5) Johnston, H. S., "Gas Phase Reaction Rate Theory," Ronald Press Co., New York, N. Y., 1966.
(6) "The Transition State," Special Publication No. 16, Chemical Society, London, England, 1962, pp. 26–27.
(7) Lumry, R., in "The Enzymes," 2nd ed., Boyer, P. D., Lardy, H., and Myrbäck, K., eds., Academic Press Inc., New York, N. Y., 1959, vol. 1, chap. 4.
(8) Kreevoy, M. M., in "The Technique of Organic Chemistry," Weissberger, A., ed., Interscience Publishers, Inc., New York, N. Y., 1963, vol. 8, part II.
(9) Glasstone, S., Laidler, K. J., and Eyring, H., *op. cit.*, p. 146.
(10) Alder, M. G., and Leffler, J. E., *J. Am. Chem. Soc.*, **76**, 1425(1954).
(11) Leffler, J. E., and Grunwald, E., "Rates and Equilibria of Organic Reactions," John Wiley & Sons, Inc., New York, N. Y., 1963, chap. 9.
(12) Huyser, E. S., and VanScoy, R. S., unpublished data
(13) Leffler, J. E., *J. Org. Chem.*, **20**, 1202(1955).
(14) *Ibid.*, **31**, 533(1966).
(15) Petersen, R. C., Markgraf, J. H., and Ross, S. D. *J. Am. Chem. Soc.*, **83**, 3819(1961).
(16) Petersen, R. C., *J. Org. Chem.*, **29**, 3133(1964).
(17) Frost, A. A., and Pearson, R. G. "Kinetics and Mechanism," 2nd ed., John Wiley & Sons, Inc., New York N. Y., 1961, p. 136.
(18) Linderstrøm-Lang, K. U., and Schellman, J. A., in "The Enzymes," 2nd ed., Boyer, P. D., Lardy, H., and

[9] For an introduction, see *Reference 55*.

Myrbäck, K., eds., Academic Press Inc., New York, N. Y., 1959, vol. 1, chap. 10.
(19) Duncan, J. F., and Kepert, D. L., in "The Structure of Electrolyte Solutions," Hamer, W. J., ed., John Wiley & Sons, Inc., New York, N. Y., 1959.
(20) Brown, R. F., *J. Org. Chem.*, **27**, 3015(1962).
(21) Belleau, B., Chemical Biology Seminar, University of Kansas, Lawrence, Kan., October 1966.
(22) Kavanau, J. L., "Water and Solute–Water Interactions," Holden-Day, Inc., San Francisco, Calif., 1964.
(23) Hepler, L. G., *J. Am. Chem. Soc.*, **85**, 3089(1963).
(24) Hammett, L. P., "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1941.
(25) Taft, R. W., Jr., in "Steric Effects in Organic Chemistry," Newman, M. S., ed., John Wiley & Sons, Inc., New York, N. Y., 1956.
(26) Schowen, R. L., Mitton, C. G., and Shapley, J., Abstracts of 152nd Meeting of the American Chemical Society, New York, N. Y., September 1966, paper S139.
(27) Hansch, C., and Fujita, T., *J. Am. Chem. Soc.*, **86**, 1616(1964).
(28) Arnett, E. M., and Burke, J. J., *ibid.*, **88**, 4308(1966).
(29) Andrews, L. J., and Keefer, R. M., "Molecular Complexes in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, chap. IV.
(30) Covitz, F., and Westheimer, F. H., *J. Am. Chem. Soc.*, **85**, 1773(1963).
(31) Davies, M., in "Hydrogen Bonding," Hadzi, D., and Thompson, H. W., eds., Pergamon Press, New York, N. Y., 1959.
(32) Davies, M., and Thomas, D. K., *J. Phys. Chem.*, **60**, 763, 767(1956).
(33) Flett, M., *J. Soc. Dyers Colourists*, **68**, 59(1952).
(34) Ackermann, R. J., Thorn, R. J., and Winslow, G. H., in "Physical Chemistry in Aerodynamics and Space Flight," Myerson, A. L., and Harrison, A. C., eds., Pergamon Press, Oxford, England, 1961.
(35) Kurz, J. L., *J. Am. Chem. Soc.*, **85**, 987(1963).
(36) Bruice, T. C., and Benkovic, S. J., "Bioorganic Mechanisms," W. A. Benjamin, New York, N. Y., 1966, vol. 1, pp. 242 *ff*.
(37) Jencks, W. P., *Ann. Rev. Biochem.*, **32**, 639(1963).
(38) Eyring, H., Lumry, R., and Spikes, J. D., in "The Mechanism of Enzyme Action," McElroy, W. D., and Glass, B., eds., Johns Hopkins Press, Baltimore, Md., 1954.
(39) Westheimer, F. H., *Advan. Enzymol.*, **24**, 441(1962).
(40) Bruice, T. C., and Pandit, U. K., *Proc. Natl. Acad. Sci. (U. S.)*, **46**, 402(1960).
(41) Bell, R. P., "Acid-Base Catalysis," Oxford University Press, Oxford, England, 1949.
(42) Bell, R. P., "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959.
(43) Leffler, J. E., and Grunwald, E., *op cit.*, pp. 235 *ff*. and 369 *ff*.
(44) Leffler, J. E., *Science*, **117**, 340(1953).
(45) Ashmore, P. G., "Catalysis and Inhibition of Chemical Reactions," Butterworths, London, England, 1963, chap. 1.
(46) Schmid, H., and Bauer, G., *Monatsh. Chem.*, **96**, 1503(1965).
(47) Swain, C. G., Kuhn, D. A., and Schowen, R. L., *J. Am. Chem. Soc.*, **87**, 1553(1965).
(48) do Amaral, L., Sandstrom, W. A., and Cordes, E. H., *ibid.*, **88**, 2225(1966).
(49) Reimann, J. E., and Jencks, W. P., *ibid.*, **88**, 3973 (1966).
(50) Schrödinger, E., "What is Life?" Cambridge University Press, Cambridge, England, 1944.
(51) Elsasser, W. M., "The Physical Foundation of Biology," Pergamon Press, New York, N. Y., 1958.
(52) Brillouin, L., "Science and Information Theory," 2nd ed., Academic Press Inc., New York, N. Y., 1962.
(53) "Information Theory and Biology," Quastler, H., ed., University of Illinois Press, Urbana, Ill., 1953.
(54) Schaltegger, H., *Chimia (Aarau)*, **20**, 197, 237(1966).
(55) Stetlow, R. B., and Pollard, E. C., "Molecular Biophysics," Addison-Wesley, Reading, Mass., 1962, chap. 3.
(56) Rapp, J. R., Niemann, C., and Hein, G. E., *Biochemistry*, **5**, 4100(1966).

Research Articles

Simultaneous Determination of Dissolution and Partitioning Rates *In Vitro*

By P. J. NIEBERGALL, M. Y. PATIL, and E. T. SUGITA

An *in vitro* method is presented for the simultaneous determination of the dissolution and partitioning rates of drugs. The kinetics of the system is described, and a number of methods are given for evaluating the rate constants. The effects of stirring rate and temperature upon the rate constants were investigated and found to be in agreement with what would be expected for diffusion-controlled processes.

THE DISSOLUTION rate of a drug may have a marked effect upon the absorption of the drug from a solid dosage form. This has led to an increasing interest in developing *in vitro* dissolution rate tests that can be correlated with *in vivo* absorption rate studies for possible use in quality control or for use in setting official standards. In addition, dissolution rates have been used to evaluate formulation variables under the assumption that all other things being equal, the drug formulation that dissolves most rapidly would have the greatest chance of clinical success.

The over-all absorption process for solid drugs, however, consists of the dissolution step followed by drug partitioning into an essentially lipid barrier. The three phase "rocking apparatus," developed by Doluisio and Swintosky (1), appears to have great promise in studying the effects of additives or of molecular modification upon the partitioning rates of drugs. This apparatus, however, cannot be used to investigate the dissolution process, and therefore is useful only for drugs in solution. Thus, the over-all process of absorption

Received February 20, 1967, from the Department of Pharmacy, The Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication May 5, 1967.

Myrbäck, K., eds., Academic Press Inc., New York, N. Y., 1959, vol. 1, chap. 10.
(19) Duncan, J. F., and Kepert, D. L., in "The Structure of Electrolyte Solutions," Hamer, W. J., ed., John Wiley & Sons, Inc., New York, N. Y., 1959.
(20) Brown, R. F., *J. Org. Chem.*, **27**, 3015(1962).
(21) Belleau, B., Chemical Biology Seminar, University of Kansas, Lawrence, Kan., October 1966.
(22) Kavanau, J. L., "Water and Solute–Water Interactions," Holden-Day, Inc., San Francisco, Calif., 1964.
(23) Hepler, L. G., *J. Am. Chem. Soc.*, **85**, 3089(1963).
(24) Hammett, L. P., "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1941.
(25) Taft, R. W., Jr., in "Steric Effects in Organic Chemistry," Newman, M. S., ed., John Wiley & Sons, Inc., New York, N. Y., 1956.
(26) Schowen, R. L., Mitton, C. G., and Shapley, J., Abstracts of 152nd Meeting of the American Chemical Society, New York, N. Y., September 1966, paper S139.
(27) Hansch, C., and Fujita, T., *J. Am. Chem. Soc.*, **86**, 1616(1964).
(28) Arnett, E. M., and Burke, J. J., *ibid.*, **88**, 4308(1966).
(29) Andrews, L. J., and Keefer, R. M., "Molecular Complexes in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, chap. IV.
(30) Covitz, F., and Westheimer, F. H., *J. Am. Chem. Soc.*, **85**, 1773(1963).
(31) Davies, M., in "Hydrogen Bonding," Hadzi, D., and Thompson, H. W., eds., Pergamon Press, New York, N. Y., 1959.
(32) Davies, M., and Thomas, D. K., *J. Phys. Chem.*, **60**, 763, 767(1956).
(33) Flett, M., *J. Soc. Dyers Colourists*, **68**, 59(1952).
(34) Ackermann, R. J., Thorn, R. J., and Winslow, G. H., in "Physical Chemistry in Aerodynamics and Space Flight," Myerson, A. L., and Harrison, A. C., eds., Pergamon Press, Oxford, England, 1961.
(35) Kurz, J. L., *J. Am. Chem. Soc.*, **85**, 987(1963).
(36) Bruice, T. C., and Benkovic, S. J., "Bioorganic Mechanisms," W. A. Benjamin, New York, N. Y., 1966, vol. 1, pp. 242 *ff*.
(37) Jencks, W. P., *Ann. Rev. Biochem.*, **32**, 639(1963).
(38) Eyring, H., Lumry, R., and Spikes, J. D., in "The Mechanism of Enzyme Action," McElroy, W. D., and Glass, B., eds., Johns Hopkins Press, Baltimore, Md., 1954.
(39) Westheimer, F. H., *Advan. Enzymol.*, **24**, 441(1962).
(40) Bruice, T. C., and Pandit, U. K., *Proc. Natl. Acad. Sci. (U. S.)*, **46**, 402(1960).
(41) Bell, R. P., "Acid-Base Catalysis," Oxford University Press, Oxford, England, 1949.
(42) Bell, R. P., "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959.
(43) Leffler, J. E., and Grunwald, E., *op cit.*, pp. 235 *ff*. and 369 *ff*.
(44) Leffler, J. E., *Science*, **117**, 340(1953).
(45) Ashmore, P. G., "Catalysis and Inhibition of Chemical Reactions," Butterworths, London, England, 1963, chap. 1.
(46) Schmid, H., and Bauer, G., *Monatsh. Chem.*, **96**, 1503(1965).
(47) Swain, C. G., Kuhn, D. A., and Schowen, R. L., *J. Am. Chem. Soc.*, **87**, 1553(1965).
(48) do Amaral, L., Sandstrom, W. A., and Cordes, E. H., *ibid.*, **88**, 2225(1966).
(49) Reimann, J. E., and Jencks, W. P., *ibid.*, **88**, 3973 (1966).
(50) Schrödinger, E., "What is Life?" Cambridge University Press, Cambridge, England, 1944.
(51) Elsasser, W. M., "The Physical Foundation of Biology," Pergamon Press, New York, N. Y., 1958.
(52) Brillouin, L., "Science and Information Theory," 2nd ed., Academic Press Inc., New York, N. Y., 1962.
(53) "Information Theory and Biology," Quastler, H., ed., University of Illinois Press, Urbana, Ill., 1953.
(54) Schaltegger, H., *Chimia (Aarau)*, **20**, 197, 237(1966).
(55) Stetlow, R. B., and Pollard, E. C., "Molecular Biophysics," Addison-Wesley, Reading, Mass., 1962, chap. 3.
(56) Rapp, J. R., Niemann, C., and Hein, G. E., *Biochemistry*, **5**, 4100(1966).

Research Articles

Simultaneous Determination of Dissolution and Partitioning Rates *In Vitro*

By P. J. NIEBERGALL, M. Y. PATIL, and E. T. SUGITA

An *in vitro* method is presented for the simultaneous determination of the dissolution and partitioning rates of drugs. The kinetics of the system is described, and a number of methods are given for evaluating the rate constants. The effects of stirring rate and temperature upon the rate constants were investigated and found to be in agreement with what would be expected for diffusion-controlled processes.

THE DISSOLUTION rate of a drug may have a marked effect upon the absorption of the drug from a solid dosage form. This has led to an increasing interest in developing *in vitro* dissolution rate tests that can be correlated with *in vivo* absorption rate studies for possible use in quality control or for use in setting official standards. In addition, dissolution rates have been used to evaluate formulation variables under the assumption that all other things being equal, the drug formulation that dissolves most rapidly would have the greatest chance of clinical success.

The over-all absorption process for solid drugs, however, consists of the dissolution step followed by drug partitioning into an essentially lipid barrier. The three phase "rocking apparatus," developed by Doluisio and Swintosky (1), appears to have great promise in studying the effects of additives or of molecular modification upon the partitioning rates of drugs. This apparatus, however, cannot be used to investigate the dissolution process, and therefore is useful only for drugs in solution. Thus, the over-all process of absorption

Received February 20, 1967, from the Department of Pharmacy, The Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication May 5, 1967.

is generally studied *in vitro* as two separate processes, each of which might be rate limiting.

It is conceivable that certain factors might increase the dissolution rate of a drug while simultaneously decreasing its partitioning rate, and possibly reverse the importance of each process in the absorption of the drug. For example, *in vitro* dissolution rate studies have shown that surfactants are capable of increasing the dissolution rate of drugs (2, 3). It has also been shown, however, that surfactants can decrease the *in vivo* absorption of a drug such as salicylic acid (4), the absorption of which would be expected to be rate controlled by the dissolution process. The authors decided, therefore, to investigate the possibility of simultaneously determining the *in vitro* drug dissolution and partitioning rates in a single system.

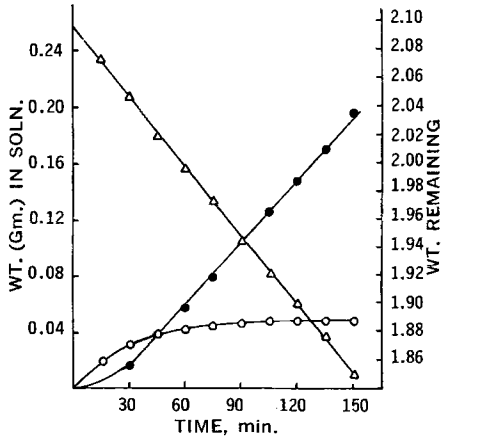


Fig. 1—Dissolution and partitioning rates of salicylic acid at 100 r.p.m. and 30°. Key: △, weight remaining in tablets; ○, weight in pH 2.00 buffer; ●, weight in octanol.

EXPERIMENTAL

Reagents—Potassium chloride, hydrochloric acid, sodium hydroxide, and salicylic acid were of analytical reagent grade obtained from the J. T. Baker Co. Standard grade *n*-octyl alcohol was obtained from Matheson Coleman and Bell. This grade octanol did not interfere with the spectrophotometric determination of salicylic acid.

Apparatus—The apparatus chosen for the study consisted of a 500-ml. round bottom, three-necked standard taper flask fitted with a mercury seal stirrer in the center opening. A 1-in. three-blade propeller was attached to the bottom of the stirring shaft which was inserted into the chuck of a variable speed stirrer.[1] The bottom edge of a second 1-in. three-blade propeller was placed on the stirring shaft exactly 3 cm. above the upper edge of the lower propeller. When 250 ml. of the aqueous phase was added to the flask, the upper edge of the bottom propeller was 2 cm. below the liquid surface. A large diameter glass tube was inserted into one of the remaining standard taper openings, and the closure sealed with aluminum foil. Octyl alcohol (250 ml.) was added through the remaining opening which was closed with a standard taper glass stopper. The lower edge of the upper propeller was 2 cm. above the octanol–water interface.

Procedure—Hard, nondisintegrating tablets of salicylic acid were prepared on a Carver model B laboratory press, without the aid of a lubricating agent. Four tablets were weighed and used in each study. In no instance was the weight loss permitted to extend beyond 10% (2.5% per tablet), thus enabling the assumption of constant surface area.

The apparatus described above was placed in a water bath until both liquid phases had reached constant temperature. The stirrer was started and adjusted to the desired speed, which was measured with a tachometer. The four tablets were introduced into the pH 2.00 buffer solution (hydrochloric acid–potassium chloride, Clark-Lubs, U.S.P. XVII) through the glass tube. One-

[1] Model 102, Talboys Instrument Co., Emerson, N. J.

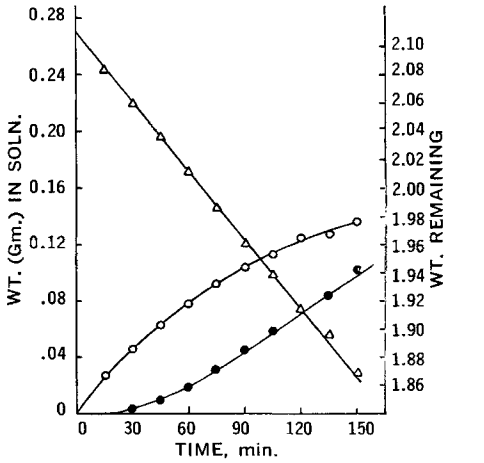


Fig. 2—Dissolution of salicylic acid into 0.1% w/v solution of polysorbate 80 at pH 2.00 and partitioning into octanol. Stirring rate was 100 r.p.m. at 30°. Key: △, weight remaining in tablets; ○, weight in polysorbate 80 solutions; ●, weight in octanol.

milliliter samples of both phases were obtained at 15-min. intervals and assayed at 298 mμ using a Beckman model DU spectrophotometer. The samples were diluted when necessary with either the pH 2.00 buffer or octanol. A total of 10 samples was taken for each run.

RESULTS

Kinetic Analysis of the Data—Typical results obtained are shown in Figs. 1 and 2. The simplest mechanism to be expected in a system such as we have described might be:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

in which A represents the drug in the tablets, B represents the drug in the aqueous phase, and C represents the drug in the octanol. In this study,

the amount of drug in each compartment is given as weight, rather than concentration, to aid in the evaluation of the constants. This causes no difficulty due to the nature of the dissolution process, and because the volumes in compartments B and C are kept constant. The following equations would describe the kinetics of the proposed mechanism:

$$-\frac{dA}{dt} = k_1 (W_s - B) \quad \text{(Eq. 1)}$$

$$\frac{dB}{dt} = k_1 (W_s - B) - k_2 B \quad \text{(Eq. 2)}$$

$$\frac{dC}{dt} = k_2 B \quad \text{(Eq. 3)}$$

in which W_s is the weight of drug needed to saturate the aqueous phase, and the other terms have the meanings previously given. The solubility of the salicylic acid in the pH 2.00 buffer and in octanol at various temperatures is given in Table I. In the early stage of the dissolution process, $W_s \gg B$ and:

$$-\frac{dA}{dt} = k_1 W_s \quad \text{(Eq. 4)}$$

Thus, a plot of weight remaining *versus* time should give a straight line, as shown in Figs. 1 and 2.

The weight of drug in the aqueous phase as a function of time can be obtained from the following:

$$\frac{dB}{dt} = k_1 W_s - k_1 B - k_2 B = K - K' B \quad \text{(Eq. 5)}$$

in which $K = k_1 W_s$ and $K' = k_1 + k_2$. The use of an integrating factor, and the condition that at time zero, $B = 0$, gives:

$$B = \frac{K}{K'} (1 - \exp.^{-K't}) \quad \text{(Eq. 6)}$$

The method of Guggenheim (5) can be used to obtain K'. Plots for typical data utilizing this method are shown in Figs. 3 and 4. Once the value of K' is obtained by least squares evaluation of the lines, B can be plotted according to Eq. 6 to yield a straight line with a slope equal to K/K'. A knowledge of W_s then enables k_1 and k_2 to be evaluated. Representative plots of Eq. 6 are shown in Figs. 5 and 6.

Steady-State Treatment—The data shown in Fig. 1 indicate that a steady state may be obtained rather quickly in the aqueous phase. If this assumption can be made:

$$\frac{dB}{dt} = 0 = k_1 (W_s - B) - k_2 B \quad \text{(Eq. 7)}$$

TABLE I—SOLUBILITY OF SALICYLIC ACID IN pH 2.00 BUFFER AND IN OCTANOL AT VARIOUS TEMPERATURES

Temp., °C.	Phase	Solubility, Gm./250 ml.
30	pH 2.00 buffer	0.56
30	Octanol	29.44
32.5	pH 2.00 buffer	0.68
32.5	Octanol	31.88
35	pH 2.00 buffer	0.75
35	Octanol	34.32

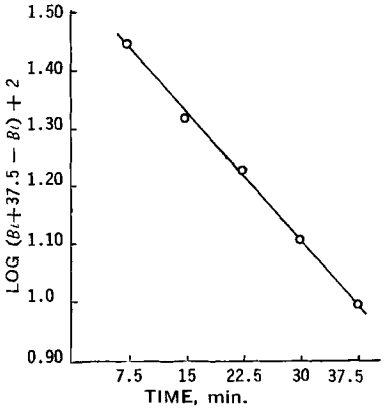


Fig. 3—Guggenheim plot for the appearance of salicylic acid in the pH 2.00 buffer. Stirring rate was 100 r.p.m. at 30°.

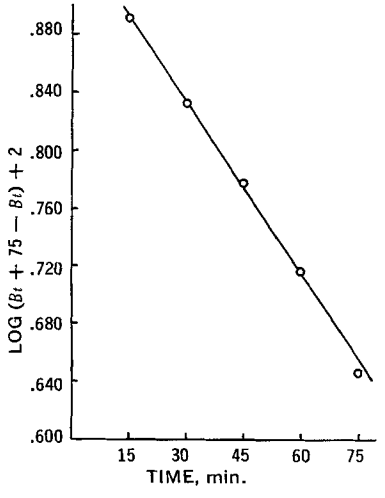


Fig. 4—Guggenheim plot for the appearance of salicylic acid in the 0.1% w/v polysorbate 80 solution at pH 2.00. Stirring rate was 100 r.p.m. at 30°.

and therefore:

$$k_2 B = k_1 (W_s - B) \quad \text{(Eq. 8)}$$

Thus, Eq. 3 becomes:

$$\frac{dC}{dt} = k_2 B = k_1 (W_s - B) = \frac{-dA}{dt} \quad \text{(Eq. 9)}$$

in which B is the steady-state weight of drug in the aqueous phase. The rate of change of C with time thus becomes constant and is equal to the dissolution rate $-dA/dt$ of the tablets. Division of dC/dt by $(W_s - B)$ yields k_1, while division of dC/dt by B yields k_2. The values of k_1 and k_2, evaluated by the various methods for the data shown in Fig. 1, are given in Table II.

The fact that the rate of appearance of the drug in the lipid phase becomes equal to the dissolution rate of the drug when B reaches a steady state is of great potential value in dissolution rate studies. This would be useful, for example, for studies involving poorly water-soluble drugs which would re-

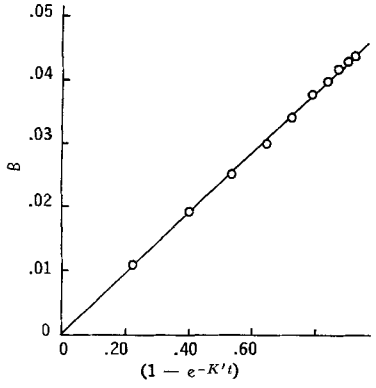


Fig. 5—Plot of Eq. 6 for the appearance of salicylic acid in the pH 2.00 buffer. Stirring rate was 100 r.p.m. at 30°.

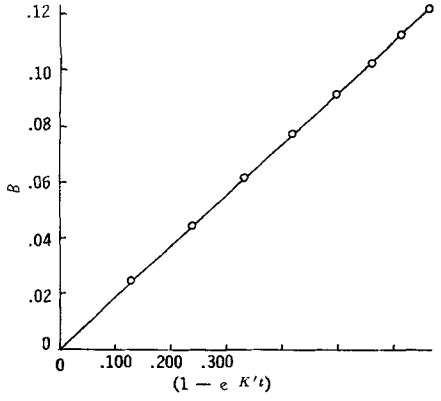


Fig. 6—Plot of Eq. 6 for the appearance of salicylic acid in the 0.1% w/v polysorbate 80 solution at pH 2.00. Stirring rate was 100 r.p.m. at 30°.

TABLE II—COMPARISON OF k_1 AND k_2 OBTAINED BY VARIOUS METHODS

Constant, min.$^{-1}$	Method		
	Eq. 6	Eq. 4	Eq. 9
k_1	0.00293	0.00292	0.00291
k_2	0.0316	.....	0.0314

quire large volumes of dissolution medium to obtain meaningful results. Large, and sometimes unwieldy, volumes of dissolution medium are also used in dissolution rate studies to prevent build up of the drug in the dissolution medium so the assumption of $W_s \gg B$, or its equivalent in concentration terms, can safely be assumed. The presence of the lipid phase acting as a sink, as it did in these studies, would obviate this difficulty. In some instances, the assay for a drug in the aqueous phase might be difficult or perhaps time consuming in that an extraction into a lipid phase is necessary. The ability to measure dissolution rates by analyzing a lipid phase, as done in these studies, would then be of great value.

Effect of Stirring Rate—The effect of stirring rate upon both constants was determined from 50–

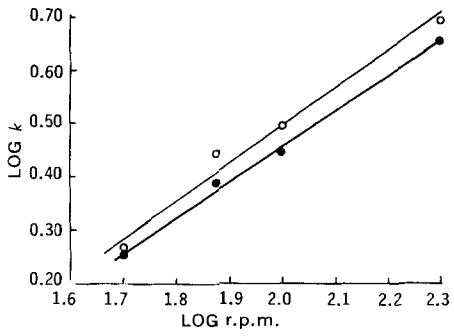


Fig. 7—Effect of stirring rate upon k_1 *and* k_2 *at 30°. Key: ●, (log* k_1*) + 3; ○, (log* k_2*) + 2.*

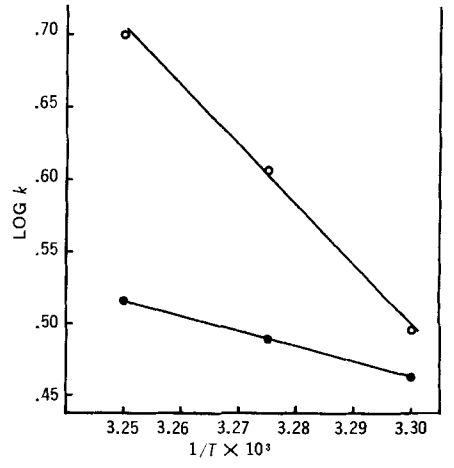


Fig. 8—Arrhenius plots for k_1 *and* k_2 *at a stirring rate of 100 r.p.m. Key: ●, (log* k_1*) + 3; ○, (log* k_2*) + 2.*

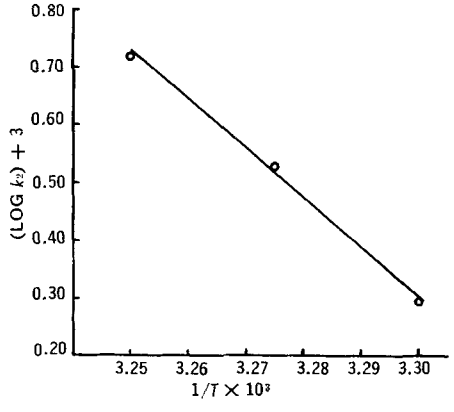


Fig. 9—Arrhenius plot for the effect of temperature upon corrected k_2 *at a stirring rate of 100 r.p.m.*

200 r.p.m. at 30 ± 0.05° using the steady-state equations. The relationship between rate constants and stirring rate may be given by:

$$k = a\,(\text{r.p.m.})^b \qquad \text{(Eq. 10)}$$

in which a and b are constants. The constant b has been found to range from zero for a nondiffusion-

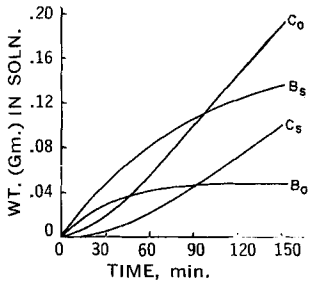


Fig. 10—Effect of polysorbate 80 upon the dissolution and partitioning rates of salicylic acid at a stirring rate of 100 r.p.m. and 30°. Key: B_0 represents the dissolution into pH 2.00 buffer, B_s represents the dissolution into the polysorbate 80 solution, C_0 represents the partitioning into octanol in the absence of the polysorbate 80, and C_s represents the partitioning into octanol in the presence of polysorbate 80.

controlled process to approximately one for a diffusion-controlled process (6). Plots of log k *versus* log (r.p.m.) are shown in Fig. 7. The constant, b, was found to be 0.67 and 0.70 for k_1 and k_2, respectively, indicating both processes are diffusion controlled.

Effect of Temperature—The effect of temperature upon both rate constants was determined at 100 r.p.m. through the temperature range of 30–35° ± 0.05° using the steady-state equations. The data are plotted according to the Arrhenius relationship in Fig. 8. The energy term associated with k_1 was found to be 4,690 cal./mole, which is in good agreement with the values of 4,446–4,548 cal./mole reported by Edwards (7) for the energy of diffusion within this temperature range. The energy term associated with k_2, however, was found to be 19,100 cal./mole which would indicate a nondiffusion-controlled process. This is not in agreement with the findings of the stirring rate data. The basic mechanism was therefore modified to include a diffusion process for the transport of solute from the aqueous to the lipid phase:

$$\frac{dC}{dt} = k_2 B (W_c - C) \qquad \text{(Eq. 11)}$$

in which W_c represents the weight of drug needed to saturate the lipid phase. Table I and Figs. 1 and 2 show that for these studies $W_c \gg C$ and:

$$\frac{dC}{dt} = k_2 B W_c = k_2' B \qquad \text{(Eq. 12)}$$

in which $k_2' = k_2 W_c$. This modification would not change the kinetic picture, as given by Eqs. 1–9, or the stirring rate data. It merely indicates that our previously determined k_2 probably contains the term W_c. A plot of the corrected k_2 *versus* temperature is shown in Fig. 9. The energy term associated with the corrected k_2 was found to be 4,640 cal./mole, which is in good agreement with what would be expected for a diffusion-controlled reaction. The modification, as shown by Eq. 11, accounts for dC/dt decreasing as C approaches W_c and for its becoming equal to zero when C becomes equal to W_c. This is more realistic than Eq. 3 which indicates that the transfer of solute into the lipid phase would continue at a constant rate, and does not provide for the eventual saturation of the lipid phase.

Effect of Polysorbate 80[2]—Sufficient polysorbate 80 was added to the pH 2.00 buffer to give a final concentration of 0.20% w/v. The data were obtained in the usual manner for a stirring rate of 100 r.p.m. at 30 ± 0.05°. The results are shown in Fig. 10. The dissolution rate, as shown by the plots of B *versus* time, is indeed increased in the presence of the surfactant, in agreement with the literature (2,3). The partitioning rate, however, as shown by the plots of C *versus* time is decreased in qualitative agreement with the findings of Levy and Reuning (4). Thus, the value of this system for a screening procedure, as opposed to the usual dissolution rate studies, becomes readily apparent. The exact mechanism for the effect of surfactants upon the dissolution and partitioning rates of drugs is currently being studied and will be reported in a future communication.

REFERENCES

(1) Doluisio, J. T., and Swintosky, J. V., *J. Pharm. Sci.*, **53**, 597(1964).
(2) Levy, G., and Gumtow, R. H., *ibid.*, **52**, 1139(1963).
(3) Wurster, D. E., and Seitz, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 335(1960).
(4) Levy, G., and Reuning, R. H., *J. Pharm. Sci.*, **53**, 1471(1964).
(5) Guggenheim, E. A., *Phil. Mag.*, **2**, 538(1926).
(6) Polli, G. P., Ph.D. Thesis, University of Wisconsin, Madison, Wis., 1960, p. 11.
(7) Edwards, L. J., *Trans. Faraday Soc.*, **47**, 1191(1951).

[2] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

Quantitative Estimation of Diosgenin in *Dioscorea* Tubers by Densitometric Thin-Layer Chromatography

By G. BLUNDEN, R. HARDMAN*, and J. C. MORRISON

Thin-layer chromatography was employed to separate diosgenin from other plant constituents present in *Dioscorea* tuber extracts. The diosgenin was rendered visible by antimony trichloride and the colored spot estimated by scanning with a photoelectric densitometer attached to an integrating logarithmic recorder. Diosgenin could be estimated with an experimental error of approximately 7 per cent when applied in concentrations between 10 and 75 mcg. Assay results obtained for diosgenin by this method were slightly lower than those obtained by conventional infrared spectrophotometry, since the 25 D-spirosta-3,5-diene formed during the extraction of diosgenin was not estimated in the chromatographic procedure.

STEROIDAL SAPOGENINS are of considerable economic importance as precursors of the pharmacologically active steroids, including the oral contraceptives and corticosteroids. The most important steroidal sapogenin is diosgenin, which is obtained commercially from the tubers of several *Dioscorea* species.

Diosgenin is usually assayed by gravimetric (1, 2) or infrared techniques (3, 4); the naturally occurring saponin is acid-hydrolyzed and the sapogenin then extracted with a hydrocarbon solvent. The gravimetric methods are applicable to plant material with high sapogenin content containing little contaminating matter, but are less satisfactory where the proportion of contaminants is high, or when a sensitive method is required for studying the factors affecting the yield of sapogenin from a given source, or screening botanical specimens for steroidal sapogenins. In these circumstances infrared spectrometric methods are more suitable. Based on the spirostan structure of the sapogenin, they are specific for steroidal sapogenins, but take no account of the nature of the steroidal nucleus; the 3,5-diene of diosgenin, for example, being assayed as diosgenin. Furthermore, the authors have found that nonsapogenin compounds extracted by the sapogenin solvent, both saponifiable and nonsaponifiable, can also interfere with the infrared assay method and cause high results. Consequently, it was felt that a convenient analytical technique, suitable for small quantities of plant material and overcoming some of the disadvantages of the existing methods, would be valuable. The authors report the development of such a technique and its application, in the first instance, to the assay of diosgenin in tubers of species of *Dioscorea*. Extension of the method to other species and morphological parts, such as seeds, will be reported.

Received March 10, 1967, from the School of Pharmacy, College of Technology, Portsmouth, Hampshire, England.

Accepted for publication May 19, 1967.

The authors thank Mr. A. Wood, Ministry of Overseas Development, Tropical Products Institute, London, England, for the infrared spectrometric assays and Mr. A. G. Kenyon, Mr. J. Parham, and Miss A. P. Wilson for tubers or seeds.

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EXPERIMENTAL

Development and Treatment of the Chromatogram—Glass plates, 15 × 20 cm., were spread with a layer of Silica Gel G to a thickness of 250 μ according to the method of Stahl (5) and air-dried before use.

Known quantities of diosgenin, dissolved in chloroform, were applied to the plates by micropipets and the chromatogram developed with *n*-hexane–ethyl acetate (4:1 v/v) using the supersaturated method of Stahl (6). When the solvent had advanced 15 cm. from the starting line, the plates were removed, dried, and double developed with the same solvent system. Following development, the plates were dried and sprayed uniformly with a solution of 3 Gm. of antimony trichloride in 1 ml. of concentrated hydrochloric acid (7). The sapogenins were then located by heating the plates evenly at 100° in a circulating air stream until the diosgenin spot was developed to a green-black color. This normally took 30 min.

After the plates had cooled, the absorbances of the spots were estimated by a Vitatron densitometer,[1] using a slit width of 20 mm. by 2 mm. and a mercury lamp source. The values so obtained were calculated by an automatic integrator. Spots were scanned in the direction opposite to that of solvent flow and erratic readings from extraneous light were eliminated by the use of a light-tight box.

Preparation of the Standards—Pure diosgenin was applied to thin-layer plates in concentrations ranging from 5 mcg. to 100 mcg. Following development, the spots produced were scanned and a linear relationship between concentration and absorbance was established for concentrations of diosgenin between 10 mcg. and 75 mcg.

Preparation of Plant Material—Diosgenin was extracted from *Dioscorea* tubers of known weight and moisture content (4). This involved maceration of the fresh tuber, incubation of the disintegrated material, acid-hydrolysis of the saponins by refluxing with 2 *N* hydrochloric acid for 2 hr., and extraction of diosgenin from the dry, acid-insoluble

[1] Fison's Scientific Apparatus Ltd., Loughborough, Leicestershire, England.

residue with petroleum ether (40–60°). After evaporating to dryness, the extract was dissolved in chloroform and made to volume. Dried, powdered tuber was first incubated with water before being treated by the above procedure.

Known volumes of the *Dioscorea* extract were applied to prepared thin-layer plates along with a standard diosgenin solution. The applied spots of the plant and standard solutions were alternated on the plate to minimize the effects of variation in thickness and opacity of the adsorbent layer. The diosgenin content of the *Dioscorea* extract was calculated relative to the standard solution, and the resulting value obtained from the mean of six plates, each containing two spots of the plant extract.

To determine the recovery of diosgenin added to a plant extract, 5 Gm. of a powdered edible *Dioscorea* tuber was extracted and assayed as above. To this extract of known volume, 40 mg. of diosgenin was added and the chromatographic assay carried out to estimate the recovery of the added diosgenin. The assay was repeated following the addition of 40 mg. cholesterol to the *Dioscorea* extract containing the added diosgenin, to show that this sterol did not interfere with the recovery of diosgenin. Cholesterol is a fairly common plant sterol (8) which occurs in *Dioscorea* tubers (9) and co-chromatographs with the common phytosterols β-sitosterol and stigmasterol in the silica gel system used. These procedures were then repeated using an assayed sample of dried, powdered *D. floribunda* Mart. et Gal. tuber.

Similarly, to determine the extent of breakdown of the sapogenin during extraction, 40 mg. of diosgenin was refluxed with 100 ml. of 2 *N* hydrochloric acid for 2 hr. and subsequently extracted with chloroform. This solution was then assayed and compared with an appropriate standard of untreated diosgenin.

Samples of the extracted *Dioscorea* tubers were assayed by the thin-layer chromatographic and infrared spectroscopic methods for purposes of comparison.

RESULTS

Relationship Between Spot Absorbance and Weight of Diosgenin—The relationship between the logarithm of the weight applied and the square root of the absorbance of the spot produced was found to be linear for weights of diosgenin from 10 mcg. to 75 mcg. (Fig. 1 and Table I).

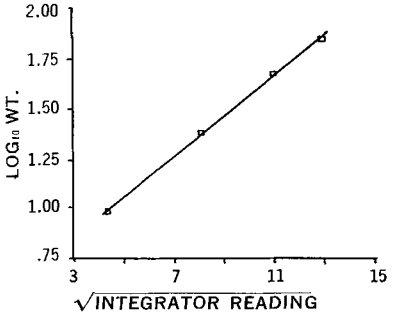


Fig. 1—Relationship between spot absorbance and weight of diosgenin.

TABLE I—RELATIONSHIP BETWEEN WEIGHT OF DIOSGENIN APPLIED AND ABSORBANCE OF THE SPOT, EXPRESSED AS A READING FROM THE AUTOMATIC INTEGRATOR

Diosgenin Wt.[a], mcg.	Log_{10} Wt.	Integrator Reading	Integrator √Reading
5	0.6990	13.0	3.61
10	1.0000	18.0	4.24
25	1.3979	64.0	8.00
50	1.6990	117.0	10.82
75	1.8751	163.0	12.77
100	2.0000	250.0	15.81

[a] Mean results from 6 plates.

TABLE II—QUANTITATIVE RESULTS FOR DIOSGENIN RECOVERY

Applied mcg.	Diosgenin Recovered mcg.	Diosgenin Recovered %[a]	No. of Estimations
20	20.32 ± 1.4	101.6 ± 6.9	40
40	40.08 ± 2.9	100.2 ± 7.3	40
60	58.2 ± 4.4	97.0 ± 7.4	40

[a] Recovered per cent is expressed as the mean of all determinations ± one standard deviation.

TABLE III—COMPARISON OF DENSITOMETRIC AND INFRARED ASSAY PROCEDURES

Plant Source	% Diosgenin m.f.b.[a] Densitometric	% Diosgenin m.f.b.[a] Infrared
D. floribunda sample *1*	3.91	3.95
D. floribunda sample *2*	4.25	4.55
D. floribunda sample *3*	2.38	2.40
D. sylvatica sample *1*	2.50	2.80
D. sylvatica sample *2*	3.65	3.85
D. deltoidea sample *1*	3.06	3.40
D. deltoidea sample *2*	3.14	3.35

[a] Moisture free basis.

The percentage error in the method was calculated by applying 20, 40, and 60 mcg. of diosgenin to thin-layer plates and estimating the variation observed in spot intensity. Ten chromatograms each of four spots were prepared in these determinations (Table II).

Estimation of Diosgenin in Dioscorea Tubers—The diosgenin content of the tuber of the edible *Dioscorea* species was found by the densitometric method to be 0.1%. When diosgenin was added to this extract, a recovery of 99.5% of the added diosgenin was obtained. Similarly, a recovery of 101% of the added diosgenin was obtained when estimated in the presence of an equal quantity of cholesterol. Similar results were obtained with a sample of *D. floribunda* tuber assayed by the same procedure.

Comparison of Densitometric and Infrared Assays—Comparison of assay results obtained from *D. floribunda*, *D. sylvatica* Ecklon., and *D. deltoidea* Wall. using both the densitometric and infrared methods is given in Table III.

DISCUSSION

Purdy and Truter (10) found that the relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced gave a linear relationship. This linear relationship was found to hold for the logarithm of the weight of diosgenin applied and the absorbance of the spot produced for diosgenin from 10 mcg. to 75 mcg. The results obtained show that densitometric thin-layer chromatography provides a suitable method for the assay of diosgenin in *Dioscorea* tubers.

In this study a number of experimental factors were found to be important in obtaining reproducible results. For example, plates of uniform gel thickness were essential, since variations in the layer produced variations in the amount of light transmitted through the plate, which affected the result recorded by the densitometer (11). Therefore, plates were selected which were uniform in appearance. It was also important to spray the plates evenly and ensure even heating to avoid development of uneven color intensity by the spots. All spots were scanned in the same direction, namely opposite to that in which the plates were developed, since considerable variations were obtained when the spots were scanned in different directions.

Using the solvent syftem *n*-hexane–ethyl acetate, diosgenin was separated from the other constituents in the *Dioscorea* extract. It was, however, observed that the sterol spot of higher R_f value and another spot of lower R_f value were sufficiently close to the diosgenin to interfere with accurate densitometric estimation. This was particularly noticeable when the proportion of sterol to diosgenin was high. This difficulty was easily overcome by double development of the chromatogram in *n*-hexane–ethyl acetate, when the diosgenin spot was well separated from the two interfering spots.

To determine the sensitivity of the procedure, known weights of diosgenin were added to *Dioscorea* tuber extracts of known diosgenin concentration. In other tests, equal weights of cholesterol and diosgenin were added to the *Dioscorea* extracts but in all cases quantitative recoveries of the added diosgenin were recorded, showing that the diosgenin was separated from the other plant constituents which did not interfere with the assay. This was also verified by the close correlation between the infrared assay results and the quantitative thin-layer chromatographic results. The results from the infrared method were always slightly higher than those obtained by quantitative thin layer. This was as expected, as during the extraction procedure of diosgenin, a certain quantity of 25 D-spirosta-3,5-diene is formed (12) on heating diosgenin with 2 *N* hydrochloric acid. The diene is estimated with the diosgenin in the infrared method, but by thin-layer chromatography the diene is well separated from diosgenin and is not estimated. In test experiments, by heating diosgenin with boiling 2 *N* hydrochloric acid for 2 hr., the degree of decomposition was estimated as being between 5.1 and 7.3%.

Using the solvent system described, diosgenin is not separated from its isomer yamogenin, hence the yamogenin present in the samples will be estimated as diosgenin. This is also the case with the infrared method of assay (4).

A major advantage of a quantitative thin-layer method for estimating diosgenin is that the quantity of plant material required is very small. One gram of dry, powdered tuber, of diosgenin content 2.5%, moisture free basis, has been successfully assayed.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and hexane–ethyl acetate (4:1 v/v) as the solvent system has been devised for the separation and analysis of diosgenin in *Dioscorea* tubers. It was demonstrated that a linear relationship exists between the logarithm of the weight of diosgenin applied and the square root of the absorbance of the spot produced. Quantitative evaluation was achieved by using a photoelectric densitometer coupled to an integrating logarithmic recorder which computed the absorbance of the spot. The experimental error was found to be approximately 7%. The technique gave quantitative results when diosgenin was applied in concentrations between 10 and 75 mcg. The results were compared with those obtained by infrared spectrometry and were found to be slightly lower due to the nonestimation of 25 D-spirosta-3,5-diene by the quantitative thin-layer method. Several experimental factors which influence the accuracy of the method are discussed.

REFERENCES

(1) Rothrock, J. W., Hammes, P., and McAleer, W. J., *Ind. Eng. Chem. Intern. Ed.*, **49**, 186(1957).
(2) Morris, M. P., Roark, B., and Cancel, B., *J. Agr. Food Chem.*, **6**, 856(1958).
(3) Wall, M. E., Eddy, R. C., McClennan, M. L., and Klumpp, M. E., *Anal. Chem.*, **24**, 1337(1952).
(4) Blunden, G., and Hardman, R., *J. Pharm. Pharmacol.*, **15**, 273(1963).
(5) Stahl, E., *Pharmazie*, **11**, 635(1956).
(6) Stahl, E., *Arch. Pharm.*, **292**, 411(1959).
(7) Nakao, T., Hirai, M., and Yoshizawa, N., *Tokyo Jikeikai Ika Daigaku Zasshi*, **73**, 1575(1958); through *Chem. Abstr.*, **53**, 22198(1959).
(8) Bennett, R. D., Ko, S.-T., and Heftmann, E., *Phytochemistry*, **5**, 231(1966).
(9) Bennett, R. D., and Heftmann, E., *ibid.*, **4**, 577(1965).
(10) Purdy, S. J., and Truter, E. V., *Analyst*, **87**, 802 (1962).
(11) Morrison, J. C., and Orr, J. M., *J. Pharm. Sci.*, **55**, 936(1966).
(12) Peal, W., *Chem. Ind. (London)*, **44**, 1451(1957).

Protection of Sympathetic α-Receptors *In Vivo*

By F. P. LUDUENA and M. J. BRANIN

An intravenous dose of 100 mcg./Kg. of phenoxybenzamine in rats completely blocked the lethal effect of 1 mg./Kg. of epinephrine injected intravenously 3 hr. later. Alpha-sympathetic receptors were protected against the effect of phenoxybenzamine by the simultaneous injection of competitive α-adrenolytics. If the α-receptor protection was maximal, a 100 per cent mortality followed the injection of the 1 mg./Kg. dose of epinephrine. Graded degrees of receptor protection were obtained by the administration of graded doses of α-adrenolytics. A rank correlation coefficient of 0.902 was obtained between the α-receptor protective $dose_{50}$ and the adrenolytic $dose_{50}$ of 17 compounds tested including *dextro*-isoproterenol and *dextro*-hyoscyamine.

BETA-HALOALKYLAMINES produce a long-lasting inactivation of the sympathetic α-receptors (1). In 1949 Seed and McKay (2) demonstrated that previous administration of piperoxan in the anesthetized dog prevented the development of the adrenolytic action of *N*-(2-chloroethyl)dibenzylamine.[1] Nickerson and Gump (3) reported that the injection of epinephrine in anesthetized cats reduced the developing of the α-receptor blocking effect of β-haloalkylamines suggesting a certain degree of α-receptor protection. Clear evidence of α-receptor protection *in vitro* by both agonists and antagonists was provided by the results reported by Furchgott (4).

The experiments described below were carried out in order to quantitate α-receptor protection activity *in vivo* and to determine the relationship between this activity and adrenolysis.

METHODS

All injections were made in the tail vein of male white rats weighing approximately 100 Gm. Doses were expressed in terms of the bases.

Adrenolytic Activity—The technique, reported previously (5), consists of injecting intravenously a dose of epinephrine (200 mcg./Kg.) simultaneously (procedure *A*) with one of three or four graded doses of the experimental compound and plotting per cent survival against the dose on probit-log paper; in some experiments the compound was injected approximately 5 min. before epinephrine administration (procedure *B*).

Sympathetic α-Receptor Protection Test—Alpha-receptors were protected by adrenolytics against the long-lasting inactivation produced by phenoxybenzamine (0.1 mg./Kg.) injected intravenously. The "protectors" were injected in the same solution (procedure *C*) or 5 min. before phenoxybenzamine (procedure *D*). After an interval of 3 hr. a dose of epinephrine of 1 mg./Kg. was injected intravenously. If the sympathetic α-receptor had been protected and the adrenolytic effect of the "protector" had worn off, epinephrine would reach the unchanged α-receptor causing all the rats to die. With graded doses of the protector, the higher the α-receptor protective effect the higher the mortality. The ED_{50} was obtained by plotting mortality against dose on probit-log paper (Fig. 1).

RESULTS

In an effort to develop a test to measure the ability of certain compounds to protect sympathetic α-receptors from the inactivating action of phenoxybenzamine, the following variables were studied: dose of phenoxybenzamine, dose of epinephrine, dose of the α-receptor "protector," and the time intervals between the administration of the "protector" and phenoxybenzamine and between the administration of phenoxybenzamine and epinephrine. The results obtained using phentolamine as the "protector" are summarized in Table I.

Experiment *1* showed that the 20-min. interval between injections of phenoxybenzamine and epinephrine was too short, as 80% mortality resulted. When the interval was increased to 40 min. in experiment *2*, only 30% of the animals died, indicating that a higher proportion of α-receptors had been inactivated at the time of epinephrine injection. Using the time interval of 40 min. between the injection of graded doses of phenoxybenzamine and a dose of 0.20 mg./Kg. of epinephrine ($2.7 - 5 \times LD_{50}$), the adrenolytic ED_{50} of phenoxybenzamine was determined to be 0.014 ± 0.003 mg./Kg.[2] Further experiments with increasing doses of epinephrine (experiments *3–7*) indicated that once the adrenolytic ED_{100} of phenoxybenzamine had been reached further increases in the dose of epinephrine could not surmount the blockade of α-receptors.

The effect of the time interval between phenoxybenzamine and epinephrine administrations is shown in the results of experiments *8–11*. When the doses of phenoxybenzamine were 0.1 mg./Kg., high doses of epinephrine partially surmounted the blockade 7 hr. after phenoxybenzamine (experiment *10*) and killed all the rats 11 hr. later (experiment *11*). While the time parameter remained constant (18 hr.) in experiments *11*, *12*, and *13*, the dose of epinephrine was varied. Both 5.0 mg./Kg. (experiment *11*) and 1 mg./Kg. (experiment *12*) of epinephrine resulted in 100% mortality. However, at a still lower dose of epinephrine (0.20 mg./Kg., experiment *13*) none of the treated rats died, demonstrating that even at this prolonged time interval there was still some α-receptor blockade produced by phenoxybenzamine. Experiments *14*, *15*, and *16* showed that the adrenolytic effect of phentolamine (5 mg./Kg.), a competitive adrenolytic, did not last

Received February 9, 1967, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144
Accepted for publication May 17, 1967.

[1] Dibenamine.

[2] Only one of the four doses of phenoxybenzamine used to calculate the ED_{50} is presented in Table I.

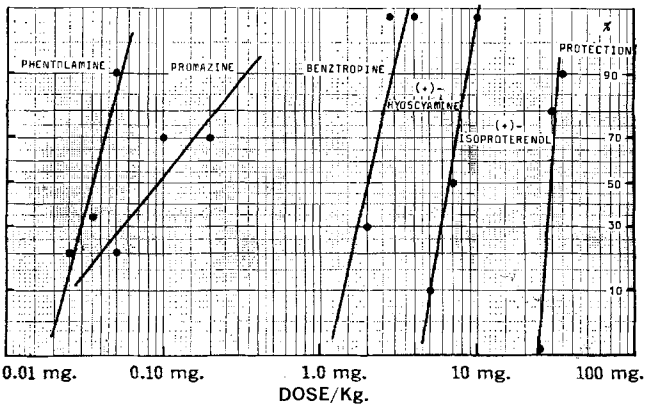


Fig. 1—Dose-effect curves of adrenolytics. Protection of α-sympathetic receptors in rats against the inactivation produced by intravenous phenoxybenzamine (0.1 mg./Kg.). Per cent protection = the per cent mortality following an intravenous high dose of epinephrine (1 mg./Kg.) injected 3 hr. after phenoxybenzamine.

TABLE I—INFLUENCE OF PHENTOLAMINE ON THE ADRENOLYTIC ACTIVITY OF PHENOXYBENZAMINE (PH) IN RATS[a]

Expt.	PH Dose, mg./Kg.	Phentolamine Dose, mg./Kg.	Epinephrine (E) Dose, mg./Kg.	Interval Between PH and E	No. of Rats	No. of Rats Killed	% Mortality
1	0.025	...	0.2	20 min.	10	8	80
2	0.025	...	0.2	40 min.	10	3	30
3	0.20	...	1.0	40 min.	10	0	0
4	0.20	...	5.0	40 min.	10	0	0
5	0.10	...	10.0	40 min.	6	0	0
6	0.10	...	20.0	40 min.	5	0	0
7	0.05	...	20.0	40 min.	5	1	20
8	0.10	...	5.0	3 hr.	5	0	0
9	0.10	...	5.0	5 hr.	4	0	0
10	0.10	...	5.0	7 hr.	10	3	30
11	0.10	...	5.0	18 hr.	5	5	100
12	0.10	...	1.0	18 hr.	5	5	100
13	0.10	...	0.2	18 hr.	5	0	0
14	...	5 (5 min.)[b]	5.0	...	5	3	60
15	...	5 (1 hr.)[b]	5.0	...	5	4	80
16	...	5 (3 hr.)[b]	5.0	...	10	10	100
17	0.10	5[c]	5.0	5 hr.	10	10	100
18	0.10	5	5.0	5 hr.	10	10	100
19	0.10	1	5.0	5 hr.	5	5	100
20	0.10	0.2	5.0	5 hr.	7	7	100
21	0.10	0.05	5.0	5 hr.	10	10	100
22	0.10	0.025	5.0	5 hr.	10	7	70
23	0.10	0.050	1.0	3 hr.	10	9	90
24	0.10	0.035	1.0	3 hr.	10	3	30
25	0.10	0.025	1.0	3 hr.	10	2	20

[a] Adrenolytic ED_{50} of phenoxybenzamine, 40 min. before epinephrine: 0.014 ± 0.003 mg./Kg. [b] Time interval between phentolamine and epinephrine. [c] Phentolamine was injected 15 min. before phenoxybenzamine; in experiments *18* to *25* the two drugs were injected in the same solution.

3 hr. against a high dose of epinephrine (5 mg./Kg.). The injection of phentolamine together with phenoxybenzamine abolished the long-acting adrenolytic action of the latter (experiments *18–25;* experiment *9 versus* experiments *18–20*). It was clear that at the doses used there was a "protection" of the sympathetic α-receptors. Experiments *22–25* showed that graded doses of phentolamine produced graded degrees of receptor protection and that an ED_{50} value could be obtained.

On the basis of the results obtained with phentolamine, the procedure previously described was adopted to test the α-receptor protective activity of a series of adrenolytics. Compounds were chosen which had weak, moderate, and strong adrenolytic activity.

The doses of phenoxybenzamine and epinephrine (0.1 and 1 mg./Kg., respectively) and the 3-hr. interval between the two injections, which the authors adopted to test the 17 compounds in the correlation study, may not be the most satisfactory. For example, although the adrenolytic effect of phentolamine disappears within 3 hr., the effect of trifluoperazine and tolazoline lingers beyond the 3-hr. interval giving false α-receptor "protection" readings. For this reason these two compounds could not be tested by the method described above. Another difficulty was the result of differences in onset of action between some adrenolytics and phenoxybenzamine. In order to demonstrate "protective" activity compounds I and III had to be injected 5 min. before phenoxybenzamine (Table II, procedure *D*). The time of onset of action might have been reduced by increasing the dose of the "protector." However, in the case of compounds I and III the increase in dosage prolonged

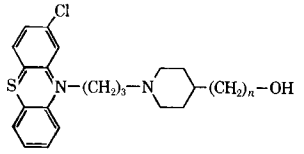
TABLE II—RELATIONSHIP BETWEEN ADRENOLYTIC AND SYMPATHETIC α-RECEPTOR PROTECTIVE ACTIVITIES IN RATS

Compd.	Form	Adrenolytic Activity ED_{50}, mg./Kg.	Sympathetic α-Receptor Protective Activity ED_{50}, mg./Kg.
I	HCl	0.020 ± 0.002[a]	0.032 ± 0.006[a]
Phentolamine	HCl	0.022 ± 0.004	0.035 ± 0.003
II	HCl	0.024 ± 0.012	0.090 ± 0.042
III	Maleate	0.033 ± 0.006[a]	0.037 ± 0.010[a]
Perphenazine	Base	0.036 ± 0.007	0.255 ± 0.053
IV	Base	0.040 ± 0.008	0.034 ± 0.007
Promazine	HCl	0.041 ± 0.010	0.094 ± 0.025
V	Base	0.048 ± 0.009	0.053 ± 0.012
VI	Base	0.062 ± 0.011	0.039 ± 0.013
Phenindamine	Hydrogen tartrate	0.080 ± 0.028	0.350 ± 0.083
Piperoxan	HCl	0.230 ± 0.054	3.85 ± 0.57
Yohimbine	HCl	0.60 ± 0.041	3.35 ± 0.47
Benztropine	Methanesulfonate	1.14 ± 0.21	2.07 ± 0.26
VII	HCl	8.3 ± 1.3	7.3 ± 1.3
(+)-Hyoscyamine	1,2-Oxobornanesulfonate	8.5 ± 1.5	6.75 ± 0.45
VIII	HCl	10.4 ± 1.9	9.2 ± 1.3
(+)-Isoproterenol	*d*-Bitartrate	12.2 ± 2.4	29.5 ± 0.95

[a] These compounds were administered approximately 5 min. before epinephrine (procedure *B*, adrenolytic test) and phenoxybenzamine (procedure *D*, sympathetic α-receptor protective test).

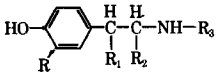
TABLE III—CHEMICAL STRUCTURE

Phenothiazines



Compd.	*n*	Compd.	*n*
I	3	IV	1
II	2	V	0
III	6	VI	4

Hydroxyphenyl Amines



		R	R_1	R_2	R_3	Salt
VII	(±)-	OH	OH	H	$-CH_2-CH_2-CH_2-CH_3$	HCl
VIII		OH	H	H	$-CH(CH_3)_2$	HCl
IX	(+)-	OH	OH	H	$-CH_2-CH_3$	HCl
X	(+)-	H	OH	H	$-CH(CH_3)_2$	*l*-Bitartrate
XI	(±)-	OH	OH	H	$-CH(CH_3)-CH_3$	Methanesulfonate
XII	(−)-	OH	OH	H	$-CH(CH_3)-CH_3$	*d*-Camphorsulfonate
XIII	(±)-	OH	OH	Et	$-CH(CH_3)_2$	HCl

their adrenolytic effect beyond the 3-hr. interval. The adrenolytic activity of these two compounds was also determined in the same manner; *i.e.*, by injecting the compound 5 min. before epinephrine. It is interesting to note that the adrenolytic ED_{50} values obtained in this manner (procedure *B*, see under *Methods*) were lower than those obtained by the standard method (procedure *A*). The values in mg./Kg. were for compound I: 0.020 ± 0.002 (*B*) *versus* 0.032 ± 0.008 (*A*); and for compound

TABLE IV—EFFECT OF (−) AND (+)-EPINEPHRINE MIXED WITH PHENOXYBENZAMINE

First Injection			2nd Injection 3 hr. Later		
Phenoxybenzamine Dose, mg./Kg.	Agonist	Dose, mg./Kg.	(−)-Epinephrine Dose, mg./Kg.	No. of Rats Inj.	No. Killed
0.2	(−)-Epinephrine	0.05	0.2	10	0
0.1	(−)-Epinephrine	0.05	5.0	10	0
0.1[a]	(−)-Epinephrine	0.2	5.0	8	0
0.2	(+)-Epinephrine	1.0	5.0	10	0
0.1	(+)-Epinephrine	4.0	5.0	10	0
0.1[a]	(+)-Epinephrine	12.0	5.0	4	0

[a] Several rats died following the first injection.

III: 0.033 ± 0.006 (*B*) *versus* 0.11 ± 0.022 (*A*). It is obvious that the full effect of these adrenolytics could not develop when injected simultaneously with epinephrine.

Correlation Study—The degree of association between adrenolytic activity (ED_{50}) and sympathetic α-receptor protective activity (ED_{50}) of 17 compounds was determined using the Spearman rank correlation method (6). The rank correlation coefficient found was 0.902. The ED_{50} values obtained are listed in Table II and the chemical structures of some of the compounds are shown in Table III.

In addition to some standard adrenolytics, a few weak adrenolytics were tested in order to widen the activity range of the groups of compounds studied. Among the latter (+)-hyoscyamine, which is responsible for the adrenolytic activity of atropine (7), and (+)-isoproterenol (8, 9) were found to be weak α-receptor protectors.

Other (+)-isomers of sympathomimetic amines were tested, but since the two ED_{50} values could not be obtained, they were not included in the correlation study. The *dextro*-isomer of *N*-ethyl norepinephrine (compound IX, Table III) was found to have a very weak adrenolytic activity (17.5 ± 8.9 mg./Kg.). Partial protection (37.5% mortality) of α-receptors was obtained at a dose of 80 mg./Kg. Higher doses were not tested. The other compound (X, Table III), the *dextro*-isomer of the *meta*-desoxy analog of isoproterenol, protected α-receptors (ED_{50}: 40 ± 3.2 mg./Kg.), but its adrenolytic ED_{50} could not be determined by the standard method. However, in rats receiving 40 mg./Kg. in the same intravenous injection with epinephrine, the LD_{50} of epinephrine was increased to 0.1 ± 0.011 mg./Kg. from a control value of 0.05 ± 0.008 mg./Kg.

Effect of Agonists on the Adrenolytic Activity of Phenoxybenzamine—Adrenergic agonists in high concentrations protect sympathetic α-receptors from the inactivating action of *N*-(2-chloroethyl)-dibenzylamine *in vitro* (4). The authors tested the effect of (−) and (+)-epinephrine mixed with another haloalkylamine, phenoxybenzamine, to determine whether agonists could occupy a sufficiently large proportion of receptors to allow epinephrine injected 3 hr. later to produce its lethal effect (Table IV).

The results in Table IV show that the isomers of epinephrine could not protect the α-receptors. If the dose of the agonist injected together with phenoxybenzamine was increased, some of the animals died. From these experiments it may be concluded that if the proportions of α-receptors occupied by the agonists in competition with phenoxybenzamine are sufficiently large, the rats die, and that in the animals that survive (Table IV) an insufficient number of receptors were protected from the action of phenoxybenzamine.

Effect of Catecholamines with No α-Receptor Activity—Since in experiments with the *dextro*-isomers of catecholamines, very large doses had been used, it was of interest to test as controls, compounds which have strong β-receptor agonist activity and no action on α-receptors. Compound XII, the *levo* form, is one of the most potent β-receptor agonists. At a dose of 20 mg./Kg. it had no adrenolytic activity when injected together with epinephrine (0.2 mg./Kg.). Injected with phenoxybenzamine (20 mg./Kg.) it had no α-receptor protective activity. On the other hand, the racemic form (compound XI) had an approximate adrenolytic ED_{50} of 15 mg./Kg. and an α-receptor protective ED_{50} of 17.8 ± 2.4 mg./Kg., indicating that in both tests the *dextro*-isomer was responsible for the observed activities. Compound XIII at 50 mg./Kg. had neither adrenolytic activity nor any protective effect on the receptor. Since XIII is the racemic form of the compound, it may be concluded that α-ethyl substitution in the molecule of (+)-isoproterenol abolishes its α-receptor blocking activity.

DISCUSSION

The results reported above have demonstrated that complete protection of the sympathetic α-receptor *in vivo* can be obtained by the injection of adrenolytics in intravenous doses as low as 0.1 mg. (or less)/Kg. The correlation study indicated a close relationship between adrenolytic and receptor protective activity. This strongly suggests that the degree of affinity of the competitive adrenolytic for the sympathetic α-receptor is involved whether the compound blocks the access of epinephrine (adrenolytic test) or phenoxybenzamine (α-receptor protective test) to the receptors.

The results obtained with the α-receptor protective test confirm the findings indicating that (+)-isoproterenol is a sympathetic α-receptor blocker (8, 9) against the negative results reported by Levy and Ahlquist (10). It has been reported (11) that in the series of *N*-alkyl analogs of norepinephrine the ethyl, propyl, and isopropyl analogs have intrinsic activity between 1 and 0 (partial agonism) and that the same was true for the *N*-*tert*-butyl and the *N*-propyl analogs of octopamine. When the adrenolytic activity of (+)-isoproterenol was discovered, it was suggested (8) that the partial agonism of isoproterenol shown by the racemic form was due to the mixture of an agonist (*levo*-isomer)

and an antagonist (*dextro*-isomer). The results obtained with (+)-isoproterenol and compounds IX, XI, XII, and XIII strongly suggest that the same explanation applies to all the compounds in the sympathomimetic amine series reported as partial agonists by Ariëns and Simonis (11).

CONCLUSIONS

Phentolamine, trifluoperazine, promazine, phenindamine, benztropine, and other competitive sympathetic α-receptor blockers injected intravenously with phenoxybenzamine in rats blocked the long-acting adrenolytic effect of the latter. A highly significant correlation was found between the sympathetic α-receptor protective and adrenolytic activities of 17 compounds.

REFERENCES

(1) Nickerson, M., *Pharmacol. Rev.*, **11**, 27(1949).
(2) Seed, J. C., and McKay, E. A., *Proc. Soc. Exptl. Biol. Med.*, **70**, 724(1949).
(3) Nickerson, M., and Gump, W. J., *J. Pharmacol. Exptl. Therap.*, **97**, 25(1949).
(4) Furchgott, R. F., *ibid.*, **111**, 265(1954).
(5) Luduena, F. P., O'Malley, E., and Oyen, I. H., *Arch. Intern. Pharmacodyn.*, **122**, 111(1959).
(6) Siegel, S., "Nonparametric Statistics," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 202.
(7) Luduena, F. P., and Branin, M. J., *J. Pharm. Sci.*, **55**, 280(1966).
(8) Ariëns, E. J., *Biochem. Pharmacol.*, **8**, 115(1961).
(9) Luduena, F. P., *Arch. Intern. Pharmacodyn.*, **137**, 155(1962).
(10) Levy, B., and Ahlquist, R. P., *J. Pharmacol. Exptl. Therap.*, **133**, 202(1961).
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By B. R. BAKER and J. L. HENDRICKSON

Twenty-six selected compounds were investigated as inhibitors of xanthine oxidase, the enzyme that can also detoxify 6-mercaptopurine; these compounds consisted of 19 purines, four pyrimidines, two 8-azapurines, and one imidazole. Among the inhibitors that complexed to xanthine oxidase as well or better than the substrate, hypoxanthine, were thioguanine, adenine, and 6,8-dihydroxy-2-methylthiopurine (XXVI). The larger 2-benzylthio-6,8-dihydroxypurine (XXVII) was synthesized and found to inhibit equally as well as XXVI. Then 2-benzylthiohypoxanthine (XXVIII) and 8-benzylthiohypoxanthine (XXIX) were synthesized; these two compounds were complexed elevenfold and threefold better, respectively, to the enzyme than the substrate. Thus the enzyme showed bulk tolerance for the benzylthio group of XXVII–XXIX; the benzylthio group is a logical group for placement of electrophilic groups to give candidate active-site-directed irreversible inhibitors of xanthine oxidase.

XANTHINE OXIDASE is a catabolic enzyme involved in degradation of purines in a cell; the enzyme normally oxidizes hypoxanthine (I) and xanthine (II) to uric acid (III) (1, 2); the latter is then either excreted or further catabolized depending upon the species. The enzyme can also oxidize 2-hydroxypurine (IV) or 8-hydroxypurine to uric acid (III) (3) and adenine (V) to 6-amino-2,8-dihydroxypurine (VI) (4). The enzyme slowly oxidizes 4-hydroxypyrazolo-[3,4-*d*]pyrimidine (IX) to the 4,6-dihydroxy derivative (X), both of which are good inhibitors of xanthine oxidase (5, 6); in fact, IX is now marketed[1] for the treatment of gout since IX slows formation of uric acid. Other known inhibitors of xanthine oxidase are an assortment of N^6-substituted 2-hydroxyadenines (XI) and S^6-substituted 2-hydroxy-6-mercaptopurines (XII) (7).

Of interest to cancer chemotherapy is the oxidation of the anticancer agent, 6-mercaptopurine (VII), to thiouric acid (VIII) (8–10) by xanthine oxidase in mammals, thus detoxifying the drug. It has been proposed (11) that the selective action on cells by 6-mercaptopurine (VII) may be due in part to selective detoxification; if a cell can activate 6-mercaptopurine (VII) to its lethal ribonucleotide (12–14), but due to a lack of sufficient xanthine oxidase cannot detoxify VII by oxidation to the nontoxic thiouric acid, then the cell will be killed. Those normal and cancer cell lines that can oxidize VII to VIII would be less affected by VII; thus some cancer cell lines that can activate 6-mercaptopurine (VII) to the ribonucleotide may not respond to the drug due to the rapid detoxification of VII. (Scheme I.)

Received February 27, 1967, from the Department of Chemistry, University of California, Santa Barbara, CA 93106
Accepted for publication April 24, 1967.
This work was generously supported by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.
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Previous paper: Baker, B. R., and Coward, J. K., *J. Heterocyclic Chem.*, **4**, 202(1967).
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and an antagonist (*dextro*-isomer). The results obtained with (+)-isoproterenol and compounds IX, XI, XII, and XIII strongly suggest that the same explanation applies to all the compounds in the sympathomimetic amine series reported as partial agonists by Ariëns and Simonis (11).

CONCLUSIONS

Phentolamine, trifluoperazine, promazine, phenindamine, benztropine, and other competitive sympathetic α-receptor blockers injected intravenously with phenoxybenzamine in rats blocked the long-acting adrenolytic effect of the latter. A highly significant correlation was found between the sympathetic α-receptor protective and adrenolytic activities of 17 compounds.

REFERENCES

(1) Nickerson, M., *Pharmacol. Rev.*, **11**, 27(1949).
(2) Seed, J. C., and McKay, E. A., *Proc. Soc. Exptl. Biol. Med.*, **70**, 724(1949).
(3) Nickerson, M., and Gump, W. J., *J. Pharmacol. Exptl. Therap.*, **97**, 25(1949).
(4) Furchgott, R. F., *ibid.*, **111**, 265(1954).
(5) Luduena, F. P., O'Malley, E., and Oyen, I. H., *Arch. Intern. Pharmacodyn.*, **122**, 111(1959).
(6) Siegel, S., "Nonparametric Statistics," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 202.
(7) Luduena, F. P., and Branin, M. J., *J. Pharm. Sci.*, **55**, 280(1966).
(8) Ariëns, E. J., *Biochem. Pharmacol.*, **8**, 115(1961).
(9) Luduena, F. P., *Arch. Intern. Pharmacodyn.*, **137**, 155(1962).
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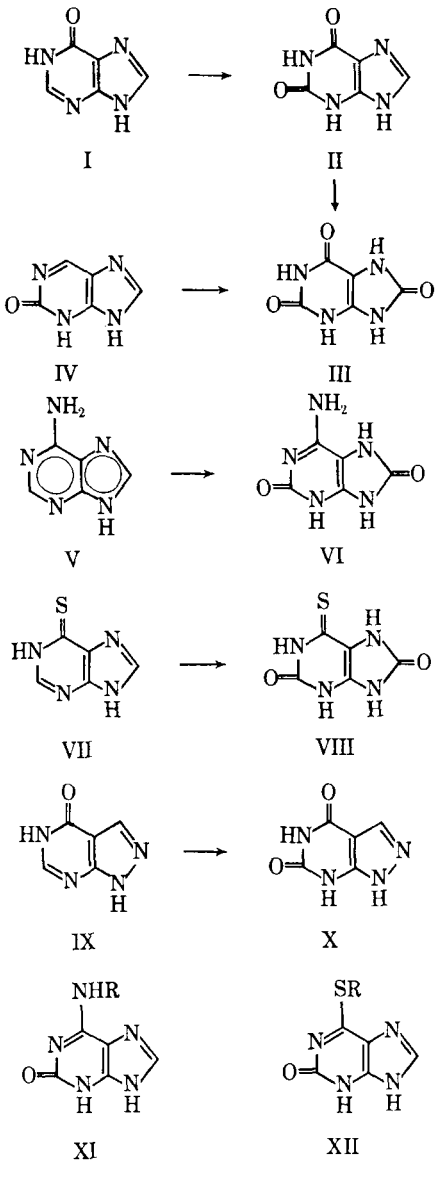
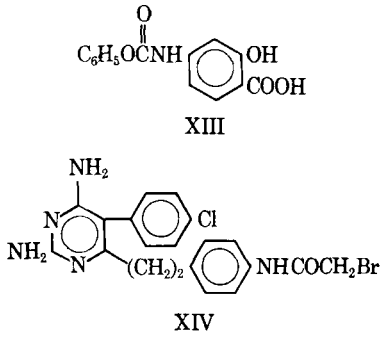


Scheme I

An inhibitor that could block xanthine oxidase in a cancer cell line with minimal blockade of the xanthine oxidase in normal cells could be a useful adjunct to treatment with 6-mercaptopurine. Reversible inhibitors rarely show sufficient tissue specificity to be of direct chemotherapeutic use without relying on unpredictable secondary considerations for selectivity such as an impaired active-transport system (15) or a lowered amount of detoxification enzyme (11, 16). In contrast, active-site-directed irreversible enzyme inhibitors can show tissue specificity since this type of inhibitor has an extra dimension of specificity not present in reversible inhibitors (14, 17, 18). For example, XIII can inactivate the lactic dehydrogenase from skeletal muscle, but not the lactic dehydrogenase from heart, and *vice versa* with the 5-isomer of XII (17, 19, 20). Another example is XIV, which will inactivate dihydrofolic reductase from *E. coli* B, but not pigeon liver (21).



In order to design an active-site-directed irreversible inhibitor of an enzyme, the following *modus operandi* has been developed (17, 19).

Phase I—The binding points on a reversible inhibitor should be determined, at least to the extent that substrate properties are destroyed, but binding is not; some binding points can be eliminated if compensated by a structural change that will give stronger binding at some other point in the molecule.

Phase II—The determination of areas on the inhibitor where bulky groups can be placed without interfering with the ability of the inhibitor to complex with the enzyme, known as either bulk tolerance within the enzyme–inhibitor complex or as a noncontact area between enzyme and inhibitor within the complex.

Phase III—A covalent bond-forming group should then be placed on a noncontact area of the inhibitor; if the dimension between the reversible complexing region on the enzyme and an enzymic nucleophilic group are correct, then a facile neighboring group reaction will occur between the inhibitor and enzyme that leads to selective inactivation of the enzyme by formation of a covalent bond.

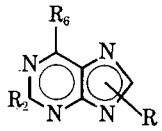
Phase IV—The irreversible inhibitor from phase III is then modified to take advantage of the bridge principle of specificity (18–20) until the desired tissue specificity for the given enzyme is achieved.

Initial studies on phases I and II are the subjects of this paper.

DISCUSSION

Since xanthine oxidase can oxidize purines at the 2-, 6-, or 8-position, either several catalytic sites are present on the enzyme with one mode of purine binding or the purines can bind in one of several different

TABLE I—INHIBITION OF XANTHINE OXIDASE BY



Compd.	R_2	R_6	R	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$[a]	Source
XV	SH	OH	8-OH	110	14	Aldrich
XVI	SH	OH	8-SH	12	1.5	NBC
XVII	SH	SH	8-SH	20	2.5	Aldrich
XVIII	NH_2	OH	8-OH	31	3.8	Aldrich
XIX	NH_2	NH_2	H	34[c]	4.2	NBC
XX	NH_2	OH	H	40[d]	4.9	NBC
XXI	NH_2	SH	H	7.7	0.95	NBC
XXII	H	Cl	H	100	12	BW
XXIII	H	n-C_4H_9S-	H	260[e]	32	Aldrich
XXIV	NH_2	OH	9-Me	58	7.1	Robins
XXV	NH_2	OH	7-Me	~1000[f]	120	Robins
XXVI	CH_3S	OH	8-OH	11	1.4	Aldrich
XXVII	$C_6H_5CH_2S$-	OH	8-OH	10	1.2	Exptl.
XXVIII	$C_6H_5CH_2S$-	OH	H	0.75[g]	0.093	Exptl.
XXIX	H	OH	8-$SCH_2C_6H_5$	2.8[g]	0.34	Exptl.
XXX	H	NH_2	H	5.6[h]	0.69	NBC
XXXI	NH_2	SCH_3	H	330	41	Sigma
XXXII	NH_2	NHC_6H_5	H	25	3.1	Robins
XXXIII	.....8-Azaguanine.....			7.5	0.93	NBC
XXXIV	.....8-Azaxanthine.....			84	10	NBC

[a] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition; $K_m = 8.5 \times 10^{-6}$ M has been reported (22). [b] Sources: Aldrich, Aldrich Chemical Co.; NBC, Nutritional Biochemical Corp.; BW, Burroughs Wellcome Co.; Robins, a gift from Professor Roland K. Robins; Exptl., see under *Experimental*; Sigma, Sigma Chemical Co. [c] Previously reported (22) as a substrate with 5% of the rate of hypoxanthine and $K_m = 7.8 \times 10^{-6}$ M. [d] Previously reported (23) as an inhibitor, but the relative amount of binding to the enzyme was not recorded. [e] Estimated from 20% inhibition at a concentration of 60 μM, the limit of solubility. [f] Estimated from 11% inhibition at a concentration of 200 μM. [g] No substrate properties at 100 μM. [h] Previously reported (22) as a substrate with 1% of the rate of hypoxanthine ($K_m = 12 \times 10^{-6}$ M) and as an inhibitor of hypoxanthine with $K_i = 11 \times 10^{-6}$ M.

rotomeric conformations and the enzyme has only one catalytic site; the latter seems a more plausible concept and has been previously invoked to explain the oxidation of aminopurines by xanthine oxidase (3). The concept of several rotomeric binding configurations has also been invoked for (*a*) binding to chymotrypsin (24, 25), (*b*) the binding of adenosine and adenylic acid to certain enzymes (26), and (*c*) the binding of pyrimidines to dihydrofolic reductase (27-31). When different rotomers of a molecule can bind to an enzyme which depends upon the substituents on the inhibitor, the exact groups of the inhibitor complexing to the enzyme are difficult to ascertain with reasonable probability (30, 31). Therefore, with xanthine oxidase it is possible to make some generalizations about binding, but what specific groups of the inhibitor are complexed to the enzyme is fraught with uncertainty at this time. However, these generalizations are sufficient to lead to phase II for construction of candidate active-site-directed irreversible inhibitors of xanthine oxidase. The following generalizations from the data in Table I seem reasonable.

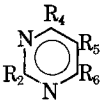
A—If no hydroxyl, thiol, or amino group is present on the purine, binding is poor; note that 6-chloropurine (XXII) and 6-butylmercaptopurine (XXIII) are complexed poorly to the enzyme, but hypoxanthine (the substrate), and adenine (XXX) are complexed well; 6-mercaptopurine (VII) is a known substrate (8-10).

B—A thiol group gives better binding than a hydroxyl group in some cases; note that thioguanine (XXI) binds fivefold better than guanine (XX), and XVI is a tenfold better inhibitor than XV. However, 2,6,8-trimercaptopurine (XVII) is not a better inhibitor than 2,8-dimercaptohypoxanthine (XVI). *S*-Methylation of thioguanine (XXI) to XXXI is extremely detrimental to binding, indicating that NHC=S moiety of thioguanine (XXI) is involved in binding to the enzyme.

C—More than one hydroxyl group can be detrimental to binding. Note that 2-benzylthiohypoxanthine (XXVIII) is a thirteenfold better inhibitor than its 8-hydroxy derivative (XXVII). If two hydroxyl groups are already present, an additional thiol can be detrimental to binding; note that 2-methylthio-6,8-dihydroxypurine (XXVI) is a tenfold better inhibitor than 2-thio-6,8-dihydroxypurine (XV). An exception occurs with aminopurines; 8-hydroxyguanine (XVIII) is a slightly better inhibitor than guanine (XX) and adenine (XXX) is a considerably better inhibitor than guanine (XX).

D—Guanine (XX) is a reasonably good inhibitor of xanthine oxidase, being complexed one-fifth as well as the substrate, hypoxanthine. Introduction of the 8-aza group, as in 8-azaguanine (XXXIII) gives a fivefold increment in binding. Replacement of the 6-hydroxyl group of guanine (XX) by amino (XIX) or introduction of a 9-methyl (XXIV) gives little change in binding; contrariwise, introduction of the 7-methyl group (XXV) is severely detrimental to binding.

TABLE II—INHIBITION OF XANTHINE OXIDASE BY



Compd.	R_2	R_4	R_5	R_6	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$[a]	Source[b]
XXXV	NH_2	OH	H	CH_3	130	16	Aldrich
XXXVI	OH	OH	H	CH_3	>5000	>620	NBC
XXXVII	NH_2	OH	H	NH_2	460	57	Aldrich
XXXVIII	OH	OH	NH_2	H	>2000	>250	EKC
XXXIX	..5-Aminoimidazole-4-carboxamide..				950	120	Aldrich

[a] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition; see under *Experimental*. [b] Aldrich Aldrich Chemical Co.; NBC, Nutritional Biochemicals Corp.; EKC, Eastman Kodak Co.

Since 2-methylthio-6,8-dihydroxypurine (XXVI) was complexed to the enzyme nearly as well as the substrate, hypoxanthine, bulk tolerance at the 2-position was investigated. 2-Benzylthio-6,8-dihydroxypurine (XXVII) was complexed as effectively as the 2-methylthiopurine (XXVI) thus showing tolerance for the bulk of the *S*-benzyl group within the enzyme–inhibitor complex. Removal of the 8-hydroxyl group of (XXVII) to give 2-benzylthiohypoxanthine (XXVIII) gave the most effective inhibitor in Table I, XXVIII being complexed elevenfold better than the substrate hypoxanthine. 8-Benzylthiohypoxanthine (XXIX) was not so good an inhibitor as its 2-isomer (XXVIII), but XXIX was still complexed threefold better than hypoxanthine; thus, there is also bulk tolerance for an 8-benzylthio group within the enzyme–inhibitor complex. Introduction of an N^6-phenyl group (XXXII) on 2,6-diaminopurine (XIX) did not decrease binding, indicating bulk tolerance for this phenyl group.

Compounds XXVII, XXVIII, and XXIX are good candidates for phase III studies on the construction of active-site-directed irreversible inhibitors of xanthine oxidase; attachment of covalent forming groups to these three compounds for phase III studies is underway.

Table II lists the inhibition results with four pyrimidines and one imidazole; none were sufficiently good inhibitors to warrant further pursuit. However, an interesting contrast can be noted on the relative binding of 5-aminoimidazole-4-carboxamide (XXXIX) and guanine (XX) to xanthine oxidase and guanase (32). Guanine (XX) complexes twice as well as XXXIX to guanase, but 24-fold better than XXXIX to xanthine oxidase; thus, the mode of binding of guanine to the two enzymes is clearly different.

EXPERIMENTAL

Methods

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Thin-layer chromatograms were run on Brinkmann Silica Gel GF in 3:1 chloroform–ethanol and spots were detected by visual examination under ultraviolet light. The analytical samples moved as a single spot and had infrared spectra in agreement with their assigned structures.

2 - Benzylmercapto - 6, 8 - dihydroxypurine (XXVII)—To a solution of 553 mg. (3 mmoles) of 6,8-dihydroxy-2-mercaptopurine (XV) in 3 ml. of 1 *N* aqueous sodium hydroxide was added 30 ml. of water and 0.35 ml. (3 mmoles) of benzyl chloride. After being stirred at ambient temperature for 5 hr., the mixture was acidified with acetic acid. The product was collected on a filter, then washed successively with hot water, acetone, and hot ethanol. The crude product was dissolved in dilute aqueous sodium hydroxide, then the solution was clarified with decolorizing carbon and acidified with acetic acid to give 406 mg. (49%) of product. Recrystallization from *N,N*-dimethylformamide gave 224 mg. (27%) of a white solid, m.p. over 300°. $\lambda_{max.}$ (pH 1): 278 mμ; (pH 13): 284 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4O_2S$: C, 52.5; H, 3.68; N, 20.4. Found: C, 52.7; H, 3.80; N, 20.3.

2-Benzylmercapto-6-hydroxypurine (XXVIII)—Reaction of 505 mg. (3 mmoles) of 6-hydroxy-2-mercaptopurine with benzyl chloride, as described for the preparation of XXVII, gave after recrystallization from aqueous 2-methoxyethanol 195 mg. (26%) of white powder, m.p. 263–265°. $\lambda_{max.}$ (pH 1): 272 mμ; (pH 13): 280 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4OS$: C, 55.8; H, 3.91; N, 21.7. Found: C, 55.9; H, 3.99; N, 21.9.

8-Benzylmercapto-6-hydroxypurine (XXIX)—This was prepared from 6-hydroxy-8-mercaptopurine hydrate in the same fashion as XXVIII; yield, 295 mg. (40%), m.p. 273–274° $\lambda_{max.}$ (pH 1): 280 mμ; (pH 13): 284 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4OS$: C, 55.8; H, 3.91; N, 21.7. Found: C, 55.9; H, 3.92; N, 21.8.

Inhibition of Xanthine Oxidase (2)—Xanthine oxidase from milk was obtained as a 5–10 unit suspension per ml. of 60% saturated ammonium sulfat from Nutritional Biochemicals Corp.; 1 unit supposedly converts 1 μmole of xanthine to uric acid per minute. For 1 day's assay, 0.05 ml. of enzyme was diluted with 2.95 ml. of buffer. Tris buffer (0.05 *M*), pH 7.4, was employed. Hypoxanthine was stocked as a 50 m*M* solution in 1 *N* aqueous sodium hydroxide; for 1 day's assays the stock solution was diluted 1:100 with buffer to give an 0.5 m*M* solution. Inhibitors XXIII–XXV, XXVII–XXIX, and XXXII were dissolved in dimethylsulfoxide and di-

luted with the same; the remainder of the inhibitors were dissolved in 10 mM aqueous sodium hydroxide and diluted with water.

In a 3-ml. cell were placed 2.70 ml. of buffer, 0.30 ml. of dimethylsulfoxide, and 50 μl. of 0.5 mM hypoxanthine. The solution was vigorously shaken for about 20 sec. to absorb air. Then 50 μl. of diluted enzyme solution was added and the rate of increase in absorbance at 290 mμ was noted with a Gilford recording spectrophotometer; sufficient enzyme was employed to give about 0.01 absorbance units change per minute. If the inhibitor was dissolved in water, up to 0.3 ml. could be used in the cell by decreasing the buffer by the corresponding amount. If the inhibitor was dissolved in dimethylsulfoxide, up to 0.30 ml. could be used, keeping the total volume of dimethylsulfoxide at 0.30 ml. Sufficient inhibitor was used to give 30–70% inhibition; the μM concentration of inhibitor necessary for 50% inhibition ($V_0/V_I = 2$) was determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration (33).

The cell concentration of hypoxanthine was 8.1 μM; the velocity was the same in the presence or absence of 10% dimethylsulfoxide.

REFERENCES

(1) Kalckar, H. M., *J. Biol. Chem.*, **167**, 429(1947).
(2) Horecker, B. L., and Heppel, L. A., "Methods in Enzymology," Colowick, S. P., and Kaplan, N. O., eds., Academic Press, Inc., New York, N. Y., 1955, vol. II, p. 482.
(3) Bergmann, F., Levin, G., Kwietny-Gourin, H., and Ungar, H., *Biochim. Biophys. Acta*, **47**, 1(1961).
(4) Wyngaarden, J. B., and Dunn, J. T., *Arch. Biochem. Biophys.*, **70**, 150(1957).
(5) Elion, G. B., Kovensky, A., Hitchings, G. H., Metz, E., and Rundles, R. W., *Biochem. Pharmacol.*, **15**, 863(1966).
(6) Rundles, R. W., Wyngaarden, J. B., Hitchings, G. H., Elion, G. B., and Silberman, H. R., *Trans. Assoc. Am. Physicians*, **76**, 126(1963).
(7) Leonard, E. O., Orme-Johnson, W. H., McMurtray, R. R., Skinner, C. G., and Shive, W., *Arch. Biochem. Biophys.*, **99**, 16(1962).
(8) Elion, G. B., Bieber, S., and Hitchings, G. H., *Ann. N.Y. Acad. Sci.*, **60**, 297(1954).
(9) Hamilton, L., and Elion, G. B., *ibid.*, **60**, 304(1954).
(10) Loo, T. L., Michael, M. E., Garceau, A. J., and Reid, J. C., *J. Am. Chem. Soc.*, **81**, 3039(1959).
(11) Baker, B. R., *Cancer Chemotherapy Rept.*, **4**, 1(1959).
(12) Brockman, R. W., *Clin. Pharmacol. Therap.*, **2**, 237(1961).
(13) Brockman, R. W., and Chumley, S., *Biochim. Biophys. Acta*, **95**, 365(1965).
(14) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. I.
(15) Werkheiser, W. C., *Proc. Am. Assoc. Cancer Res.*, **3**, 371(1962).
(16) Potter, V. P., *Univ. Mich. Med. Bull.*, **23**, 401(1957).
(17) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(18) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).
(19) *Reference 14*, chap. IX.
(20) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(21) Baker, B. R., and Jordaan, J. H., *ibid.*, **56**, 660(1967).
(22) Wyngaarden, J. B., *J. Biol. Chem.*, **224**, 453(1957).
(23) Coombs, H. I., *Biochem. J.*, **21**, 1259(1927).
(24) Hein, G. E., and Niemann, C., *J. Am. Chem. Soc.*, **84**, 4495(1962).
(25) Jones, J. B., Niemann, C., and Hein, G. E., *Biochemistry*, **4**, 1735(1965).
(26) Leonard, N. J., and Laursen, R. A., *ibid.*, **4**, 354, 365 (1965).
(27) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).
(28) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
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(30) Baker, B. R., and Shapiro, H. S., *J. Pharm. Sci.*, **55**, 308(1966).
(31) Baker, B. R., Lourens, G. J., and Jordaan, J. H., *J. Heterocyclic Chem.*, **4**, 39(1967).
(32) Baker, B. R., *J. Med. Chem.*, **10**, 59(1967).
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Irreversible Enzyme Inhibitors XCIII

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By B. R. BAKER

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THE PREVIOUS PAPER of this series discussed the possible chemotherapeutic use for a blockade of xanthine oxidase (1): since 6-mercaptopurine can be detoxified by oxidation to the nontoxic 6-thiouric, an active-site-directed irreversible inhibitor that is sufficiently tissue specific on a given tumor line would be a useful adjunct to 6-mercaptopurine therapy. The normal substrates of xanthine oxidase, hypoxanthine and xanthine, are highly polar molecules; therefore, if a hydrophobic region could be detected by purines substituted by hydrocarbon groups, this hydrophobic region would most probably be just adjacent to the active site, but not part of

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Accepted for publication April 24, 1967.
This work was generously supported by grant CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
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luted with the same; the remainder of the inhibitors were dissolved in 10 mM aqueous sodium hydroxide and diluted with water.

In a 3-ml. cell were placed 2.70 ml. of buffer, 0.30 ml. of dimethylsulfoxide, and 50 μl. of 0.5 mM hypoxanthine. The solution was vigorously shaken for about 20 sec. to absorb air. Then 50 μl. of diluted enzyme solution was added and the rate of increase in absorbance at 290 mμ was noted with a Gilford recording spectrophotometer; sufficient enzyme was employed to give about 0.01 absorbance units change per minute. If the inhibitor was dissolved in water, up to 0.3 ml. could be used in the cell by decreasing the buffer by the corresponding amount. If the inhibitor was dissolved in dimethylsulfoxide, up to 0.30 ml. could be used, keeping the total volume of dimethylsulfoxide at 0.30 ml. Sufficient inhibitor was used to give 30–70% inhibition; the μM concentration of inhibitor necessary for 50% inhibition ($V_0/V_I = 2$) was determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration (33).

The cell concentration of hypoxanthine was 8.1 μM; the velocity was the same in the presence or absence of 10% dimethylsulfoxide.

REFERENCES

(1) Kalckar, H. M., *J. Biol. Chem.*, **167**, 429(1947).
(2) Horecker, B. L., and Heppel, L. A., "Methods in Enzymology," Colowick, S. P., and Kaplan, N. O., eds., Academic Press, Inc., New York, N. Y., 1955, vol. II, p. 482.
(3) Bergmann, F., Levin, G., Kwietny-Gourin, H., and Ungar, H., *Biochim. Biophys. Acta*, **47**, 1(1961).
(4) Wyngaarden, J. B., and Dunn, J. T., *Arch. Biochem. Biophys.*, **70**, 150(1957).
(5) Elion, G. B., Kovensky, A., Hitchings, G. H., Metz, E., and Rundles, R. W., *Biochem. Pharmacol.*, **15**, 863(1966).
(6) Rundles, R. W., Wyngaarden, J. B., Hitchings, G. H., Elion, G. B., and Silberman, H. R., *Trans. Assoc. Am. Physicians*, **76**, 126(1963).
(7) Leonard, E. O., Orme-Johnson, W. H., McMurtray, R. R., Skinner, C. G., and Shive, W., *Arch. Biochem. Biophys.*, **99**, 16(1962).
(8) Elion, G. B., Bieber, S., and Hitchings, G. H., *Ann. N.Y. Acad. Sci.*, **60**, 297(1954).
(9) Hamilton, L., and Elion, G. B., *ibid.*, **60**, 304(1954).
(10) Loo, T. L., Michael, M. E., Garceau, A. J., and Reid, J. C., *J. Am. Chem. Soc.*, **81**, 3039(1959).
(11) Baker, B. R., *Cancer Chemotherapy Rept.*, **4**, 1(1959).
(12) Brockman, R. W., *Clin. Pharmacol. Therap.*, **2**, 237(1961).
(13) Brockman, R. W., and Chumley, S., *Biochim. Biophys. Acta*, **95**, 365(1965).
(14) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. I.
(15) Werkheiser, W. C., *Proc. Am. Assoc. Cancer Res.*, **3**, 371(1962).
(16) Potter, V. P., *Univ. Mich. Med. Bull.*, **23**, 401(1957).
(17) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(18) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).
(19) *Reference 14*, chap. IX.
(20) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(21) Baker, B. R., and Jordaan, J. H., *ibid.*, **56**, 660(1967).
(22) Wyngaarden, J. B., *J. Biol. Chem.*, **224**, 453(1957).
(23) Coombs, H. I., *Biochem. J.*, **21**, 1259(1927).
(24) Hein, G. E., and Niemann, C., *J. Am. Chem. Soc.*, **84**, 4495(1962).
(25) Jones, J. B., Niemann, C., and Hein, G. E., *Biochemistry*, **4**, 1735(1965).
(26) Leonard, N. J., and Laursen, R. A., *ibid.*, **4**, 354, 365 (1965).
(27) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).
(28) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
(29) *Reference 14*, chap. X.
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the active site. It is outside the active site where evolutionary changes of amino acids could more easily occur and still maintain the integrity of the active site (2). Such hydrophobic regions adjacent to the active site have been discovered on dihydrofolic reductase (3–5), guanase (6, 7), and thymidine phosphorylase (8, 9); the exploitation of hydrophobic regions adjacent to the active site for the design of reversible (5, 10) and irreversible inhibitors (5, 11) has been discussed. Therefore, a search for a hydrophobic bonding region on xanthine oxidase was initiated and the results are the subject of this paper.

DISCUSSION

It was noted in the previous paper that 8-azaguanine (II) was complexed as well as the substrate, hypoxanthine, and guanine (I) was complexed one-fifth as well (Table I). Since substitution of the 9-hydrogen of guanine (I) by methyl (III) gave less than a twofold loss in binding, variation of the 9-substituent of guanine was investigated for detection of hydrophobic bonding; these compounds were kindly supplied by Professor Roland K. Robins (12).

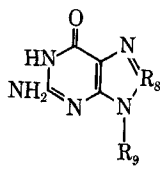
The 9-(*n*-amyl)-substituent of IV and the cyclohexyl substituent of V gave no appreciable increase in binding over guanine, and the 9-benzyl substituent (VI) gave only about twofold increase in binding. A large increment in binding was observed with the in-plane 9-phenyl substituent of VII, VII being complexed 100-fold better than guanine (I) and 140-fold better than 9-methylguanine (III). 9-(*p*-Chlorophenyl)guanine (VIII) also showed hydrophobic bonding to the enzyme with a 22-fold increment in binding over that of guanine (I), but which was less than that noted with 9-phenylguanine (VI). Similarly, the 9-(*p*-chlorophenyl) substituent (IX) of 8-azaguanine showed a 31-fold increment in binding over the parent 8-azaguanine (II). Thus, IX is the most potent inhibitor in Tables I and II, being complexed 33-fold better than the substrate, hypoxanthine which has $K_m = 7.8 \times 10^{-6}$ *M* (13).

Two guanine derivatives with an 8-phenyl (X) and 8-phenylpropyl (XI) substituent (6) were investigated for hydrophobic bonding; both showed about a fivefold increment in binding over guanine (I), but this increment was considerably less than that observed with the 9-phenyl substituent (VII).

Through a generous gift from Professor E. C. Taylor, the 4-mercaptopyrazolo[3,4-*d*]pyrimidines with hydrocarbon groups at the 2-position (14), were studied; these were of interest since 4-hydroxypyrazolo[3,4-*d*]pyrimidine is a known substrate and inhibitor (15) of xanthine oxidase and since hydrophobic bonding had been detected with 9-phenylguanine (Table I).

The 2-methyl derivative (XII) (Table II) was complexed fifteenfold less than hypoxanthine, the substrate. When the 2-methyl group of XII was replaced by the in-plane *p*-nitrophenyl group (XIII), a 38-fold increment in binding was observed; this is similar to the 31-fold increment observed in replacing the 9-methyl group (III) on guanine by *p*-chlorophenyl (VIII), but not so good as the increment in 9-phenylguanine (VII).

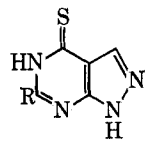
Table I—Inhibition of Xanthine Oxidase by



Compd.	R_8	R_9	μM Concn. for 50% Inhibition[a]	$[I/S]_{0.5}$[b]	Source
I	CH	H	40[d]	4.9	NBC
II	N	H	7.5[d]	0.93	NBC
III	CH	CH_3	58[d]	7.1	Robins
IV	CH	*n*-C_5H_{11}-	35	4.3	Robins
V	CH	Cyclohexyl-	55	6.8	Robins
VI	CH	$C_6H_5CH_2$-	23	2.9	Robins
VII	CH	C_6H_5-	0.41	0.051	Robins
VIII	CH	*p*-ClC_6H_4-	1.8	0.22	Robins
IX	N	*p*-ClC_6H_4-	0.25	0.030	Robins
X	CC_6H_5	H	7.4	0.92	(6)
XI	$C(CH_2)_2C_6H_5$	H	8.4	1.0	(6)

[a] Xanthine oxidase from bovine milk (Nutritional Biochemicals Corp.) was assayed with 8.1 μM hypoxanthine in Tris buffer (pH 7.4) containing 10% dimethylsulfoxide as previously described (1). [b] The ratio of the concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition. [c] Sources: NBC, Nutritional Biochemicals Corp.; Robins, a gift from Professor Roland K. Robins (12). [d] Data from *Reference 1*.

Table II—Inhibition of Xanthine Oxidase by



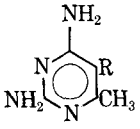
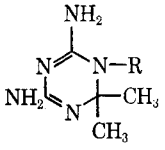
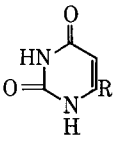
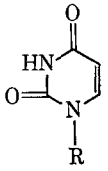
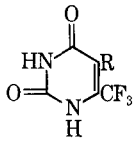
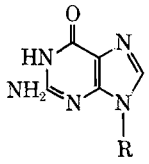
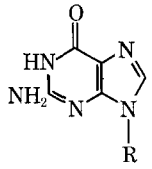
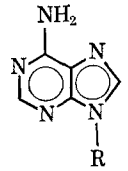
Compd.	R	μM Concn. for 50% Inhibition[a]	$[I/S]_{0.5}$[b]	Hansch π-Constant for φ Substituent[c]
XII	CH_3	120	15	
XIII	*p*-$NO_2C_6H_4$-	3.3	0.39	−0.28
XIV	*p*-$NH_2C_6H_4$-	32	4.0	−1.23
XV	*p*-$CH_3OC_6H_4$-	2.5	0.31	−0.02

[a] See *Footnote a*, Table I; these compounds were a gift from Professor E. C. Taylor (14). [b] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition. [c] A measurement of hydrophobic character on a log scale, a minus value being more polar than hydrogen; data from *Reference 24*.

The *p*-methoxyphenyl substituent (XV) was complexed as well as the *p*-nitrophenyl substituent (XIII); these results indicate that there is no donor-acceptor character in the mode of complexing of the phenyl group in this series to the enzyme, else considerable difference in binding should have been seen due to the strong electron-donating methoxyl group and the strong electron-withdrawing nitro group. Replacement of the *p*-methoxy group of XV with *p*-amino (XIV) gave a thirteenfold loss in binding. This result could be due to repulsion of the weakly basic amino group from an electron-donor site on the enzyme or due to the difference in polarity between amino on one hand and methoxy and nitro on the other; note in Table II that the amino group is by far the most polar of the three groups.

That an in-plane phenyl group can give hydrophobic bonding on either the 9-position or "2" position of a purine or related compound agrees with the postulate of Bergmann *et al.* (16) that various rotomers of the purine can bind to the active site of xanthine oxidase. Strong hydrophobic bonding can be determinate on which rotomer binds to the enzyme, as previously postulated with binding to chymotrypsin (17, 18), dihydrofolic reductase (4, 5, 19, 20), some adenylate using enzymes (21), and adeno-

TABLE III—HYDROPHOBIC BONDING TO SOME SELECTED ENZYMES BY HETEROCYCLES WITH HYDROCARBON SUBSTITUENTS

Enzyme	Source	Heterocycle	R for Max. Hydrophobic Bonding	Binding Increment	Ref.
Dihydrofolic reductase	Pigeon liver		$C_6H_5(CH_2)_4$-	40,000	(3, 5)
			$C_6H_5(CH_2)_4$-	2000[a]	(3, 5)
			C_6H_5-	700[a]	(3, 5)
			i-C_5H_{11}-	1300[a]	(3, 5)
			m-$C_6H_4(CH_2)_4C_6H_5$	20,000	(29)
Thymidine phosphorylase	*E. coli* B		$C_6H_5CH_2$-	18	(8, 9)
			$C_6H_5(CH_2)_5$-	100[b]	(8, 9)
			$C_6H_5(CH_2)_4$-	9[b]	(30)
Guanase	Rabbit liver		C_6H_5-	28	(6, 7)
Xanthine oxidase	Bovine milk		C_6H_5-	100[c]	
Succinoadenylate kinosynthetase	*E. coli* B		$C_6H_5CH_2$-	11	(28)

[a] Included for comparison. [b] Higher phenylalkyl analogs not yet investigated, but lower analogs were less effective. [c] Data from Table I.

sine deaminase (22). By substitution of the phenyl group on various purines and related heterocycles, such as those in Tables I and II, it should then be possible to study the mode of binding of other substituents on the heterocyclic ring such as OH, NH, and SH; if good phenyl binding is observed compared to the corresponding methyl or benzyl purine, then it can be logically assumed that the phenyl group anchors one rotomer to the enzyme, but with two "flip" configurations (5, 20).

Also of interest would be to establish whether the phenyl group complexes to the enzyme only by hydrophobic bonding and the accompanying van der Waal's forces—as seen with dihydrofolic reductase (5, 23)—or if there is additional donor-acceptor character in the phenyl ring (unlikely). Furthermore, the use of these phenyl heterocycles for construction of active-site-directed irreversible inhibitors is worthy of pursuit.

Some seven enzymes using a heterocyclic substrate have been investigated in this laboratory for hydrophobic bonding by hydrocarbon groups attached to the heterocycle. Hydrophobic bonding has yet to be detected with thymidine kinase (9, 25, 26) and thymidylate synthetase (27) even though such a search was performed; weak hydrophobic bonding with 9-alkyl and 9-aralkyladenines to succinoadenylate kinosynthetase has been observed (28); strong hydrophobic bonding with 6-aralkyluracils to thymidine phosphorylase (8, 9), with 9-phenylguanines to guanase (6), and with 9-phenylpurines and related heterocycles to xanthine oxidase has been observed. The strongest hydrophobic bonding observed in this set of seven enzymes was with dihydrofolic reductase (3, 5, 23) where the in-plane phenyl group gives a 700-fold increment and the phenylbutyl group gives a 40,000-fold increment in binding.

These comparisons are summarized in Table III. Note that the type of hydrocarbon giving maximum hydrophobic bonding varies with the enzyme and with the position of the hydrophobic group on the inhibitor; for example, the in-plane phenyl group gives maximum hydrocarbon bonding with guanase and xanthine oxidase, but higher phenylalkyl groups are more effective on the other enzymes. Also note that the benzyl group on the 6-position of uracil gives maximum hydrocarbon interaction, but that higher phenylalkyl groups are more effective on the 1- and 5-positions of uracil. Although the 9-phenyl group on guanine is the most effective for hydrocarbon interaction with guanase and xanthine oxidase, substitution on the phenyl group has shown that the enzymic environment in the region of 9-phenyl bonding is not the same with the two enzymes; for example, a *p*-carboxylate on the 9-phenyl group aids binding to xanthine oxidase, but is extremely detrimental to binding to guanase (31).

REFERENCES

(1) Baker, B. R., and Hendrickson, J. L., *J. Pharm. Sci.*, **56**, 955(1967).

(2) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. IX.

(3) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).

(4) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *ibid.*, **55**, 295(1966).

(5) *Reference 2*, chap. X.

(6) Baker, B. R., and Santi, D. V., *J. Med. Chem.*, **10**, 62(1967).

(7) *Reference 2*, chap. V.

(8) Baker, B. R., and Kawazu, M., *J. Med. Chem.*, **10**, 311(1967).

(9) *Reference 2*, chap. IV.

(10) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 470(1966).

(11) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417(1966).

(12) Koppel, H. C., and Robins, R. K., *J. Am. Chem. Soc.*, **80**, 2751(1958); Koppel, H. C., O'Brien, D. E., and Robins, R. K., *ibid.*, **81**, 3049(1959); Noell, W. C., and Robins, R. K., *J. Med. Pharm. Chem.*, **5**, 558(1962).

(13) Wyngaarden, J. B., *J. Biol. Chem.*, **224**, 453(1953).

(14) Taylor, E. C., and Zoltewicz, J. A., *J. Am. Chem. Soc.*, **83**, 248(1961).

(15) Elion, G. B., Kovensky, A., Hitchings, G. H., Metz, E., and Rundles, R. W., *Biochem. Pharmacol.*, **15**, 863(1966).

(16) Bergmann, F., Levin, G., Kwietny-Gourin, H., and Ungar, H., *Biochim. Biophys. Acta*, **47**, 1(1961).

(17) Hein, G. E., and Niemann, C., *J. Am. Chem. Soc.*, **84**, 4495(1962).

(18) *Reference 2*, chap. III.

(19) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).

(20) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).

(21) Leonard, N. J., and Laursen, R. A., *Biochemistry*, **4**, 354, 365(1965).

(22) *Reference 2*, chap. XII.

(23) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 355(1965).

(24) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).

(25) Baker, B. R., Schwan, T. J., and Santi, D. V., *J. Med. Chem.*, **9**, 66(1966).

(26) Baker, B. R., and Schwan, T. J., *ibid.*, **9**, 73(1966).

(27) *Reference 2*, chap. XI.

(28) Baker, B. R., and Erickson, E. H., unpublished data.

(29) Baker, B. R., Ho, B.-T., and Lourens, G. J., *J. Pharm. Sci.*, **56**, 737(1967).

(30) Baker, B. R., and Kawazu, M., unpublished data.

(31) Baker, B. R., and Wood, W. F., unpublished data.

Synthesis of 1-Methylpyrazolidine and Some of Its Derivatives

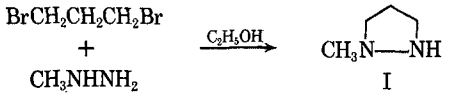
By MILTON J. KORNET

1-Methylpyrazolidine has been prepared *via* a two-step reaction sequence from ethyl acrylate and methylhydrazine, and also directly from trimethylene bromide and methylhydrazine. The *N*-methylpyrazolidino group was substituted for the dialkylamino group in a limited number of clinically effective agents. These derivatives are to be screened for pharmacologic activity.

RECENTLY, a series of esters which has a *N*-alkylated pyrazolidinyl group substituted for a dialkylamino group has been synthesized and found to possess local anesthetic activity (1). One of the compounds, 3-(*N*,*N'*-diethylpyrazolidinyl)methyl *p*-(*n*-butylamino) benzoate was found to be six times as effective as piperocaine[1] when tested on earthworms of genus *Lumbricus*. In view of these encouraging results, it appeared desirable to extend this work and to incorporate the pyrazolidine ring into other types of drug molecules. To this end, the previously unknown 1-methylpyrazolidine has been synthesized, and substituted for the customary dialkylamino group, as a *N*-methylpyrazolidino group, in a number of clinically active agents.

DISCUSSION

1-Methylpyrazolidine (I) was obtained *via* a two-step reaction sequence from methylhydrazine and ethyl acrylate. The intermediate product obtained by distillation was not characterized, but was reduced directly by lithium aluminum hydride and afforded 1-methylpyrazolidine (I) in an over-all yield of 68%. Presumably the intermediate is predominantly 1-methyl-3-pyrazolidinone rather than the 2-methyl isomer. Reduction of either isomer would give 1-methylpyrazolidine. The above structure is proposed for the intermediate because reaction of methylhydrazine with methacrylic acid reportedly gave 1,4-dimethyl-3-pyrazolidinone as the sole product (2). The structure of I is supported by its elemental analysis and infrared spectrum. With excess phenyl isothiocyanate, I forms a monophenylthiourea derivative. Further support for structure I is an alternate one-step synthesis from methylhydrazine and trimethylene bromide. (Scheme I.)



Scheme I

The infrared spectrum of the product obtained by this pathway was identical to that of the product obtained *via* the methylhydrazine–ethyl acrylate route. Furthermore, no depression was observed when a melting point determination was performed on a mixture of the phenylthiourea derivatives of the two products, and the infrared spectra of these derivatives were identical (see under *Experimental*). Because of a higher yield and purer product, 1-methylpyrazolidine (I) is best prepared *via* the longer two-step route.

The preparation of the *N*-methylpyrazolidino analog of the local anesthetic lidocaine[2] was attempted by reacting *N*-chloroacetyl-2,6-dimethylaniline with excess 1-methylpyrazolidine. A crystalline salt having the correct elemental analysis was obtained in 92% yield. However, the salt is not the desired 2-(2-methylpyrazolidino)-2′,6′-dimethylacetanilide hydrochloride but is instead a hydrazinium chloride, 1-methyl-1-[2-(2′,6′-dimethylanilino)-2-oxoethyl]pyrazolidinium chloride (II). The infrared spectrum of the salt supports structure II since it revealed the absence of absorption in the NH^+ region. Treatment of an aqueous solution of the salt with sodium bicarbonate failed to evolve carbon dioxide, behavior which rules out a hydrochloride salt. Furthermore, an aqueous solution of the salt reacted with silver oxide to precipitate silver chloride and gave a strongly alkaline solution (pH ~ 11, Hydrion E paper) indicating the conversion of a hydrazinium chloride to a hydrazinium hydroxide. This observation is in agreement with the report that hydrazinium hydroxides are slightly less basic than quaternary ammonium hydroxides (3). Studies concerned with the alkylation of alkylhydrazines have shown that substitution occurs at the substituted nitrogen atom to give good yields of the trialkylated quaternary hydrazinium halide unless the alkyl halide contains bulky groups (4, 5). Thus α-bromopropiophenone reacts with 1,1-dimethylhydrazine at the methylated nitrogen atom, resulting in the formation of a quaternary hydrazinium bromide product (6). In view of these earlier findings, it is not surprising that II is the product of the reaction between *N*-methylpyrazolidine and *N*-chloroacetyl-2,6-dimethylaniline.

The *N*-methylpyrazolidino analog of an aminoalkyl ether type of antihistaminic agent, of which chlordiphenhydramine[3] is the prototype, was realized in the following way. Reaction of I with ethyl acrylate proceeded smoothly and afforded the adduct, 1-(2-ethoxycarbonylethyl)-2-methylpyrazolidine (IV) in 85% yield. Lithium aluminum hydride reduction of IV gave an 87% yield of the

Received December 5, 1966, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506

Accepted for publication May 5, 1967.

This investigation was supported in part by grant GB-3331 from the National Science Foundation, Washington, D.C.

[1] 3-(2-Methylpiperidino)propyl benzoate.

[2] 2-(Diethylamino)-2′,6′-acetoxylidide.

[3] 2-(*p*-Chlorobenzhydryloxy)-*N*-dimethylethylamine.

corresponding alcohol (V), which was condensed with *p*-chlorobenzhydryl chloride to give the ether, 1-[3-(*p*-chlorobenzhydryloxy)propyl]-2-methylpyrazolidine (VI). The preparation of a pyrazolidinoethyl ether was abandoned in favor of a pyrazolidinopropyl ether when it was found that I could be converted to 1-(2-hydroxyethyl)-2-methylpyrazolidine (III) in only 9% yield by reaction with ethylene oxide.

Cyanoethylation of 1-methylpyrazolidine proceeded readily and in 77% yield to give the nitrile, 1-(2-cyanoethyl)-2-methylpyrazolidine (VII) which was reduced by lithium aluminum hydride and afforded the propylamine derivative (VIII) in 83% yield. The latter compound, upon reaction with *S*-methylisothiourea sulfate, was converted into the guanidino derivative, 1-(3-guanidinopropyl)-2-methylpyrazolidine sulfate (IX). Compound IX may be regarded as a relative of the hypotensive agent guanethidine.[4]

1-Nicotinoyl-2-methylpyrazolidine (X), related to the respiratory stimulant nikethamide,[5] was prepared from 1-methylpyrazolidine and ethyl nicotinate using sodium methoxide as the catalyst. In addition, I reacted with ethyl chloroformate and gave 1-ethoxycarbonyl-2-methylpyrazolidine (XI). This compound bears structural resemblances to 1-ethoxycarbonyl-4-methylpiperazine, a potent filaricidal agent (7). The above transformations are summarized in Scheme II. The derivatives described in this paper are to be screened for pharmacological activity.

EXPERIMENTAL[6]

1-Methylpyrazolidine (I)—*Method A*—To a solution of 50.6 Gm. (1.10 moles) of methylhydrazine in 25 ml. of absolute ethanol was added dropwise a solution of 100 Gm. (1.00 mole) of ethyl acrylate in 65 ml. of absolute ethanol with stirring (magnetic) and ice-bath cooling. The reaction mixture was stirred and allowed to come to room temperature overnight, and refluxed for 24 hr. The ethanol was distilled *in vacuo* on a water bath, and the residue was distilled and gave 98.6 Gm. of a nearly colorless liquid, b.p. 90–110° (0.7 mm.). The liquid was partially dissolved in 500 ml. of anhydrous ether and the resulting mixture (2-phase) was added dropwise to a suspension of 70.0 Gm. (1.85 moles) of $LiAlH_4$ in 1300 ml. of anhydrous ether with stirring (mechanical). After refluxing for 18 hr. the complex was decomposed with 40% aqueous KOH, with cooling of the reaction mixture by means of a brine and ice cooling mixture. The ether layer was separated and the inorganic sludge was washed twice with 100-ml. portions of ether. The combined ether layers were dried ($MgSO_4$) and the filtered solution was distilled through a Todd column (12 × 90 mm.) packed with glass helices (4 mm. diameter). After removal of the ether fraction and a small fraction of b.p. 78° (probably ethanol), the product distilled and gave 58.9 Gm. (68.5% yield based on ethyl acrylate) of a colorless liquid, b.p. 118° (739 mm.); n_D^{20} 1.4561; $\lambda_{max.}^{film}$ 3.06 μ (NH). In addition, 10 Gm. of a higher boiling residue remained which was not further investigated.

Anal.—Calcd. for $C_4H_{10}N_2$: C, 55.77; H, 11.70; N, 32.52. Found: C, 55.73; H, 11.62; N, 32.58.

A phenylthiourea derivative (8) was prepared and recrystallized from 95% ethanol, m.p. 90–91°.

Anal.—Calcd. for $C_{11}H_{15}N_3S$: C, 59.69; H, 6.83; N, 18.99. Found: C, 59.58; H, 6.72; N, 18.93.

The dihydrobromide was recrystallized from absolute ethanol–ether, m.p. 181–182.5°.

Anal.—Calcd. for $C_4H_{10}N_2 \cdot 2HBr$: C, 19.37; H, 4.88. Found: C, 19.83; H, 5.16.

Method B—To a mechanically-stirred solution of 34.5 Gm. (0.75 mole) of methylhydrazine in 100 ml. of 98% ethanol was added 75.7 Gm. (0.375 mole) of trimethylene bromide in small portions over a period of 30 min. with cooling by means of a cold water bath. The mixture was stirred for 1 hr. at room temperature and refluxed for 17 hr. The clear yellow solution was treated with 45 Gm. (0.8 mole) of KOH pellets with stirring whereupon KBr precipitated. After stirring for 1 hr. the reaction mixture was filtered and the filtrate was distilled through the Todd column described above. The fraction of b.p. 75–84° was collected and discarded. The contents of the still pot was cooled, dissolved in 250 ml. of tetrahydrofuran, and dried over KOH pellets overnight. After filtering, the solution was distilled through the Todd column and afforded 11 Gm. (34%) of a fraction, b.p. 107–115° (735 mm.). Redistillation (without a fractionating column) from KOH pellets gave 8.5 Gm. of liquid, b.p. 110–116° (735 mm.). The infrared spectrum (film) of the product obtained by method *B* was the same as the I.R. spectrum of the product obtained by method *A*, except that the intensity of the NH band was somewhat weaker in the spectrum of the product obtained *via* method *B*. The phenylthiourea derivative was prepared, m.p. 90–91°. A mixed melting point with the phenylthiourea derivative obtained by method *A* was 90–91°, and the infrared spectra of the two derivatives were identical.

1-Methyl-1-[2-(2′,6′-dimethylanilino)-2-oxoethyl]pyrazolidinium chloride (II)—To a solution of 8.25 Gm. (0.096 mole) of 1-methylpyrazolidine (I) in 35 ml. of dry benzene was added 7.90 Gm. (0.040 mole) of *N*-chloroacetyl-2,6-dimethylaniline (9) and the mixture was refluxed for 1.75 hr. The mixture was cooled and filtered to give 10.5 Gm. (92.5%) of a white crystalline salt, m.p. 189–197°. Two recrystallizations from absolute ethanol afforded the analytically pure compound, m.p. 200–201.5°; $\lambda_{max.}^{Nujol}$ 6.0 μ (C=O), 3.2 μ (NH).

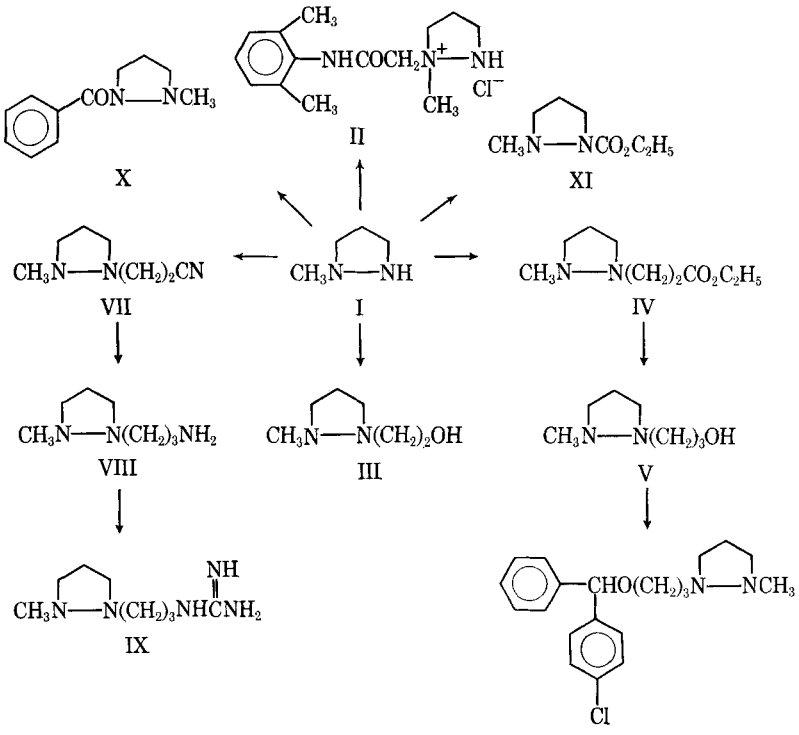
Anal.—Calcd. for $C_{14}H_{22}ClN_3O$: C, 59.25; H, 7.81. Found: C, 59.19; H, 7.83.

1-(2-Hydroxyethyl)-2-methylpyrazolidine (III)—To a solution of 17.2 Gm. (0.2 mole) of 1-methylpyrazolidine (I) in 40 ml. of methanol was added dropwise a solution of 13.3 Gm. (0.30 mole) of ethylene oxide in 65 ml. of anhydrous ether over a period of 30 min. with ice-salt bath cooling. The stoppered reaction mixture was allowed to reach room temperature overnight and was stored for 4 days. An additional 3 ml. of ethylene oxide was added and the reaction mixture was refluxed for 5 hr. using a dry ice–acetone condenser and allowed to stand overnight. The solvents were removed on a water bath *in vacuo* and the residue was fractionally distilled to give 2.4 Gm. (9.2%) of a colorless liquid,

[4] [2-(Octahydro-1-azocinyl)ethyl]guanidine.

[5] *N,N*-Diethylnicotinamide.

[6] Melting points were determined with the Fisher-Johns melting point apparatus and are corrected. Infrared spectra were recorded on a Beckman IR 8 spectrophotometer using sodium chloride optics. Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerland.



Scheme II

b.p. 78–83° (28 mm.); n_D^{20} 1.4432. The picrate was prepared and recrystallized from absolute ethanol, m.p. 146.5–148.5°.

Anal.—Calcd. for $C_{12}H_{17}N_5O_8$: N, 19.49. Found: N, 19.31.

1-(2-Ethoxycarbonylethyl)-2-methylpyrazolidine (IV)—A solution of 13.7 Gm. (0.137 mole) of ethyl acrylate in 16 ml. of absolute ethanol was added dropwise to a stirred (magnetic) solution of 11.8 Gm. (0.137 mole) of 1-methylpyrazolidine (I) in 6 ml. of absolute ethanol with ice-bath cooling. After stirring overnight at room temperature the reaction mixture was refluxed on the steam bath for 44 hr. The ethanol was removed (rotary evaporator) and the residue was distilled to give 21.7 Gm. (85%) of a colorless liquid, b.p. 105° (8 mm.); b.p. 60° (0.08 mm.); n_D^{20} 1.4533. In a second experiment with a 20-hr. reflux period the yield was 21.8 Gm. (85.4%).

Anal.—Calcd. for $C_9H_{18}N_2O_2$: C, 58.04; H, 9.74. Found: C, 58.19; H, 9.77.

A picrate derivative was prepared and recrystallized from absolute ethanol, m.p. 118–119°.

Anal.—Calcd. for $C_{15}H_{21}N_5O_9$: C, 43.37; H, 5.10; N, 16.86. Found: C, 43.52; H, 5.07; N, 17.00.

1-(3-Hydroxypropyl)-2-methylpyrazolidine (V)—A solution of 20.6 Gm. (0.111 mole) of 1-(2-ethoxycarbonylethyl)-2-methylpyrazolidine (IV) in 75 ml. of anhydrous ether was added to a suspension of 4.20 Gm. (0.111 mole) of $LiAlH_4$ and the resulting mixture was refluxed for 17 hr. After decomposing the complex with 40% aqueous KOH (about 50 ml.), the inorganic salts were extracted with ether and the combined ether layers were dried ($MgSO_4$). The spent drying agent was filtered and the filtrate was distilled at atmospheric pressure to remove the ether. The residue was distilled and afforded 13.97 Gm. (87.5%) of a colorless oil, b.p. 112° (11 mm.); n_D^{20} 1.4788; $\lambda_{max.}^{film}$ 2.97 μ (broad H-bonded OH).

Anal.—Calcd. for $C_7H_{16}N_2O$: C, 58.30; H, 11.18. Found: 58.36; H, 11.56.

1-[3-(p-Chlorobenzhydryloxy)propyl]-2-methylpyrazolidine (VI)—A solution of 11.8 Gm. (0.050 mole) of *p*-chlorobenzhydryl chloride in 75 ml. of dry xylene was added dropwise with stirring (mechanical) to a refluxing mixture of 7.2 Gm. (0.05 mole) of 1-(3-hydroxypropyl)-2-methylpyrazolidine (V) and 5.3 Gm. (0.05 mole) of anhydrous sodium carbonate in 30 ml. of dry xylene. The mixture was refluxed for 27 hr. After cooling, the reaction mixture was extracted several times with 10% aqueous HCl and the combined extracts were made alkaline with 40% aqueous NaOH and extracted with chloroform. The chloroform solution was dried ($MgSO_4$) and distilled at atmospheric pressure to remove the chloroform. The residue was distilled and gave 2.0 Gm. (11.6%) of a pale yellow oil, b.p. 169° (0.1 mm.); n_D^{20} 1.5615; $\lambda_{max.}^{film}$ 9.2 μ (COC), 13.3, 14.3 μ (monosubstituted benzene).

Anal.—Calcd. for $C_{20}H_{25}ClN_2O$: C, 69.65; H, 7.31; N, 8.12. Found: C, 69.67; H, 7.40; N, 8.13.

1-(2-Cyanoethyl)-2-methylpyrazolidine (VII)—To 12.9 Gm. (0.15 mole) of 1-methylpyrazolidine (I) was added dropwise 7.59 Gm. (0.15 mole) of acrylonitrile with stirring (magnetic) and ice-bath cooling. The resulting mixture was stirred at room temperature overnight and then heated on the steam bath for 2 hr. The yellow mixture was

distilled and afforded 15.96 Gm. (76.6%) of a colorless liquid, b.p. 128–130° (26 mm.); b.p. 64° (0.17 mm.); n_D^{20} 1.4663; $\lambda_{max.}^{film}$ 4.44 μ (C≡N).

Anal.—Calcd. for $C_7H_{13}N_3$: C, 60.40; H, 9.41; N, 30.19. Found: C, 60.38; H, 9.64; N, 29.98.

A picrate derivative was prepared and recrystallized from absolute ethanol, m.p. 151–152.5°.

Anal.—Calcd. for $C_{13}H_{16}N_6O_7$: C, 42.39; H, 4.38. Found: C, 42.31; H, 4.90.

1-(3-Aminopropyl)-2-methylpyrazolidine (VIII)—A solution of 10.0 Gm. (0.0719 mole) of 1-(2-cyanoethyl)-2-methylpyrazolidine (VII) in 50 ml. of anhydrous ether was added dropwise with stirring (mechanical) to a suspension of 5.0 Gm. (0.132 mole) of $LiAlH_4$ in 450 ml. of anhydrous ether. The mixture was refluxed overnight and cooled, and the complex was decomposed with 40% aqueous KOH. The ether layer was decanted, the inorganic sludge was extracted twice with ether, and the combined ether layers were dried ($MgSO_4$). The drying agent was filtered and the ether distilled on a water bath at atmospheric pressure. The residue was distilled to give 8.56 Gm. (83.1%) of a colorless liquid, b.p. 45° (0.25 mm.); n_D^{20} 1.4777; $\lambda_{max.}^{film}$ 3.02 μ (NH).

Anal.—Calcd. for $C_7H_{17}N_3$: C, 58.70; H, 11.96; N, 29.34. Found: C, 58.59; H, 12.28; N, 29.30.

A dipicrate derivative was prepared and recrystallized from 95% ethanol, m.p. 175.5–176.5°.

Anal.—Calcd. for $C_{19}H_{23}N_9O_{14}$: C, 37.94; H, 3.85. Found: C, 38.07; H, 3.96.

1-(3-Guanidinopropyl)-2-methylpyrazolidine Sulfate (IX)—A mixture of 5.0 Gm. (0.035 mole) of 1-(3-aminopropyl)-2-methylpyrazolidine (VIII) and 8.8 Gm. (0.032 mole) of *S*-methylisothiourea sulfate (10) in 35 ml. of 95% ethanol was refluxed with stirring for 6 hr. After cooling, the reaction mixture was filtered and the solid obtained (2.66 Gm.) was identified as *S*-methylisothiourea sulfate. The filtrate was evaporated *in vacuo* and the oily residue was dried (P_2O_5) in an evacuated desiccator for 3 days. Trituration with anhydrous ether gave a hygroscopic crystalline solid which was filtered and redried (P_2O_5). The dried solid weighed 6.0 Gm. (61%) and was recrystallized by dissolving it in water and adding absolute ethanol to the cloud point, m.p. 223.5–224°.

Anal.—Calcd. for $C_8H_{19}N_5 \cdot H_2SO_4$: C, 33.91; H, 7.47; N, 24.72. Found: C, 34.02; H, 7.47; N, 24.70.

1-Nicotinoyl-2-methylpyrazolidine (X)—A solution of 4.3 Gm. (0.05 mole) of 1-methylpyrazolidine (I), 7.55 Gm. (0.05 mole) of ethyl nicotinate (11), and 53 ml. of hexane was dried by azeotropic distillation using a Dean-Stark trap. After 1 hr., 0.3 Gm. of anhydrous sodium methoxide was added and the refluxing was continued for 19 hr. The hexane was distilled *in vacuo* on a water bath. About 10 ml. of water was added, and the mixture was extracted three times with chloroform. The chloroform solution was dried ($MgSO_4$) and the solvent was removed *in vacuo* on a water bath. The residue was distilled and gave 5.1 Gm. (53%) of a colorless oil, b.p. 124° (0.08 mm.). The oil crystallized upon standing, m.p. 50.5–52.5°; $\lambda_{max.}^{Nujol}$ 6.2 μ (C=O).

Anal.—Calcd. for $C_{10}H_{13}N_3O$: C, 62.81; H, 6.85; N, 21.97. Found: C, 62.62; H, 6.97; N, 21.90.

A dipicrate was prepared and recrystallized from aqueous ethanol, m.p. 167–168°.

Anal.—Calcd. for $C_{22}H_{19}N_9O_{15}$: C, 40.69; H, 2.95; N, 19.41. Found: C, 40.76; H, 3.29; N, 19.43.

1-Ethoxycarbonyl-2-methylpyrazolidine (XI)—In a 250-ml. three-necked flask provided with a mechanical stirrer and cooled by an ice–salt mixture were placed 25 ml. of ether and 25.8 Gm. (0.1 mole) of a 33% aqueous 1-methylpyrazolidine solution. When the stirred mixture had cooled to 5°, 10.9 Gm. (0.1 mole) of ethyl chloroformate was added without allowing the temperature to rise above 5°. After all of the chloroformate had been added, a solution of 4.0 Gm. (0.1 mole) of NaOH in 6 ml. of water was added dropwise keeping the temperature below 5°. The reaction mixture was stirred for 10 min. longer, the ether layer was separated, and the aqueous solution was extracted with 15 ml. of ether. The combined ether layers were dried ($MgSO_4$) and the ether was distilled using a rotary evaporator. The residue was distilled and afforded 3.03 Gm. (19.2%) of a colorless liquid, b.p. 118° (18 mm.); n_D^{20} 1.4630; $\lambda_{max.}^{film}$ 5.92 μ (C=O).

Anal.—Calcd. for $C_7H_{14}N_2O_2$: C, 53.14; H, 8.92; N, 17.71. Found: C, 53.24; H, 8.93; N, 17.57.

REFERENCES

(1) Kornet, M. J., *J. Med. Chem.*, **9**, 493(1966).
(2) Wadsworth, W. S., Jr., *J. Org. Chem.*, **31**, 1704 (1966).
(3) Sisler, H. H., Omietanski, G. M., and Rudner, B., *Chem. Rev.*, **57**, 1025(1957).
(4) Westphal, O., *Ber.*, **74**, 759(1941).
(5) Fischer, E., *Ann.*, **199**, 317(1879).
(6) Benoit, G., *Bull. Soc. Chim.*, **6**, 708(1939).
(7) Stewart, H. W., Turner, R. J., Denton, J. J., Kushner, S., Bancone, L. M., McEwen, W. L., Hewitt, R. I., and Subba Row, Y., *J. Org. Chem.*, **13**, 134(1948).
(8) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," John Wiley & Sons, Inc., New York, N. Y., 1956, p. 227.
(9) Foye, W. O., Levine, H. B., and McKenzie, W. L., *J. Med. Chem.*, **9**, 61(1966).
(10) Shildneck, P. R., and Windus, W., "Organic Syntheses," coll. vol. II, John Wiley & Sons, Inc., New York, N. Y., 1957, p. 411.
(11) Kloetzel, M. C., and Chubb, F. L., *J. Am. Chem. Soc.*, **79**, 4226(1957).

Medicinal Folklore Evaluation I

Alleged Androgenic and Aphrodisiac Action of Pega Palo (*Rhynchosia pyramidalis*)

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Pega palo is a plant that has received recent and widespread publicity as an alleged aphrodisiac and androgenic agent. One literature report claims clinical efficacy for these effects; however, other investigators could not confirm these activities in laboratory animals. In the present investigation, using a variety of biological test procedures, neither androgenic activity nor an aphrodisiac effect could be demonstrated in laboratory animals.

RHYNCHOSIA PYRAMIDALIS (Lam.) Urb. (*Leguminosae*), is a woody vine found extensively in the Dominican Republic and in Haiti. Dominican folklore and tradition, dating to pre-Columbian times, reveal that alcoholic extracts prepared from the stems of this plant have been used empirically for the purpose of increasing libido and strengthening of sexual performance in the male (1–3). More recently, several popular magazines and newspapers have reported on the more sensational aspects of the biological activity associated with *R. pyramidalis* (2). The use of pega palo (virility vine) in the United States for these purposes has been widely publicized, and its availability to the general public advertized in newspapers and magazines. Since labeling of such material failed to indicate adequate directions for use, and since scientific evidence establishing any specific biological activity was not available, and because new drug applications had not been filed, both the Federal Food and Drug Administration and the U. S. Post Office Department have prevented further distribution of pega palo through interstate commerce (1).

Objective evidence concerning the alleged aphrodisiac and/or androgenic effect of *R. pyramidalis* is restricted to two reports. In 1960, Danowski and Price (4) determined that the inclusion of 5% *R. pyramidalis* in the diet of normal and hypophysectomized rats for 26 and 56 days, respectively, resulted in observations of no appreciable effect on the testes, prostate, or seminal vesicles of treated animals. On the other hand, Sobá *et al.* (3) reported that in a controlled clinical study, which involved 50 cases of sexual impotence, an androgenic effect was observed in 82% of the patients receiving a pega palo tincture.

Folkers and Unna (6) have reported that in old seeds of *R. pyramidalis*, traces of alkaloids could be detected. Furthermore, they indicated that extracts from these seeds possessed some curare-like activity. More recently, Ristić and Thomas (7) have isolated an alkaloid (picrate, m.p. 132°), which by thin-layer chromatography was shown to be different from yohimbine. Santesson (8) examined the closely related *Rhynchosia phaseoloides* and found it to contain alkaloids and glucosides, but further indicated that it was devoid of curare-like activity in the frog. No other reports in the literature have been found concerning the phytoconstituents of *Rhynchosia* species.

The controversy in the literature regarding the biological activity of *R. pyramidalis*, together with a desire to explore the value of this plant as a source of new medicinal agents, prompted the evaluation of extracts of the vine of *R. pyramidalis* through a variety of biological screening tests.

EXPERIMENTAL

Materials—The plant material used in this investigation was the vine of *R. pyramidalis* (Lam.) Urb. (*Leguminosae*), collected in Haiti during September of 1961 and supplied by S. B. Penick & Co., New York, N. Y. A voucher specimen has been deposited there.

Preparation of Extracts for Biological Testing—A defatted ethanol extract was utilized for the antimicrobial test, the antitumor test, and for the mouse behavior evaluation. The method for preparing this extract has been previously described (5).

According to Sobá *et al.* (3), the native custom in Haiti for preparing pega palo tincture is to steep the dried vine in rum for several days. In the clinical studies conducted by this group, a 15% "hydro-alcoholic" extract of the vine was used (5).[1] Extracts utilized in the present investigation

Received March 23, 1967, from the Department of Pharmacognosy and the †Department of Psychology, University of Pittsburgh, Pittsburgh, PA 15213

Accepted for publication May 19, 1967.

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This investigation was supported in part by grants MH-1951 and NB-04400 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and from the Riker Laboratories, Northridge, Calif.

[1] Identified as Tintura Pega Palo Fortidum, Laboratorio Quimico Dominicano, Ciudad Trujillo, D.N., Dominican Republic.

for the androgenic activity assays, and for the sexual behavior studies were prepared in the following manner.

In a typical extraction, 125 Gm. of coarsely powdered vine was moistened with 50% (w/v) ethanol and allowed to stand for 1 hr. The moistened drug was then packed into a suitable cylindrical glass percolator, sufficient 50% ethanol was added to cover the drug, and the mixture was allowed to macerate for 24 hr. Percolation was then initiated at a moderate rate until 1000 ml. of percolate was collected. The tincture prepared in this manner was then reduced to a volume of 250 ml. using a flash evaporator (*in vacuo*, 40°), thus removing any ethanol that might alter the biological activity of active constituents in the vine when evaluated in animals. A cloudy aqueous suspension resulted, which was of high viscosity and required no suspending agent. This extract, which contained the extractives of 0.5 Gm. of vine in each 1.0 ml., was labeled extract *A*, and was used in all evaluations requiring an oral route of administration. In experiments involving intraperitoneal administration of the extract, a less concentrated aqueous suspension was prepared by dilution with water so that each 1.0 ml. was equivalent to the extractives from 0.13 Gm. of vine (extract *B*).

General Biological Evaluation—Using methods previously described for screening native plants (5), a defatted ethanol extract of *R. pyramidalis* vine was evaluated for activity against 16 bacterial animal pathogens and saprophytes, three fungal animal pathogens and saprophytes, four bacterial plant pathogens, 15 fungal plant pathogens and material degrading organisms, and three protozoa. Using the agar diffusion technique, the extract was found to be inhibitory for *Mycobacterium avium* and *Lactobacillus casei* at the 100 mcg./ml. level. No activity was demonstrated against any of the other test organisms.

Similarly, the extract was devoid of activity against the C-1498 leukemia, P-1534 leukemia, adenocarcinoma 755, mecca lymphosarcoma, and the X-5563 myeloma, all in mice (5). In the general mouse behavior evaluation (5), the defatted ethanol extract was classified as having weak central nervous system depressant properties.

Assay for Androgenic Activity—Extract *B* (0.13 Gm./ml.) was administered i.p. daily to each of six albino Wistar male rats, previously castrated in the usual manner. Injections were initiated 24 hr. following the surgical procedure, and continued for nine successive days at a dose of 5.0 ml./Kg. (0.65 Gm./Kg.). No unusual effects were noted in the treated animals during this period of time. A second group of six rats were similarly castrated and received daily i.p. injections (5.0 ml./Kg.) of normal saline solution. Twenty-four hours following the final injection, all animals were sacrificed, the seminal vesicles and prostate gland removed from each animal, blotted between paper to remove seminal fluid and excess moisture, and carefully weighed.

The androgenic activity of extracts can be ascertained by noting the lack of any significant increment in relative weights of the prostate and seminal vesicles over saline-treated controls. The results are presented in Table I.

Following autopsy of the animals, it was observed that the peritoneum of each extract-treated rat was colored dark brown. The absence of androgenic activity, together with this observation, presented the possibility of hindered absorption of active principles in the extract due to the presence of tannins. In addition, Sobá *et al.* (3) have reported that administration of pega palo tincture to humans over extended periods of time usually gave more satisfactory results. This seemed to apply in this study as an additional explanation for the negative results, since nine doses could have been inadequate. The authors therefore modified the initial androgenic assay by: (*a*) utilizing a more concentrated extract (extract *A*), (*b*) administering the extract at three different dosage levels (5.0 ml./Kg., 10.0 ml./Kg., and 20.0 ml./Kg.), (*c*) giving the extract orally by means of a suitable stomach tube, and (*d*) continuing the extract administration for a longer period of time (single daily doses for 79 successive days).

TABLE I—EFFECT OF *R. pyramidalis* EXTRACT *B* ON THE WEIGHT OF CASTRATE RAT SEMINAL VESICLES AND PROSTATE GLAND

Animal	Dose,[a] ml./Kg.	Type Extract	Prostate Wt., mg./ 100 Gm. Body Wt.	Seminal Vesicles Wt., mg./ 100 Gm. Body Wt.	Body Wt. at Autopsy
1	5.0	*B*[b]	35	32	177
2	5.0	*B*	22	33	160
3	5.0	*B*	37	37	200
4	5.0	*B*	29	36	171
5	5.0	*B*	24	37	178
			—	—	—
			$\bar{x}$ 31	35	178
7	5.0	Saline	31	36	195
8	5.0	Saline	34	38	175
9	5.0	Saline	41	37	163
10	5.0	Saline	45	38	183
11	5.0	Saline	21	33	174
12	5.0	Saline	28	33	162
			—	—	—
			$\bar{x}$ 33	36	175

[a] Nine successive daily i.p. injections were made. [b] Extract *B* contained the extractives from 0.13 Gm. of vine in each 1.0 ml.

The results of this study, utilizing extract *A* (0.5 Gm./ml.) are presented in Table II and substantiate the negative findings of the initial study. It was further observed that no unusual gross effects were seen in the drug-treated animals during the 79 days of the study.

Representative tissue specimens from the prostate, seminal vesicles, penis, bladder, kidney, and liver were removed from several drug-treated and control rats at autopsy and these were examined for evidence of pathological or abnormal effects. No evidence of abnormal tissue was observed in the specimens examined.

Effect of Extract A on the Sexual Behavior of Normal Rats—Twenty hooded male rats, 120–150 days of age, were randomly assigned to experimental and control groups. Following a series of five 1 hr.–once weekly copulation tests with receptive females, the drug phase of the study was initiated. Experimental rats received extract *A* daily by stomach tube (4.4 ml./Kg., equivalent to 2.2 Gm./Kg.), while controls were given an equivalent volume of water by stomach tube. Weekly, hour-long copulation tests with receptive females were continued for 20 weeks, with the 10th and 15th test following initiation of drug administration being

TABLE II—EFFECT OF *R. pyramidalis* EXTRACT *A* ON THE WEIGHT OF CASTRATE RAT SEMINAL VESICLES AND PROSTATE GLAND

Animal	Dose,[a] ml./Kg.	Type Extract[b]		Prostate Wt., mg./100 Gm. Body Wt.	Seminal Vesicles Wt., mg./100 Gm. Body Wt.	Body Wt. at Autopsy
1	5.0	*A*		222	95	413
2	5.0	*A*		178	107	486
3	5.0	*A*		137	84	365
4	5.0	*A*		153	95	353
			$\bar{x}$	173	95	404
5	10.0	*A*		217	89	487
6	10.0	*A*		187	73	380
7	10.0	*A*		125	101	417
8	10.0	*A*		233	121	393
			$\bar{x}$	191	96	419
9	20.0	*A*		175	101	424
10	20.0	*A*		165	94	435
11	20.0	*A*		163	101	418
12	20.0	*A*		265	95	457
13	20.0	*A*		165	65	393
14	20.0	*A*		261	100	361
			$\bar{x}$	199	93	415
15	10.0	Water		180	100	428
16	10.0	Water		237	107	443
17	10.0	Water		153	78	405
18	10.0	Water		148	116	355
19	10.0	Water		177	105	360
			$\bar{x}$	179	101	398

[a] Seventy-nine successive single oral daily doses. [b] Extract *A* contained the equivalent of 0.5 Gm. of extractives in each 1.0 ml.

satiation tests. Satiation tests have no specific time limit, but continue until the male spends 30 min. without making a sexual response. During the time course of the experiment, males were kept in isolation except during the actual sexual tests.

Measures taken during sexual behavior tests included latency to first mount, number of incomplete mounts (no intromission), number of complete mounts (intromissions), number of ejaculations, length of refractory periods (time from ejaculation to the next sexual response), and amount of time spent in actual sexual behavior. For all of the measures taken, *t* tests were run between experimental and control groups with separate tests run for the pre-drug tests and the total post-drug tests. Also, in case the results of drug administration were cumulative, or gradual in onset, post-drug tests were split into first 10 test and last 10 test categories. These data were then compared within and between control and experimental groups.

No significant ratios were found for any of the above measures or comparisons. It is therefore concluded that the administration of *R. pyramidalis* extract *A* to male rats under these conditions, and at the dosage level indicated, has no significant effect on any of the sexual parameters measured.

Phytochemical Screening—Using the methods of Wall *et al.* (9–11), *R. pyramidalis* vine was found to contain trace amounts of alkaloids, tannins of the catechin type, saponins, and organic acids and phenols. Flavonoids could not be detected using these methods.

Examination of R. pyramidalis Alkaloids—Using the U.S.P. assay for belladonna alkaloids, *R. pyramidalis* vine was found to contain 0.19% of total crude alkaloids (as strychnine) (12).

A thin-layer chromatogram of the total crude alkaloids from *R. pyramidalis* vine was prepared as follows. One hundred grams of powdered vine was moistened with 28% NH_4OH, air-dried, and continuously extracted with chloroform. The chloroform extract was concentrated to 10.0 ml. Twenty microliters was applied to a Silica Gel G plate (0.25 mm.), prepared in the usual manner, and eluted with methanol. After air-drying the plate, it was sprayed with Dragendorff's reagent. Three typical orange-colored alkaloid spots were noted at R_f 0.08, 0.22, and 0.35.

DISCUSSION

The results of this study clearly indicate that the *R. pyramidalis* extracts utilized were devoid of androgenic activity as evidenced by an absence of accessory organ stimulation in castrate rats. These findings are in agreement with those of Danowski and Price (4), who also used rats as assay animals. However, both our results and those of Danowski and Price are contradictory to those reported by Sobá *et al.* (3), who evaluated pega palo tincture in 50 clinical cases of sexual impotence with partial or complete loss of libido and erections. On the other hand, the paper by Sobá *et al.* (3) is entitled "Androgenic Action of Pega Palo," but the biological activity described in this paper alluded to a restoration of libido and erections. These workers performed no assays to indicate that their extracts contained truly androgenic materials. Furthermore, although the authors mention that, "For comparison, in many cases, a placebo was substituted for the Pega Palo," (3), no details are given concerning the type of placebo used, or the results obtained with the placebo preparation. In addition, it was reported that 50 cases of sexual impotence with partial or complete loss of libido and erections were included in the study, and their conclusions indicated that 41 patients were restored to normal activity during the period of medication, whereas nine (making a total of 50) showed no increase in libido or erectile power. If these 50 patients constituted the entire group studied, and no statements to the contrary were made, it is difficult to ascertain how a placebo preparation was used for comparison.

When plant material is being evaluated for biological activity, it is often difficult to make valid comparisons between the results obtained in any two different laboratories, even under ideal conditions. One must consider not only variations in the test methods but also variability in the chemical constituents of investigational plant material due to ecological factors, the age of the plant at harvest, methods of processing the collected specimens, and the possibility of working with chemical races of the same species. Further complicating factors, which appear to apply in the present situation, are the possibility of improper identification of the material being investigated, as well as the problem of selecting the correct plant material when reference is made only to the vernacular name.[2]

Danowski and Price (4) in their studies utilized a sample of plant material identified only as "Pega Palo." An authentic voucher specimen of their

[2] Sobá *et al.* (3) have pointed out that two unrelated low shrubs are also sometimes referred to by the vernacular names "pega pallo" and "pega palo," but these resemble *R. pyramidalis* neither in appearance nor herbal properties.

material was not made (13). Thus the distinct possibility exists that their plant material was not, in reality, *R. pyramidalis*. Along similar lines, no mention was made in the paper of Sobá *et al.* (3) that they deposited a voucher specimen representative of their investigational material for reference purposes.

With regard to chemical variability, the authenticated material gave definite positive tests for alkaloids (*vide supra*), which has been duplicated and confirmed by the studies of Ristić and Thomas (7). The material studied by Sobá *et al.* (3) was devoid of alkaloids, although their test methods were not defined.

SUMMARY

The vine of *R. pyramidalis* (Lam.) Urb. (*Leguminosae*), popularly known as pega palo and used in folkloric medicine as an aphrodisiac, has been the subject of the present investigation. Extracts from this plant were found to be devoid of androgenic activity and did not significantly alter the sexual behavior of normal rats when the following measures were taken: latency to first mount, number of incomplete mounts (no intromission), number of complete mounts (intromission), number of ejaculations, length of refractory periods (time from ejaculation to the next sexual response), and amount of time spent in actual sexual behavior.

In a general biological screening of a defatted ethanol extract of *R. pyramidalis* vine, inhibitory activity was observed against *Mycobacterium avium* and *L. casei* (*in vitro*). The extract was devoid of antitumor activity, and in gross observation studies using mice, central nervous system depression was observed.

The vine was found to contain three alkaloids by means of thin-layer chromatography, comprising 0.19% of total crude bases. Tannins, saponins, and organic acids and phenols were also found to be present, but tests for the presence of flavonoids were negative.

REFERENCES

(1) Anon., *J. Am. Med. Assoc.*, **165**, 695(1957).
(2) Landman, D., *True, The Man's Magazine*, **39**, 46 (1958).
(3) Sobá, J. G., Molano, E. E. S., and Damrau, F., *J. Natl. Med. Assoc.*, **52**, 25(1960).
(4) Danowski, T. S., and Price, L., *Endocrinology*, **66**, 788(1960).
(5) Farnsworth, N. R., Henry, L. K., Svoboda, G. H., Blomster, R. N., Yates, M. J., and Euler, K. L., *Lloydia*, **29**, 101(1966).
(6) Folkers, K., and Unna, K., *J. Am. Pharm. Assoc., Sci. Ed.*, **28**, 1019(1939).
(7) Ristić, S., and Thomas, A., *Arch. Pharm.*, **295**, 510 (1962).
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Trifluoromethyl analogs of amphetamine (I) and norephedrine (II) were synthesized for pharmacological evaluation. They showed no activity in profile tests for anorectic activity (I), antiemetic activity (I), CNS activity (I), MAO-inhibitory activity (I, II), and pressor activity (II).

THE SYNTHESIS and pharmacological investigation of organic fluorine compounds has led to the development of such important drugs as the fluorinated inhalation anesthetics (1), the fluorinated corticosteroids (2), the trifluoromethylphenothiazine tranquilizers and the trifluoromethylthiazide diuretics (1, 2). A number of compounds containing the trifluoromethyl group have been found to possess anesthetic, ataractic, and antiemetic (1), diuretic (1, 3), antihistaminic (4), tumor inhibitory (5, 6), and antimicrobial (7, 8), hypotensive (9), and spasmolytic (10) properties.

The trifluoromethyl group is particularly suited to replace the methyl group in known pharmacologically active compounds, both because of the similarity in physical properties of the two classes of compounds and because of the unique chemical and physiological stability of the trifluoromethyl group. The pronounced electron-withdrawing properties of the group will also be expected to play a part in modifying the activity of the prototype compounds.

The authors have now carried out a synthesis of 2-amino-3-phenyl-1,1,1-trifluoropropane (I),

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REFERENCES

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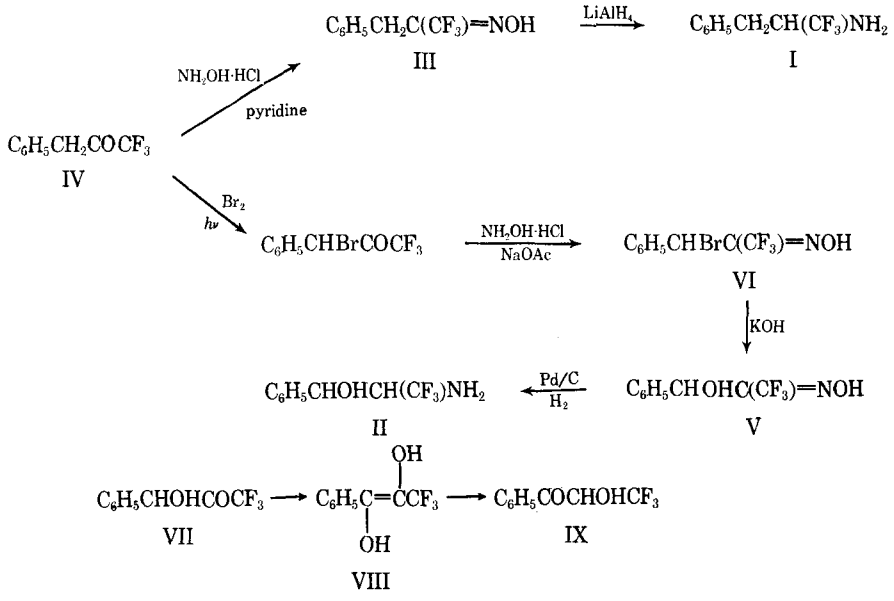
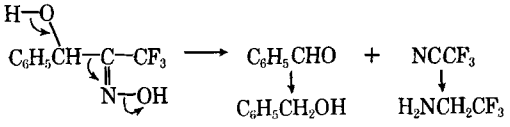


Scheme I

the trifluoromethyl analog of amphetamine (11), by a new sequence in order to make available material for additional pharmacological investigation. Also, the synthesis of the trifluoromethyl analog of norephedrine, *i.e.*, 2-amino-3-hydroxy-3-phenyl-1,1,1-trifluoropropane (II) has been performed.

Compound I was synthesized by reduction of the oxime III with $LiAlH_4$ rather than the previously used method (11) of reductive alkylation of ammonia by the ketone IV. Compound I was also obtained from 2-amino-3-(4-chlorophenyl)-1,1,1-trifluoropropane by dehalogenation (12). The norephedrine analog (II) was obtained by reduction of the oxime of the α-hydroxy ketone (V). The parent ketone, phenyl trifluoroacetyl carbinol (VII), is unstable and undergoes spontaneous enolization to the enediol (VIII), which may further ketonize to the stable benzoyl trifluoromethyl carbinol (IX) (11). The oxime (V) was therefore prepared from the oxime of the bromoketone (VI), by hydrolysis of the bromine atom with ethanolic KOH. Reduction to the amine (II) was effected by catalytic hydrogenation. (Scheme I.)

Reduction with $LiAlH_4$ gave a complex mixture of products, with several runs having varying compositions. Compound II was obtained in a maximum yield of 18%, the other major products being benzylamine, benzyl alcohol, and trifluoroethylamine. Rearrangement and fragmentation of oximes under the conditions of the $LiAlH_4$ reduction are well known (13), and α-hydroxyoximes are particularly susceptible in this respect (14, 15). Benzylamine presumably arises



Scheme II

by a rearrangement of the oxime to an α-hydroxyamide (16), $C_6H_5CHOHNHCOCF_3$, with subsequent reduction and elimination of the trifluoroacetyl group (17). The formation of benzyl alcohol and trifluoroethylamine is somewhat easier to rationalize; a concurrent electron transfer (15) will give benzaldehyde and trifluoroacetonitrile, which will be further reduced to benzyl alcohol and trifluoroethylamine, respectively. (Scheme II.)

The amphetamine analog (I), in standard tests (18),[1] showed no anorectic activity at 25 mg./Kg. (p.o.) in the rat and 10 mg./Kg. in the dog, no antiemetic activity in the dog at 25 mg./Kg., no conditioned escape response blocking action in the rat at 50 and 200 mg./Kg., no tryptamine potentiation in the rat at 100 mg./Kg., and no depletion of catecholamines in the brain and heart of the rat at 100 mg./Kg. (i.p.). The *p*-chloroamphetamine analog showed no anorectic activity in the rat at 29 mg./Kg. (p.o.) and did not deplete heart or brain catecholamines in the rat at 100 mg./Kg. (i.p.). The norephedrine analog (II) showed only 1/300th of the pressor activity of norephedrine itself in the pentobarbitalized rat, and did not deplete brain or heart catecholamines in the rat at 100 mg./Kg. (i.p.).

[1] The authors thank Smith Kline & French Laboratories for the pharmacological data.

EXPERIMENTAL

Benzyl trifluoromethyl ketone, *p*-chlorobenzyl trifluoromethyl ketone, and 3-bromo-3-phenyl-1,1,1-trifluoropropanone were prepared according to Nes and Burger (11).

α-p-Chlorophenyltrifluoroacetylacetonitrile had m.p. 79.5–80.5° (benzene).

Anal.—Calcd. for $C_{10}H_5ClF_3NO$: C, 48.50; H, 2.04; N, 5.66. Found: C, 48.42; H, 2.14; N, 5.70.

p-Chlorobenzyl trifluoromethyl ketone had b.p. 65–66° (0.2 mm.).

Anal.—Calcd. for $C_9H_6ClF_3O$: C, 48.55; H, 2.72. Found: C, 48.66; H, 3.09.

The 2,4-dinitrophenylhydrazone, prepared by mixing ethanolic solutions of the ketone and 2,4-dinitrophenylhydrazine with shaking was recrystallized as needles from ethanol, m.p. 111–112°.

Anal.—Calcd. for $C_{15}H_{10}ClF_3N_4O_4$: C, 44.73; H, 2.50; N, 13.91. Found: C, 45.16; H, 2.50; N, 13.90.

The semicarbazone, prepared by refluxing the ketone, sodium acetate, and semicarbazide hydrochloride in ethanol, was recrystallized as plates from dilute ethanol, m.p. 135–136°.

Anal.—Calcd. for $C_{10}H_9ClF_3N_3O$: C, 42.94; H, 3.24; N, 15.03. Found: C, 42.92; H, 3.19; N, 15.28.

The oxime, prepared by refluxing the ketone and $NH_2OH \cdot HCl$ in ethanol and pyridine (1:1), was recrystallized as needles from petroleum ether (b.p. 30–60°), m.p. 60.5–61.5°.

Anal.—Calcd. for $C_9H_7ClF_3NO$: C, 45.57; H, 2.98; N, 5.90. Found: C, 45.56; H, 3.18; N, 5.84.

2-Amino-3-(4-chlorophenyl)-1,1,1-trifluoropropane—To a stirred suspension of $LiAlH_4$ (5.7 Gm., 0.15 mole) in dry ether (100 ml.) was added dropwise a solution of *p*-chlorobenzyl trifluoromethyl ketoxime (23.5 Gm., 0.1 mole) in dry ether (100 ml.). The mixture was heated under reflux for 5 hr., and excess $LiAlH_4$ was destroyed by careful addition of water. Then 10% NaOH (100 ml.) was added, the solid material was filtered off, and the clear yellow filtrate dried ($MgSO_4$). Removal of ether gave a semisolid. The colorless hydrochloride was prepared in ether–petroleum ether, and was purified by sublimation at 150° (1 mm.), m.p. 209–211°, yield 19.0 Gm. (86%).

Anal.—Calcd. for $C_9H_9ClF_3N \cdot HCl$: C, 41.56; H, 3.87; N, 5.39. Found: C, 41.32; H, 3.97; N, 5.42.

The *N*-acetyl derivative, obtained by the Schotten-Baumann method using acetic anhydride, crystallized from dilute ethanol as needles, m.p. 171–172°.

Anal.—Calcd. for $C_{11}H_{11}ClF_3NO$: C, 49.74; H, 4.18; N, 5.27. Found: C, 49.84; H, 4.05; N, 5.41.

2-Amino-3-phenyl-1,1,1-trifluoropropane (11) was prepared in 65% yield from the oxime (III) by a similar method, m.p. of hydrochloride 204–205°. [Lit. (11) m.p. 203–206°.] It was also prepared by the following method.

A mixture of 2-amino-3-(4-chlorophenyl)-1,1,1-trifluoropropane (10 Gm.), methanol (120 ml.), 5% Pd/C (4 Gm.), and KOH (10 Gm.) was shaken at room temperature and 2.8 Kg./cm.^{2} for 5 hr. The catalyst was filtered off and methanol removed under reduced pressure. The residue was taken up in ether, washed with water, and dried ($MgSO_4$). The hydrochloride was identical with that prepared above. Yield, 6.2 Gm. (72%).

3-Bromo-3-phenyl-1,1,1-trifluoropropanone Oxime (VI)—To a solution of hydroxylamine hydrochloride to (40 Gm.) and sodium acetate (40 Gm.) in water (300 ml.) was added all at once 3-bromo-3-phenyl-1,1,1-trifluoropropanone (20 Gm.), and the mixture was shaken vigorously for 10 min. Precipitation of the oxime began immediately and was complete after 30 min. Recrystallization from chloroform gave needles, m.p. 100–102°. Yield, 19 Gm. (85%). $\nu_{max.}^{KBr}$ 3305 (OH), 1695 (C=N, weak) cm.$^{-1}$.

Anal.—Calcd. for $C_9H_7BrF_3NO \cdot H_2O$: C, 36.02; H, 3.09; N, 4.67. Found: C, 36.15; H, 3.02; N, 4.51.

3-Hydroxy-3-phenyl-1,1,1-trifluoropropanone Oxime (V)—To a solution of VI (30 Gm., 0.1 mole) in ethanol (100 ml.) was added dropwise, with stirring and cooling, ethanolic KOH (5.6 Gm. of KOH, 0.1 mole), and the mixture was stirred at room temperature for 2 hr. The precipitated KBr was filtered off and washed thoroughly with ethanol. Most of the ethanol was removed by evaporation under reduced pressure, and water (50 ml.) was added to precipitate the oxime as an oil, which was extracted with ether (2 × 150 ml.) and dried ($MgSO_4$). The ether was removed to yield a yellow oil, which was crystallized from benzene–petroleum ether (b.p. 30–60°), m.p. 90–91.5°, yield, 20 Gm. (90%). $\nu_{max.}^{KBr}$ 3505 (OH), 3250 (OH), 1660 (C=N, weak) cm.$^{-1}$.

Anal.—Calcd. for $C_9H_8F_3NO_2$: C, 49.33; H, 3.68; N, 6.39. Found: C, 49.18; H, 3.70; N, 6.47.

2-Amino-3-hydroxy-3-phenyl-1,1,1-trifluoropropane (II)—A mixture of the oxime (V) (8.8 Gm., 0.04 mole) and 5% Pd/C (4.4 Gm.) in glacial acetic acid (100 ml.) and concentrated H_2SO_4 (8.8 Gm., 0.09 mole) was shaken at room temperature under 2.8 Kg./cm.^{2} of hydrogen. Hydrogen absorption began at once and the theoretical uptake was complete within 2 hr. After the catalyst had been removed by filtration, the clear, colorless filtrate was treated with a solution of NaOH (3.6 Gm., 0.09 mole) in water (15 ml.), with cooling, and the precipitated sodium sulfate was removed by filtration. Acetic acid was removed under reduced pressure, and the residue was made basic with 50% aqueous KOH (cooling). The mixture was extracted with ether (3 × 50 ml.) and dried ($MgSO_4$). Dry HCl was passed into the ethereal solution, and the collected solid was recrystallized from ethanol–petroleum ether (b.p. 30–60°), m.p. 212–214°. Yield, 6.2 Gm. (65%). $\nu_{max.}^{KBr}$ 3445 (OH), 2880 (NH_3^+) cm.$^{-1}$.

Anal.—Calcd. for $C_9H_{10}F_3NO \cdot HCl$: C, 44.74; H, 4.59; N, 5.80. Found: C, 44.49; H, 4.81; N, 5.76.

Hydrogenation in AcOH–anhydrous HCl could not be achieved, a situation also observed in the reduction of 2-indanone oxime (19).

The *N*-acetyl derivative, prepared by boiling the amine with acetic anhydride for 30 min. and then pouring into cold water, was recrystallized from dilute ethanol as needles, m.p. 128.5–130°.

Anal.—Calcd. for $C_{11}H_{12}F_3NO_2$: C, 53.65; H,

4.50; N, 5.69. Found: C, 53.48; H, 4.62; N, 5.80.

REFERENCES

(1) Yale, H. L., *J. Med. Pharm. Chem.*, **1**, 121(1959).
(2) Herr, F., in "Aromatic Fluorine Compounds," Pavlath, A. E., and Leffler, A. J., eds., A. C. S. Monograph 155, Reinhold Publishing Co., New York, N. Y., 1962, p. 682.
(3) Holdrege, C. T., Babel, R. B., and Cheney, L. C., *J. Am. Chem. Soc.*, **81**, 4807(1959).
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(16) Gaylord, N. G., "Reduction with Complex Metal Hydrides," Interscience Publishers, Inc., New York, N. Y., 1956, p. 756.
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(18) "Evaluation of Drug Activities: Pharmacometrics," Bacharach, A. L., and Laurence, D. R., eds., Academic Press Inc., New York, N. Y., 1964.
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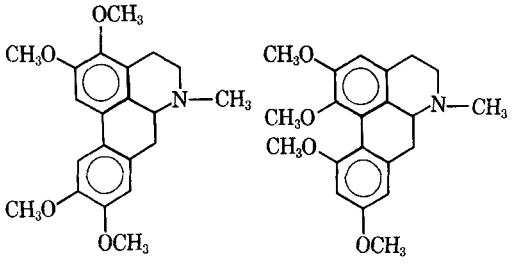

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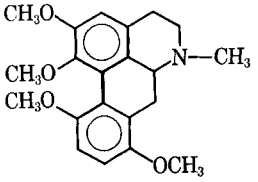

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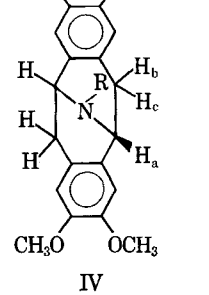

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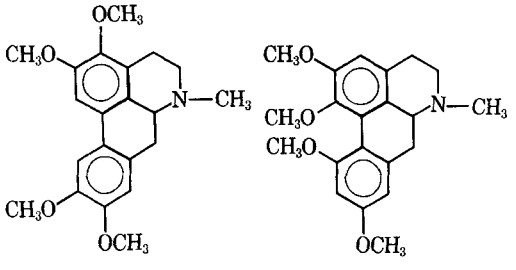

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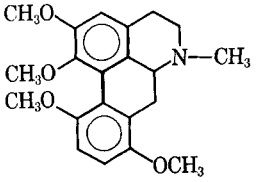

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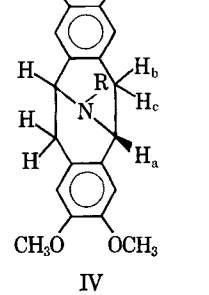

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ether seemed indicated [as discussed in an earlier communication from these laboratories (3)]. *O*-Ethylnorargemonine, obtained by diazoethylation, was oxidized under mild conditions with manganese dioxide in diluted sulfuric acid, a procedure that previously had yielded 4,5-dimethoxy-*N*-methylphthalimide and 4,5-dimethoxyphthalic acid from argemonine (2). The oxidation yielded four products—namely, 4,5-dimethoxyphthalic acid and its *N*-methylimide as well as 4-ethoxy-5-methoxyphthalic acid and its *N*-methylimide. On this basis, structures I, II, and III were all shown to be untenable (3). Reassurance, however, of the $C_{20}H_{23}NO_4$ molecular formula of norargemonine was obtained by mass spectral analysis, and the molecular formula of argemonine, $C_{21}H_{25}NO_4$, was confirmed as well. The mass spectrum of argemonine showed a large peak at *m/e* 204, corresponding to a *N*-methyl-6,7-dimethoxyisoquinolinium cation (5). For a more extensive treatment of the mass spectra of the pavine type alkaloids, see *Reference 5*.

At this point, nuclear magnetic resonance (NMR) studies were initiated which, aside from showing a far more symmetrical absorption pattern than expected, indicated the presence of *two pairs* of identical aromatic protons, rather than the *three* nonidentical protons expected in an aporphine formulation. This rendered untenable any aporphine formulation and a review of all previously known naturally-occurring alkaloidal types based on the isoquinoline moiety indicated that none of them could be entertained as structural candidates. The high degree of symmetry of the NMR spectrum suggested a symmetrical molecule, and yet the optical activity suggested asymmetry. Both of these requirements could be sustained in the *N*-methylpavine (IV, R = CH_3) structure, since, in spite of the obvious symmetry of the molecule, the asymmetric bridgehead carbons would have the same relative configuration and thus contribute optical activity. The NMR spectrum was explicable in light of the structure of IV (R = CH_3) and is, in part, an ABX system (6) in which protons H_a couple only with H_b[1] and appear as a doublet centering at 5.92 τ (J_{ab} = 6 c.p.s.).

Protons H_c couple only with protons H_b and are a doublet centering at 7.38 τ (J_{bc} = 17 c.p.s.). Protons H_b are split by H_a and H_c and are a pair of doublets centering at 6.47 and 6.58 τ. The aromatic protons are singlets of intensity two each at 3.24 and 3.41 τ and the methoxyl groups are singlets at 6.08 and 6.16 τ. The *N*-methyl group is a singlet at 7.43 τ. Comparison of the spectrum with that of (±)-pavine[2] (IV, R = H) indicated a remarkable similarity, except that the bridgehead protons are shifted downfield to a doublet centering at 5.55 τ and protons H_c occur as a doublet shifted downfield to 7.25 τ. The *N*-methyl group is absent, of course, and the NH appears as a singlet at 7.87 τ.

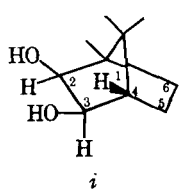
In order to permit direct comparison with authentic *N*-methylpavine it was synthesized by the usual procedure (7) from *N*-methyl-1,2-dihydropapaverine to provide the racemic base. The NMR spectrum of the synthetic base was superimposable on that of argemonine, indicating structural identity. The racemic base was then deracemized by conversion to the bitartrate salts of (+)-L- and (−)-D-tartaric acid. The levorotatory form, obtained from the (−)-D-bitartrate salt, was identical to natural argemonine in every respect.

The possibility that argemonine, isolated from plant material, was an artifact arising from the cyclization of *N*-methyl-1,2-dihydropapaverine due to acid treatment during isolation was considered. It seemed unlikely in view of the work of Knabe *et al.* (8), who showed that dilute acid treatment of this compound results in migration of the 1-homoveratryl group to the 3-position, rather than ring closure. Furthermore, Schermerhorn and Soine (1) had isolated argemonine earlier in a procedure scrupulously avoiding mineral acids. However, to eliminate any doubt on this point, a sample of *N*-methyl-1,2-dihydropapaverine was dissolved in 10% tartaric acid and the ultraviolet spectra of samples withdrawn at various times over a period of 72 hr. were observed. No conversion to *N* methylpavine was noted.

Argemonine, therefore, is the naturally-occurring form of *N*-methylpavine, and this is the first instance of this tetracyclic system being observed in a plant alkaloid. Further examples of the pavine types of alkaloids have been recently described in the literature (5, 9).

In addition, the absolute configuration of argemonine has been determined (10).

[1] This phenomenon has been investigated by Anet, F. A. L. [*Can. J. Chem.*, 39, 789(1961), and references contained therein] as applied to the bicyclo[2.2.1]heptane system. Thus, 2-exo,3-exo-camphane-2,3-diol (*i*) exhibits a bridgehead coupling constant of zero ($J_{3,4}$ = 0) with the adjacent proton, as predicted by the Karplus equation [Karplus, M., *J. Chem. Phys.*, 30, 11(1959)] where the dihedral angle is 79°.



i

[2] Kindly supplied by Professor A. R. Battersby, University of Liverpool, Liverpool, England.

EXPERIMENTAL

Oxidation of O-Ethylnorargemonine—Norargemonine (1 Gm., 0.003 mole) was dissolved in a mixture of 200 ml. of dichloromethane and 50 ml. of absolute methanol. The solution was cooled in an ice bath to 5° and to it was added an excess of diazoethane[3] (1.68 Gm., 0.03 mole) in ether. The mixture was then allowed to come to room temperature gradually over a period of several hours, after which the solvent was stripped off on a steam bath. The residue was dissolved in 100 ml. of 2% (w/v) hydrochloric acid and then alkalinized with enough 10% (w/v) sodium hydroxide solution to make the final solution about 4% (w/v) in sodium hydroxide. The alkaline solution was extracted with several 50-ml. portions of ether until the extracts were negative to Valser's reagent. The combined ethereal extracts were washed twice with 50-ml. portions of distilled water, dried over anhydrous sodium sulfate, and then stripped of solvent under reduced pressure to leave an amorphous yellow residue. The crude ethylated product, without further purification, was dissolved in 100 ml. of 2.5% (w/v) sulfuric acid and refluxed with 1 Gm. of manganese dioxide for 5 hr. The reaction mixture was filtered while hot, cooled, and then extracted for 3 days continuously with ether in a liquid–liquid extractor. The ethereal extract was dried over anhydrous magnesium sulfate and then stripped of solvent to leave a yellowish residue. The residue was warmed with 5% (w/v) sodium carbonate solution (20 ml.) upon which a portion of the residue dissolved, the remaining residue being termed the "neutral fraction" and the dissolved portion the "acidic fraction."

Acidic Fraction—The sodium carbonate solution was acidified with 5% (w/v) hydrochloric acid and then extracted continuously with ether in a liquid–liquid extractor for 2 days. The ethereal extract was dried and stripped of solvent, the residue was dissolved in 5 ml. of 33% aqueous ethylamine, and the solution was concentrated to dryness. The residue was heated at 180° for 10 min. and then sublimed at 170–185°/1 mm. to yield 50 mg. of sublimate. The sublimate was crystallized from methanol to yield 20 mg. of white needles, m.p. 223–224°, which failed to depress the melting point of authentic 4,5-dimethoxy-*N*-ethylphthalimide. The mother liquor from the first crystallization was allowed to stand and slowly concentrate, upon which a white crystalline residue, m.p. 189–195°, separated (14 mg.). Recrystallization of this residue yielded 5 mg. of product, m.p. 200–202°, which failed to depress the melting point of authentic 4-ethoxy-5-methoxy-*N*-ethylphthalimide.[4]

Neutral Fraction—This residue was washed with water, dried, and sublimed at 170–185°/0.1 mm. to give a yellowish-white sublimate with a melting range of 200–256°. Crystallization from ethanol yielded 10 mg. of white crystals, m.p. 263–264°, which failed to depress the melting point of authentic 4,5-dimethoxy-*N*-methylphthalimide. The mother liquor, on slow concentration, yielded a white crystalline residue, m.p. 180–200°, which on continued recrystallization yielded 6 mg. of white crystals, m.p. 189–191°. A mixed melting point determination with authentic 4-ethoxy-5-methoxy-*N*-methylphthalimide[5] failed to depress the melting point.

N-Methyl-1,2-dihydropapaverine—Prepared according to the method of Schopf and Thierfelder (11), m.p. 128–130°. [Lit. (11) m.p. 129–130°.]

(±)-N-Methylpavine—This was prepared from *N*-methyl-1,2-dihydropapaverine by the method of Battersby and Binks (7), m.p. 138–140°. [Lit. (7) m.p. 135–140°.]

(+)-N-Methylpavine-(+)-L-bitartrate—(+)-L-Tartaric acid (1.5 Gm., 0.01 mole) was dissolved with heat in 35 ml. of ethanol and added while hot to a hot solution of (±)-*N*-methylpavine (3.55 Gm., 0.01 mole) in ethanol (40 ml.). On cooling, copious crystallization occurred to yield 3.1 Gm. of white crystals. These were recrystallized several times from ethanol to yield 0.4 Gm. of white crystals, m.p. 228–230° (with effervescence and previous softening at 221°); $[\alpha]_D^{29}$ +174.8° (c 2.0, H_2O).

Anal.—Calcd. for $C_{25}H_{31}NO_{10}$: C, 59.40; H, 6.18. Found: C, 59.12; H, 6.31.

(+)-N-Methylpavine—(+)-*N*-Methylpavine-(+)-L-bitartrate (0.3 Gm.) was dissolved in about 25 ml. of water and then alkalinized with 5 ml. of 5% (w/v) aqueous sodium hydroxide. The alkaline solution was then extracted with three portions (15 ml.) of dichloromethane and the combined extracts washed once in a separator with 15 ml. of water. The dichloromethane extract was then dried over anhydrous magnesium sulfate and stripped of solvent to leave a clear glassy, noncrystalline residue. This residue was recrystallized from a dilute ethanolic solution to yield prisms (60 mg.), m.p. 133–145° (with effervescence), $[\alpha]_D^{25}$ +207.8° (c 2.0, C_2H_5OH). When these crystals were powdered and dried in vacuum over phosphorus pentoxide at 100° the melting point changed to 151–153°. Argemonine (20 mg.) and (+)-*N*-methylpavine (20 mg.), both in the dried form, were each dissolved in sufficient dichloromethane to effect complete solution and the solutions combined. The solvent was stripped from the combined solution to leave a colorless gum which solidified and crystallized when rubbed with a small amount of ether, m.p. 136–139°. The melting point was not depressed on admixture with authentic (±)-*N*-methylpavine.

(−)-N-Methylpavine-(−)-D-bitartrate—*From (±)-N-Methylpavine*—This salt was prepared in exactly the same manner as described for (+)-*N*-methylpavine-(+)-L-bitartrate except for the use of (−)-D-tartaric acid ("unnatural") instead of the natural (+)-L-tartaric acid, m.p. 227–230°, $[\alpha]_D^{25}$ −176.5° (c 2.0, H_2O).

From Argemonine—Argemonine (0.355 Gm., 0.001 mole) was dissolved in ethanol, and to this solution, while hot, was added 10 ml. of a hot ethanolic solution containing 0.150 Gm. (0.001 mole) of (−)-D-tartaric acid. On cooling, a copious gelatinous residue formed which became crystalline on agitation and warming. Recrystallization of the white crystalline residue from ethanol yielded 350 mg. of crystalline rosettes, m.p. 230–232° (with effervescence and softening at 225°); $[\alpha]_D^{23}$ −188.8°

[3] Prepared from Ethyl Diazald, Aldrich Chemical Co., Milwaukee, Wis.

[4] Prepared by permanganate oxidation of *O*-ethylated boldine and subsequent conversion of the acidic fraction to the *N*-ethylimide.

[5] Prepared by permanganate oxidation of *O*-ethylated boldine and subsequent conversion of the acidic fraction to the *N*-methylimide.

(c 2.5, H_2O). A mixed melting point determination with the same salt prepared by the first method showed no depression of melting point.

Anal.—Calcd. for $C_{25}H_{31}NO_{10}$: C, 59.40; H, 6.18. Found: C, 59.26; H, 6.28.

(−)-N-Methylpavine—The preparation of this base was carried out in exactly the same manner as for (+)-*N*-methylpavine from its bitartrate salt. The product, after recrystallization from aqueous ethanol, possessed a melting point of 130–140° (with effervescence); $[\alpha]_D^{25}$ −209° (c 1.0, C_2H_5OH). On drying the crystals in the same way as for the (+)-rotatory base, the white powder melted at 152–153°. A mixed melting point determination as well as infrared comparison with authentic dried argemonine base showed them to be completely identical.

Spectra

Nuclear Magnetic Resonance—Spectra were obtained in $CDCl_3$ on a Varian A-60 spectrometer using tetramethylsilane as the internal standard.

Mass—Spectra were obtained on a C.E.C.-103-C instrument at 200° using a metal inlet at 70 e.v.

Ultraviolet—*N* - Methyl - 1,2 - dihydropapaverine (100 mg.) was dissolved in 100 ml. of 10% (w/v) aqueous tartaric acid. At various time intervals over a 72-hr. period the spectrum of the solution was examined over the wavelength range of 220–370 mμ to determine whether the characteristic absorption bands for argemonine were appearing in the 275–295-mμ region. The analysis was carried out by diluting 1 ml. of the sample solution to 100 ml. with water and placing this solution in the sample cell. The reference cell was filled with a solution obtained by diluting 1 ml. of the 10% aqueous tartaric acid solution (without sample) to 100 ml. The measurements were carried out on a Bausch & Lomb model 505 recording spectrophotometer and showed no absorption in the region mentioned although maxima developed at 250 and 312 mμ.

REFERENCES

(1) Schermerhorn, J. W., and Soine, T. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 19(1951).
(2) Kier, L. B., and Soine, T. O., *J. Pharm. Sci.*, **50**, 321(1961).
(3) Soine, T. O., and Kier, L. B., *ibid.*, **51**, 1196(1962).
(4) Shamma, M., *Experientia*, **18**, 64(1962).
(5) Manske, R. H. F., Shin, K.-H., Battersby, A. R., and Shaw, D. F., *Can. J. Chem.*, **4**, 2183(1965); Stermitz, F. R., and Sieber, J. N., *J. Org. Chem.*, **31**, 2925(1966).
(6) Pople, J. A., Schneider, W. G., and Bernstein, H. J., "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 132; Jackman, L. M., "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, New York, N. Y., 1959, p. 90.
(7) Battersby, A. R., and Binks, R., *J. Chem. Soc.*, **1955**, 2888.
(8) Knabe, J., and Kubitz, J., *Arch. Pharm.*, **297**, 129 (1964).
(9) Lu, S.-T., and Lan, P.-K., *Yakugaku Zasshi*, **86**, 177(1966).
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By MAHMOUD M. ABDEL-MONEM* and TAITO O. SOINE

The structures of three products encountered during structural studies on argemonine and (±)-*N*-methylpavine (I) have been determined. The first of these, obtained by hydrochloric acid treatment of 3-hydroxy-2′:3′:2″:3″-tetramethoxy-1:2-5:6-dibenzoöcta-1:5:7-triene (II), has been determined to be 3-chloromethyl-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4:6-triene. The second, known as argemoninic acid and resulting from permanganate oxidation of II, has been shown to be 2:2′-dicarboxy-4:5:4′:5′-tetramethoxybenzil. The third product, resulting from alkaline ethyl chloroformate treatment of I, has been shown to be the corresponding *N*-carbethoxy derivative of pavine.

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In the course of the early structural work on argemonine a number of degradation products were obtained as shown in Scheme I.

Received February 3, 1967, from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455
Accepted for publication May 4, 1967.
Presented to the Scientific Section, A.PH.A., Detroit meeting, March 1965.
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REFERENCES

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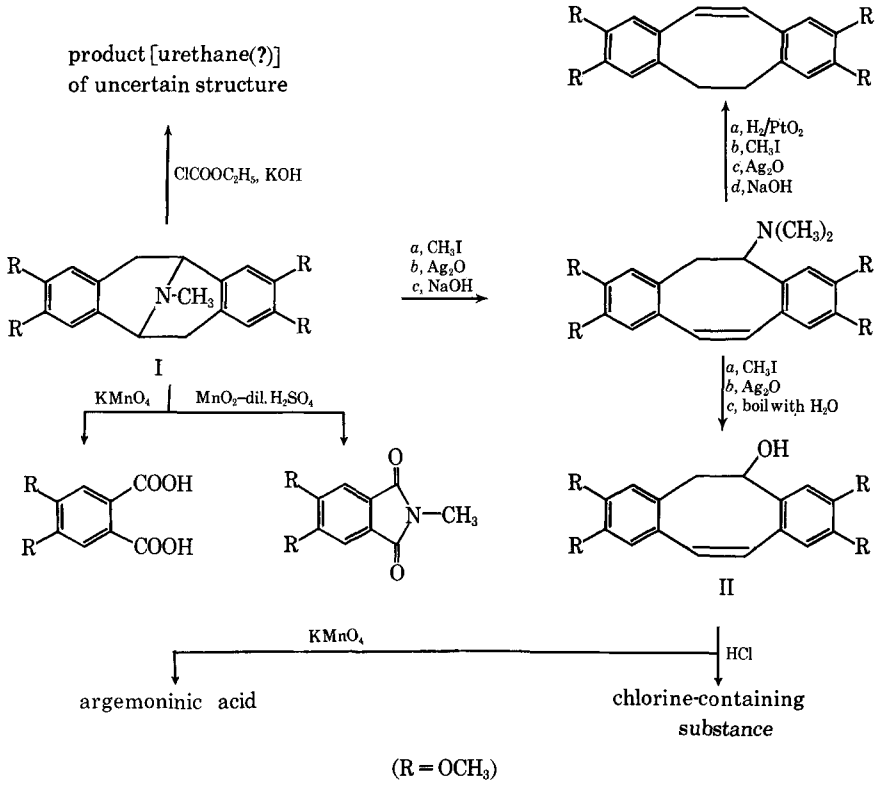


Scheme I

Some of these had been reported earlier by Pyman (7) as well as by Battersby and Binks from their somewhat parallel studies on racemic *N*-methylpavine. In considering the possible structural assignments to the degradation products it appeared that some of them needed additional structural definition. The present work describes the structural elucidation of the three principal degradation products: (*a*) the chlorine-containing substance, (*b*) argemoninic acid, and (*c*) the ethyl chloroformate reaction product.

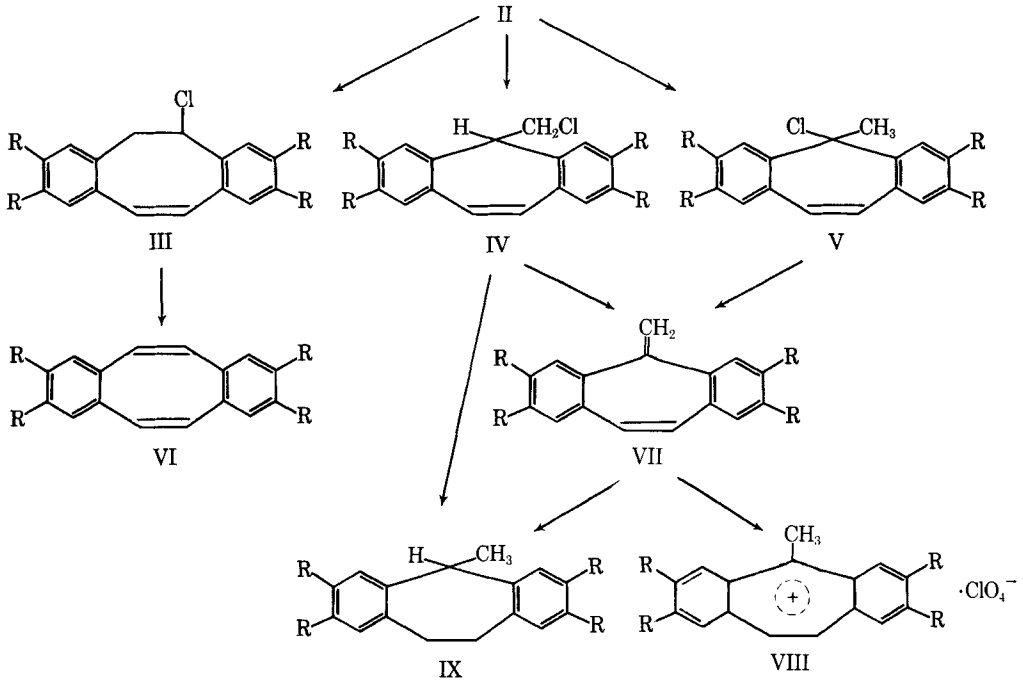
DISCUSSION

The Chlorine-Containing Substance—(±)-*N*-Methylpavine (I), when subjected to two steps of the Hofmann exhaustive methylation procedure, produced a nitrogen-free alcohol (II). The structure of II had been determined unambiguously (6) by Battersby and Binks as 3-hydroxy-2′:3′:-2″:3″-tetramethoxy-1:2-5:6-dibenzocycloöcta-1:5:7-triene. When II was treated with acetyl chloride by Pyman (7) a chlorine-containing compound, $C_{20}H_{21}ClO_4$, was produced which was assigned the structure III by Battersby and Binks. (Scheme II.)

(−)-*N*-Methylpavine (*i.e.*, argemonine), when subjected to a similar degradation, produced trimethylamine and a dextrorotatory nitrogen-free substance which was evidently an optically-active isomer of II (4). Treatment of an ethanolic solution of the latter with concentrated hydrochloric acid produced a chlorine-containing substance which had identical properties with the chlorine-containing substance reported by Pyman and to which Battersby and Binks had assigned structure III.

In considering the possible reactions of hydrochloric acid with II there appeared to be three possible structures (III, IV, and V) for the chlorine-containing compound. From a comparison of the ultraviolet spectra of the two compounds, *i.e.*, the alcohol (II) and the chloro-compound, it was obvious that there had been a bathochromic shift in the maxima of the latter compared to II, a shift that suggested a more significant change in the nature of the chromophore than would be expected in passing from II to III. In contrast, structures IV and V were both more reasonable on the basis of the ultraviolet data because the rearrangement of the middle ring from a cycloöctatriene to a cycloheptatriene system would be expected to cause the two aromatic rings to become more coplanar.

The chloro-compound, when refluxed in 2-picoline or alcoholic sodium hydroxide, provided a halogen-free substance analyzing for $C_{20}H_{20}O_4$, indicating that dehydrohalogenation had taken place. Structure III, on dehydrohalogenation, could be expected to produce a tetramethoxydibenzocycloöctatetraëne (VI). The latter, however, is a known compound (7) and was not the product of the reaction. On the other hand, structures IV and V under the conditions of the reaction would give the same product, namely VII. In order to gain more information about the dehydrohalogenated product, it was subjected to nuclear magnetic resonance (NMR) study.



Scheme II

The NMR spectrum of this compound showed six distinct singlets integrating for a total of 20 protons. Aromatic protons with an intensity of two each were found as singlets at 3.26 and 3.32 τ. The singlets for the methoxyl protons integrating for six each were found at 6.06 and 6.10 τ. A singlet at 3.06 τ, integrating for two protons, was assigned to a pair of olefinic protons conjugated to aromatic rings. The difference in the chemical shift of this pair of protons, compared to the almost equivalent pair of olefinic protons in *cis*-stilbene (3.45 τ), was attributed to the greater coplanarity of the benzene rings and the consequent deshielding effect. A singlet at 4.47 τ integrated for two protons and was assigned to a pair of terminal methylene protons. The NMR data, therefore, excluded structure VI but was compatible with structure VII.

Structure VII would be expected to form a tropylium salt on treatment with acids and it was found that the dehydrohalogenation product did, indeed, form a stable perchlorate with the correct analysis (VIII).

Hydrogenation of the dehydrohalogenation product provided a tetrahydro derivative, $C_{20}H_{24}O_4$, a product that would be expected from VII and which would be represented by structure IX. This derivative, on C-methyl analysis, showed one terminal methyl group as expected. The NMR spectrum showed the aromatic protons as singlets integrating for two each at 3.23 and 3.36 τ. The methoxyl protons gave singlets integrating for six each at 6.13 and 6.17 τ. A singlet at 6.90 τ, integrating for four protons, was assigned to the four equivalent benzylic protons of the ethylene bridge. The protons of the terminal methyl group appeared as a doublet centered at 8.32 τ with an integration of three protons (J = 7.5 c.p.s.). A quartet centered at 5.80 τ, integrating for one proton, was assigned to the benzylic proton (J = 7.5 c.p.s.) adjacent to the methyl group.

Compound IX had been synthesized unequivocally by Battersby and Binks (8) and the reported melting point coincided with that of the substance obtained in the present study by hydrogenation of VII. From the above data it appears that the dehydrohalogenation product can be assigned structure VII and its hydrogenation product, structure IX.

Although the structures of VII and IX appear unequivocal at this point, the structure of the chlorine-containing substance had not necessarily been proven since the possibility of rearrangement of III during dehydrohalogenation, although seemingly remote, could not be ignored. This possibility was dismissed by hydrogenolysis of the chloro-compound to yield IX. Since no rearrangement would be anticipated under these conditions, the chloro-compound would of necessity have structure IV or V. Because it was sparingly soluble in virtually all solvents which could be used for NMR analysis except pyridine, it was studied in the latter solvent. The methoxyl protons appeared as singlets at 6.15 and 6.17 τ. The chloromethyl group appeared as a doublet centered at 5.96 τ (J = 8 c.p.s.). A triplet centered at 5.40 τ (J = 8 c.p.s.) was assigned for the benzylic proton coupled to the protons of the chloromethyl group. The spectrum, in general, was not very clear and integration was not possible. Nevertheless, it appeared certain that the chloro-compound did not have structure V since its NMR spectrum should have

shown a singlet corresponding to the terminal methyl, this singlet together with the methoxyl proton peaks being the only ones expected in the region of 4.00 to 10.00 τ. Such absorption was not observed and, coupled with the observation (4) that C-methyl was not found by analysis, the evidence appears conclusive that the chlorine-containing substance may be represented by structure IV rather than III as suggested earlier by Battersby and Binks.

Argemoninic Acid—When II was oxidized with potassium permanganate, an optically inactive acid was obtained which was named argemoninic acid. The acid was tentatively identified in the earlier studies as a tetramethoxybiphenyltricarboxylic acid, based on an erroneous aporphine formulation of argemonine (4). Following elucidation of the correct structure (5), it became obvious that the proposed structure for the acid was incorrect. Furthermore, additional analyses suggested $C_{20}H_{18-20}O_{10}$ as a more reasonable empirical formula, in keeping with neutral equivalent determinations which gave values of 215 and 219 (theory, 209 and 210).

Likely structures for argemoninic acid, based on the known structure of II, would be X and XI with the structure XII, which conceivably could arise from X by benzilic acid rearrangement, being untenable on the basis of analytical and neutral equivalent data. The indophenine test for 1,2-diketones (9) proved to be positive and was substantiated by hydrogen peroxide oxidation of argemoninic acid to yield the expected *m*-hemipinic acid (XIV) isolated as its *N*-methylimide (XV). These results suggested X as the most likely structural possibility.

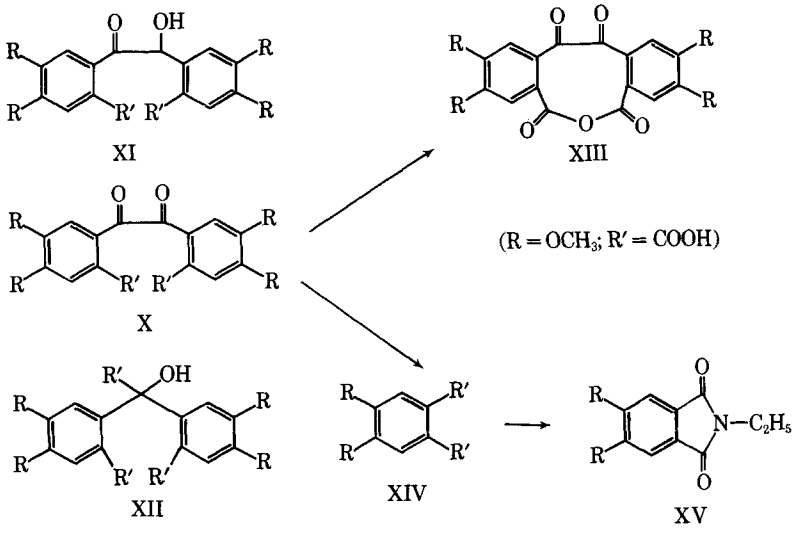
To further eliminate the possibility of a benzoin structure such as in XI, the infrared spectra of the acid and its triethylamine salt were examined. In the case of the acid, a broad band due to carboxylic group hydroxyl at about 3400 cm.$^{-1}$ precluded making a decision, but the triethylamine salt, on the other hand, while showing carbonyl absorption at 1690 cm.$^{-1}$ (α-diketone) and 1750 cm.$^{-1}$ (carboxylate) failed to show absorption in the hydroxyl region.

The NMR spectrum of argemoninic acid was determined in two solvent systems: (*a*) sodium bicarbonate in deuterium oxide and (*b*) sodium methoxide in dimethylsulfoxide. The spectrum of the acid in the first system was quite simple, showing only three distinct peaks corresponding to aromatic protons, methoxyl protons, and water. The aromatic protons appeared as a singlet at 2.90τ with an intensity of four protons, whereas the methoxyl protons were singlets at 6.04 and 6.18 τ with an intensity of six protons each. A large peak at 5.3τ was attributed to water. However, the possibility that the acid had still other spectral features that were masked by the water peak could not be ruled out. Inspection of the NMR spectrum in the second system showed the aromatic protons as a pair of singlets at 2.75 and 2.90τ. The methoxyl peaks at 6.13τ were complex due to interference of the methoxyl peak from sodium methoxide but, nevertheless, the spectrum failed to show peaks in the 3.0 to 6.0τ region and, therefore, strongly pointed to structure X.

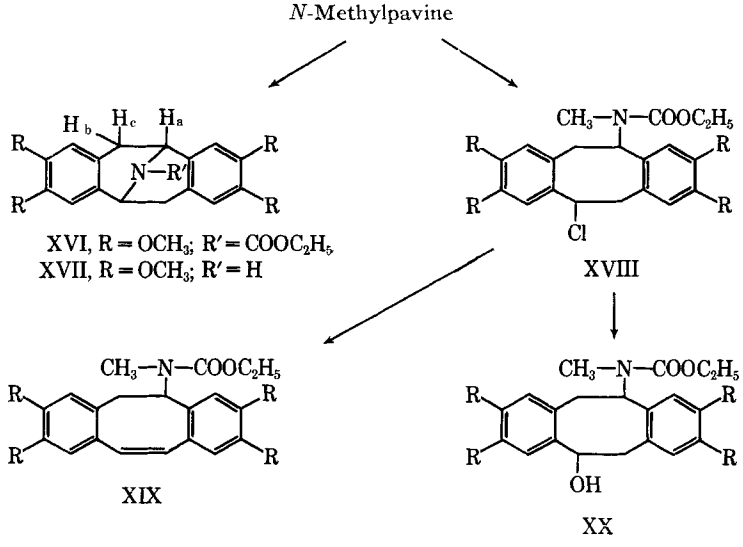
An interesting confirmation of structure X was also achieved by taking advantage of the fact that Kier and Soine (4) had previously shown that the acid, at its melting point, effervesced and liberated a mole of water. The most likely possibilities would have been formation of an anhydride from X or an anhydride or lactone from XI. The solid residue from such a melt analyzed for $C_{20}H_{16}O_9$. Its infrared spectrum showed no hydroxyl absorption but did show carbonyl absorptions at 1690 cm.$^{-1}$ (α-diketone) and at 1750 and 1790 cm.$^{-1}$ (anhydride). If argemoninic acid had structure XI it would have been impossible for it to form an anhydride *or* a lactone without leaving one hydroxyl group. The structure of the compound resulting from the melting of argemoninic acid can, therefore, be assigned as XIII and the structure of argemoninic acid is X. (Scheme III.)

The Ethyl Chloroformate Reaction Product—Subjection of *N*-methylpavine to the action of ethyl chloroformate in the presence of alkali according to the method of Gadamer and Knoch (10) yielded a neutral product (11). This reaction, which specifically cleaves benzylic carbon-to-nitrogen bonds (12), ordinarily provides a urethane as the end product and the reaction has been used to some extent in alkaloidal structural work. It fails occasionally, as in the case of tetrahydroberberine (12) but with aporphines, for example, the reaction proceeds with facility. Since earlier tentative conclusions as to the structure of argemonine indicated that it might be an aporphine (4) this reaction was employed during the structural studies. In light of the now known structure of argemonine as (−)-*N*-methylpavine, it is apparent that there are *two* benzylic carbon-to-nitrogen bonds in the molecule, and therefore the fact that a neutral product was obtained was of considerable interest from the standpoint of its structure.

Analysis of the product, resulting in a probable formula of $C_{23}H_{27}NO_6$, rendered untenable the product that might have resulted from a simple cleavage of a benzylic carbon-to-nitrogen bond such as XVIII or structures XIX and XX which could result from XVIII. The ultraviolet spectrum of the compound further supported the assumption that no cleavage had occurred, since the spectrum was virtually identical with that of *N*-methylpavine and unlike that of II, and further, the infrared spectrum failed to show hydroxyl absorption. The loss of —CH_3 during the reaction together with gain of —$COOC_2H_5$ appeared compatible with the analytical data and the neutral character of the product and thus suggested structure XVI as the most likely possibility. On this basis, the compound was subjected to lithium aluminum hydride reduction, since such reductions of urethanes have been reported by Knabe (13) and others (14) to yield an *N*-methyl group. The reduction process yielded a basic compound which was isolated as the methiodide and which proved to be identical in melting point and mixed melting point with *N*-methylpavine methiodide. Unfortunately, the methiodide melts with decomposition, and therefore further evidence of the identity of the two products seemed desirable. The infrared spectrum of the methiodide of reduced XVI was found to be identical with the spectrum of *N*-methylpavine methiodide, as were the X-ray powder diffraction patterns. In addition, alkaline hydrolysis of the suspected



Scheme III



Scheme IV

urethane yielded a new basic substance which was identical in all respects to (±)-pavine. The NMR spectrum of this new basic substance was also identical in all respects to that of (±)-pavine (5). (Scheme IV.)

Examination of the NMR spectrum of the ethyl chloroformate reaction product was entirely compatible with structure XVI. The aromatic protons were singlets at 3.28 and 3.47τ with an intensity of two each, whereas the methoxyl protons were singlets at 6.12 and 6.20τ with an intensity of six each. The protons of the methyl group of the ethoxy moiety appeared as a triplet centering at 8.72τ (J = 7 c.p.s.) with an intensity of three protons and the methylene protons of this group were found as a quartet centering at 5.79τ (J = 7 c.p.s.) with an intensity of two protons. The bridgehead protons appeared as a broad peak at 4.47τ with an intensity of two protons. As in the case of (−)-*N*-methylpavine (5), protons H_c coupled only with protons H_b and were seen as a doublet centering at 7.24τ (J_{bc} = 17 c.p.s.). Protons H_b were split by H_a and H_c and were observed as a pair of doublets at 6.41 and 6.68τ.

On the basis of the above evidence, structure XVI can be assigned to the product of the ethyl chloroformate reaction.

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover capillary melting point apparatus. Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, or by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. The infrared spectra were determined as Nujol and halocarbon mulls by the Infrared Laboratory, School of Chemistry,

University of Minnesota, using a Perkin-Elmer 521 grating infrared spectrophotometer. Ultraviolet spectra were determined in ethanolic solution using a Bausch & Lomb Spectronic 505 recording spectrophotometer. Nuclear magnetic resonance spectra were determined through the courtesy of Dr. William Schwabacher, School of Chemistry, University of Minnesota, using a Varian Associates A-60 instrument.

N-Methylpavine (I)—This substance was prepared essentially following the method of Battersby and Binks (6), m.p. 139–140°. [Lit. m.p. 135–140° (6).]

N-Methylpavinemethine Methiodide—This derivative of I was prepared according to the procedure of Kier and Soine (4) and was used without further purification.

3-Hydroxy-2′:3′:2″:3″-tetramethoxy-1:2-5:6-dibenzocyclooöcta-1:5:7-triene (II)—A suspension of *N*-methylpavinemethine methiodide (10 Gm.) in water (200 ml.) was treated with freshly prepared silver oxide (from 5 Gm. of silver nitrate). The reaction mixture was kept in an ice bath and stirred until a filtered portion failed to give a test for iodide ion with silver nitrate T.S. The precipitated silver iodide and excess silver oxide were removed by filtration and the filtrate was then boiled for 30 min., during which time a white crystalline precipitate formed. This precipitate was separated by filtration and recrystallized from a methanol–water mixture. The dried crystals (4.97 Gm.) melted at 142–147°. [Lit. (5) m.p. 145–146°.] $\lambda_{min.}$ 267.5 mμ (log ε 3.97); $\lambda_{max.}$ 220 (4.49), 292 mμ (4.05) in ethanol.

3-Chloromethyl-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4:6-triene (IV)—A solution of II (300 mg.) in ethanol (30 ml.) was cooled in an ice bath and then concentrated hydrochloric acid (15 ml.) was added dropwise. The solution acquired a transient pink to violet color which disappeared on shaking. The solution was then refluxed for 20 min. on a steam bath. On cooling, a white crystalline material separated which was removed by filtration (270 mg.) and recrystallized from hot glacial acetic acid. The dried crystals melted at 219–220°. [Lit. (4) m.p. 219–220°.] $\lambda_{min.}$ 265 mμ (log ε 4.54); $\lambda_{max.}$ 238 (4.54), 316 mμ (4.15) in ethanol.

Anal.—Calcd. for $C_{20}H_{21}ClO_4$: C, 66.57; H, 5.87; Cl, 9.83. Found: C, 66.65; H, 5.77; Cl, 9.55.

3-Methylene-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4:6-triene (VIII)—This substance was obtained from IV by two different methods.

Dehydrohalogenation Using 2-Picoline—A solution of IV (250 mg.) in 2-picoline (25 ml.) was refluxed under a nitrogen atmosphere for 7 days. The solution was then treated with 1% hydrochloric acid (80 ml.) and extracted with three successive portions (50 ml.) of ether. The combined ethereal extracts were washed successively with 1% hydrochloric acid (20 ml.) and distilled water (20 ml.). The ether extract was then dried with anhydrous sodium sulfate and, following removal of the drying agent by filtration, was evaporated to dryness to yield a crystalline residue. Recrystallization from ethanol yielded 126 mg. of yellowish plates, m.p. 226–229°. Further recrystallization from the same solvent provided faintly yellow crystals, m.p. 233.5–234.5°, which gave a negative halogen test.

Anal.—Calcd. for $C_{20}H_{20}O_4$: C, 74.06; H, 6.35. Found: C, 73.81; H, 6.34.

Dehydrohalogenation with Alcoholic Sodium Hydroxide—A solution of IV (300 mg.) in ethanol (300 ml.) was treated with sodium hydroxide pellets (3 Gm.) and refluxed for 24 hr. The ethanol was evaporated and the residue was treated with 100 ml. of boiling water, allowed to cool, and then filtered. The precipitate was then recrystallized from the least amount of boiling ethanol and colorless platelets (258 mg.), m.p. 234–236°, were obtained. The product gave no depression in melting point with the material obtained through the use of 2-picoline.

3-Methyl-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4-diene (IX) from VII—Palladium-on-carbon catalyst (10%, 400 mg.) was added to a solution of VII (200 mg.) in ethanol (200 ml.), and the mixture was then shaken with hydrogen under atmospheric pressure until the uptake of hydrogen stopped. The hydrogenated mixture was then heated to boiling and filtered to remove the catalyst. The filtrate was evaporated to dryness under reduced pressure and the residue recrystallized from the least amount of hot ethanol to obtain a crystalline precipitate (82.5 mg.), m.p. 128–129°. [Lit. (6) m.p. 126–128°.]

Anal.—Calcd. for $C_{20}H_{24}O_4$: C, 73.14; H, 7.36; C-methyl, 4.37. Found: C, 73.37; H, 7.13; C-methyl, 5.34.

3-Methyl-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4-diene (IX) from IV—Palladium-on-carbon catalyst (10%, 1.0 Gm.) was added to a solution of IV (400 mg.) in ethanol (300 ml.) and the mixture was then shaken with hydrogen under atmospheric pressure until the uptake of hydrogen stopped. The hydrogenated mixture was heated to boiling and filtered to remove the catalyst. The filtrate was evaporated to dryness and the residue recrystallized from the least amount of ethanol to yield a crystalline precipitate (79 mg.), m.p. 128–129°, which gave no depression of melting point with the product from the previous experiment.

3-Methylene-2′:3′:2″:3″-tetramethoxy-1:2-4:5-dibenzocyclohepta-1:4:6-triene Perchlorate (VIII)—Perchloric acid (60%, 2 ml.) was added dropwise to a hot solution of VII (100 mg.) in glacial acetic acid (200 ml.). The solution was boiled, filtered, and allowed to stand overnight in the refrigerator to yield a brown crystalline precipitate (75 mg.). This material was recrystallized from glacial acetic acid to yield 10 mg. of product, m.p. 350°.

Anal.—Calcd. for $C_{20}H_{21}ClO_8$: C, 56.54; H, 4.98. Found: C, 56.47; H, 4.96.

Argemoninic Acid (X)—A suspension of II (1.0 Gm.) in distilled water (75 ml.) was warmed and potassium permanganate solution (5%, 75 ml.) was added slowly. The mixture was heated on a steam bath with occasional stirring for 1 hr., cooled, and the excess permanganate reduced with methanol. The suspension was cooled, acidified with 30 ml. of 50% sulfuric acid, and the dark color of manganese dioxide discharged by the careful addition of solid sodium bisulfite. A fine white

precipitate remained which was removed by filtration (899 mg.). The precipitate was recrystallized from methanol to give white crystals, m.p. 254–255.5°, with effervescence. $\lambda_{min.}$ 246 mμ (log ε 4.03), 281 (3.91); $\lambda_{max.}$ 229 (4.71), 264 (4.21), and 295 (4.06).

Anal.—Calcd. for $C_{20}H_{18}O_{10}$: C, 57.41; H, 4.33; neut. equiv. (for 2 COOH), 209.18. Found: C, 57.12; H, 4.51; neut. equiv. 215, 219.

Argemoninic Acid Anhydride (XIII)—X (300 mg.) was heated under a nitrogen atmosphere to the melting point, when effervescence was observed followed by solidification of the melt. The residue was cooled and recrystallized from ethyl acetate several times to obtain white needles, m.p. 280–281°.

Anal.—Calcd. for $C_{20}H_{16}O_9$: C, 60.00; H, 4.02. Found: C, 59.76; H, 4.00.

N-Ethyl-m-hemipinimide (XV)—A solution of X (100 mg.) in glacial acetic acid (20 ml.) was treated with 30% hydrogen peroxide (10 ml.) and refluxed for 4 hr. The cooled solution was finally evaporated to dryness. The residue was treated with ethylamine solution (33%, 4 ml.) and again taken to dryness, heated at 180° for 10 min., and then sublimed at 180°/5 mm. The white sublimate was recrystallized from ethanol to yield 10 mg. of white needles, m.p. 227–229°, which failed to depress the melting point of an authentic sample of *N*-ethyl-*m*-hemipinimide.

N-Carbethoxypavine (XVI)—A solution of I (1.5 Gm.) in chloroform (30 ml.) was treated at room temperature with ethyl chloroformate (4 ml.) and aqueous potassium hydroxide solution (15%, 40 ml.). The reaction mixture was shaken for 10 min., additional ethyl chloroformate (5 ml.) added, and shaking continued for 30 min. The addition of ethyl chloroformate (5 ml.) and aqueous potassium hydroxide (40 ml.) was repeated three times at 30-min. intervals, after which the reaction mixture was allowed to stand overnight. The chloroformic layer was separated, the aqueous layer was washed with a little chloroform, and the washing added to the original chloroformic solution. On shaking the chloroformic solution with 20 ml. of dilute (3.7%) hydrochloric acid, the acid extract was found to be positive to Valser's reagent. The chloroform layer, therefore, was extracted with successive portions of hydrochloric acid until the acid extract failed to give a turbidity with the reagent. The chloroform solution was then washed with distilled water dried over anhydrous sodium sulfate, and evaporated to dryness. Crystallization was effected from ethanol to obtain large cubical crystals which were recrystallized from the same solvent, m.p. 191–192°. The yield was 1.18 Gm. (68% of theory).

Anal.—Calcd. for $C_{23}H_{27}NO_6$: C, 66.81; H, 6.58; N, 3.38. Found: C, 66.92; H, 6.41; N, 3.61.

Pavine (XVII)—A solution of XVI (400 mg.) in ethanol (100 ml.) was treated with potassium hydroxide pellets (10 Gm.) and the solution then refluxed for 48 hr. The mixture was then concentrated and, finally, evaporated to dryness following which water (10 ml.) was added and distilled off to remove any residual ethanol. The residue was suspended in water and extracted with three successive portions (50 ml.) of chloroform. The combined chloroformic extract was washed with water and then extracted with successive 50-ml. portions of 3.7% hydrochloric acid until the acid extracts gave a negative test with Valser's reagent. The combined acid extracts were washed with chloroform (20 ml.) and rendered alkaline by the careful addition of 20% sodium hydroxide solution following which the alkaline solution was extracted with successive 50-ml. portions of chloroform until the chloroform extracts gave a negative test with Valser's reagent. The combined chloroformic extracts were washed with water, filtered, and dried over anhydrous sodium sulfate. The dried extract was evaporated to dryness and the residue treated with absolute ethanol (10 ml.) and the ethanol then evaporated to remove traces of moisture. The dry residue was crystallized from xylene and then recrystallized from ethanol to provide 20 mg. of product, m.p. 198–199°. [Lit. (6) m.p. 199–200°.]

N-Methylpavine Methiodide from XVI—A solution of XVI (232 mg.) in dry tetrahydrofuran (40 ml.) was added dropwise to a suspension of lithium aluminum hydride (290 mg.) in dry tetrahydrofuran (50 ml.). After the addition was complete, the reaction mixture was refluxed for 10 hr., following which the excess lithium aluminum hydride was decomposed by the careful addition of moist ether. After filtering the reaction mixture, the precipitate was washed with tetrahydrofuran and the washings added to the filtrate. The filtrate was treated with enough ether (50 ml.) to complete the separation of an aqueous layer which was removed, washed with ether, and the ether washings added to the original ether layer. The ethereal solution was dried with anhydrous sodium sulfate, the drying agent removed by filtration, and the solvent removed by distillation. The dry residue was then dissolved in ethyl acetate, filtered, and treated with methyl iodide (1 ml.). The methiodide salt which separated was filtered off and recrystallized from ethanol, m.p. 265–266°. The yield of product was 214 mg. (61.4% of theory) and failed to depress the melting point of authentic *N*-methylpavine methiodide.

REFERENCES

(1) Soine, T. O., and Gisvold, O., *J. Am. Pharm. Assoc., Sci. Ed.*, **33**, 185(1944).
(2) Schermerhorn, J. W., and Soine, T. O., *ibid.*, **40**, 19(1951).
(3) Kier, L. B., and Soine, T. O., *ibid.*, **49**, 187(1960).
(4) Kier, L. B., and Soine, T. O., *J. Pharm. Sci.*, **50**, 321(1961); Soine, T. O., and Kier, L. B., *ibid.*, **51**, 1196 (1962).
(5) Martell, M. J., Jr., Soine, T. O., and Kier, L. B., *J. Am. Chem. Soc.*, **85**, 1022(1963).
(6) Battersby, A. R., and Binks, R., *J. Chem. Soc.*, **1955**, 2888.
(7) Pyman, F. L., *ibid.*, **107**, 176(1915).
(8) Battersby, A. R., and Binks, R., *ibid.*, **1955**, 2896.
(9) Feigl, F., "Spot Tests in Organic Analysis," 7th ed., Elsevier Publishing Co., New York, N. Y., 1966, p. 325.
(10) Gadamer, J., and Knoch, F., *Arch. Pharm.*, **259**, 148(1921).
(11) Soine, T. O., and Kier, L. B., unpublished data.
(12) Knabe, J., *Arch. Pharm.*, **289**, 479(1956).
(13) Von Bruchhausen, F., and Knabe, J., *ibid.*, **287**, 601(1954).
(14) Dannley, R. L., Lukin, M., and Shapiro, J., *J. Org. Chem.*, **20**, 92(1955).

Hydrolysis of Solubilized Esters

By P. B. SHETH* and E. L. PARROTT

The solubilizing ability of four nonionic surface-active agents is quantitatively compared. The critical micelle concentration (CMC) of the surface-active agents was evaluated from the solubility curves. The effect of the surface-active agents on the rate of hydrolysis of benzocaine and homatropine was investigated. At a concentration of surface-active agent less than the CMC, the surface-active agents do not significantly affect the rate of hydrolysis of the esters. At a concentration exceeding the CMC, the surface-active agents enhance the stability, due to micellar interaction.

THE PHENOMENON of micellar solubilization in aqueous systems and its applications to pharmaceuticals has received considerable attention (1–5). The nonionic surface-active agents have been most frequently used in the solubilization of drugs which possess a low water solubility (6, 7). Their use has been presumably due to their lower toxicity and irritation in comparison to ionic surface-active agents (8–10).

Studies of several readily oxidizable materials solubilized by ionic and nonionic surface-active agents have shown the solubilized preparation to be less easily oxidized than emulsions or simple aqueous dispersions (11–13). Riegelman reported that ionic and nonionic surface-active agents provide a different degree of protection from base hydrolysis (14).

This investigation was undertaken to determine the effectiveness of several nonionic surface-active agents in solubilizing and in retarding the alkaline hydrolysis of benzocaine and homatropine. As several mechanisms for increasing the stability of benzocaine to hydrolytic degradation have been studied, benzocaine was chosen for this investigation with the thought that a comparison with previous reports would be of interest (14–16). In pharmaceuticals, basic nitrogen-containing drugs, *i.e.*, amines and alkaloids, are generally used in the salt form in order to enhance the water solubility and/or stability of the drug. In product development it may occasionally be necessary to prepare a clear liquid pharmaceutical in a pH range at which these drugs exist in the base form. In such products the utilization of the phenomenon of micellar formation could possibly increase the apparent solubility and stability of the drug; to study this possibility homatropine was chosen.

Received December 27, 1966, from the College of Pharmacy, University of Iowa, Iowa City, IA 52240

Accepted for publication May 11, 1967.

Abstracted in part from a dissertation presented by Pravin B. Sheth to the Graduate College, University of Iowa, in partial fulfillment of Doctor of Philosophy degree requirements.

This project was supported in part by research grant 1573 from Ciba Pharmaceutical Co., Summit, N. J.

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EXPERIMENTAL

Materials—The surface-active agents were commercial lots which were used without further purification: polyoxyethylene lauryl ether[1] (PLE); oxyethylene polymer containing 80% polyoxyethylene[2] (POE), approximate mol. wt. 8700; a product formed by the addition of propylene oxide to ethylenediamine followed by the addition of ethylene oxide[3] (POEEO); a condensation product of *tert*-octylphenol with ethylene oxide[4] (OPEO), average mol. wt. 2800; homatropine;[5] and benzocaine N.F.

Solubility Studies—An excess of benzocaine and aqueous solutions of the surface-active agents were rotated in 50-ml. amber bottles in a constant-temperature bath at 30 ± 0.1° for 24 hr. A portion was withdrawn from each bottle with a pipet fitted with a cotton filter. After dilution with distilled water, the absorbance was measured with a Beckman DU spectrophotometer, and the solubility of the benzocaine was determined by means of a calibration curve.

In the analysis of samples containing surface-active agents, with the exception of OPEO solutions, water was used as a blank, since the samples were diluted at least 500-fold and there was no absorption by the surface-active agents. OPEO absorbs strongly in the region of 280 mμ near the maximum for benzocaine. In the assay of solutions containing OPEO, a blank of the OPEO solution, diluted in the same manner as the sample, was used to compensate for absorption by the surface-active agent.

The concentration of a surface-active agent at which micelles are formed is known as the critical micelle concentration. As shown in Figs. 1–4, the apparent solubility of benzocaine was plotted against the concentration of the surface-active agent. The inflection in the solubility curve indicates micelle formation (17).

Hydrolysis of Benzocaine—For studies in 3% POE, 75 ml. of an aqueous 4% POE solution was pipeted into a 6-oz. bottle containing 1 mg. of benzocaine in 5 ml. of solution. For studies in 0.005% POE, 5 ml. of an aqueous 0.1% POE solution was pipeted into a 6-oz. bottle containing 70 ml. of distilled water and 5 ml. of a solution containing 1 mg. of benzocaine. The solution was placed in a 30° constant-temperature bath, and

[1] Marketed as Brij 35 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Pluronic F 68 by Wyandotte Chmeical Corp., Wyandotte, Mich.

[3] Marketed as Tetronic 908 by Wyandotte Chemical Corp., Wyandotte, Mich.

[4] Marketed as Triton WR-1339 by Winthrop Laboratories, New York, N. Y.

[5] Obtained from K & K Laboratories, Inc.

when thermal equilibrium was attained, 20 ml. of 0.2 *N* sodium hydroxide solution at 30° was added to give a 0.04 *N* hydroxyl ion concentration. At definite intervals of time, samples were withdrawn from the solution which was maintained at 30°. The absorbance of the sample was measured immediately at 284 and 265 mμ, and the concentration of benzocaine was determined by use of simultaneous equations obtained from standard absorption curves (14). With samples containing 3% POE, a blank of a solution of 3% POE and 0.04 *N* hydroxyl ion concentration was used; with samples containing 0.005% POE, a blank of a solution containing 0.005% POE and 0.04 *N* hydroxyl ion concentration was used.

The hydrolysis of benzocaine in water, PLE, and POEEO was followed in a similar manner. With OPEO, a direct ultraviolet method was not satisfactory due to strong absorbance of the surface-active agent at 284 mμ (6). A chloroform extraction procedure was used. The absorption maximum of benzocaine in chloroform was 279 mμ.

Hydrolysis of Homatropine—One hundred milligrams of homatropine was dissolved in 100 ml. of solvent medium, *i.e.*, water, 0.005 or 3% solution of surface-active agent, at 30°. The first 30-ml. sample was withdrawn, designated as the zero time, and analyzed for homatropine. The solution was maintained at 30°, and subsequent 10-ml. samples were withdrawn at definite intervals of time and were analyzed by the procedure of Patel and Lemberger (18).

RESULTS AND DISCUSSION

In dilute aqueous solutions in which the surface-active agent is molecularly dispersed, compounds of low water solubility, such as benzocaine, do not dissolve to a greater extent than they would in water; however, at concentrations of the surface-active agent in excess of the CMC, the apparent solubility is increased. When the CMC is exceeded, the apparent solubility of benzocaine is linearly dependent on the concentration of the surface-active agent. As shown in Figs. 1–4, the point of inflection in the solubility curve indicates the CMC.

The amount of benzocaine solubilized by micellar interaction is represented by the difference between the apparent solubility in a solution of a surface-active agent and the solubility of benzocaine in water. The difference between the concentration of the surface-active agent and its CMC represents the amount of surface-active agent in the micellar form. This relationship may be expressed by the equation:

$$S = S_0 + K\,(C_{\text{surfactant}} - C_{\text{CMC}}) \quad \text{(Eq. 1)}$$

in which S is the apparent solubility of a compound in the aqueous solution of a surface-active agent, S_0 is the solubility of the compound in water, $C_{\text{surfactant}}$ is the concentration of the surface-active agent, C_{CMC} is the critical micelle concentration, and K is a constant, *i.e.*, the slope of the solubility curve.

For the solubilization of benzocaine, the K values in Table I show the order of solubilizing ability of the surface-active agents to be OPEO > PLE > POEEO > POE.

The conditions of 30° and 0.04 *N* hydroxyl ion concentration for the hydrolysis of benzocaine were essentially the same as those used by previous investigators (14, 15). The initial concentration

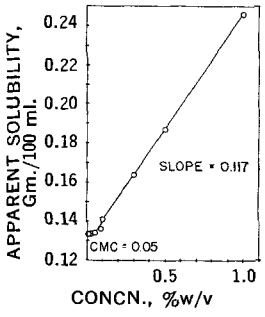


Fig. 1—Apparent solubility of benzocaine at 30° in aqueous solutions of PLE.

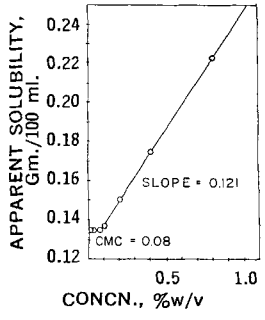


Fig. 2—Apparent solubility of benzocaine at 30° in aqueous solutions of OPEO.

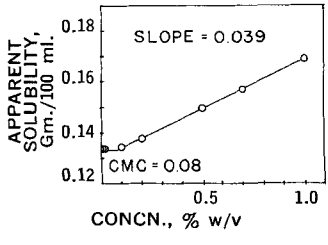


Fig. 3—Apparent solubility of benzocaine at 30° in aqueous solutions of POEEO.

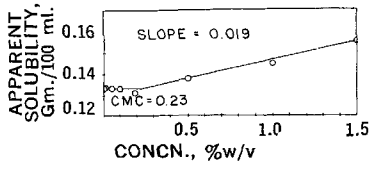


Fig. 4—Apparent solubility of benzocaine at 30° in aqueous solutions of POE.

TABLE I—SOLUBILIZING AND STABILIZING ABILITY OF SOME NONIONIC SURFACE-ACTIVE AGENTS FOR BENZOCAINE

Agent	*K*, Slope of Solubility Curve	$t_{1/2}$, in 3% Agent, min.
PLE	0.117	291
OPEO	0.121	295
POEEO	0.039	107
POE	0.019	91

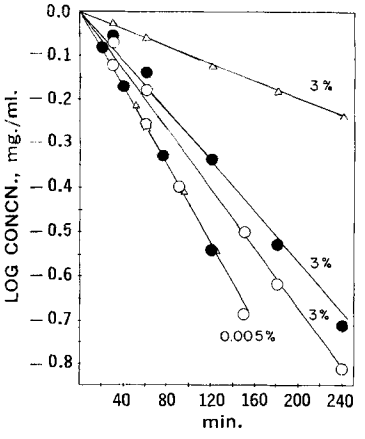


Fig. 5—A plot showing the effect of 3% concentration of surface-active agents on the rate of hydrolysis of benzocaine at 30° and 0.04 N hydroxyl ion concentration. The points on the 0.005% curve represent the hydrolysis in the presence of 0.005% surface-active agent; the line on which these points fall is drawn for the hydrolysis of benzocaine at 0.04 N hydroxyl ion concentration. Key: O, POE; ●, POEEO; △, PLE.

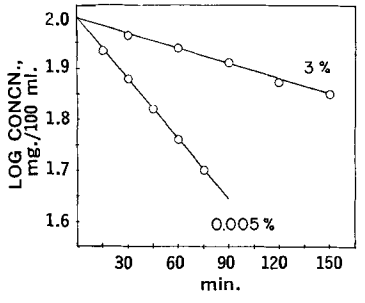


Fig. 6—A plot showing the effect of 3% OPEO on the rate of hydrolysis of benzocaine at 30° and 0.04 N hydroxyl ion concentration. The points on the 0.005% curve represent the hydrolysis in the presence of 0.005% OPEO; the line on which these points fall is drawn for the hydrolysis of benzocaine at 0.04 N hydroxyl ion concentration.

TABLE II—SPECIFIC RATE CONSTANTS AND HALF-LIFE PERIODS FOR HYDROLYSIS OF BENZOCAINE IN 0.04 *N* HYDROXYL ION CONCENTRATION AT 30° IN THE PRESENCE OF SOME NONIONIC SURFACE-ACTIVE AGENTS

Agent	% Agent	k (min.$^{-1}$)	$t_{1/2}$, min.
Water	...	0.01006	69
PLE	0.005	0.00996	70
	3.00	0.00238	291
OPEO	0.005	0.00908	76
	3.00	0.00234	295
POEEO	0.005	0.01012	69
	3.00	0.00644	107
POE	0.005	0.00989	70
	3.00	0.00759	91

of benzocaine was either 1 mg./100 ml. or 1 mg./ml. of solution. The hydrolysis of homatropine was carried out in distilled water at 30°. The experimental conditions for the hydrolysis of the esters produced an insignificant change in hydrogen ion concentration, and consequently the observed hydrolysis was pseudo first-order.

Hydrolysis of Benzocaine in the Presence of Nonionic Surface-Active Agents—The rate of hydrolysis of benzocaine was followed at 30° in the presence of four nonionic surface-active agents. As shown in Figs. 5 and 6, a plot of the logarithm of concentration of benzocaine against time is linear, designating a pseudo first-order reaction. The specific rate constant was determined by multiplying the slope of the curve by 2.303. As the hydrolysis is a pseudo first-order reaction, the half-life period was determined by dividing 0.693 by the specific rate constant (19). The specific rate constants and the half-life periods are given in Table II.

The alkaline hydrolysis was carried out in water and in a 0.005% solution of the surface-active agent, which was a concentration less than the CMC. A comparison of the half-life periods of benzocaine at 0.04 *N* hydroxyl ion concentration in water and in 0.005% surface-active agent shows that the surface-active agent *per se* does not significantly affect the stability. In Figs. 5 and 6 the points on the 0.005% curve are experimental values showing the rate of hydrolysis of benzocaine in the presence of 0.005% surface-active agent; the line upon which these points fall is drawn to show the rate of hydrolysis of benzocaine in water at 0.04 *N* hydroxyl ion concentration.

In 3% concentration of surface-active agents, the half-life periods were increased. For example, with PLE and OPEO the half-life of benzocaine was increased fourfold. As the surface-active agents exhibited no effect on the stability below their CMC, the increased stability at a concentration exceeding the CMC is attributed to micellar interaction.

This is further suggested by the stability of benzocaine in other surface-active agents. POE, which forms micelles of a low aggregation number, would theoretically provide less opportunity for micellar interaction with benzocaine; therefore, it would only slightly increase the stability of benzocaine. The experimental results concur, as the half-life of benzocaine in the presence of 3% POE is increased only 30%.

Hydrolysis of Homatropine in the Presence of Nonionic Surface-Active Agents—The rate of hydrolysis of homatropine was followed at 30° in the presence of four nonionic surface-active agents. As shown in Figs. 7 and 8 the rate of hydrolysis is first order with respect to homatropine. The observed specific rate constants and the half-life periods are given in Table III.

When the hydrolysis was carried out at a concentration of 0.005% surface-active agent, the rate of degradation was the same as that in distilled water. In Figs. 7 and 8, the points on the 0.005% curve are experimental values showing the rate of hydrolysis of homatropine in the presence of 0.005% surface-active agent; the line upon which these points fall is drawn to show the rate of hydrolysis of homatropine in water.

In 3% concentrations of OPEO and PLE, the half-life periods were increased approximately twofold. In 3% concentrations of POE and

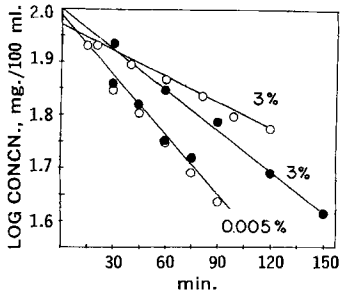


Fig. 7—A plot showing the effect of 3% of surface-active agents on the rate of hydrolysis of homatropine at 30°. The points on the 0.005% curve represent the hydrolysis in the presence of 0.005% surface-active agent; the line on which these points fall is drawn for the hydrolysis of homatropine in water. Key: ○, PLE; ●, POE.

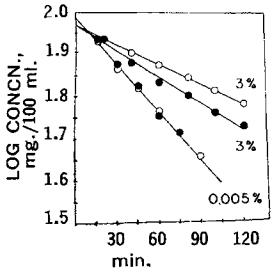


Fig. 8—A plot showing the effect of 3% of surface-active agent on the rate of hydrolysis of homatropine at 30°. The points on the 0.005% curve represent the hydrolysis in the presence of 0.005% surface-active agent; the line on which these points fall is drawn for the hydrolysis of homatropine in water. Key: ○, OPEO; ●, POEEO.

TABLE III—SPECIFIC RATE CONSTANTS AND HALF-LIFE PERIODS FOR HYDROLYSIS OF HOMATROPINE AT 30° IN THE PRESENCE OF SOME NONIONIC SURFACE-ACTIVE AGENTS

Agent	% Agent	k (min.$^{-1}$)	$t_{1/2}$, min.
Water	...	0.00897	77
OPEO	0.005	0.00885	78
	3.00	0.0046	150
PLE	0.005	0.00938	74
	3.00	0.00483	143
POEEO	0.005	0.00897	77
	3.00	0.00552	125
POE	0.005	0.0091	76
	3.00	0.00552	125

POEEO, the half-life periods are increased approximately 60%.

It has been suggested that solubilization occurs by (*a*) solution of the solubilizate within the interior of the micelle, (*b*) adsorption on the surface of the micelle, and (*c*) penetration into the palisade layer of the micelle (14). When a nonpolar compound is solubilized within the lipophilic interior of the micelle, the system is presumably more stable because the attacking species cannot readily contact the solubilized compound. If a compound is solubilized by penetration into the palisade layer of the micelle, it is generally susceptible to attack and degradation, although to a lesser extent than a compound solubilized by surface adsorption on the micelle.

In comparing the effect of the nonionic surface-active agents on the hydrolysis of benzocaine and homatropine, the greater stabilization of benzocaine may be attributed to the deeper penetration of the less polar benzocaine into the palisade layer of the micelles. The more polar homatropine only superficially penetrates the palisade layer, or is adsorbed onto the surface of the micelle; thus, homatropine is exposed to the hydroxyl ions and its stability is not markedly enhanced by solubilization.

A comparison of the effect of the four nonionic surface-active agents on the solubilization and the stability of benzocaine, as given in Table I, indicates that the stability increase by solubilization reflects the solubilizing ability of the surface-active agent. PLE and OPEO have the same solubilizing ability, and at 3% concentration both increase the half-life of benzocaine approximately fourfold. POE, which has the least solubilizing ability, only slightly increases the half-life of benzocaine. POEEO is intermediate in its solubilizing ability and enhancement of stability.

The limited solubilization of benzocaine by POE mirrors its low aggregation number and limited incorporation of benzocaine in the micelle. POE does not appreciably retard the hydrolysis of benzocaine, which is adsorbed on the surface of the micelle and is exposed to the attacking hydroxyl ions.

REFERENCES

(1) Mulley, B. A., in "Advances in Pharmaceutical Sciences," Bean, H. S., Beckett, A. H., and Carless, J. E., eds., Academic Press Inc., New York, N. Y., 1964.
(2) McBain, M. E. L., and Hutchinson, E., "Solubilization and Related Phenomena," Academic Press Inc., New York, N. Y., 1955.
(3) Boon, P. F. G., Coles, C. L. J., and Tait, M., *J. Pharm. (London)*, **13**, 200T(1961).
(4) Davis, J. G., *J. Appl. Bacteriol.*, **25**, 195(1962).
(5) Swarbrick, J., *J. Pharm. Sci.*, **54**, 1229(1965).
(6) Guttman, D. E., Hamlin, W. E., Shell, J. W., and Wagner, J. G., *ibid.*, **50**, 305(1961).
(7) Johnson, R. H., U. S. pat. 2,880,130 and 2,880,138 (1959).
(8) Stüpel, H., *Soap Perfumery Cosmetics*, **28**, 58, 300 (1955).
(9) Schwartz, A. M., Perry, J. W., and Berch, J., "Surface-Active Agents and Detergents," vol. II, Interscience Publishers, New York, N. Y., pp. 368–384.
(10) Krehl, W. A., Cowgill, G. R., and Whedon, A. D., *J. Nutr.*, **55**, 35(1955).
(11) Coles, C. L. J., and Thomas, D. F. W., *J. Pharm. Pharmacol.*, **4**, 898(1952).
(12) Carless, J. E., and Nixson, J. R., *ibid.*, **12**, 348(1960).
(13) Carless, J. E., and Mitchell, A. G., *ibid.*, **14**, 46(1962).
(14) Riegelman, S., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 339(1960).
(15) Higuchi, T., and Lachman, L., *ibid.*, **44**, 521(1955)
(16) Lachman, L., Guttman, D. E., and Higuchi, T., *ibid.*, **46**, 36(1957).
(17) Preston, W. C., *J. Phys. Colloid Chem.*, **52**, 85(1948).
(18) Patel, J. L., and Lemberger, A. P., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 878(1958).
(19) Daniels, F., and Alberty, R. A., "Physical Chemistry," 3rd ed., John Wiley & Sons, Inc., New York, N. Y., 1966, p. 329.

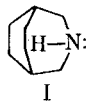
Potential Anti-Infective Agents I

Quinoline, Phenolic, and β-Aminoketone Derivatives

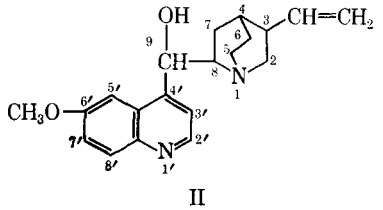
By ROBERT ARMEN MAGARIAN* and W. LEWIS NOBLES

Mannich bases were prepared from quinolinols, isoquinolinols, phenols, biphenols, and ketones in order to have their antimicrobial properties evaluated.

THE USE OF 3-azabicyclo[3.2.2]nonane (I), also known as AZBN, in the Mannich reaction was reported by Blanton and Nobles in 1962 (1). In biological screening, some of these Mannich bases were found to possess marked antimicrobial activity (2). As an extension of this study, AZBN, along with other bicyclic and heterocyclic amines, was condensed with moieties of known anti-infective agents utilizing the Mannich reaction (3) in order to study their antimicrobial properties, *i.e.*, antimalarial, antiamebic, and related effects. Quinine (II), a quinolylcarbinolamine containing a methoxy group on the quinoline nucleus linked by a secondary carbinol to a quinuclidine ring bearing a vinyl group, is an antimalarial that has been utilized since the year 1630 (4). This structure (II) served to provide the fountainhead of ideas for this work when a search of the literature revealed that many known anti-infectives have resulted from condensation of quinoline and phenolic compounds with aminomethyl moieties. Since AZBN is a bicyclic amine, it is conceivable that this amine can be useful—along with other bicyclic and heterocyclic amines—in place of the quinuclidine moiety in quinine. With this in mind, a number of substituted phenols and compounds structurally related to quinine were synthesized; more specifically, the products were prepared by the condensation of quinolinols, isoquinolinols, phenols, biphenols, and ketones with 37% aqueous formaldehyde (formalin), and cyclic amines.



I



II

Quinine (6′-methoxy-3-vinyl-9-rubanol)

DISCUSSION

The *o*- and *p*-hydrogens in phenols are sufficiently active to react in the Mannich reaction (5). From 8-quinolinol and 5-isoquinolinol (Table I), mono- and disubstituted products are expected, while only a mono-substituted product is obtainable with 5-chloro-8-quinolinol. Bruson (6), Burckhalter and associates (7), and Burke (8) have carried out Mannich reactions with phenols in which an *ortho* or *para* position was blocked by a chloro or bromo group. From the reaction of equimolar amounts of 2-phenylphenol, formaldehyde, and diethylamine, Burckhalter (9) was able to prove that the Mannich condensation takes place preferentially in the *ortho* position as compared to the *para* position.

It is interesting to note that the disubstituted Mannich product of 8-quinolinol (compound *2*, Table I) was not obtainable when 8-quinolinol was refluxed in absolute ethanol with 2 moles each of formaldehyde and AZBN. This disubstituted compound could only be obtained when equimolar amounts of formaldehyde and AZBN were reacted with the monosubstituted Mannich product III in absolute ethanol.

The solubility of aminomethylphenols in dilute alkali has served also as an aid in the confirmation of structure. Burckhalter and co-workers (7, 9), reported that 4-phenyl, 4-alkyl, or 4-halo-substituted 2-(dialkylaminomethyl) phenols are insoluble in 5% sodium hydroxide solution at room temperature, whereas the isomeric and analogous 4-(dialkylaminomethyl) phenols are soluble. The work of Julia and Tchernoff (10) is in agreement with that of Burckhalter and associates. They concluded that solubility in alkali apparently depends on the position of the amino group; the phenolic properties of the compound are possibly masked by the amino groups.

Millon's test has been utilized to identify an unsubstituted *ortho* position in phenolic derivatives (9). This test is used with monohydroxy phenols, tyrosine, tyrosine-containing proteins, phenolic acids, and other compounds that have a phenolic group with an open *ortho* position (11). The procedure involves the addition of 50 mg. of the phenolic derivative to 1 ml. of Millon's reagent. The test tube is placed in a beaker of water and heated to boiling. A red color indicates an open *ortho* position. (See Table II.)

Received December 29, 1966, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677

Accepted for publication May 17, 1967.

Abstracted in part from a thesis submitted by Robert A. Magarian to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.

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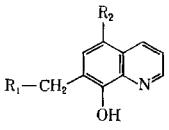
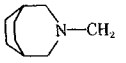
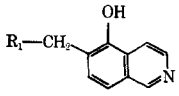
TABLE I—MANNICH BASES (QUINOLINOL AND ISOQUINOLINOL)

No.	R_1	R_2	Yield, %	M.p.,[a] °C.	Formula	Anal.[b] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
											
1	AZBN	H	80	130–132[c]	$C_{18}H_{22}N_2O$	76.56	76.61	7.85	7.88	9.92	9.88
2	AZBN		76	156–158[d]	$C_{27}H_{37}N_3O$	77.28	77.11	8.89	8.90	10.02	10.24
3	AZBN	Cl	73	112–114[e]	$C_{18}H_{21}ClN_2O$[g]	68.24	68.05	6.68	6.61	8.84	8.64
											
4	AZBN	...	30	186.5–188[f]	$C_{18}H_{22}N_2O$	76.56	76.82	7.85	8.85	9.92	9.76

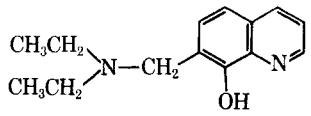
[a] Melting points are corrected. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. [c] Recrystallized from absolute ethanol–water; [d] pyridine–water; [e] acetone; [f] absolute ethanol. [g] *Anal.*—Calcd. for Cl: 11.19. Found: 11.06.

8-Quinolinols have been found to be effective against bacteria (12, 13), and considerable evidence points to direct antiamebic activity by compounds of this type (13). One such compound is shown in structure III (14).

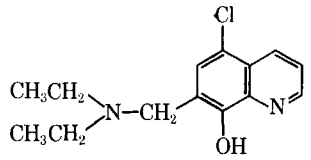
Various 7-(mono- and di-alkylaminomethyl)-8-quinolinols have also been prepared and evaluated by others as amebicides in the hope that the introduction of this solubilizing group would confer promising activity against amebic hepatitis (13–19). Although this new type of 8-quinolinol conferred only partial suppression of the hepatic lesion in hamsters (13), the activity of many of these compounds *in vitro* and in experimental intestinal amebiasis is equivalent to or exceeds that of the clinically-used iodinated 8-quinolinols (13, 18). One of the most promising members of this series is 5-chloro-7-diethylaminomethyl-8-quinolinol (IV) which was synthesized by Burckhalter and co-workers (14) and was found to be effective in human intestinal amebiasis (13).

There is a possibility that such derivatives could also be antifungal, since quinoline derivatives frequently possess such a property. This fact led to the preparation of the compounds in Table I. The effectiveness of diallylbiphenols, biphenols, and phenolic derivatives as medicinal agents was established by Burckhalter (7) when he found that certain α-dialkylamino-*o*-cresols (V, VI) possessed antimalarial activity. This work aroused interest in the synthesis of derivatives using various amine components along with the phenolic-types mentioned above in the Mannich reaction.

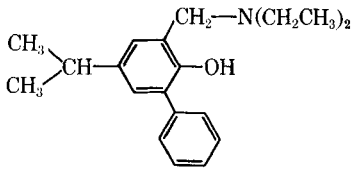
The products shown in Tables III, IV, V, and VI possibly offer the basis for a good study in structure–activity relationship, since they can be viewed as compounds that resemble 2,2′-diallyl-4,4′-biphenol (VII) with or without all the functional groups. The antibacterial activity of the Mannich bases derived from *p*-nitroacetophenone (20) led to the preparation of four β-aminoketones (Table VII) to be tested for possible anti-infective activity.



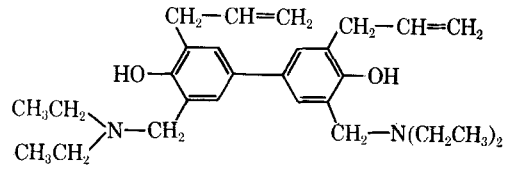
III



IV



V



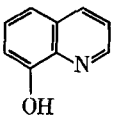
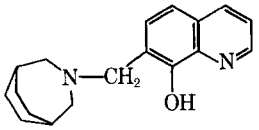
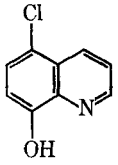
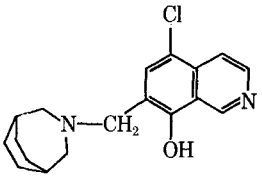
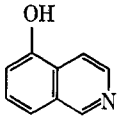
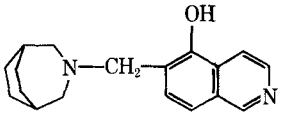
VI

Also, there was interest in seeing what activity the 3-azaspiro[5.5]undecane may possess, since Grogan (21) and co-workers have prepared a number of different azaspirane derivatives that were found to be of interest in medicinal chemistry.

EXPERIMENTAL

Experimental Results—The procedure of Blanton

TABLE II—MANNICH BASES (DIALLYLBIPHENOLS)

Compd.	Solubility in 5% Sodium Hydroxide	Millon's Test (Color)
	Soluble	Positive—blood red
	Insoluble	Negative—dark green
	Soluble	Positive—dark red
	Insoluble	Negative—light orange
	Soluble	Positive—blood red
	Insoluble	Negative—light orange

and Nobles (1) was essentially followed, except that 37% aqueous formaldehyde (formalin) was employed rather than paraformaldehyde and the amine hydrochloride was used only in the synthesis of the β-aminoketones. The starting materials were available commercially.[1]

RESULTS AND DISCUSSION

Conclusions regarding the reactants used were drawn from this study based on the reaction conditions employed. The phenolic and quinolinolic derivatives reacted with facility when the free amine was used; however, Mannich products were virtually impossible to obtain when the amine hydrochloride was used. For example, 4,4′-biphenol and 3-azabicyclo[3.2.1]octane hydrochloride were refluxed together with 37% aqueous formaldehyde; instead of isolating the Mannich product, the starting materials were obtained. The results were similar with 4-phenylphenol. Conversely, the Mannich ketones were relatively easy to obtain when the amine hydrochloride was used. If the free base were used, however, the ketonic product was not isolated; when AZBN, as the free base, was refluxed with *p*-nitroacetophenone and formalin, a novel di-substituted substance was isolated (22).

Aqueous formaldehyde (37%) was employed in all reactions and it was found to be as effective as paraformaldehyde; the latter appears more often in the literature when the Mannich reaction is discussed. The reason that paraformaldehyde is used more often may be attributed to its use with organic solvents, whereas a condensation reaction involving 37% aqueous formaldehyde is ordinarily carried out by shaking or stirring the reactants in the absence of an organic solvent (3). When aqueous formaldehyde was used with 2-allyl-4-methylphenol and 1-(*p*-chlorophenyl)-2-methylpiperazine in only 10 ml. of absolute ethanol as solvent, the desired Mannich base was not isolated in spite of shaking, stirring, and finally refluxing for 2.5 hr. Instead, a bis-amine (VIII) was isolated as the product. The white crystalline solid was thus obtained in 70% yield and had a melting point of 162–164° (corrected). Recrystallization from acetone–water resulted in a product melting at 164–166° (corrected). The conclusion that the product was a bis-amine was based on elemental analyses and infrared spectral data. The following analytical values were obtained.

Anal.—Calcd. for CHN: C, 63.79; H, 6.98; N, 12.93. Found: C, 63.94; H, 6.91; N, 12.12.

It is not uncommon to find bis-amines as products in the Mannich reaction (3). Other bis-type Mannich products have also been reported. The isolation of a methylene-bis-indanedione (IX) involving indanedione, amine, and formaldehyde has been accomplished in this laboratory by Blanton, Magarian, and Varma with secondary amines of varying strength (23). Also, Sam and Thompson (24) isolated a methylene-bis-thiaindanone (X).

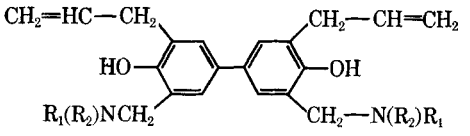
Absolute ethanol was employed in all the reactions with aqueous formaldehyde, and the latter did not appear to encumber the reaction. Also paraformaldehyde is normally used in the synthesis of ketonic Mannich bases (Table VII); however, aqueous formaldehyde was used and the yields were relatively good.

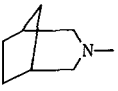
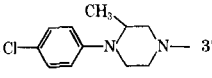
The Mannich products in most cases were easy to obtain, but, in nearly all instances exclusive of the ketonic Mannich bases, the products were difficult to recrystallize. Perhaps this could be attributed to a complex mixture of many products. In some cases, the reaction products (compounds *15* and *17*) obtained from these phenolic compounds were difficult to crystallize. They were dissolved in ethyl ether and extracted with 10% aqueous hydrochloric acid solution. The aqueous solution was neutralized with 10% sodium carbonate solution which was then extracted with ethyl ether and the extract dried over anhydrous magnesium sulfate. The drying agent was removed by filtration and anhydrous hydrogen chloride was bubbled through the anhydrous ether solution to yield a solid. In other instances (compound *19*), 20% aqueous hydrochloric acid provided the right environment to form

[1] The biphenol, 2,2′-diallyl-4,4′-biphenol, was supplied through the courtesy of Dow Chemical Co., Midland, Mich., and the amine, 3-azabicyclo[3.2.1]octane hydrochloride, was prepared by N. D. Potti of this laboratory.

a crystalline hydrochloride from a sticky residue. In another situation a viscous residue (compound *23*) was dissolved in ethyl ether and anhydrous hydrogen chloride bubbled through the solution. This precipitated an oil that crystallized from acetone when placed in the refrigerator overnight.

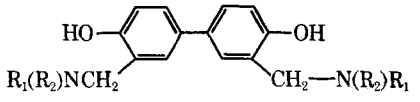
TABLE III—MANNICH BASES (DIALLYLBIPHENOLS)

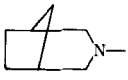
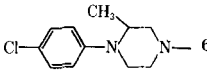
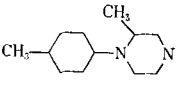


No.	R_1 / R_2	Yield, %	M.p.,[a] °C.	Formula	Anal.[b] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
5	AZBN	38	183–185[c]	$C_{36}H_{48}N_2O_2$	79.96	80.03	8.95	9.16	5.18	5.11
6		50	104–106[d]	$C_{32}H_{44}N_2O_2$	78.65	79.24	9.07	9.20	5.73	5.80
7		38	151–152[e]	$C_{34}H_{44}N_2O_2$	79.65	79.79	8.65	8.78	5.46	5.71
8		37	211–212.5[f]	$C_{42}H_{48}Cl_2N_4O_2$	70.87	71.62	6.80	6.92	7.87	8.03
9		39	141–142[e]	$C_{40}H_{56}N_2O_2$	80.49	80.73	9.46	9.53	4.69	4.77

[a] Melting points are corrected. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. [c] Recrystallized from pyridine–water; [d] isopropanol; [e] absolute ethanol–water; [f] benzene–absolute ethanol.

TABLE IV—MANNICH BASES (BIPHENOLS)

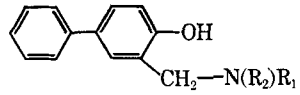
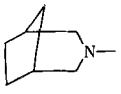
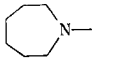
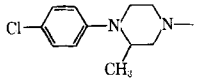


No.	R_1 / R_2	Yield, %	M.p.,[a] °C.	Formula	Anal.[b] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
10	AZBN	63	241–242[c]	$C_{30}H_{40}N_2O_2$	78.22	78.05	8.75	8.84	6.08	6.22
11		86	198.5–200.5[d]	$C_{28}H_{36}N_2O_2$	77.74	77.59	8.39	8.41	6.48	6.72
12		63	116–118[e]	$C_{26}H_{36}N_2O_2$	76.43	76.72	8.88	9.05	6.86	7.16
13		56	132–134[f]	$C_{22}H_{28}N_2O_2$	74.97	75.15	8.01	7.92	7.95	8.26
14		66	275–277[d]	$C_{36}H_{40}Cl_2N_2O_2$	68.45	68.38	6.38	6.46	8.87	8.75
15		98	271.5–273.5[f]	$C_{38}H_{46}N_4O_2$	77.25	77.53	7.85	8.00	9.48	9.70
16		86	220–222[d]	$C_{34}H_{48}N_2O_2$	79.02	78.88	9.36	9.21	5.42	5.62

[a] Melting points are corrected. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. [c] Recrystallized from pyridine; [d] pyridine–absolute ethanol; [e] isopropanol; [f] pyridine–water.

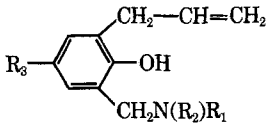
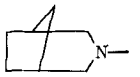
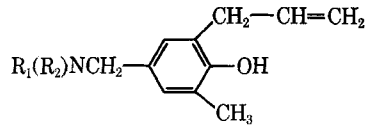
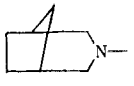
The β-amino ketones were, in general, prepared by refluxing the appropriate ketone, 37% aqueous formaldehyde, and amine hydrochloride in absolute ethanol. The solvent was removed on a rotary

TABLE V—MANNICH BASES (PHENYLPHENOLS)

No.	R_1, R_2	Yield, %	M.p.,[a] °C.	Formula	Anal. C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
										
17	AZBN	65	133–134.5[c]	$C_{21}H_{25}NO$	82.04	82.09	8.20	8.19	4.56	4.61
18		59	102–104[c]	$C_{20}H_{23}NO$	81.87	81.65	7.90	7.86	4.77	5.03
19		69	182.5–184.5[d,f]	$C_{19}H_{24}ClNO$	71.77	71.60	7.61	7.91	4.40	3.79
20		46	147.5–149.0[e]	$C_{24}H_{25}ClN_2O$	73.36	73.25	6.41	6.38	7.13	7.48
21		67	99–101	$C_{23}H_{29}NO$	82.34	82.15	8.71	8.56	4.17	4.18
22	AZBN	33	120–122[c]	$C_{21}H_{25}NO$	82.05	82.08	8.20	8.18	4.56	4.61

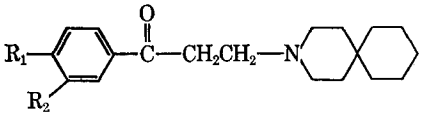
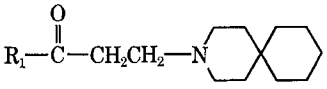
[a] Melting points are corrected. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. [c] Recrystallized from absolute ethanol; [d] isopropanol, [e] absolute ethanol–water. [f] Hydrochloride.

TABLE VI—MANNICH BASE (2-ALLYLPHENOL DERIVATIVES)

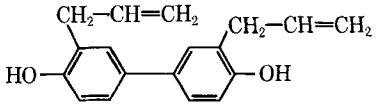
No.	R_1, R_2	R_3	Yield, %	M.p.,[a] °C.	Formula	Anal.[b] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
											
23	AZBN	H	34	218–220[c,f]	$C_{18}H_{26}ClNO^g$	68.21	68.56	8.58	8.68	4.49	4.25
24	AZBN	CH_3	64	92–94[d]	$C_{19}H_{27}NO$	79.95	79.89	9.54	9.52	4.91	4.93
25		CH_3	44	188–188.5[e,f]	$C_{18}H_{26}ClNO$	70.22	70.49	8.51	8.61	4.55	5.20
											
26	AZBN		40	236.5–237[d,f]	$C_{19}H_{28}ClNO$	70.89	71.20	8.77	8.64	4.35	4.26
27			38	220.5–221.5[e,f]	$C_{19}H_{26}ClNO$	70.22	70.19	8.51	8.52	4.55	4.59

[a] Melting points are corrected. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. [c] Recrystallized from methanol–ether; [d] absolute ethanol; [e] absolute ethanol–ether. [f] Hydrochloride. [g] Calculated for 0.5 mole of water.

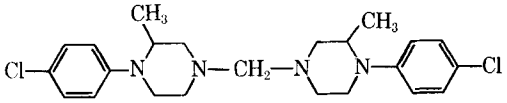
TABLE VII—MANNICH BASES (β-AMINO KETONES)

No.	R_1	R_2	Yield, %	M.p.,[a] °C.	Formula	Anal.[b] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
											
28	NO_2	H	59	222.5–224.5	$C_{19}H_{27}ClN_2O_3$	62.18	62.11	7.42	7.42	7.63	7.49
29	F	H	97	216.5–218.5	$C_{19}H_{27}FClNO$	67.13	67.32	8.00	8.30	4.12	4.15
30	H	NO_2	41	220.5–222	$C_{19}H_{27}ClN_2O_3$	62.18	62.33	7.42	7.45	7.63	7.80
											
31			93	220.5–222.5	$C_{17}H_{26}ClNOS$	62.25	62.54	7.99	8.06	4.27	4.13

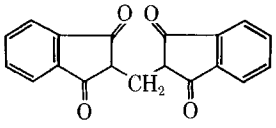
[a] Melting points are corrected. Mannich bases in this table were recrystallized from absolute ethanol. All compounds exist as the hydrochloride. [b] Microanalyses were conducted by Alfred Bernhardt Microanalytisches Laboratorium, Max Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany.



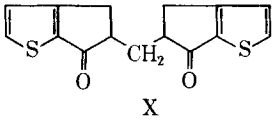
VII



VIII



IX



X

evaporator and the product was recrystallized from absolute ethanol. The reactions proceeded smoothly and the yields were consistently good. These Mannich products were easy to recrystallize in comparison with the phenolic derivatives.

Although the condensations in the Mannich reaction proceed much faster in the higher-boiling solvents, and the formation of certain by-products, obtained by prolonged heating in ethanol, is avoided (3), it is believed that the condensation in ethanol is less subject to side reactions associated with the instability of the aminoketone salts at the higher temperatures (25). The time required for a Mannich reaction depends, among other things, upon the nature of the starting materials and upon the boiling point of the solvent (3); it was found that these reactions in absolute ethanolic solution yielded the desired products after the mixtures had been refluxed for 2–3 hr. An increase in reflux time was of no avail in producing better yields.

Biological Evaluation—When the results of the antibacterial screening tests are available, they will be presented in connection with a subsequent article in this series.

REFERENCES

(1) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 878(1962).
(2) *Ibid.*, **53**, 521(1964).
(3) Blicke, F. F., "Organic Reactions," vol. 1, John Wiley & Sons, New York, N. Y., 1942, p. 303.
(4) DiPalma, J. R., "Drill's Pharmacology in Medicine," McGraw-Hill Book Co., New York, N. Y., 3rd ed., 1965, p. 1376.
(5) Blicke, F. F., "Organic Reactions," vol. 1, John Wiley & Sons, New York, N.Y., 1942, p. 311.
(6) Bruson, H. A., *J. Am. Chem. Soc.*, **58**, 1741(1936).
(7) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Holcomb, W. F., and Rowlins, A. L., *ibid.*, **68**, 1894(1946).
(8) Burke, W. J., *ibid.*, **71**, 609(1949).
(9) Burckhalter, J. H., *ibid.*, **72**, 5309(1950).
(10) Julia, M., and Tchernoff, G., *Bull. Soc. Chim. France*, 830(1955).
(11) Cheronis, N. D., and Entrikin, J. B., "Semimicro Qualitative Organic Analysis," Interscience Publishers, Inc., New York, N. Y., 2nd ed., 1961, p. 273.
(12) Albert, A., Rubbo, S. D., Goldacre, R. J., and Balfour, B. J., *Brit. J. Exptl. Pathol.*, **28**, 69(1947).
(13) Thompson, P. E., Reinertson, J. W., Bayles, A., McCarthy, D. A., and Elslager, E. F., *Am. J. Trop. Med. Hyg.*, **4**, 224(1955).
(14) Burckhalter, J. H., and Edgerton, W. H., *J. Am. Chem. Soc.*, **73**, 4837(1957).
(15) Burckhalter, J. H., U. S. pat. *2,746,963(1956)*.
(16) Burckhalter, J. H., Stephens, V. C., Scarborough, H. C., Jr., Briniger, W. S., and Edgerton, W. H., *J. Am. Chem. Soc.*, **76**, 4902(1954).
(17) Edgerton, W. H., and Burckhalter, J. H., *ibid.*, **74**, 5209(1952).
(18) Schraufstatter, E., and Bock, M., U. S. pat. 2,770,619 (1956).
(19) Sen, A. B., and Arora, S. L., *J. Indian Chem. Soc.*, **35**, 613(1958).
(20) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 86.
(21) Grogan, C. H., Geschickter, C. F., Freed, M. E., and Rice, L. M., *J. Med. Chem.*, **8**, 62(1965).
(22) Magarian, R. A., and Nobles, W. L., *J. Pharm. Sci.*, **56**, 1003(1967).
(23) Varma, R., and Nobles, W. L., *ibid.*, **55**, 1451(1966).
(24) Sam, J., and Thompson, H. C., *ibid.*, **53**, 535(1964).
(25) Ruberg, L., and Small, L., *J. Am. Chem. Soc.*, **63**, 736 (1941).

Antibacterial and Antifungal Properties of β-Naphthol Derivatives VI

By M. L. KHORANA*, S. Y. PANDIT†, and A. D. PISHAWIKAR‡

The bacteriostatic and fungistatic activities of a number of β-naphthol, 2-hydroxy-6-naphthoic acid, and 2-hydroxy-3-naphthoic acid derivatives are reported against *Staphylococcus aureus, Salmonella typhosa,* and *Aspergillus niger.* Selected compounds from the initial screening have been further tested for their activity against *Staphylococcus albus, Salmonella paratyphi* A, *S. paratyphi* B, *Escherichia coli, Shigella dysenteriae, Trichophyton rubrum, Trichophyton gypseum, Candida albicans,* and *Candida tropicalis.* Few compounds exhibited activity against the selected Gram-negative organisms at concentrations below 50 mcg./ml. For *S. aureus* and *S. albus,* the bacteriostatic concentrations of 6-alkyl-, 3-alkyl-, and 1,6-dialkyl-β-naphthols, esters of 2-hydroxy-6-naphthoic acid, and substituted anilides of 1,6-dibromo-, 1-nitro-, and 1-nitro-6-bromo-2-hydroxy-3-naphthoic acid are between 1 and 10 mcg./ml. Marked inhibition of the few fungus pathogens tested is shown by 3-propyl, 3-butyl, 1-methyl-6-ethyl-, 1-methyl-6-propyl- and 1,6-diethyl-β-naphthol, and alkyl esters of 2-hydroxy-6-naphthoic acid.

ANTI-INFECTIVE ACTIVITY of a number of β-naphthol derivatives has been reported previously (1–5) from this laboratory. As a further extension of this work another 200 derivatives have been prepared and tested. The syntheses of these compounds have already been published (6–11).

In the preliminary screening all the compounds were tested against *S. aureus*, *S. typhosa*, and *A. niger*. Various derivatives of 3-keto- (9), 6-keto- β-naphthols (7), 6-naphthyl-alkyl acids (8, 11), dinaphthols (7), *etc.*, are not included in this publication as they showed little activity in the screening.

EXPERIMENTAL

Bacteriostatic Activity

The serial broth dilution method of McKee, Rake, and Menzel (12) was followed, using the organisms *S. aureus* (FDA 209) and *S. typhosa* (Lister). Different concentrations of the test compounds were prepared by adding the requisite quantities of alcoholic (95%) stock solutions to double strength broth to make up the volume to 5 ml. Stock solutions were so prepared that in no case did the quantity of the alcoholic solution added to broth exceed 0.1 ml. A similar quantity of alcohol alone added to the broth did not show bacteriostasis. The results were observed after incubation at 37° for 24 hr. and further confirmed after 48 hr. The minimum inhibition concentrations, in mcg./ml., are recorded in Table I.

Fungistatic Activity

Screening for fungistatic activity against *A. niger* was done following the agar cup-plate (13) as well as the agar-streak (14) methods. The results of the former method gave an idea of the power of diffusion of the substances; that of the latter gave the minimum inhibition concentrations.

Agar Cup-Plate Method (13)—One per cent alcoholic solutions of the compounds were added to prepared cups and the plates were incubated for 48 hr. at 25–27°. The average diameters of the zones of complete inhibition have been recorded in Table I. Alcohol alone gave no zone of inhibition.

Agar Streak Method (14)—Spore suspensions of test organisms were streaked on Sabouraud's agar plates containing the test substances in concentrations of 200, 100, 50, 10, and 5 mcg./ml. Control plates containing alcohol exhibited no inhibition. Results were noted after incubation for 5 days at 25–27° and these have been recorded in Table I.

Antimicrobial Spectra—Compounds which were fairly active in the preliminary screening were next tested against organisms such as *S. aureus, S. albus, S. typhosa, S. paratyphi* A, *S. paratyphi* B, *S. dysenteriae,* and *E. coli.* The results of these tests are presented in Table II.

Similarly, the antifungal activity of these compounds was determined against *T. rubrum* and *T. gypseum* by the agar-streak method, and against *C. tropicalis* and three strains of *C. albicans* by both the serial broth dilution and the agar-streak methods. The methods used were essentially the same as described above except that Sabouraud's glucose broth and glucose agar were used as the culture media.

DISCUSSION

Alkylation increases the bacteriostatic activity of β-naphthol. Compared to 6-alkyl derivatives reported in a previous publication (4), the corresponding 1,6-dialkyl derivatives now tested are more active. The lower members of 1,6-dialkyl derivatives, such as 1-methyl-6-ethyl-, 1,6-diethyl-, and

Received June 13, 1966, from the Department of Chemical Technology, University of Bombay, Bombay, India.
Accepted for publication January 20, 1967.
Abstracted in part from the theses submitted by S. Y. Pandit and A. D. Pishawikar to the University of Bombay in fulfillment of Doctor of Philosophy degree requirements in the Faculty of Technology.
Cultures were obtained from Haffkine Institute, Bombay, K. E. M. Hospital, Bombay, and Indian Institute of Science, Bangalore, India.
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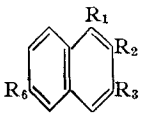
† Present address: Wyeth Laboratories Ltd., Bombay, India.
‡ Present address: Biological Evans Pvt. Ltd., Hyderabad, India.
Previous paper: Baichwal, R. S., Baichwal, M. R., and Khorana, M. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 537 (1958).

TABLE I—BACTERIOSTATIC AND FUNGISTATIC ACTIVITIES OF β-NAPHTHOL DERIVATIVES



Compd.				Activity Against: *S. aureus*[a]	*S. typhosa*[a]	*A. niger*[b]	*A. niger*[a]
	Alkyl-β-naphthols (R_2 = OH)						
	R_1	R_3	R_6				
1	H	H	H	200	100	43	50
2	H	H	(*iso*)C_5H_{11}	10	100	12	[c]
3	H	H	(*iso*)C_6H_{13}	5	100	13.5	[c]
4	H	H	C_7H_{15}	5	100	12	[c]
5	H	CH_3	H	50	100	28	100
6	H	C_2H_5	H	20	20	40	10
7	H	C_3H_7	H	10	20	30	10
8	H	C_4H_9	H	2	20	28	10
9	CH_3	H	H	100	100	42	50
10	C_2H_5	H	H	100	100	45	50
11	C_3H_7	H	H	100	100	40	50
12	CH_3	H	CH_3	20	100	24	50
13	CH_3	H	C_2H_5	10	20	35	20
14	CH_3	H	C_3H_7	5	200	25	20
15	CH_3	H	C_4H_9	2	200	23	200
16	CH_3	H	C_5H_{11}	2	200	18	200
17	CH_3	H	C_6H_{13}	1	200	15	[c]
18	C_2H_5	H	C_2H_5	10	10	35	20
19	C_2H_5	H	C_3H_7	2	10	24	50
20	C_2H_5	H	C_4H_9	2	[c]	19	200
21	C_2H_5	H	C_5H_{11}	1	[c]	15	[c]
22	C_2H_5	H	C_6H_{13}	1	[c]	12	[c]
	Bromo-alkyl-β-naphthols (R_2 = OH)						
	R_1	R_3	R_6				
23	Br	Br	C_2H_5	10	100	28	100
24	Br	Br	C_3H_7	5	100	28	100
25	Br	Br	C_4H_9	2	100	28	[c]
26	Br	Br	C_5H_{11}	10	100	16	[c]
27	Br	Br	C_6H_{13}	10	100	16	[c]
	Ethers (R_6 = H)						
	R_1	R_2	R_3				
28	H	OCH_3	H	200	200	18	[c]
29	CH_3	OCH_3	H	50	100	32	50
30	C_2H_5	OCH_3	H	10	[c]	18	[c]
31	C_2H_5	OC_2H_5	H	10	[c]	20	[c]
32	H	OCH_3	COC_2H_5	5	5	[d]	[c]
	Aldehydes and Derivatives (R_6 = H)						
	R_1	R_2	R_3				
33	H	OH	CHO	50	20	23	100
34	H	OH	CH=N—$C_6H_4Cl(4')$	200	200	[d]	200
35	H	OH	CH=N—$C_6H_4NO_2(4')$	200	200	[d]	200
36	NO_2	OH	CHO	50	50	25	100
37	NO_2	OH	CH=N—C_6H_4Cl	200	200	[d]	[c]
38	H	OH	CH=CH—$COCH_3$	50	200	[d]	[c]
	Esters of 2-Hydroxy-6-naphthoic Acid (R_3 = H)						
	R_1	R_2	R_6				
39	H	OH	$COOCH_3$	50	50	24	100
40	H	OH	$COOC_2H_5$	25	25	22	100
41	H	OH	$COOC_3H_7$	25	25	20	100
42	H	OH	$COOC_4H_9$	25	100	22	...[e]
43	H	OH	$COOC_3H_7$(*iso*)	25	100	18	...
44	H	OH	$COOC_4H_9$(*iso*)	25	100	20	...
45	H	OH	$COOC_6H_5$	50	100	18	...
46	H	OH	$COOC_6H_4OH$ (4′)	100	100	18	...
47	H	OH	$COOC_6H_3OH(3')Cl(4')$	100	100	18	...
48	CH_3	OH	$COOCH_3$	[c]	[c]	14	...
49	CH_3	OH	$COOC_2H_5$	[c]	[c]	22	...
50	CH_3	OH	$COOC_3H_7$	[c]	[c]	14	...
51	CH_3	OH	$COOC_3H_7$(*iso*)	10	[c]	18	...
52	CH_3	OH	$COOC_4H_9$(*iso*)	5	[c]	17	...
53	H	OH	COOH	200	[c]	[d]	...
54	H	OCH_3	COOH	[c]	[c]	[d]	...
55	H	$OCOCH_3$	COOH	200	[c]	[d]	...
56	CH_3	OH	COOH	[c]	[c]	17	...

(*Continued on next page.*)

TABLE I—(*Continued.*)

Compd.	Anilides of 2-Hydroxy-3-naphthoic Acid (R_2 = OH)			Activity Against			
				S. aureus[a]	*S. typhosa*[a]	*A. niger*[a]	*A. niger*[b]
	R_1	R_3	R_6				
57	Br	COOH	Br	[c]	[c]	...	[c]
58	Br	$CONH_2$	Br	100	100	12	[c]
59	Br	CONH—C_6H_5	Br	50	200	...	[c]
60	Br	$CONHC_6H_4OCOCH_3(4')$	Br	25	100	...	[c]
61	Br	$CONHC_6H_4NO_2(4')$	Br	1	100	...	[c]
62	Br	$CONHC_6H_4Cl(4')$	Br	0.5	100	...	[c]
63	Br	$CONHC_6H_3OH(3')$-$COCH_3(4')$	Br	25	100	...	[c]
64	Br	$CONHC_6H_3NO_2(3')CH_3(4')$	Br	2	100	...	[c]
65	Br	$CONHC_6H_4COOC_2H_5(4')$	Br	1	100	14	...
66	Br	$CONHC_4H_3S$	Br	25	100	16	...
67	Br	$CONHC_5H_4N$	Br	25	100	12	....
68	NO_2	COOH	H	[c]	200	...	...
69	NO_2	$CONH_2$	H	200	100	14	...
70	NO_2	$CONHC_6H_5$	H	25	100	20	...
71	NO_2	$CONHC_6H_4NO_2(4')$	H	0.5	100	24	100
72	NO_2	$CONHC_6H_4Cl(4')$	H	25	100	...	...
73	NO_2	$CONHC_6H_4COCH_3(4')$	H	50	100	16	...
74	NO_2	$CONHC_6H_3NO_2(3')CH_3(4')$	H	1	100	...	250
75	NO_2	$CONHC_4H_3S$	H	50	100	20	250
76	NO_2	$CONHC_5H_4N$	H	100	100	14	250
77	NO_2	$CONHC_6H_4COOC_2H_5(4')$	H	50	100	16	...
78	NO_2	COOH	Br	[c]	[c]	[d]	[c]
79	NO_2	$CONHC_6H_5$	Br	10	200	[d]	[c]
80	NO_2	$CONHC_6H_4Cl(4')$	Br	10	200	[d]	[c]
81	NO_2	$CONHC_6H_4Cl(3')$	Br	100	200	[d]	[c]
82	NO_2	$CONHC_6H_4Cl(2')$	Br	200	200	[d]	[c]
83	NO_2	$CONHC_6H_4NO_2(4')$	Br	10	200	[d]	[c]
84	NO_2	$CONHC_6H_4NO_2(3')$	Br	100	200	[d]	[c]
85	NO_2	$CONHC_6H_5OCH_3(4')$	Br	10	200	[d]	[c]

[a] Minimum inhibition concentration (MIC) in mcg./ml. [b] Average diameter of zone of inhibition in mm. with 1% w/v solution. [c] MIC more than 200 mcg./ml. [d] No zone of inhibition. [e] Not tested.

1-ethyl-6-propyl-β-naphthol (compounds *13*, *18*, *19*) are active against all the microorganisms tested, while the higher homologs, such as 1-methyl-6-hexyl-, 1-ethyl-6-pentyl-, and 1-ethyl-6-hexyl-β-naphthol (compounds *17*, *21*, *22*) show specific activity against *S. aureus* and *S. albus* only. The position of the alkyl group has a marked influence. The 3-alkyl derivatives possess activity against a wider microbial spectra than the 1-alkyl-, 6-alkyl-, or 1,6-dialkyl derivatives. Of all the alkyl derivatives screened, 3-butyl-β-naphthol (compound *8*), is the most active compound.

Introduction of bromine in 6-alkyl-β-naphthols shows variable effects. The activity increases as the chain-length is increased to butyl (compound *25*) and then drops down. In general, bromination does not appear to enhance considerably the antimicrobial activity of the parent alkyl compounds.

Conversion of β-naphthol to alkyl ethers has been reported (4) to depress the activity. However, the ethers of 1-alkyl-β-naphthols now tested show enhanced activity especially against *S. aureus* and *S. albus* (compounds *29*, *30*, *31*).

All the ketonaphthols, their methyl ethers, and bromo derivatives tested show poor activity; the only exception is 3-propionyl-2-methoxy-naphthalene (compound *32*) and this compound is bacteriostatic at a concentration of 5 mcg./ml. against *S. aureus* and *S. albus* and some Gram-negative organisms.

2-Hydroxy-3-naphthaldehyde (compound *33*) is much more active than 2-naphthol, itself, against the few Gram-positive and Gram-negative organisms tested. However, introduction of an additional nitro group, conversion of the aldehydes to anils or chalkones depresses the activity considerably.

Introduction of a carboxyl group in β-naphthol has been reported (3) to reduce its bacteriostatic activity. The activity of 1-methyl-2-naphthol (compound *9*) is similarly reduced by the introduction of a carboxyl group (compound *56*). However, esterification of the carboxyl group appears to enhance the antimicrobial activity. Among the straight-chain alkyl esters of 2-hydroxy-6-naphthoic acid studied, the bacteriostatic activity increases until the propyl (compound *41*) derivative is reached, and then it drops. The branched-chain esters are as active as the straight-chain esters against *S. aureus* and *S. albus* (Table II).

The anilides of 2-hydroxy-3-naphthoic acid have been reported (4) to be bacteriostatic at low concentrations against Gram-positive organisms. Various substituted anilides of 1,6-dibromo-, 1-nitro-, and 1-nitro-6-bromo-2-hydroxy-3-naphthoic acids, now studied, exhibit marked activity against *S. aureus* and *S. albus*. The activity varies considerably with the position and nature of the substituent on the aniline ring; in general, the *para*-substituted

TABLE II—ANTIBACTERIAL ACTIVITY OF SELECTED COMPOUNDS[a]

Compd.[b]	*S. aureus*	*S. albus*	*S. typhosa*	*S. paratyphi* A	*S. paratyphi* B	*E. coli*	*S. dysenteriae*
2	10	10	[d]	[d]	[d]	[d]	[d]
3	5	5	[d]	[d]	[d]	[d]	[d]
4	5	5	[d]	[d]	[d]	[d]	[d]
6	20	20	20	20	20	50	50
7	10	10	20	20	20	50	50
8	2	5	20	20	20	50	50
13	10	10	20	50	50	[d]	[d]
14	5	10	[d]	[d]	[d]	[d]	[d]
15	2	5	[d]	...[e]	...	...	...
16	2	2	[d]	...	...	...	...
17	1	1	[d]	...	...	...	...
18	10	10	10	50	50	[d]	[d]
19	2	5	10	...	...	...	...
20	2	2	[c]	...	...	...	...
21	1	1	[c]	...	...	...	...
22	1	1	[c]	...	...	...	...
24	5	5	[d]	[d]	[d]	[d]	[d]
25	2	5	[d]	[d]	[d]	[d]	[d]
32	5	5	5	10	20	20	20
33	50	50	20	20	20	50	20
39	50	50	50	50	50	[d]	50
40	25	25	25	50	50	[d]	50
41	25	25	25	50	50	[d]	50
42	25	25	[d]	...	...	...	...
43	25	25	[d]	...	...	...	...
44	25	25	[d]	...	...	...	...
51	10	10	[c]	...	...	...	...
52	5	5	[c]	...	...	...	...
60	25	25	[c]	...	...	...	...
61	1	1	[c]	...	...	...	...
62	0.5	0.5	[d]	...	...	...	...
65	1	1	[d]	...	...	...	...
71	0.5	0.5	[d]	[d]	[d]	[d]	[d]
74	1	1	[d]	[d]	[d]	[d]	[d]

[a] Minimum inhibition concentration (MIC) in mcg./ml. [b] Numbers refer to compounds listed in Table I. [c] MIC more than 200 mcg./ml. [d] MIC more than 50 mcg./ml. [e] Not tested.

TABLE III—FUNGISTATIC ACTIVITY OF SELECTED COMPOUNDS[a]

Compd.[b]	*A. niger*	*T. rubrum*	*T. gypseum*	*C. albicans* Strain I	*C. albicans* Strain II	*C. albicans* Strain III	*C. Tropicalis*
1	50	50	50				
6	10	10	10				
7	10	5	5	50	50	20	[c]
8	10	5	5	20	20	10	[c]
13	20	50	50	50	50	10	[c]
14	20	20	20	10	10	2	[c]
18	20	50	50	50	50	5	[c]
19	50	50	50	20	50	5	[c]

[a] Minimum inhibition concentration (MIC) in mcg./ml. [b] Numbers refer to compounds listed in Table I. [c] MIC more than 50 mcg./ml.

derivatives show the highest and the *ortho*-substituted ones the lowest activity. Of the *para*-substituted compounds, the chloro and nitro derivatives (compounds *61*, *62*, *71*) are bacteriostatic at 1 mcg./ml.

Table II, which records the activity of the selected compounds against some other microorganisms, shows that while very few compounds are bacteriostatic against the four Gram-negative microorganisms used for the study below the concentration of 20 mcg./ml., several of them are active against *S. aureus* and *S. albus* at fairly low concentrations. The specificity of activity shown by the alkyl derivatives is noteworthy and further data on these compounds will be reported separately.

The fungistatic activity reported in Table I indicates that as the length of the alkyl chain in alkyl naphthols increases, the zone of inhibition against *A. niger* first increases and then decreases, the ethyl derivatives being the most active.

Of the dialkyl derivatives, 1-methyl-6-ethyl-, 1-methyl-6-propyl-, and 1,6-diethyl-β-naphthol (compounds *13*, *14*, *18*) exhibit good activity against *A. niger* and a few other fungi. Of the esters of 2-hydroxy-6-naphthoic acid, the methyl ester (compound *39*) shows the highest activity and the

ethyl and propyl esters (compounds *40*, *41*) come next. However, in general they have lower fungistatic activity than the alkyl naphthols.

Comparing the activities against all the fungi included in the tests (Table III), it is observed that while compounds *8* and *14*, 3-butyl- and 1-methyl-6-propyl-β-naphthol, are fungistatic at 20 mcg./ml., all others are fungistatic only at 50 mcg./ml.

REFERENCES

(1) Baichwal, R. S., and Khorana, M. L., *J. Sci. Ind. Res. (India)*, **11B**, 169(1952).
(2) *Ibid.*, **12B**, 43(1953).
(3) Baichwal, R. S., Baichwal, M. R., and Khorana, M. L., *Indian J. Pharm.*, **16**, 93(1954).
(4) Baichwal, R. S., Baichwal, M. R., and Khorana, M. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 603(1957).
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(8) *Ibid.*, **23**, 297(1961).
(9) Khorana, M. L., and Pandit, S. Y., *J. Indian Chem. Soc.*, **40**, 789(1963).
(10) Khorana, M. L., and Pandit, S. Y., *Indian J. Chem.*, **1**, 489(1963).
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(12) McKee, C. M., Rake, C. P., and Menzel, A. E., *J. Immunol.*, **48**, 259(1944).
(13) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 771(1939).
(14) Schamberg, J. F., and Kolmer, J. A., *Arch. Dermatol. Syphilol.*, **6**, 746(1922).

Effects of Adrenochrome Semicarbazone on Blood Loss in the Mouse

By JACK H. KEITH, WILLIAM W. KEITH, and MARY H. NELSON

A method for determining the blood loss and bleeding time of a standardized wound is described, and it is applied in examining the activities of a clinically-used hemostatic agent—adrenochrome semicarbazone. It is shown that adrenochrome semicarbazone has an effect in decreasing blood loss at doses as low as 0.1 mg./Kg., and is effective in the presence of anticoagulants. A mechanism of action is suggested.

THE DIFFICULTY in showing a decrease in blood loss, clinically or experimentally, is apparent when one attempts to produce a standard wound. Variations of up to 1000% may occur on the same animal with the same size wound.

Roskam (1) measured blood loss from rabbit ears, achieving reproducible results, but the authors found that his method gave insufficient information. The major deficiency in his procedure was the measurement of bleeding times rather than blood loss. Blood loss was assumed to parallel bleeding time, and the authors have found that this is not always true.

After many attempts at determining blood loss, with and without adrenochrome semicarbazone, it was found that blood loss could be most accurately and reproducibly measured in mice. The clipping of 1.5 cm. from the tips of the tails of animals of the same size and sex gives an easily measurable method of determining blood loss.

The mouse tail was used because the vessels are large enough to produce a bleeding condition that could be classified as traumatic hemorrhage. The major vessels in the mouse tail vary from 0.05 mm. to 0.25 mm. in diameter. During the peak period of hemorrhage, the first 30 sec., the blood loss may be as great as 0.2 ml., which would represent 10% of total blood volume.

This paper concerns the effect of adrenochrome semicarbazone upon initial blood loss from the clipped tail of the mouse, and an indication of its mechanism of action. We emphasize the term "initial blood loss," since anticoagulant-treated animals stop bleeding only for a short period, and then begin again.

METHOD

Determination of Blood Loss and Bleeding Time—Male mice[1] weighing 18–22 Gm. were used. The tail was severed 1.5 cm. from the tip with a frequently changed razor blade to ensure keenness. The animals were placed in a holder at least 5 min. before cutting. This holder consisted of a plastic vial 1 in. × 2.5 in. (Fig. 1) with holes for ventilation. In some of the experiments the tails were bled into a beaker containing 100 ml. of deionized water at 37°; 3–5 mice were bled into the same 100 ml. of water containing disodium ethylendiaminetetraacetate, 2 mg./100 ml., as an anticoagulant. The tail was observed until bleeding stopped and was then removed immediately from the water. The

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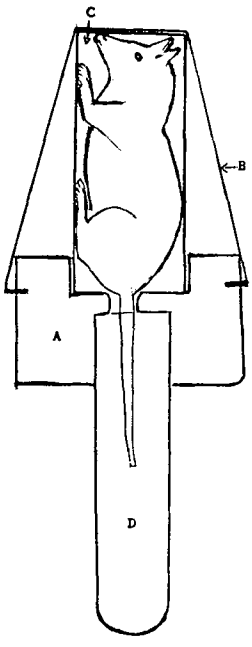


Fig. 1—Holder and tube arrangement for bleeding-time determinations. Key: A, Plexiglas block 5.5 cm.² and 3 cm. thick. When used with a 100-ml. beaker the block is 1.5 cm. thick; B, wire holder, 19 mm. for test tube and 29 mm. for mouse holder; C, plastic vial, 64 mm. × 28 mm.; D, 25-ml. test tube.

dose response experiment varied from this procedure in that the tail was left in the water for 3 min. After bleeding, 1 ml. of concentrated hydrochloric acid was added to the beaker to change the hemoglobin to acid hematin for quantitative measurement. The blood loss was determined from a curve made from a pooled sample of known quantities of blood which was read at 540 mμ on a Bausch & Lomb spectrophotometer (Spectronic 20).

In some of these tests a 25-ml. tube was used in place of the 100-ml. beaker, and only one mouse was bled into each 25 ml. of water.

The mean values in all tests were compared, using the Fisher *t* test for significance (2).

Dose Response—The drug was administered subcutaneously at 0.1, 1, and 5 mg./Kg. Fifteen minutes were allowed for drug action. The mouse tail remained in the water for 3 min.

Adrenochrome Semicarbazone and Heparin Administration—The mice were given 6 mg./Kg. sodium heparin intraperitoneally (119 units/mg.) and 30 min. was allowed for drug action. Adrenochrome semicarbazone was used at a 5 mg./Kg. dosage subcutaneously, allowing 30 min. for drug action.

Adrenochrome Semicarbazone and Bishydroxycoumarin Administration—The effects of bishydroxycoumarin in mice were investigated by administering the drug orally or intraperitoneally daily for 2 to 8 days. One group (A) was given the bishydroxycoumarin in the diet at a level of 0.015% for 8 days. In two of the groups (B), bishydroxycoumarin was given intraperitoneally, 20 mg./Kg. at 0 hr. and 10 mg./Kg. at 24 hr. The mice were bled 24 hr. after the second dose. In three other groups (C), a 10-mg. dose intraperitoneally was given at 0, 24, and 48 hr.; the mice were bled at 72 hr. Adrenochrome semicarbazone, 0.5 mg./ml., was added to the drinking water and 3 mg./Kg. was injected subcutaneously 30 min. before bleeding.

Each group—controls, bishydroxycoumarin, and bishydroxycoumarin–adrenochrome semicarbazone—contained 21 mice. Three mice were bled into the same 100 ml. of water.

Adenosine Diphosphate and Adenosine Administration—These drugs were given at 1 and 30 mg./Kg. intraperitoneally in 2% gelatin solution. Thirty minutes was allowed for drug action. Forty animals were used at each dosage level and compared with 40 controls receiving an equal volume of 2% gelatin solution.

Bishydroxycoumarin Toxicity—One hundred male mice, 22–25 Gm., were given their normal diet *ad libitum* with bishydroxycoumarin 80 mg./100 Gm. of food. Another group of 100 mice received bishydroxycoumarin, 80 mg., and adrenochrome semicarbazone, 60 mg./100 Gm. of food. The number of deaths were recorded daily for 8 days. The drug was removed from the food on the 6th day. These animals were caged in groups of 10. (See Table IV.)

In another test the mice were caged individually and fed the regular diet plus bishydroxycoumarin, 35 mg./100 Gm. of food. One group was given adrenochrome semicarbazone at a level of 20 mg./100 Gm. of food. The drugs were administered for 12 days. Each of the two groups contained 75 animals. (See Table V.)

The daily accumulative deaths were graphed using the Litchfield–Wilcoxon (3) method to analyze the LD effects for possible difference. The day of death rather than the drug consumption was used as the abscissa. The LD_{50} is based on the number of days after beginning drug administration.

Heparin Toxicity—Male mice, 22–25 Gm., were injected with adrenochrome semicarbazone subcutaneously, 5 mg./Kg. in a volume of 0.5 ml./100 Gm. body weight. The adrenochrome semicarbazone was given 5–10 min. before intravenous administration of sodium heparin. The tails were cut with a razor blade 2.5–3 cm. from the base immediately after the injection of the heparin. Those animals given only heparin were given a volume of physiological saline, 0.5 ml./100 Gm., subcutaneously. The sodium heparin was dissolved in 0.85% sodium chloride solution and administered at a volume of 1 ml./100 Gm. of body weight. The dosage levels of heparin were 500, 1000, 1500, 2000, and 3000 units/Kg. The animals, in groups of 10, were placed in plastic boxes 5 × 6 × 10 in. with shavings on the floor. The results were graphed, and the Litchfield-Wilcoxon (3) method of comparison was used to determine significance of difference in the two treatments.

RESULTS AND DISCUSSION

The end point in these experiments is easily determined, but accounting for the wide variations in blood loss is a complex, unsolved problem. The day-by-day differences in groups often reaches a multiple of three in treated and untreated alike.

Dose Response—Table I shows a dose response obtained with adrenochrome semicarbazone as seen in the mouse tail experiment. To make this test more objective, the blood from each mouse was collected for a period of 3 min. at which time the

TABLE I—EFFECT OF ADRENOCHROME SEMICARBAZONE ON BLOOD LOSS IN NORMAL MICE[a]—A DOSE RESPONSE

	Controls	Adrenochrome Semicarbazone 0.1 mg./Kg.	1 mg./Kg.	5 mg./Kg.
Blood Loss, ml.	.195	.107	.085	.087
S.D.	.130	.084	.041	.051
T Value		5.5[b]	4.5[c]	
Significance		.01	.01	

[a] Number of animals used: controls, 74; adrenochrome semicarbazone 0.1 mg./Kg., 101; 1 mg./Kg., 33; 5 mg./Kg., 39. [b] As compared to controls. [c] As compared to 0.1 mg./Kg.

TABLE II—EFFECTS OF ADRENOCHROME SEMICARBAZONE IN HEPARIN-TREATED MICE[a]

	Controls	Heparin, 6 mg./Kg.	Heparin, 6 mg./Kg., and Adrenochrome Semicarbazone, 5 mg./Kg.
Blood loss, ml.	0.142	0.114	0.086
T value		1.4[b]	2.1[c]
Significance		0.2	0.05
Bleeding time, sec.	100	138	114
T value		2.4[b]	2.5[c]
Significance		0.01	0.02

[a] Each group contained 81 animals which were bled in groups of three. [b] As compared to controls. [c] As compared to heparin alone.

TABLE III—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON LD EFFECT OF HEPARIN IN TRAUMATIZED MICE

Heparin, Units/Kg. i.v.	Heparin[a]	Heparin and Adrenochrome Semicarbazone, 5 mg./Kg.[a]
500	1/10	1/10
1000	2/10	4/10
1500	12/20	13/20
2000	15/20	17/20
3000	9/10	10/10
LD_{50}	1150 Units	1400 Units
	Potency ratio $= \frac{1400}{1150} = 1.2$	
	Factor of potency ratio = 1.22	

[a] Number dead/number used.

tail was removed from the water. The 3-min. time period is considerably longer than the normal bleeding time. The dosage of 0.1 mg./Kg. is in the same range as that used clinically.

With a drop in blood loss of 45% at 0.1 mg./Kg., it might be concluded that a lower dose would have effected a dose response. Constantinescu (4) *et al.* have found activity at much lower doses in rats. They compared blood loss from the tail in anesthetized rats, 6 groups of 40 each, and found a 64% reduction in blood loss at 5 mcg./Kg. adrenochrome semicarbazone.

Heparin—Table II shows that adrenochrome semicarbazone decreases blood loss in heparin-treated mice. Bleeding time is significantly increased by heparin, but the initial blood loss is less than that of the controls. The blood pressure is lowered by doses of heparin larger than those used in this experiment, which in itself will reduce initial blood flow. The authors have observed in previous experiments that a dose of 25 mg./Kg. of heparin will cause initial blood loss to fall to a very low level. Care was exercised in all the experiments to rule out an effect due to a drop in blood pressure. Mice given 6 mg./Kg. doses of heparin occasionally bleed to death after the tail is severed. The blood stops flowing, but a clot does not form and the bleeding resumes. If the tail is observed for long periods, there are numerous periods of spontaneous bleeding. Although there is a decrease in the initial blood loss, the LD_{50} of heparin in mice is not changed when adrenochrome semicarbazone is administered. In addition to the anticoagulant effect of heparin, which exceeds 1 hr. in these experiments, Zucker (5) and Solandt and Best (6) have shown that the activity of platelets is greatly decreased.

To determine an LD_{50} with heparin, a standard wound is produced by severing the tail 2.5–3 cm. from the base. Those animals that succumb do so in a period of 2–12 hr. The wound greatly reduces the amount of heparin necessary for a lethal dose. The authors had previously noted that doses of heparin, 4000–12,000 units/Kg., do not produce death, but often a semishock state is produced. In many mice there is a massive fluid exudate in the peritoneal cavity. Because of this fluid loss, the dosage was kept below 4000 units/Kg. and a trauma severe enough to cause death was used.

The LD_{50} for the heparin–adrenochrome combination was 1150 units/Kg., and 1400 units/Kg. for the heparin only group. (See Table III.) The difference of 350 units/Kg. in toxicity was not significant at the 95% confidence limits.

Bishydroxycoumarin—When bleeding time and blood loss is examined in groups of mice given bishydroxycoumarin and adrenochrome semicarbazone (Table VI), there is a significant decrease in blood loss in these animals as compared with bishydroxycoumarin-treated animals, with the bleeding time returned to normal. An exception was noted in group A: some of these animals had died and the remainder were in severely hemorrhagic condition. Hemorrhagic areas were present around the shoulders and testicles, and in the peritoneal cavity. The bleeding time increased significantly with bishydroxycoumarin, but blood loss did not reflect this increase. The results seen here with bishydroxycoumarin agree with those seen by Chen and Tsai (7). They state that in their animals (frogs and rabbits) given bishydroxycoumarin the initial bleeding was stopped by vasoconstriction, but bleeding started again when the constricted vessels relaxed. Without intervention death often resulted.

As indicated in Tables II and III, the failure of bishydroxycoumarin and heparin to increase the initial blood loss is a convincing demonstration of the importance of factors other than clotting mechanisms in the initial retardation of blood flow.

The ability of adrenochrome semicarbazone to reduce initial blood loss in heparin- or bishydroxycoumarin-treated animals clearly indicates activity in the absence of effective clotting components.

TABLE IV—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BISHYDROXYCOUMARIN LD_{50} EFFECT

Days[a]	Bishydroxycoumarin, 80 mg./100 Gm. Food No. Dying	A.P.D.[b]	Bishydroxycoumarin, 80 mg., and Adrenochrome Semicarbazone, 20 mg./100 Gm. Food No. Dying	A.P.D.[b]
3	5	5	7	7
4	9	14	9	16
5	8	22	14	30
6	20	42	9	39
7	10	52	12	51
8	2	54	4	55
Deaths	54		55	
Time of LD_{50}, days	7.6		7.25	
Time ratio	7.6/7.25 = 1.047			
Factor of time ratio	1.28			

[a] Number of days of drug administration. [b] Accumulative per cent deaths.

TABLE V—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BISHYDROXYCOUMARIN LD_{50} EFFECT

Days[a]	Bishydroxycoumarin 35 mg./100 Gm. Food No. Dying	A.P.D.[b]	Bishydroxycoumarin, 35 mg., and Adrenochrome Semicarbazone, 20 mg./100 Gm. Food No. Dying	A.P.D.[b]
4	3	4	6	8
5	3	8	8	18
6	24	40	26	53.5
7	1	41	1	54.5
8	2	44	4	60
9	3	59	3	64
10	17	71	3	68
11	1	72	9	79
12	1	74	1	80
Deaths	55		60	
Time of LD_{50}, days	6.7		6	
Time ratio	6.7/6 = 1.17			
Factor of time ratio	1.25			

[a] Number of days of drug administration. [b] Accumulative per cent deaths.

The toxicity of bishydroxycoumarin requires several days to be manifest. (See Tables IV and V.) Thus the drug was administered in the diet. Food consumption was normal the first 3 days with a sharp drop after that. There was no difference in quantity of food consumed by the 2 groups. The animals receiving bishydroxycoumarin, 35 mg./100 Gm. of food, were not less affected than those given 80 mg./100 Gm. of food. This was not expected, since the animals at 35 mg. were caged individually. The authors anticipated that individual contact would intensify hemorrhage, causing a more rapid death. It may be that grouping together helps the animals to maintain normal temperature. The time required for bishydroxycoumarin to produce death in 50% of the mice was not reduced by adrenochrome semicarbazone.

Since adrenochrome semicarbazone fails to reduce mortality in heparin- or bishydroxycoumarin-treated animals, the activity would have to be other than an antiheparin or increased coagulation effect. The mechanism left to consider would be an effect on increasing the efficiency of the platelet plug formation or potentiation of the local vasoconstrictor effect.

Adenosine and Adenosine Diphosphate—Stewart and Brooks (8) presented information suggesting that adrenochrome semicarbazone produced a hemostatic effect in irradiated rats by increasing the activity of platelets. In the authors' test, the effects appeared to be separate from the platelet plug effect. This is not to infer that adrenochrome semicarbazone does or does not have an effect on or in conjunction with platelets, but that adrenochrome semicarbazone will promote hemostasis in the absence of the platelet plug.

Johnson (9) *et al.* have recently observed the mechanism of hemostasis in vessels of 50–160 μ by sectioning the hemostatic plug. Their plug was composed of red cells enmeshed in fibrin. Platelet aggregation did not occur until after this plug formed.

Other evidence which helps us rule out more efficient plug formation as the basic method of adrenochrome semicarbazone activity was obtained with adenosine and adenosine diphosphate. Adenosine diphosphate, a physiological substance known to produce *in vivo* clumping of platelets (10–12) and the subsequent platelet plug, was used to test the effect of increased platelet activity. With adenosine diphosphate, the initial blood loss was not decreased. Adenosine is a competitive inhibitor (12) of adenosine diphosphate and thereby blocks the formation of the platelet plug. Adenosine did not increase the initial blood loss. These results strongly suggest that the hemostatic effect of adrenochrome semicarbazone in the experiments was not in the more rapid building of the platelet plug but on the local vascular contraction at the area of the trauma.

TABLE VI—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BLEEDING TIME AND BLOOD LOSS IN BISHYDROXYCOUMARIN-TREATED MICE

Group	Controls B.T., sec.	Controls B.L., ml.	Bishydroxycoumarin B.T., sec.	Bishydroxycoumarin B.L., ml.	Bishydroxycoumarin and Adrenochrome Semicarbazone B.T., sec.	Bishydroxycoumarin and Adrenochrome Semicarbazone B.L., ml.
A[a]	99	.392	128	.120	109	.119
B[b]	97	.200	130	.342	81	.024
B	118	.159	138	.157	105	.054
C[c]	101	.255	158	.319	123	.140
C	97	.319	138	.391	103	.239
C	128	.335	93	.178	108	.107
Av.	106	.276	130	.251	104	.114
T Value		3.8		2.5		
Significance		.01		.05		

Received bishydroxycoumarin for 8 days. [b] Bishydroxycoumarin 20 mg./Kg. at 0 hr., 10 mg./Kg. at 24 hr.; bled at 48 hr. [c] 10 mg. at 0, 24, 48 hr.; bled at 72 hr.

SUMMARY

Adrenochrome semicarbazone has been shown to decrease blood loss in normal mice whose tails have been clipped. The decrease in blood loss does not correlate with bleeding time. The bleeding times increased with anticoagulants but initial blood loss did not change significantly.

Heparin and bishydroxycoumarin have been used to deplete or block the blood stream of necessary clotting components and in these animals adrenochrome semicarbazone can be shown to decrease the blood loss when measured to cessation of the initial blood flow. Adrenochrome semicarbazone will not increase rate of survival in animals lacking a necessary amount of clotting components.

The failure of adenosine and adenosine diphosphate, heparin, and bishydroxycoumarin to influence initial blood loss indicates that in the tail vessel of the mouse the primary hemostatic consideration is that of local vasoconstriction. Adrenochrome semicarbazone appears to augment this constrictor effect.

REFERENCES

(1) Roskam, J., "L'Hemostase Spontanee," Masson & Cie Editeor, Paris, France, pp. 63–64, 81–84.
(2) Fisher, R. A., "Statistical Methods for Research Work," Oliver & Boyd, London, England, 1950, p. 122.
(3) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(4) Constantinescu, M., Stoian, F., Papa, V., and Copae, L., *Pharmazie*, **16**, 458(1961).
(5) Zucker, M. B., *Am. J. Physiol.*, **148**, 275(1947).
(6) Solandt, D., and Best, C., *Lancet*, **1**, 1042(1942).
(7) Chen, T. L., and Tsai, C., *J. Physiol.*, **107**, 280 (1948).
(8) Brooks, A. M., and Stewart, G. J., *Fed. Proc. Abstr.*, **25**, 2898(1966).
(9) Johnson, S. A., Fredell, L. M., Shepard, J. A., Tebo, T. H., Pedrson, H. J., and VanHorn, D. L., Fourteenth Annual Symposium on Blood, Wayne State University of Medicine, January 1966, p. 6.
(10) Spaet, T. H., *ibid.*, p. 16.
(11) Bambel, D. E., *ibid.*, p. 16.
(12) Haslam, R. J., *ibid.*, p. 10.

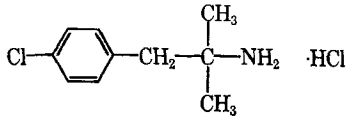
Drug Standards

Qualitative and Quantitative Tests for Chlorphentermine Hydrochloride

By EDWARD F. SALIM*, J. RITNER WEAVER†, and LESTER CHAFETZ†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

p-CHLORO-α,α-DIMETHYLPHENETHYLAMINE HYDROCHLORIDE; $C_{10}H_{14}ClN.HCl$; mol. wt. 220.14. The structural formula of chlorphentermine hydrochloride may be represented as:



Physical Properties—Chlorphentermine hydrochloride occurs as an odorless, white to off-white powder with a bitter taste, m.p. 232–235° (U.S.P. class I). It is freely soluble in water and in alcohol, sparingly soluble in chloroform, and practically insoluble in ether. The pH of a solution of chlorphentermine hydrochloride in carbon dioxide-free water (1 in 100) is between 5.0 and 6.0.

Received August 16, 1966, from the * Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication May 10, 1967.

† Warner-Chilcott Laboratories, Morris Plains, NJ 07950.

Warner-Chilcott Laboratories has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The Drug Standards Laboratory gratefully acknowledges the assistance of Miss Carolyn Damon and Miss Hannah Klein.

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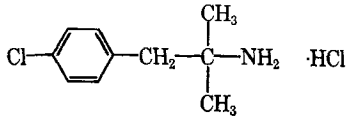

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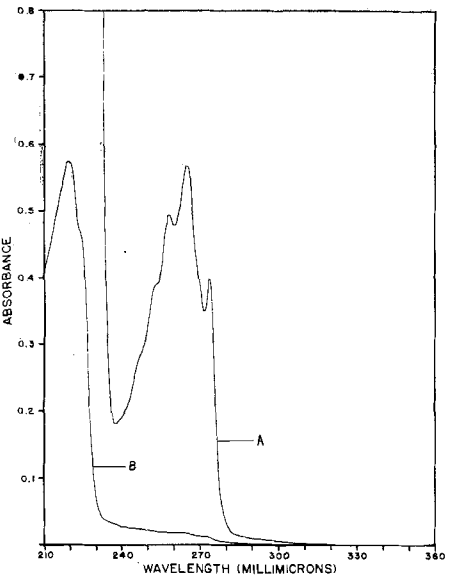


Fig. 1—Ultraviolet absorption spectra of chlorphentermine hydrochloride in water; Beckman model DK-2A spectrophotometer. Key: A, *500 mcg./ml.;* B, *10 mcg./ml.*

Identity Tests—Dissolve about 50 mg. of chlorphentermine hydrochloride in 3 ml. of water, add ammonia T.S. until basic, and filter. Acidify the filtrate with diluted nitric acid and add 1 ml. of silver nitrate T.S.: a white precipitate forms, which is insoluble in diluted nitric acid but soluble in ammonia T.S. (presence of chloride).

A 1 in 2000 solution of chlorphentermine hydrochloride in water exhibits ultraviolet maxima at about 259, 268, and 278 mμ, and absorbance minima at about 239, 261, and 275 mμ. A 1 to 50 dilution of this solution exhibits an absorbance maximum at about 220 mμ [absorptivity (a) about 52]. The spectrum at each concentration is shown in Fig. 1.

The infrared spectrum of a chlorphentermine hydrochloride dispersion in liquid petrolatum is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of chlorphentermine hydrochloride, accurately weighed, at 105° for 4 hr.: it loses not more than 1% of its weight.

Char about 1 Gm. of chlorphentermine hydrochloride, accurately weighed, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the heavy metals content of chlorphentermine hydrochloride by the U.S.P. heavy metals test, method II: the heavy metals limit for chlorphentermine hydrochloride is 20 p.p.m.

Assay—Transfer about 400 mg. of chlorphentermine hydrochloride, accurately weighed, to a tall-form 200-ml. beaker, and add 25 ml. of glacial acetic acid and 10 ml. of mercuric acetate T.S. Warm slightly to effect solution, cool, add 2 drops of crystal violet T.S., and titrate with 0.1 N acetous perchloric acid to a blue-green end point. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 N perchloric acid is equivalent to 22.01 mg. of $C_{10}H_{14}ClN.HCl$.

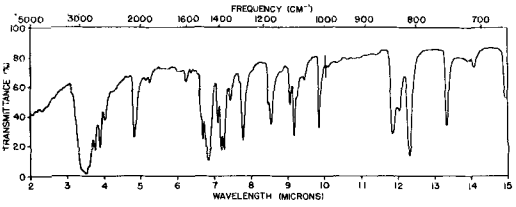


Fig. 2—Infrared spectrum of chlorphentermine hydrochloride dispersion in liquid petrolatum; Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

The amount of chlorphentermine hydrochloride found is not less than 98.0% and not more than 102.0%.

DOSAGE FORMS OF HYDROCHLORIDE CHLORPHENTERMINE

Chlorphentermine Hydrochloride Tablets

Identity Test—The filtrate from the original tablet solution obtained in the *Assay* exhibits absorbance maxima and minima at the same wavelengths as a solution of chlorphentermine hydrochloride standard in 0.01 N hydrochloric acid at a concentration of about 500 mcg./ml.

Assay—Weigh and finely powder not less than 10 chlorphentermine hydrochloride tablets. Weigh accurately a portion of the powder, equivalent to about 500 mg. of chlorphentermine hydrochloride, and transfer to a 1000-ml. volumetric flask. Add about 500 ml. of 0.01 N hydrochloric acid and warm on a steam bath for about 15 min. with intermittent agitation. Cool, dilute to volume with 0.01 N hydrochloric acid, and mix. Filter the solution through paper discarding the first 50 ml. of filtrate. Transfer 20.0 ml. of clear filtrate into a 125-ml. separator, add 1 ml. of sodium hydroxide T.S., and extract with four 20-ml. portions of ether, collecting the ether extracts in a second separator. Extract the ether phase with two 40-ml. portions of 0.01 N hydrochloric acid, combine the acid extracts in a 100-ml. volumetric flask, dilute with the acid to volume, and mix. Dilute 10.0 ml. of this solution to 100.0 ml. with the acid, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of chlorphentermine hydrochloride, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 220 mμ, with a suitable spectrophotometer, using 0.01 N hydrochloric acid as the blank. Calculate the quantity, in milligrams, of $C_{10}H_{14}ClN$ in the portion of tablets taken by the formula $50\ C\ (A_u/A_s) \times 0.834$, in which C is the exact concentration of the standard solution, in mcg./ml., A_u is the absorbance of the sample solution, A_s is the absorbance of the chlorphentermine hydrochloride standard solution, and 0.834 is the conversion factor relating chlorphentermine base to chlorphentermine hydrochloride. The amount of chlorphentermine base found is not less than 95.0% and not more than 105.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility,

melting range, reagents, *etc.*, has been used wherever feasible.

Chlorphentermine hydrochloride,[1] an anorectic agent indicated for treatment of obesity, is distinguished by a selective pattern of pharmacologic action. This pattern of activity differs qualitatively and quantitatively from the amphetamines, whose indiscriminate effects at anorectic doses on the cardiovascular system and central nervous system are well known. Unlike the amphetamines, chlorphentermine hydrochloride exhibits no adverse effect on blood pressure, heart rate, or blood sugar.

The tablet formulation is a sustained action product so prepared that the medication is released gradually and without interruption over an 8-hr. period. The tablets are labeled to contain 65 mg. of chlorphentermine base which is equivalent to 78 mg. of the hydrochloride salt.

Identity Tests—The chloride test and ultraviolet absorption spectrum are not sufficient for differentiation of chlorphentermine hydrochloride from other sympathomimetic amine hydrochlorides. However, this information together with the infrared spectrum and melting range for chlorphentermine hydrochloride does provide a satisfactory identification. Mason *et al.* (1) have published a comprehensive report dealing with the physical properties of the drug.

Quantitative Methods—The nonaqueous titration for chlorphentermine hydrochloride with perchloric acid using crystal violet T.S. gave an average value of 99.9 ± 0.2%.[2] Analysis of commercial chlorphentermine hydrochloride tablets by the spectrophotometric method gave an average value of 100.5 ± 1.5%.[2] The suitability of the procedure was verified by an average recovery of 100.2 ± 0.3%[2] for a standard chlorphentermine hydrochloride solution carried through the extractive steps as included for the tablets.

[1] Marketed as Pre-Sate by Warner-Chilcott Laboratories, Division of Warner-Lambert Pharmaceutical Co., Inc., Morris Plains, N. J.

REFERENCE

(1) Mason, M. F., Foerster, E., Patterson, W., and Drummon, W., *J. Forensic Sci.*, 11, 243(1966).

[2] Maximum deviation from the mean value.

Notes

Novel Disubstituted Mannich Product as a Potential Anti-Infective Agent

By ROBERT A. MAGARIAN and W. LEWIS NOBLES

Identification of α,α′-di-{3-(3-azabicyclo[3.2.2]-nonyl)-methyl}-*p*-nitroacetophenone is reported.

THE ISOLATION of a new disubstituted Mannich product was mentioned in a previous publication (1). The Mannich reaction, involving the reactive hydrogen compound acetophenone (I), is shown and illustrates the replacement of one of the active hydrogen atoms by an aminomethyl group which may arise from formaldehyde and the amine.

The Mannich product from a methyl ketone (II) contains labile hydrogens, and, in some cases, it is possible to proceed a step further yielding a compound with two basic groups (III) (2).

The amine hydrochloride is usually employed to provide the proper acidic environment. Cummings and Shelton (3) and Hellmann and Opitz (4) have carried out kinetic studies of the Mannich reaction and have concluded that the active aminomethylating agent is the electrophilic carbonium-immonium ion ($R_2\overset{+}{N}{-}\overset{+}{C}H_2 \leftrightarrow R_2\overset{+}{N}{=}CH_2$) which arises only under acidic conditions from the aminomethylol (*N*-hydroxymethylamine) derivative R_2NCH_2OH. The nucleophile is thought to be the enolic tautomer of the ketone which condenses with the aminomethylating agent.

When the free amine is used in equimolar amounts with the other reactants, a dibasic Mannich product may be isolated. The mechanism of the Mannich reaction has not been completely elucidated; it is considered unlikely that a single mechanism can be postulated which will include all known cases involving this reaction. In these studies, the Mannich condensation involving equimolar amounts of *p*-nitroacetophenone, 37% aqueous formaldehyde, and 3-azabicyclo[3.2.2]nonane was first conducted by modifying the conditions set forth by Blanton and Nobles (5); that is, the free base rather than the hydrochloride salt of the amine was employed.

Received December 29, 1967, from the Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Mississippi, University, MS 38677
Accepted for publication May 17, 1967.

melting range, reagents, *etc.*, has been used wherever feasible.

Chlorphentermine hydrochloride,[1] an anorectic agent indicated for treatment of obesity, is distinguished by a selective pattern of pharmacologic action. This pattern of activity differs qualitatively and quantitatively from the amphetamines, whose indiscriminate effects at anorectic doses on the cardiovascular system and central nervous system are well known. Unlike the amphetamines, chlorphentermine hydrochloride exhibits no adverse effect on blood pressure, heart rate, or blood sugar.

The tablet formulation is a sustained action product so prepared that the medication is released gradually and without interruption over an 8-hr. period. The tablets are labeled to contain 65 mg. of chlorphentermine base which is equivalent to 78 mg. of the hydrochloride salt.

Identity Tests—The chloride test and ultraviolet absorption spectrum are not sufficient for differentiation of chlorphentermine hydrochloride from other sympathomimetic amine hydrochlorides. However, this information together with the infrared spectrum and melting range for chlorphentermine hydrochloride does provide a satisfactory identification. Mason *et al.* (1) have published a comprehensive report dealing with the physical properties of the drug.

Quantitative Methods—The nonaqueous titration for chlorphentermine hydrochloride with perchloric acid using crystal violet T.S. gave an average value of 99.9 ± 0.2%.[2] Analysis of commercial chlorphentermine hydrochloride tablets by the spectrophotometric method gave an average value of 100.5 ± 1.5%.[2] The suitability of the procedure was verified by an average recovery of 100.2 ± 0.3%[2] for a standard chlorphentermine hydrochloride solution carried through the extractive steps as included for the tablets.

[1] Marketed as Pre-Sate by Warner-Chilcott Laboratories, Division of Warner-Lambert Pharmaceutical Co., Inc., Morris Plains, N. J.

REFERENCE

(1) Mason, M. F., Foerster, E., Patterson, W., and Drummon, W., *J. Forensic Sci.*, 11, 243(1966).

[2] Maximum deviation from the mean value.

Notes

Novel Disubstituted Mannich Product as a Potential Anti-Infective Agent

By ROBERT A. MAGARIAN and W. LEWIS NOBLES

Identification of α,α′-di-{3-(3-azabicyclo[3.2.2]-nonyl)-methyl}-*p*-nitroacetophenone is reported.

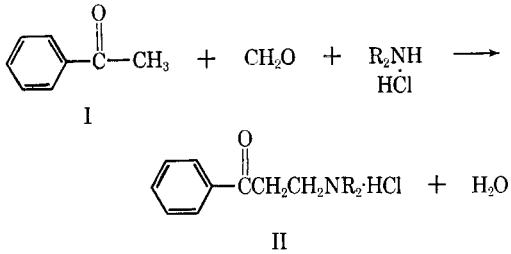
THE ISOLATION of a new disubstituted Mannich product was mentioned in a previous publication (1). The Mannich reaction, involving the reactive hydrogen compound acetophenone (I), is shown and illustrates the replacement of one of the active hydrogen atoms by an aminomethyl group which may arise from formaldehyde and the amine.

The Mannich product from a methyl ketone (II) contains labile hydrogens, and, in some cases, it is possible to proceed a step further yielding a compound with two basic groups (III) (2).

The amine hydrochloride is usually employed to provide the proper acidic environment. Cummings and Shelton (3) and Hellmann and Opitz (4) have carried out kinetic studies of the Mannich reaction and have concluded that the active aminomethylating agent is the electrophilic carbonium-immonium ion ($R_2\overset{+}{N}{-}\overset{+}{C}H_2 \leftrightarrow R_2\overset{+}{N}{=}CH_2$) which arises only under acidic conditions from the aminomethylol (*N*-hydroxymethylamine) derivative R_2NCH_2OH. The nucleophile is thought to be the enolic tautomer of the ketone which condenses with the aminomethylating agent.

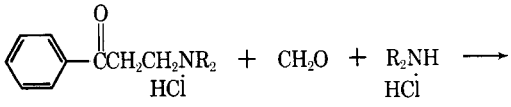
When the free amine is used in equimolar amounts with the other reactants, a dibasic Mannich product may be isolated. The mechanism of the Mannich reaction has not been completely elucidated; it is considered unlikely that a single mechanism can be postulated which will include all known cases involving this reaction. In these studies, the Mannich condensation involving equimolar amounts of *p*-nitroacetophenone, 37% aqueous formaldehyde, and 3-azabicyclo[3.2.2]nonane was first conducted by modifying the conditions set forth by Blanton and Nobles (5); that is, the free base rather than the hydrochloride salt of the amine was employed.

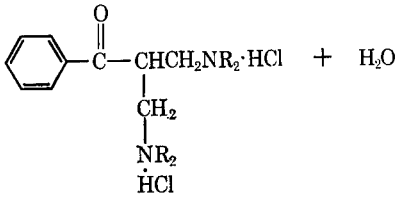
Received December 29, 1967, from the Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Mississippi, University, MS 38677
Accepted for publication May 17, 1967.



I

II





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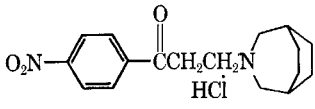

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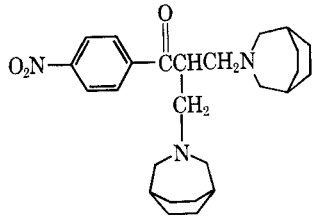

V

Consequently, the product (IV) as reported by Blanton and Nobles was not isolated. Instead, a bright yellow solid was obtained in 41% yield with a melting point of 148–152° (corrected). Recrystallization several times from absolute ethanol–acetone resulted in a product melting at 158–160° (corrected). The starting ketone melted at 79–82° (corrected) and the melting point of a mixture of the above product and the ketone was 76–79° (corrected). The product was shown to be the disubstituted derivative (V) as evidenced by elemental, infared, and nuclear magnetic resonance analyses.[1]

Anal.—Calcd. for $C_{26}H_{37}N_3O_3$ (mol. wt. 439.6): C, 71.04; H, 8.48; N, 9.56. Found: C, 70.87; H, 8.55; N, 9.46.

[1] Microanalyses were conducted by Alfred Bernhardt, Microanalytisches Laboratorium, Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany. Infrared spectra were determined on a Perkin-Elmer model 137G Infracord spectrophotometer using potassium bromide disks. The Varian A-60(mc) was employed in obtaining the nuclear magnetic resonance spectra.

The infrared spectrum showed two strong nitro bands at 1520 cm.$^{-1}$ and 1350 cm.$^{-1}$. There was a strong carbonyl band at 1675 cm.$^{-1}$. The relative proton absorption of the NMR spectrum in deuterochloroform was in agreement with the proposed structure. Neither the ketonic nor enolic tautomers seemed to predominate. The phenyl proton pattern might have been partially collapsed by this tautomeric exchange. The NMR spectrum in trifluoroacetic acid showed the low resolution expected from the Stark effect due to a doubly charged ion.

Subsequent to the discovery of the antibacterial activity of the Mannich bases derived from *p*-nitroacetophenone (6), interest was aroused in the reduction of this compound to the corresponding carbinol in order to study the effect of this variation in structure on the biological activity of such a compound when appearing as a side chain in quinoline and acridine derivatives. Consequently, product V was reduced to the corresponding carbinol by the method of Chaikin and Brown (7) as modified by Nobles (8) using sodium borohydride. The reaction proceeded smoothly, and a light yellow solid was isolated in 67% yield with a melting point of 163–165° (corrected). Recrystallization from absolute ethanol resulted in a very light yellow, almost cream-colored, solid with a melting point of 163–164° (corrected). Two additional recrystallizations yielded a white solid with a melting point of 163–164° (corrected). A 2,4-dinitrophenylhydrazine test performed on the product was negative. The melting point of a mixture of the above product and the ketone was 140–144° (corrected).

Anal.—Calcd. for $C_{26}H_{39}N_3O_3$ (mol. wt. 441.6): C, 70.71; H, 8.90; N, 9.52. Found: C, 70.19; H, 8.90; N, 9.19.

Inspection of the infrared spectrum revealed that carbonyl absorption at 1675 cm.$^{-1}$ was absent, and a hydroxy band was present at 3400 cm.$^{-1}$. Also, there were two strong nitro bands at 1520 cm.$^{-1}$ and 1350 cm.$^{-1}$. The relative proton absorption in the NMR spectrum performed in deuterochloroform showed that the disubstituted alcohol was the product. Also, the NMR spectrum indicated a reduction from two electron-withdrawing groups on a benzene ring to only one; *i.e.*, the keto and nitro groups were present in the keto compound, but after keto reduction the only electron-withdrawing group on the benzene ring was the nitro group. The NMR spectrum in trifluoroacetic acid showed low resolution due to the Stark effect from two protonated nitrogens. Due to the optically active carbinol carbon atom, the cyclohexyl ring peaks are separated farther part—103 and 113 c.p.s.

The Mannich reaction involving equimolar amounts of *p*-nitroacetophenone, 37% aqueous formaldehyde, and 3-azabicyclo[3.2.2]nonane was repeated; however, this time the hydrochloride salt of the amine was used and the desired product IV was isolated as reported by Blanton and Nobles (5).

REFERENCES

(1) Magarian, R. A., and Nobles, W. L., *J. Pharm. Sci.*, **56**, 987(1967).
(2) Blicke, F. F., "Organic Reactions," vol. 1, John Wiley & Sons, New York, N. Y., 1942, p. 304.
(3) Cummings, T. F., and Shelton, J. R., *J. Org. Chem.*, **25**, 419(1960).
(4) Hellmann, H., and Opitz, G., *Chem. Ber.*, **90**, 15 (1957).

(5) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, 51, 878(1962).
(6) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 42.
(7) Chaikin, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, 71, 122(1949).
(8) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 86.

Estimation of Amphetamine in Urine of Race Horses

By M. S. KARAWYA, M. A. EL-KIEY, S. K. WAHBA, and A. R. KOZMAN

Separation of amphetamine from urine of doped horses was achieved on anion-exchange resin (Na^+) and Silica Gel G plates. A micro method for the estimation of the free base was performed by measuring the violet color developed after nitration of the amphetamine eluted from either an ion-exchange resin column or Silica Gel G plates.

In Egypt in 1961, the menace of doping with amphetamine hovered over the horseraces and seriously threatened the results of the race. Since then, the race authorities have decided to stand against this new doping invader.

The method of analysis of amphetamine adopted by the B.P. (1) depends on steam distillation, while that of the E.P. (2) makes use of the direct extraction with ether.

The biological fluids contain normally some steam volatile and ether extractable bases (3) which may interfere in the determination of amphetamine. This fact renders the official methods not applicable for the separation and determination of amphetamine in urine of doped animals.

In an attempt to eliminate these interfering substances, ion-exchange and thin-layer chromatographic techniques were adopted.

Tampsett (4) was able to elute amphetamine on a cationic ion-exchange resin[1] column. He used 400 ml. of 8.0 *N* hydrochloric acid to achieve complete recovery. However, on trying this method, the eluate was found to be highly colored, and the colorimetric assay of the eluted amphetamine was almost impossible.

Materials—The materials used in this experiment were horse urine containing amphetamine (100 mcg./100 ml.); a column of ion-exchange resin[2] (Na^+) (1 × 15 cm.); and eluents used in the following succession: (*a*) 0.5% ammonium hydroxide, (*b*) acetone–water (3:7), (*c*) distilled water, (*d*) 10% aqueous solution of potassium chloride–1 *N* hydrochloric acid (1:1).

EXPERIMENTAL

Separation of Amphetamine on Ion-Exchange Resin (Na^+)

In the present work an anion-exchange resin[2] (Na^+) was chosen for the quantitative elution of amphetamine from horse urine.

Procedure—Add the urine sample to the resin column. Remove the impurities by washing successively with 50 ml. of each of the above-mentioned eluents (*a*), (*b*), and (*c*), and then elute the amphetamine with 150 ml. of eluent (*d*). Render the eluate alkaline with ammonium hydroxide T.S., extract the free base with chloroform (4 × 25 ml.), evaporate the chloroform, and keep the residue for the colorimetric estimation as described later. The results in Table I show the following observations.

A—The successive washing of the column with 0.5% ammonium hydroxide, acetone–water (3:7), and distilled water achieves the removal of most of the urine pigments and other impurities but not amphetamine.

B—Complete recovery of amphetamine is effected by washing the ion-exchange resin column with 150 ml. of 10% potassium chloride–1 *N* hydrochloric acid (1:1).

Separation of Amphetamine on Silica Gel G Plates

Separation of amphetamine from the saliva on silica gel plates was performed by Baeumler *et al.* (5) using triethanolamine–acetone–methanol (0.03:1:1). However, it was found difficult to separate amphetamine from urine on adopting the same procedure.

Other systems were tried and the system *n*-butanol–glacial acetic acid–water (4:1:5) proved to effect good separation of amphetamine from urine pigments, R_f values being 0.65 and 0.95, respectively.

Amphetamine is located with bromocresol blue T.S. when a blue color is obtained.

Procedure—Amphetamine is first extracted from urine before its application on the plate as follows.

Table I—Recovery of Amphetamine from Horse Urine by Columns of Ion-Exchange Resin (Na^+)

Fraction of Effluent	100 mcg./100 ml. Urine: Ion-Exchange Resin	100 mcg./100 ml. Urine: Color of Effluent
Eluent (*a*) (50 ml.)	...	Yellow
Eluent (*b*) (50 ml.)	...	Yellow
Eluent (*c*) (50 ml.)	...	Yellow
Eluent (*d*) (50 ml.)	58.20	Colorless
Eluent (*d*) (50 ml.)	30.15	Colorless
Eluent (*d*) (50 ml.)	13.20	Colorless
8 *N* HCl (400 ml.)	...	Colorless
Total	101.55	

Received January 9, 1967, from the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt, U. A. R.
Accepted for publication March 30, 1967.

[1] Dowex-50, Dow Chemical Co., Midland, Mich.
[2] Amberlite IRC-50, Rohm & Haas, Philadelphia, Pa.

(5) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, 51, 878(1962).
(6) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 42.
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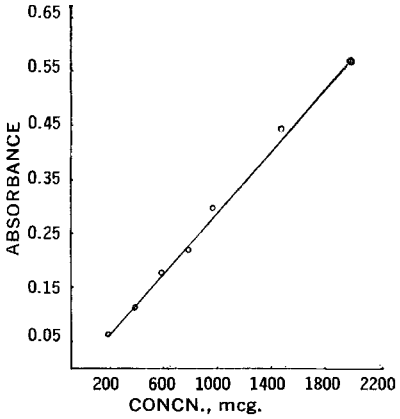


Fig. 1—Absorbance/concentration curve of amphetamine.

Introduce 50 ml. of urine to a separator and render it alkaline to litmus by the gradual addition of ammonium hydroxide T.S. solution. Extract the liberated base with chloroform (4 × 20 ml.), washing each portion with the same 5 ml. of water. Dehydrate the combined chloroformic extract with anhydrous sodium sulfate, washing the sodium sulfate with fresh chloroform and add the washing to the mother extract. Adjust the combined chloroformic extract to a suitable known volume according to the concentration of amphetamine. Another sample of urine free of amphetamine was treated and extracted as described above to be used as a blank.

By means of an Agla micrometer syringe apply the chloroform extracts of both experiment and blank on the starting line of a Silica Gel G plate, 2 cm. apart. Apply on each side of the starting line a pilot spot of a chloroformic solution of authentic amphetamine. The amphetamine content of any spot lies between 50–250 mcg. Insert the plate in the chromatographic jar and develop by the organic layer of the solvent system. In order to locate the amphetamine, spray the two peripheral pilot spots with bromocresol T.S. after covering the central part of the plate with a sheet of glass. Scrape the nonsprayed areas enclosed between and at the level of the located pilot spots with a thin spatula. Collect the powder obtained from each area on a glazed paper and transfer it quantitatively to a micro column (0.4 × 10 cm.). Wash each column with 10 ml. of ethanol.

Evaporate the ethanol cautiously, and keep the residues corresponding to experiment and blank for the colorimetric estimation.

Colorimetric Determination of the Eluted Amphetamine from Resin Columns and Silica Gel Plates

The nitration test (6) is used as the basis of the colorimetric estimation of amphetamine after making the following modification.

A 0.5% amount of potassium hydroxide in methanol–acetone (97:3) is used instead of 10% aqueous sodium hydroxide which does not allow stability of color. The procedure is run as follows.

To the residue in a test tube, add 0.1 Gm. of potassium nitrate and 2 ml. of sulfuric acid. Heat in a boiling water bath for 15 min., then cool. Transfer the liquid quantitatively with distilled water to a separator, wash the liquid with chloroform, reject the chloroform, make the aqueous solution alkaline with the gradual addition of ammonium hydroxide, and extract with chloroform. Evaporate the chloroformic extract in an evaporating dish and dissolve the residue in 10 ml. of acetone. Transfer the acetone solution to a test tube, add 2 drops of 0.5% potassium hydroxide in methanol–acetone (97:3), and allow to stand for 10 min. Measure the developed purple color in a Unicam SP 1300 colorimeter using No. 1 filter (370–515 mμ) against the blank experiment. The results are deduced from an absorbance/concentration curve (Fig. 1), from which it is clear that the color obeys Beer's law in the range of 50–250 mcg. of amphetamine.

The color reaches its maximum after 10 min. and remains stable for another 15 min.

The colorimetric method was applied to residues obtained from both ion-exchange column and chromatoplates using urine samples containing variable amounts of amphetamine. The results are shown in Table II.

From Table II, it is clear that amphetamine can be recovered quantitatively from either the ion-exchange resin (Na^+) column or Silica Gel G plate, with an error of +3.6 to +5.0 and −0.8 to +2.5%, respectively.

Tracing of Amphetamine in Urine of Doped Horses

In order to trace amphetamine in urine of doped

Table II—Recovery of Amphetamine from Ion-Exchange Column and Silica Plates

Quantity Added, mcg.	Quantity Recovered from Column	Error, %	Quantity Recovered from TLC	Error, %
100	105	+5.0	100	0.0
150	155	+3.6	152	+1.3
200	210	+5.0	205	+2.5
250	260	+4.0	248	−0.8

Table III—Excretion Rate of Amphetamine in Horse Urine

Time of Collection of Urine	Mean Vol. of Collected Urine, ml.	% with Respect to Injected Dose, 100 mg. 1st Horse	2nd Horse	3rd Horse	Mean
24 hr. before drugging	3000	..	..	..	...
3 hr. after drugging	300	16	18	17	17.0
12 hr. after drugging	400	14	15	12	13.6
24 hr. after drugging	1350	18	17	19	18.0
48 hr. after drugging	2300	6	7	8	7.0
72 hr. after drugging	3400	..	..	..	...
Total		54	57	56	55.6

horses three horses weighing between 250 and 300 Kg. were chosen. The animals were kept in a clean stable under veterinary medical observation[3] for 3 days before the administration of the drug and were examined constantly to ascertain their normal state of health. During the 24 hr. prior to administration of amphetamine, urine was collected from the three horses in leather bags tied under their bodies. Each animal was then injected subcutaneously with 100 mg. amphetamine sulfate. The urine of each animal was collected periodically after 3, 12, 24, 48, and 72 hr. by means of a sterile catheter. In addition, a clean leather bag was tied under each horse to collect any urine which might pass off between the specified collection times. The urine samples were pale yellow, turbid, and possessed a pH 5.9–6.3.

The urine samples were made alkaline with ammonium hydroxide T.S., shaken with chloroform, the extract chromatographed by TLC, and the separated amphetamine was colorimetrically assayed as described above.

The urine collected before the administration of amphetamine was used as a blank. The results are shown in Table III, from which it is clear that about 55.6% of the administered amphetamine was excreted in the urine of horses within 48 hr. after the administration of amphetamine. During this period, amphetamine could be detected by thin-layer chromatography; after 48 hr. it was hardly detectable and had almost disappeared after 72 hr.

[3] The authors thank Dr. A. Hanna, Faculty of Veterinary Medicine, Cairo University, for his valuable help.

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 43.
(2) "Egyptian Pharmacopoeia," Cairo University, Cairo, Egypt, U. A. R., 1953, p. 126.
(3) "Clinical Biochemistry," 6th ed., W. B. Saunders and Co., London, England, 1962, pp. 383–394.
(4) Tompsett, S. L., *Acta Pharmacol. Toxicol.*, **17**, 295 (1960).
(5) Baeumler, J., Brault, A. L., and Obersteg, J. I., *Schweiz. Arch. Tierheilk.*, **106**, 346(1964).
(6) Rathenasinkam, E., *Analyst*, **76**, 155(1951).

Antibacterial Activity of Some Substituted Benzofurans. Preliminary Study of Structure Activity Relationship

By P. K. SHARMA, KALA MEHTA, O. P. GUPTA, M. M. MAHAWAR, and S. K. MUKERJI

Sensitivity of three substituted nitrobenzofurans, 4-methyl-6-phenylbenzofuran, 2(*p*-nitrophenyl)benzofuran, and 2-aceto-5-methyl-7-nitrophenylbenzofuran, against *Staphylococci, Pyocyanea,* and *B. coli* was studied. A possible structure activity relationship has been worked out, which suggested that the sensitivity of benzofurans is due to nitro substitution. Sensitivity is reduced by substitution of aceto and methyl groups.

NITROFURANS have been widely studied for their antibacterial activity (1–3). A series of substituted benzofurans and nitrobenzofurans were synthesized and tested for their antibacterial activity.

The reaction of *p*-nitrobenzyl bromide with an *O*-hydroxy carbonyl compound in presence of anhydrous potassium carbonate in methanol yields a 2-(*p*-nitrophenyl) benzofuran (4, 5). This reaction was utilized to prepare a few benzofurans required for pharmacological studies.

The reaction of benzyl bromide with hydroxymethyl propiophenone and *p*-nitrobenzyl bromide with 2-hydroxypropiophenone and 2-hydroxy-3-aceto-6-methylpropiophenone in methanol, in the presence of anhydrous potassium carbonate from 45 min. to 1 hr., furnishes the respective benzyloxy and *p*-nitrobenzyloxy derivatives. When allowed to react further with anhydrous potassium carbonate in methanol under reflux (time varying from 6–9 hr.), they yield the respective benzofurans—*viz.*, 4-methyl-6-phenylbenzofuran (a pasty substance) (I), 2-(*p*-nitrophenyl) benzofuran (m.p. 193°) (II), and 2-aceto-5-methyl-7-nitrophenylbenzofuran (m.p. 68°) (III). All the three compounds are completely soluble in alcohol, acetone, and ether.

Received August 4, 1966, from R. N. T. Medical College, Udaipur, Rajasthan, India.
Accepted for publication November 7, 1966.

EXPERIMENTAL

Aqueous suspensions of these compounds were prepared in 1% polysorbate 80,[1] the wetting agent being used to compensate for low aqueous solubility. Control experiments were also performed with the same solvent.

The bactericidal property of the test agents (I, II, and III) was studied against *Staphylococci, Pyocyanea,* using nutrient agar media and *B. coli* on MacConkey's agar media, by the use of impregnated filter paper disks to determine the bacterial sensitivity for the compounds under study.

The disks were soaked in the solutions, the activity of which was to be tested, placed on the media, and incubated overnight at 37°. The diameters of the circular areas of inhibited growth were observed and percentage of inhibition was calculated.

RESULTS AND DISCUSSION

The results of these observations are presented in Table I. From the results it is evident that compound II is the most active against all three test

[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

horses three horses weighing between 250 and 300 Kg. were chosen. The animals were kept in a clean stable under veterinary medical observation[3] for 3 days before the administration of the drug and were examined constantly to ascertain their normal state of health. During the 24 hr. prior to administration of amphetamine, urine was collected from the three horses in leather bags tied under their bodies. Each animal was then injected subcutaneously with 100 mg. amphetamine sulfate. The urine of each animal was collected periodically after 3, 12, 24, 48, and 72 hr. by means of a sterile catheter. In addition, a clean leather bag was tied under each horse to collect any urine which might pass off between the specified collection times. The urine samples were pale yellow, turbid, and possessed a pH 5.9–6.3.

The urine samples were made alkaline with ammonium hydroxide T.S., shaken with chloroform, the extract chromatographed by TLC, and the separated amphetamine was colorimetrically assayed as described above.

The urine collected before the administration of amphetamine was used as a blank. The results are shown in Table III, from which it is clear that about 55.6% of the administered amphetamine was excreted in the urine of horses within 48 hr. after the administration of amphetamine. During this period, amphetamine could be detected by thin-layer chromatography; after 48 hr. it was hardly detectable and had almost disappeared after 72 hr.

[3] The authors thank Dr. A. Hanna, Faculty of Veterinary Medicine, Cairo University, for his valuable help.

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 43.
(2) "Egyptian Pharmacopoeia," Cairo University, Cairo, Egypt, U. A. R., 1953, p. 126.
(3) "Clinical Biochemistry," 6th ed., W. B. Saunders and Co., London, England, 1962, pp. 383–394.
(4) Tompsett, S. L., *Acta Pharmacol. Toxicol.*, **17**, 295 (1960).
(5) Baeumler, J., Brault, A. L., and Obersteg, J. I., *Schweiz. Arch. Tierheilk.*, **106**, 346(1964).
(6) Rathenasinkam, E., *Analyst*, **76**, 155(1951).

Antibacterial Activity of Some Substituted Benzofurans. Preliminary Study of Structure Activity Relationship

By P. K. SHARMA, KALA MEHTA, O. P. GUPTA, M. M. MAHAWAR, and S. K. MUKERJI

Sensitivity of three substituted nitrobenzofurans, 4-methyl-6-phenylbenzofuran, 2(*p*-nitrophenyl)benzofuran, and 2-aceto-5-methyl-7-nitrophenylbenzofuran, against *Staphylococci, Pyocyanea,* and *B. coli* was studied. A possible structure activity relationship has been worked out, which suggested that the sensitivity of benzofurans is due to nitro substitution. Sensitivity is reduced by substitution of aceto and methyl groups.

NITROFURANS have been widely studied for their antibacterial activity (1–3). A series of substituted benzofurans and nitrobenzofurans were synthesized and tested for their antibacterial activity.

The reaction of *p*-nitrobenzyl bromide with an *O*-hydroxy carbonyl compound in presence of anhydrous potassium carbonate in methanol yields a 2-(*p*-nitrophenyl) benzofuran (4, 5). This reaction was utilized to prepare a few benzofurans required for pharmacological studies.

The reaction of benzyl bromide with hydroxymethyl propiophenone and *p*-nitrobenzyl bromide with 2-hydroxypropiophenone and 2-hydroxy-3-aceto-6-methylpropiophenone in methanol, in the presence of anhydrous potassium carbonate from 45 min. to 1 hr., furnishes the respective benzyloxy and *p*-nitrobenzyloxy derivatives. When allowed to react further with anhydrous potassium carbonate in methanol under reflux (time varying from 6–9 hr.), they yield the respective benzofurans—*viz.*, 4-methyl-6-phenylbenzofuran (a pasty substance) (I), 2-(*p*-nitrophenyl) benzofuran (m.p. 193°) (II), and 2-aceto-5-methyl-7-nitrophenylbenzofuran (m.p. 68°) (III). All the three compounds are completely soluble in alcohol, acetone, and ether.

Received August 4, 1966, from R. N. T. Medical College, Udaipur, Rajasthan, India.
Accepted for publication November 7, 1966.

EXPERIMENTAL

Aqueous suspensions of these compounds were prepared in 1% polysorbate 80,[1] the wetting agent being used to compensate for low aqueous solubility. Control experiments were also performed with the same solvent.

The bactericidal property of the test agents (I, II, and III) was studied against *Staphylococci, Pyocyanea,* using nutrient agar media and *B. coli* on MacConkey's agar media, by the use of impregnated filter paper disks to determine the bacterial sensitivity for the compounds under study.

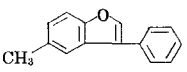
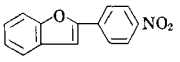
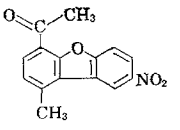
The disks were soaked in the solutions, the activity of which was to be tested, placed on the media, and incubated overnight at 37°. The diameters of the circular areas of inhibited growth were observed and percentage of inhibition was calculated.

RESULTS AND DISCUSSION

The results of these observations are presented in Table I. From the results it is evident that compound II is the most active against all three test

[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE I—RESULTS OF ANTIBACTERIAL ACTIVITY OF COMPOUNDS

No.	Compd.	Chemical Structure	Bacteria	Concn., mcg./ml.	% Inhibition of Growth[a]
I	4-Methyl-6-phenyl-benzofuran		*Staphylococci*	0.25	0
				0.50	0
				1.00	0
			Pyocyanea	0.25	0
				0.50	0
				1.00	10
			B. coli	0.25	0
				0.50	0
				1.00	0
II	2-(*p*-Nitrophenyl) benzofuran		*Staphylococci*	0.25	80
				0.50	100
				1.00	100
			Pyocyanea	0.25	50
				0.50	80
				1.00	100
			B. coli	0.25	100
				0.50	100
				1.00	100
III	2-Aceto-5-methyl-7-nitrophenyl-benzofuran		*Staphylococci*	0.25	50
				0.50	50
				1.00	100
			Pyocyanea	0.25	0
				0.50	0
				1.00	100
			B. coli	0.25	0
				0.50	50
				1.00	100

[a] No inhibition of growth was observed with control.

species of bacteria. Compound III is active against *Staphylococci* but inactive against *Pyocyanea* and *B. coli* in low concentrations (0.25 and 0.50 mcg./ml.) and sensitive in higher concentration (1.0 mcg./ml.). Compound I is practically resistant to all the three types of bacteria. This suggests that the nitro group is essential for antibacterial activity of the benzofurans (compound II) while the sensitivity is reduced by the substitution of methyl and aceto groups (compound III).

REFERENCES

(1) Saikawa, I., and Suzuki, Y., *J. Pharm. Soc. Japan*, **84**, 212, 646 (1964).

(2) Buckett, W. R., and Kidd, D., *J. Pharm. Pharmacol.*, **16**, 663 (1964).

(3) Miura, K., Ohashi, T., Matsuda, S., and Igarashi, Y., *J. Pharm. Soc. Japan*, **83**, 778(1963).

(4) Mathur, K. B. L., and Mehra, J. C., *J. Chem. Soc.*, **1960**, 1954.

(5) Deohra, D. S., and Mukerji, S. K., *J. Indian Chem. Soc.*, **40**, 817(1963).

(6) Cruickshank, R., "Hand Book of Bacteriology," E. & S. Livingstone Ltd., Edinburgh and London, 1962, 10th ed., pp. 187, 195, 217.

Solubilization of Lecithin in Antibiotic Preparations by a Nonionic Surfactant

By FRANCES W. BOWMAN, EVERETT W. KNOLL, and MIRIAM P. CALHOUN

A nonionic surfactant (alkyl phenoxy polyethoxy ethanol) solubilizes antibiotic preparations containing up to 1 per cent lecithin. Toxicity studies showed that the surfactant at a concentration of 0.1 per cent (v/v) in 0.1 per cent peptone solution (w/v) permits recovery from a low inoculum of vegetative cells when membrane filtration sterility tests are performed. The addition of the surfactant to peptone diluting fluid makes it possible to perform membrane filtration tests on many antibiotic preparations that otherwise could only be tested by the direct method. The use of surfactant–peptone diluting fluid also makes it possible to use 300 mg. from each of 20 vials instead of 50 mg. when testing oxytetracycline, chlortetracycline, or tetracycline for sterility.

In 1964, the Antibiotic Regulations were amended (1) to permit the testing of the sterility of antibiotics by membrane filtration (2), in addition to the direct test. Membrane filtration is now specified in 51 of the 121 monographs, the direct method in 41, and either in 29.

In the direct method, the antibiotic must be inactivated so that it does not interfere in the test, *e.g.*, penicillin is inactivated by the addition of the enzyme penicillinase. Thus the direct method is unsuitable for preparations containing penicillin combined with streptomycin or dihydrostreptomycin for which there are no suitable inactivators.

The membrane filtration technique, on the other hand, requires that the test material be soluble enough to pass through the pores of the membrane filter without clogging them; therefore, its use is limited to materials that are soluble or can be solubilized in some way. For example, in combination with streptomycin or dihydrostreptomycin, mentioned above, the slightly soluble procaine penicillin G is converted by the addition of penicillinase to soluble penicilloic acid until its concentration is reduced to about 5 mg./ml. At this concentration the antibiotic is dissolved (3). Since streptomycin and dihydrostreptomycin are both in solution, the formulation may then be filtered without difficulty.

However, problems are presented by formulations containing emulsifying agents, such as sodium carboxymethylcellulose or lecithin, a water-dispersible phospholipid with strongly polar groups. These compounds are used as suspending and/or homogenizing agents to produce oil-in-water emulsions of antibiotics that are rather insoluble in water. Although these agents improve the resuspendability and syringeability of the aqueous suspensions of antibiotics, the resulting suspensions are not completely soluble at the concentrations used in the membrane filtration test, and they tend to clog the filter. The clogged membrane will either stop the filtration or greatly extend the filtration time. In either case, a filtration sterility test cannot be performed.

Because of these difficulties, a study was undertaken to devise a reliable method for solubilizing injectable preparations containing lecithin. The first approach involved the use of lecithinase to hydrolyze lecithin, in analogy with the use of penicillinase to convert procaine penicillin G to a soluble substance. However, this approach was unsuccessful.

The authors then explored the use of a polyoxyethylene nonionic surfactant (alkyl phenoxy polyethoxy ethanol).[1] Russomanno and Wollish (4) reported using this surfactant to wash and remove oil from the membrane following a filtration sterility test for oils. Since lecithin is used to produce oil-in-water emulsions of antibiotics, it seemed feasible that the surfactant could be used to solubilize these emulsions so that they would be suitable for membrane filtration sterility testing. This approach proved more successful.

EXPERIMENTAL

In the usual membrane filtration sterility test, the sample is added to a sterile 500-ml. conical flask containing approximately 200 ml. of a 0.1% peptone (w/v) solution. The flask is stoppered and swirled to dissolve the drug. The solution is then filtered aseptically.

Ten different antibiotic formulations containing varying amounts of lecithin were used in the tests with the surfactant (see Table I). When 0.1% peptone alone was used as the diluting fluid, the preparations were insoluble and could not be filtered. Adding the surfactant at 0.1% (v/v) to the peptone diluting fluid solubilized each of the preparations so that they could be filtered through a 0.45-μ membrane filter. The total time required to filter the antibiotic preparation and make the three washings did not exceed 20 min.

The solubility of lecithin in 0.1% surfactant–peptone solution was determined on samples of commercial grade lecithin used by two antibiotic manufacturers. Lecithin was added to the surfactant–peptone diluting fluid at 25° until the fluid became turbid. Additional surfactant–peptone diluting fluid was added until the turbidity disappeared. This titration procedure established that 0.5 mg./ml. of lecithin is completely soluble in 0.1% peptone solution containing the surfactant at a concentration of 0.1%. Apparently the successful use of filtration sterility tests for preparations containing lecithin depends on the final concentration of lecithin in the diluting fluid after the preparation has been pooled as directed in the Antibiotic Regulations (1). If this final concentration of lecithin in surfactant–peptone diluting fluid does not exceed

Received March 1, 1967, from the Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington, DC 20204
Accepted for publication May 16, 1967.

[1] Available as Triton X-100 from Rohm and Haas, Philadelphia, Pa.

TABLE I—ANTIBIOTIC PREPARATIONS CONTAINING VARYING AMOUNTS OF LECITHIN SOLUBILIZED BY THE SURFACTANT FOR THE FILTRATION STERILITY TEST

Preparation	% Lecithin in Formulation
Procaine penicillin G in streptomycin sulfate soln. (1.0% monothioglycerol)	0.5
Procaine penicillin G and buffered sodium penicillin G for aqueous suspension	0.1
Procaine penicillin G in dihydrostreptomycin soln.	0.1
Procaine penicillin G in dihydrostreptomycin soln.	0.25
Procaine penicillin G, potassium penicillin G, and streptomycin sulfate for aqueous injection	1.0
Procaine penicillin G and buffered potassium penicillin G for aqueous injection	0.3
Procaine penicillin G, potassium penicillin G, and streptomycin sulfate for aqueous injection	0.2
Procaine penicillin in dihydrostreptomycin with prednisolone	0.28
Procaine penicillin G in aqueous suspension	1.0
Chloroprocaine penicillin O for aqueous injection	1.5

0.5 mg./ml., the solutions filter rapidly through a 0.45-μ membrane, whereas the same amount of lecithin is insoluble in peptone diluting fluid alone, and cannot be filtered satisfactorily. Furthermore, when up to 2 mg./ml. of lecithin is added to surfactant–peptone diluting fluid alone, the solution can be filtered, even though it is cloudy; yet when this concentration of lecithin is combined with actual samples containing antibiotics and other components, results are variable: sometimes the solutions can be filtered, sometimes they cannot.

The surfactant was also found to be useful in the filtration procedures for antibiotics that do not contain lecithin. In the standard filtration procedure, a total of 6 Gm. (300 mg. from each of 20 containers) of the antibiotic is added to 200 ml. of the peptone diluting fluid, resulting in a 3% solution. Some antibiotics, such as the tetracyclines, cannot be filtered at this concentration; only 50 mg. can be taken from each container instead of 300 mg., and therefore only 1 Gm., a 0.5% solution, can be tested. When surfactant was added to the peptone diluting fluid, the usual 3% solution could be filtered and the standard amount of the antibiotic could be tested for sterility.

When the filtration procedures for sterility testing were developed, 0.1% peptone was selected as the diluting fluid because it protected the vulnerable vegetative cells in an antibiotic milieu. Before proposing that the surfactant be added to this diluting fluid, it was necessary to investigate its toxicity to microorganisms. Russomanno and Wollish (4) reported that it was nontoxic when in contact with vegetative forms of bacteria for several hours. Dowben and Koehler (5), in a study on the interaction of the surfactant with bovine plasma, found that no gross structural changes occur in the protein, *i.e.*, proteins are not denatured. The following study on the survival of eight microorganisms in surfactant–peptone solution confirms these findings.

TABLE II—SURVIVAL OF ORGANISMS IN SURFACTANT–PEPTONE SOLUTIONS FOR 30 min. AT 25° AS DETERMINED BY MEMBRANE FILTRATION STERILITY TESTS

Organism	Estimated No. Cells of Inoculum	Day Showing Growth After Incubation: Thioglycollate at 32°	Day Showing Growth After Incubation: Sabouraud at 25°
Bacillus subtilis ATCC 6633	44	3	6
Bacillus cereus ATCC 11778	52	2	3
Staphylococcus aureus ATCC 6538P	132	3	6
Sarcina lutea ATCC 9341	66	3	4
Streptococcus faecalis	160	2	3
Klebsiella pneumoniae ATCC 10031	88	1	3
Pseudomonas sp. ATCC 19146	154	3	3[a]
Saccharomyces cerevisiae ATCC 9763	64	3	2

[a] pH was adjusted to 7.0 since this organism will not grow at pH 5.7 ± 0.1.

Eight organisms representing Gram-positive and negative bacilli, Gram-positive cocci, and yeasts were used. A stock suspension of each organism was prepared, and a viable cell count was made by the plate method. The stock suspensions were further diluted with 0.1% peptone so that 1 ml. contained from 40 to 160 cells. One milliliter of the dilute suspension was added to each of 10 flasks containing 200 ml. of surfactant–peptone solution, and the flasks were held at room temperature. After a 30-min. contact period, the viability of the organisms added to the surfactant–peptone was determined by the membrane filtration sterility tests (1). Table II shows that all microorganisms were recovered from small inocula in both thioglycollate and Sabouraud liquid media after incubation for 1–5 days.

REFERENCES

(1) 21 CFR, Section 141.2, *Federal Register*, **29**, 4119 (March 28, 1964).
(2) Holdowsky, S., *Antibiot. Chemotherapy*, **2**, 49(1957).
(3) Bowman, F. W., *J. Pharm. Sci.*, **52**, 705(1963).
(4) Russomanno, R., and Wollish, E. G., *ibid.*, **53**, 1538 (1964).
(5) Dowben, R. M., and Koehler, W. R., *Arch. Biochem. Biophys.*, **93**, 496(1961).

Determination of Impurities in Phenacetin by Thin-Layer Chromatography

By PAUL TURI and JERRY POLESUK

A rapid and sensitive method has been devised to detect acetanilid, *p*-chloroacetanilid, and *p*-phenetidin contaminations in phenacetin by a single test, simplifying the U.S.P. technique which involves three separate procedures. The TLC method is not only faster, but also more sensitive than the official procedures.

PHENACETIN, one of the most popular constituents in analgesic combinations,[1] may contain impurities which increase toxicity. In U.S.P. XVII, requirements have been established for three of these impurities: acetanilid and *p*-phenetidin should be absent, and the *p*-chloroacetanilid content should not exceed 0.03%. The toxic properties of these contaminants are well described in the literature.

Several useful methods (1–5) have been published on the detection of the possible impurities in phenacetin. However, some of the methods do not consider all of the above-listed significant contaminants, and others apply laborious procedures and sophisticated equipment. The authors' objective was to develop a simple, rapid, and sensitive technique for the detection of the three impurities.

EXPERIMENTAL

Apparatus—Desaga-Brinkmann TLC equipment, 8 × 4 and 8 × 8 in. glass plates coated with a 250 μ layer of fluorescent Silica Gel (GF). Short-wave U.V. lamp, Mineralight,[2] model UVS-12. Long-wave U.V. lamp, Blak-Ray,[2] model UVL-22.

Reagents—Solvents (Table I): reagent grade. Phenacetin: U.S.P. grade. Acetanilid, *p*-chloroacetanilid, *p*-phenetidin: commercially available samples were used, without further purification.

Procedure—One gram of phenacetin was shaken with 5.0 ml. of ether for 5 min. in a small glass-stoppered flask. The suspension was allowed to settle, and 50 μl. of the supernatant was spotted on a Silica Gel GF plate. As a control, ethereal solutions of acetanilid, *p*-chloroacetanilid, and *p*-phenetidin were applied, containing 0.15, 0.30, and 3.0 mcg. of each. A partial list of solvents and solvent mixtures that were utilized to develop the chromatograms is included in Table I. The solvent front was allowed to ascend 10 to 15 cm. above the spot points.

The plate was then removed from the tank and dried in air. The dried plate was exposed to iodine vapor in an appropriate tank for 20 sec., and subsequently inspected under a short-wave (253.7 mμ) U.V. lamp. The substances appeared as dark spots against a bright green fluorescent background. (Fig. 1.)

As a corroborative estimation of the *p*-chloroacetanilid, the U.S.P. detection method was also applied (6). After exposing the plate to the short-wave U.V. radiation for about 5 min., the chromatogram was inspected under a long-wave (366.0 mμ) U.V. lamp: only the *p*-chloroacetanilid spots exhibited a bright blue fluoresence. This additional testing was more selective, but the range of sensitivity was the same as that of the first observation.

DISCUSSION AND RESULTS

In attempting to develop an improved procedure for detecting certain possible impurities in phenacetin, there were basically two problems to be solved: selecting the most suitable technique, and finding the optimal conditions for both the separation and identification of the substances.

Thin-layer chromatography was selected because of its easy availability, speed, and high degree of sensitivity. Unlike gas chromatography, the TLC method can be used without lengthy preliminary steps such as column preparation and equilibration. It facilitates the estimation of as little as 0.003% *p*-chloroacetanilid (the U.S.P. sets a limit of 0.03%). Furthermore, in proving the absence of acetanilid, it is much more accurate than the official method. Koshy and his co-workers (3) found that the U.S.P. test is sensitive only to concentrations greater than 1.2%. The same authors estimated that the sensitivity of the color reaction of iodine T.S. with *p*-phenetidin was in the range of 0.003%. It was found that by the described TLC method, a *p*-phenetidin contamination corresponding to a concentration of 0.0015% is safely detectable.

In selecting the optimal solvent system, it was obvious that the major difficulty was the separation of the acetanilid and *p*-chloroacetanilid, because the solubility properties of these compounds are very similar. Strongly polar solvents (methanol, acetone) moved all the compounds too fast and resulted in practically no separation. On the other hand, a solvent containing 90% petroleum ether left all substances at the starting line. It was found that ethyl ether mixed with a small volume (0.5–1.0%) of nonpolar solvent yielded a safe and reproducible separation of the four compounds. An ether–hexane (99:1) mixture proved suitable because of the comparatively speedy development (about 30 min.), and because of the availability of these solvents in reagent grade purity.

The detection of the phenacetin and its impurities can be achieved by alternate methods. Szász and his co-workers (1) exposed the plates to freshly generated nitrogen monoxide gas and subsequently sprayed the chromatogram with a 1% solution of β-naphthol in 2 *N* sodium hydroxide. The products (acetanilid was not included) appear in different colors; nevertheless, the limit of detection for *p*-chloroacetanilid is 0.1%. Other papers refer to the use of a spray reagent consisting of 3% potassium permanganate in strong sulfuric acid, or, in the

Received February 6, 1967, from the Analytical Laboratories, Sandoz Pharmaceuticals, Hanover, NJ 07936
Accepted for publication April 5, 1967.
The authors thank Mr. Donald Cooper for technical assistance in the course of the experimental work.

[1] "American Drug Index," Wilson, C. O., and Jones, T. E., eds., J. B. Lippincott Co., Philadelphia, Pa., 1961, lists 119 phenacetin-containing combinations.

[2] Ultra-Violet Products, Inc., San Gabriel, Calif.

TABLE I—TLC OF PHENACETIN AND ITS IMPURITIES IN DIFFERENT SOLVENT SYSTEMS

Solvent System	*Rf* Phenacetin	*p*-Phenetidin	*p*-Chloroacetanilid	Acetanilid
Pet. ether–chloroform, 9:1	0.00	0.00	0.00	0.00
Chloroform	0.05	0.23	0.09	0.09
Isopropyl ether	0.10	0.47	0.13	0.17
Ether–hexane, 90:10	0.20	0.47	0.23	0.27
Ether–pet. ether, 99:1	0.25	0.54	0.31	0.35
Ether–hexane, 99:1	0.27	0.53	0.32	0.36
Ether	0.30	0.67	0.37	0.37
Cyclohexane–acetone–diisobutylketone–methanol–water, 100:80:30:5:1[a]	0.40	0.55	0.45	0.49
Acetone	0.73	0.77	0.77	0.77
Methanol	0.75	0.70	0.80	0.80
Methanol–water, 3:1	0.90	0.90	0.90	0.90

[a] See *Reference 5*.

case of *p*-chloroacetanilid only, to the exposure to short-wave U.V. radiation followed by an examination under long-wave U.V. radiation (5, 6). It was found that by utilizing the fluorescent silica gel plate, no spray is necessary in order to detect the spots, and the use of the U.V. irradiation technique is not limited to the *p*-chloroacetanilid identification, but also facilitates the rapid and simultaneous examination of all four compounds. (The 20-sec. iodine exposure is necessary in order to make the *p*-phenetidin spot visible.)

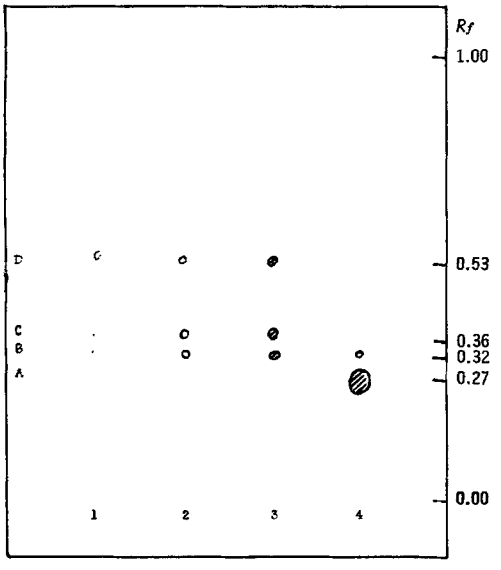


Fig. 1—Typical TLC of phenacetin (A), p-*chloroacetanilid* (B), *acetanilid* (C), *and* p-*phenetidin* (D). *Key:* 1—B, C, *and* D, *0.15 mcg. each;* 2—B, C, *and* D, *0.30 mcg. each;* 3—B, C, *and* D, *3.0 mcg. each;* 4—*phenacetin sample* (*containing 1.5 mcg. of* p-*chloroacetanilid in the spotted volume*).

SUMMARY

The separation, identification, and estimation of possible phenacetin impurities listed in the U.S.P. XVII monograph have been investigated.

A thin-layer chromatographic technique has been developed which offers a rapid identification of acetanilid, *p*-chloroacetanilid, and *p*-phenetidin impurities, and facilitates the near quantitative evaluation of their amounts. Limits of detection are 0.003% for acetanilid and *p*-chloroacetanilid, and 0.0015% for *p*-phenetidin.

The method presents a favorable alternative for the official limit test and two additional qualitative assays, by replacing them with a single, rapid, and highly sensitive procedure.

REFERENCES

(1) Szász, G., Vámos, F., and Végh, A., *Acta Pharm. Hung.*, **35**, 241(1965).
(2) Fresen, J. A., *Pharm. Weekblad*, **99**, 829(1964).
(3) Koshy, K. T., Wickersham, H. C., and Duvall, R. N., *J. Pharm. Sci.*, **54**, 1547(1965).
(4) Crummett, W. B., Simek, J., and Stenger, V. A., *Anal. Chem.*, **36**, 1834(1964).
(5) Savidge, R. A., and Wragg, J. S., *J. Pharm. Pharmacol.*, **17**, Suppl. 60S(1965).
(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 451.

Atropine Antagonism of Carbachol-Induced Hyperglycemia

By A. E. WADE

Hyperglycemia was induced with carbachol in rats, rabbits, and dogs, and the effect in rabbits was shown to be dose related. In albino rabbits with negligible plasma esterase activity, atropine sulfate effectively prevented hyperglycemia following 25 mcg./Kg. of carbachol, and the effect appeared to be dose dependent. In rabbits with moderate esterase levels, atropine sulfate dosage as high as 0.8 mg./Kg. had little inhibitory effect on carbachol hyperglycemic or cholinomimetic responses. This plasma esterase was not effectively blocked *in vivo* with 20 mg./Kg. of 2-(diethylamino)ethyl-2,2-diphenylvalerate (proadifen) HCl (SKF 525-A). However, this dose of proadifen HCl did block carbachol hyperglycemia in rabbits with negligible esterase activity. Carbachol hyperglycemia was similarly prevented by atropine in rats and dogs.

HEPATIC PHOSPHORYLASE ACTIVITY glycogenolysis, and the hyperglycemia resulting from the administration of exogenous epinephrine are reduced by certain adrenergic blocking agents (1–8) and certain indirectly acting sympathomimetics (2). The biochemical site of action of these dissimilar agents appears to be different (9), some suppressing synthesis of adenosine-3′,5′-monophosphate and others affecting reactions subsequent to this.

Catecholamines may be liberated from the adrenal medulla as a result of hypoglycemia (10) or by drugs such as acetylcholine, carbachol, histamine, nicotine, physostigmine, or reserpine (11, 12). Suppression of catecholamine mobilization from adrenal stores by these stimuli has been accomplished with chronic administration of reserpine and monoamine oxidase inhibitors, by ganglionic blocking agents, and in certain instances by atropine, chlorpromazine, guanethidine, and tetracaine (12).

Atropine has been shown to reduce blood levels of catecholamines (13), block the pressor response of neostigmine (14–16) and other ganglionic stimulants (16, 17), and depress the response of the nictitating membrane to nerve stimulation (18). The site of action is presumably at the ganglion. Atropine in large doses has adrenolytic activity, reducing many effects of exogenous epinephrine and sympathetic nerve stimulation (19, 20).

The effects of atropine on carbachol hyperglycemia in rabbits, rats, and dogs are enumerated in this report.

EXPERIMENTAL

The animals used in this study included female albino rabbits of the California strain, weighing 1.2 to 5.3 Kg.; female Sprague-Dawley rats, weighing 170–251 Gm.; and mongrel dogs of both sexes, weighing 8–14.5 Kg.

Serum esterase levels of the rabbits were determined by the method outlined previously (21). The serum esterase level was considered negligible if, under the conditions of this assay, less than 0.06 μ-mole procaine was hydrolyzed per ml. serum per hour at 26 ± 1°. Following an 18–24-hr. fast, rabbits were anesthetized with i.p. injections of a solution of Na phenobarbital (100 mg./Kg.) and Na pentobarbital (15 mg./Kg.) dissolved in 50% propylene glycol. Approximately 1 hr. after anesthesia was established, 0.9% NaCl solution, atropine sulfate, or 2-(diethylaminoethyl)-2,2-diphenylvalerate (proadifen) HCl (SKF 525-A)[1] was administered i.v. Blood samples of 0.6 to 1.0 ml. were removed by cardiac puncture 10 min. after such pretreatment and 10 min. prior to the intravenous carbachol.[2] Additional blood samples were obtained at 15, 30, and 60 min. following carbachol. The samples were mixed with dry sodium citrate and immediately placed in an ice–salt bath until deproteinized.

Rats were fasted overnight and anesthetized with i.p. injections of 25 mg./Kg. Na pentobarbital and 75 mg./Kg. Na phenobarbital in 50% propylene glycol. Blood samples (0.5 ml.) were removed by cardiac puncture 20 min. prior to the subcutaneous administration of 0.9% NaCl or atropine $SO_4^=$ and 35 min. following the subcutaneous administration of 25 mcg. of carbachol per Kg. body weight.

Dogs were fed 18–20 hr. prior to i.p. anesthesia with a solution of Na pentobarbital (10 mg./Kg.) and Na phenobarbital (120 mg./Kg.) in 50% propylene glycol, and 3 hr. later atropine $SO_4^=$ (0.4 mg./Kg.) was administered subcutaneously to the experimental group. Blood samples were withdrawn from the cephalic vein 20 min. prior to subcutaneous carbachol (25 mcg./Kg.) administration, and at 10, 20, and 50 min. after carbachol. In some experiments epinephrine HCl (10 mcg./Kg.) was administered 120 min. following carbachol in order to validate the animals' ability to mobilize hepatic glycogen.

Glucose content of 0.1-ml. samples of deproteinized blood was determined in duplicate by the method of Washko and Rice (22) using a prepared enzyme reagent.[3]

Drug effects were based on the quantitative change in mg. per cent of glucose from pre-carbachol injection values. Each point on the dose-response curves representing total measured response to a given treatment was derived by calculating the area under the graph produced by plotting change in glucose content (mg. %) during the 60 min. post-carbachol. One factor analysis of variance was conducted on all data using standard electronic data processing equipment by the University of Georgia Bureau of Statistics. Differences between groups were calculated at the 5% level using the Duncan multiple range test.

Received March 1, 1967, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication May 17, 1967.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by the University of Georgia General Research Budget.

[1] SKF 525-A was graciously supplied by Smith, Kline & French Laboratories, Philadelphia, Pa.

[2] Marketed as Carcholin by Merck & Co., Rahway, N. J.

[3] Marketed as Glucostat by Worthington Biochemical Corp., Freehold, N. J.

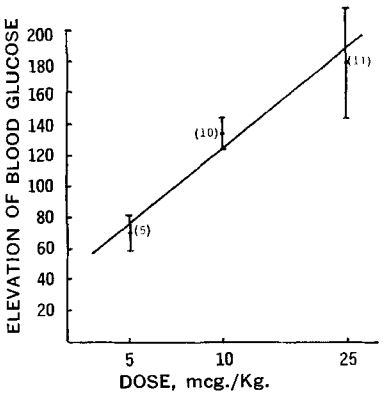


Fig. 1—Total blood glucose elevation during 60-min. post-carbachol in rabbits. Values and bars indicate mean ± S.E. Numbers in parentheses indicate number of animals per dose.

RESULTS

Carbachol Hyperglycemia—The log dose-response curve for carbachol-induced hyperglycemia over the 60-min. period following i.v. carbachol administration in rabbits is il ustrated in Fig. 1. The response is clearly dose dependent, and although 25 mcg./Kg. of carbachol appeared to be supramaximal in some animals, this dose produced hyperglycemia significantly greater than that resulting from 5 mcg./Kg. The hyperglycemia was significant for all doses of carbachol at 15 and 30 min. post-injection, but at the 60-min. period the blood glucose concentration had returned to a level not significantly higher than the controls administered 0.9% NaCl solution. All doses produced profound parasympathomimetic actions, but seldom produced death if infused over a 60–90-sec. period.

Atropine Inhibition of Carbachol Hyperglycemia—The inhibitory effect of atropine sulfate on the hyperglycemia induced by carbachol in rabbits exhibiting negligible serum esterase activity is recorded in Table I. This effect of atropine appeared to be dose related, since 0.05 mg./Kg. produced 70.8% inhibition of the carbachol hyperglycemia and 0.10 mg./Kg. resulted in a 92% inhibition. Doses of 0.2 and 0.4 mg./Kg. produced inhibition statistically indistinguishable from that produced by 0.1 mg./Kg. All doses of atropine sulfate above 0.05 mg./Kg. maintained blood glucose levels in the presence of carbachol that were not statistically different from the nontreated (saline injected) controls.

The mean hyperglycemic response to carbachol was less pronounced in atropinized rabbits having innately high serum esterase levels than in nonatropinized controls; however, this effect was not significant (Fig. 2). Data are also presented in Fig. 2 which suggest that proadifen HCl produces an effect resembling that of atropine but that it does not effectively block the esterase responsible for atropine hydrolysis. This ineffectiveness as a blocking agent was explained, at least in part, by *in vitro* experiments which demonstrated that proadifen HCl is inactivated by rabbit serums having atropinesterase activity. *In vivo* studies in rabbits with negligible esterase levels demonstrated the atropine-like effect of proadifen HCl on carbachol-induced hyperglycemia (Fig. 3).

In the rat, atropine sulfate (0.05 to 0.4 mg./Kg.) produced complete blockade of the hyperglycemia of carbachol. This blockade, measured at 50–55 min. following subcutaneously administered atropine, was not evident at 80 min. post-atropine.

The effect of atropine sulfate in the dog is depicted in Fig. 4. Complete blockade of carbachol hyperglycemia with this dose of atropine sulfate seems evident, although statistically significant changes in blood glucose concentrations were not measured until 50 min. post-carbachol. It became evident early in this study with dogs that glycogen reserves are quickly depleted and that heavy feeding 14–16 hr. prior to the experiments supplied insufficient glycogen reserves to allow consistent hyperglycemia following carbachol injections. Priming doses of orally administered dextrose (3 Gm./Kg.) approximately 3 hr. prior to the experiments alleviated this difficulty and provided sufficient hepatic glycogen to allow measurable hyperglycemia in response to epinephrine in both atropinized and control animals.

DISCUSSION

Carbachol induces hyperglycemia in rabbits, rats, and dogs which is probably due to the liberation of epinephrine from adrenal medullary stores. The data reported here indicate that the mechanism by which atropine inhibits this hyperglycemia is similar in these animals. Although the site of action was not specifically located, it seems reasonable to assume that atropine blocks at a point preceding the release of epinephrine, since it was shown that atropine did not prevent the hyperglycemia caused by a dose of 10 mcg./Kg. of epinephrine in dogs that had failed to respond to carbachol.

TABLE I—EFFECT OF ATROPINE $SO_4^=$ ON CARBACHOL (25 mcg./Kg. i.v.) HYPERGLYCEMIA IN RABBITS WITH NEGLIGIBLE SERUM ESTERASE ACTIVITY

No. of Animals in Group	Pretreatment	Change in Blood Glucose (mg. %) from Pre-Carbachol Levels— 15 min.	30 min.	60 min.
11	Saline	59.43 ± 9.70	55.89 ± 13.58	36.68 ± 8.88
8	Atropine $SO_4^=$ 0.05 mg./Kg.	12.46 ± 5.51[a]	12.31 ± 4.66[a]	21.89 ± 8.98
6	Atropine $SO_4^=$ 0.10 mg./Kg.	6.25 ± 7.18[a]	4.18 ± 5.48[a]	1.90 ± 4.89[a]
5	Atropine $SO_4^=$ 0.20 mg./Kg.	3.28 ± 4.80[a]	2.36 ± 3.44[a]	3.74 ± 4.73[a]
6	Atropine $SO_4^=$ 0.40 mg./Kg.	4.05 ± 1.86[a]	2.57 ± 3.95[a]	1.35 ± 3.80[a]
7	No carbachol-saline	−2.21 ± 5.13[a]	−2.17 ± 3.60[a]	1.09 ± 9.05[a]

[a] Significantly different from carbachol controls.

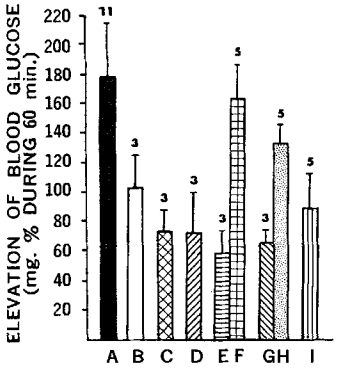


Fig. 2—Effect of atropine SO_4^- and atropine SO_4^- with proadifen HCl (20 mg./Kg.) on the hyperglycemia of carbachol (25 mcg./Kg.) in rabbits with innately high serum esterase levels. Vertical bars represent standard errors of the mean. The number above each bar indicates number of animals per group. Key: A, *control;* B, *proadifen HCl;* C, *proadifen HCl + atropine SO_4^- (0.05 mg./Kg.);* D, *proadifen HCl + atropine SO_4^- (0.10 mg./Kg.);* E, *proadifen HCl + atropine SO_4^- (0.20 mg./Kg.);* F, *atropine SO_4^- (0.20 mg./Kg.);* G, *proadifen HCl + atropine SO_4^- (0.40 mg./Kg.);* H, *atropine SO_4^- (0.40 mg./Kg.);* I, *atropine SO_4^- (0.80 mg./Kg.).*

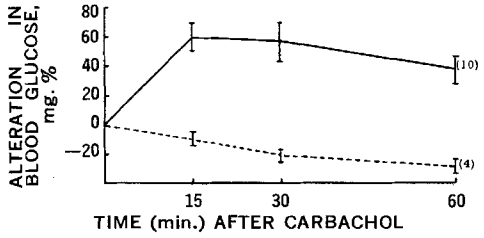


Fig. 3.—The effect of proadifen HCl (20 mg./Kg. i. v.) on carbachol (10 mcg./Kg.) hyperglycemia in rabbits with negligible serum esterase. Numbers in parentheses indicate number of animals per group. Key: ————, *control;* – – – – – –, *proadifen HCl pretreated.*

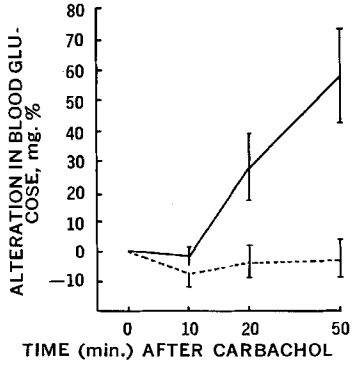


Fig. 4—The effect of atropine SO_4^- (0.4 mg./Kg. s. c.) on carbachol (25 mcg./Kg. s. c.) in the dog. The values and bars represent mean ± S. E. of 4 experiments. Key: ————, *control;* – – – – – –, *atropine pretreatment.*

The enzyme inhibitor proadifen HCl has been reported to inhibit human serum procainesterase activity (23). This inhibition was verified during an *in vitro* study which demonstrated that 1×10^{-4} *M* proadifen HCl reduced procaine hydrolysis by human serum by about 61%. *In vitro* experiments indicated, however, that proadifen HCl at concentrations of 1.4×10^{-3} *M* does not inhibit hydrolysis of atropine by esterase of rabbit serum, and in fact serves as a substrate for this enzyme. The fact that proadifen HCl does not effectively block atropinesterase activity was verified in the *in vivo* experiments in rabbits with high serum esterase levels. The anticholinergic activity of proadifen HCl is well known and its atropine-like activity in blocking carbachol hyperglycemia was demonstrated in these studies. Thus, the antihyperglycemic activity of atropine and proadifen HCl in rabbits with high esterase activity appears to be no more than an additive effect of the two drugs.

If the antihyperglycemic action of atropine is present in man, and if it is capable of inhibiting epinephrine release from the adrenal medulla by stimuli other than carbachol (*i.e.*, hypoglycemia), then two distinct possibilities of clinical importance appear likely. Cholinergic blocking drugs may find use (*a*) in controlling the hyperglycemia of the select group of patients having normal insulin levels but transiently high concentrations of circulating catecholamines, the release of which is triggered by an oversensitive sympathetic hyperglycemic reaction, and (*b*) as insulin-sparing agents for diabetics receiving oral hypoglycemics, by reducing the antagonistic effect of the adrenal medulla. This second point may be doubly important in view of the fact that certain of the oral hypoglycemic agents stimulate epinephrine release from the adrenal medulla (24).

SUMMARY

Carbachol in doses of 5, 10, and 25 mcg./Kg. i.v. produced peak elevations in blood glucose concentration in rabbits within 15 min.

This hyperglycemia was effectively blocked by doses of atropine sulfate as low as 0.05 mg./Kg. in both rabbits with negligible serum esterase activity and in rats. Atropine antagonism of carbachol hyperglycemia was also evident in dogs.

The enzyme inhibitor proadifen HCl (20 mg./Kg. i.v.) completely antagonized the hyperglycemia of carbachol (10 mcg./Kg. i.v.) in rabbits with negligible serum esterase activity, but was ineffective in blocking the esterase of rabbit serum responsible for atropine hydrolysis.

REFERENCES

(1) Nickerson, M., *Pharmacol. Rev.*, **11**, 443(1959).
(2) Haugaard, N., and Hess, M. E., *ibid.*, **17**, 27(1965).
(3) McCutcheon, R. S., *J. Pharmacol. Exptl. Therap.*, **136**, 209(1962).
(4) Elder, J. T., *Intern. J. Neuropharmacol.*, **3**, 295(1964).
(5) Vizi, E. S., Pogatsa, G., and Kaldor, A., *J. Pharm. Pharmacol.*, **17**, 805(1965).
(6) Lei, B. W., and McCutcheon, R. S., *J. Pharm. Sci.* **53**, 503(1964).
(7) Schwartz, N. B., *Am. J. Physiol.*, **203**, 525(1962).
(8) Kvam, D. C., Riggilo, D. A., and Lish, P. M., *J. Pharmacol. Exptl. Therap.*, **149**, 183(1965).
(9) Northrop, G., and Parks, R. E., Jr., *ibid.*, **145**, 87 (1964).
(10) Armin, J., and Grant, R. T., *J. Physiol.*, **149**, 228 (1959).
(11) Greenberg, R., and Toman, J. E. P., *Arch. Intern. Pharmacodyn.*, **159**, 87(1966).
(12) Garrett, J., *Actualities Pharmacol.*, **18**, 69(1965).

(13) Unghuary, L., Hovanyi, M., and Farkas, F., *Pharm. Zentralhalle*, **104**, 27(1965); through *Intern. Pharm. Abstr.*, **3**, (January 1966).
(14) Long, J. P., and Eckstein, J. W., *J. Pharmacol. Exptl. Therap.*, **133**, 216(1961).
(15) Hilton, J. G., *ibid.*, **132**, 23(1961).
(16) Steinberg, M., and Hilton, J. G., *Tex. Rept. Biol. Med.*, **24**, 222(1966).
(17) Roszkowski, A. P., *J. Pharmacol. Exptl. Therap.*, **132**, 156(1961).
(18) Reas, H. W., and Tsai, T. H., *ibid.*, **152**, 186(1966).
(19) Bussell, L. J., *ibid.*, **69**, 128(1940).
(20) Luduena, F. P., and Branin, M.-J., *J. Pharm. Sci.*, **55**, 280(1966).
(21) Daniell, H. B., Wade, A. E., and Millikan, F. F., *ibid.*, **53**, 1341(1964).
(22) Washko, M. E., and Rice, E. W., *Clin. Chem.*, **7**, 542(1961).
(23) Netter, K. J., *Arch. Exptl. Pathol. Pharmakol.*, **235**, 498(1959).
(24) Dulin, W. E., Morley, E. H., and Nezamis, J. E., *Proc. Soc. Exptl. Biol. Med.*, **93**, 132(1956).

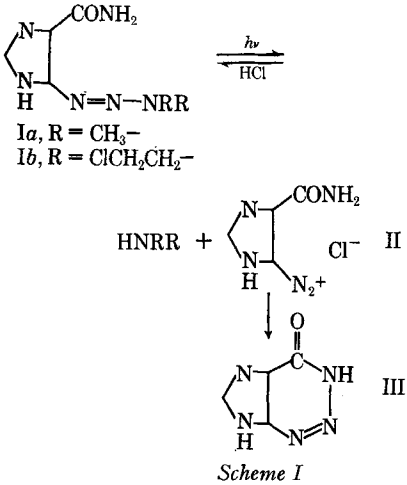
Colorimetric Determination of Dialkyltriazenoimidazoles

By TI LI LOO and ELIZABETH A. STASSWENDER

A colorimetric method has been developed for the determination of dialkyltriazenoimidazoles in plasma and urine.

RECENTLY a series of dialkyltriazenoimidazoles (I) has been synthesized as potential antitumor agents (1). Several of these have shown promise as useful drugs in cancer chemotherapy, notably 5-(dimethyltriazeno)-imidazole-4-carboxamide (I*a*) (DIC, NSC-45388) and 5-[di(β-chloroethyl) triazeno]-imidazole-4-carboxamide (I*b*) (NSC-82196) (2,3). The former is presently under clinical trial. Concurrently, pharmacologic studies on these new agents in man and animals are also in progress in our laboratories. For such studies a simple, sensitive, and specific method for the quantitative determination of the dialkyltriazenoimidazoles is desirable. The method described below appears to meet this demand.

The dialkyltriazenoimidazoles undergo photodecomposition in dilute acid to 5-diazoimidazole-4-carboxamide (II) and a corresponding dialkylamine according to Scheme I.



Scheme I

In aqueous solutions II readily cyclizes by intramolecular coupling to 2-azahypoxanthine (III), imidazo-[4,5-*d*]-*v*-triazin-4(3*H*)-one (1). However, in the presence of an aromatic amine such as *N*-(1-naphthyl)ethylenediamine (Bratton-Marshall reagent) (4), coupling takes place with the formation of an intensely colored azo-dye. Under rigidly controlled experimental conditions with an excess of the coupling amine, the competing intramolecular coupling to III could be held to a constant minimum; thus the intensity of the color adequately measures the concentration of I. Because the photodecomposition is a general reaction of I, the colorimetric method is expected to be applicable to all dialkyltriazenoimidazoles, although the present work concerns mainly I*a* and I*b*.

EXPERIMENTAL

Apparatus

A standard colorimeter (for example, Bausch & Lomb Spectronic 20) is required for measurement of absorbance of 0.4 ml. to 2 ml. of solution.

The light source is a long-wave (maximal radiation at 366 mμ) ultraviolet lamp (Blak-Ray lamp, model XX-15, supplied by Ultraviolet Products, Inc., San Gabriel, Calif., equipped with two General Electric F15 T8-BLB 15-w. bulbs).

A wooden rack holds the test tubes (Pyrex 75 × 10 mm.) containing drug solutions rigidly vertical and at a fixed distance of 2 cm. from the light bulbs. The lamp is positioned so that an entire row of test tubes may receive the same maximal exposure to ultraviolet radiation.

Reagents

Bratton-Marshall Reagent—Aqueous solutions of *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.2% for aqueous and plasma assays, but 1% for urine assays.

DIC Standards—A stock solution of DIC, 10.0 mg./ml. in 0.1 *N* hydrochloric acid is prepared and stored in the dark under refrigeration. It is stable for at least 1 month. Dilutions are made of stock with 0.1 *N* hydrochloric acid, plasma, or urine.

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PROCEDURES

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(13) Unghuary, L., Hovanyi, M., and Farkas, F., *Pharm. Zentralhalle*, **104**, 27(1965); through *Intern. Pharm. Abstr.*, **3**, (January 1966).
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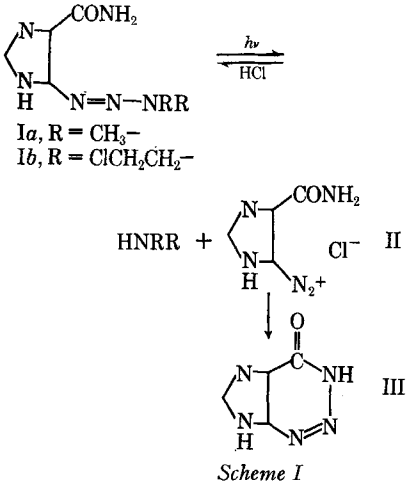

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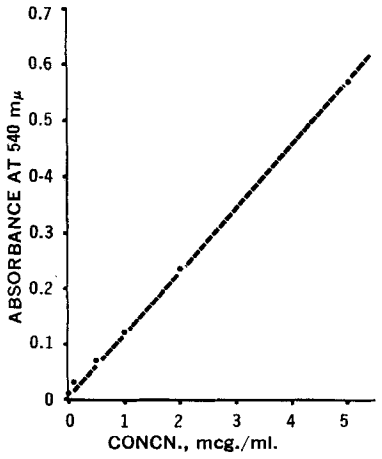


Fig. 1—Linearity between absorbance and concentration of DIC, 5-(dimethyltriazeno)-imidazole-4-carboxamide, NSC-45388.

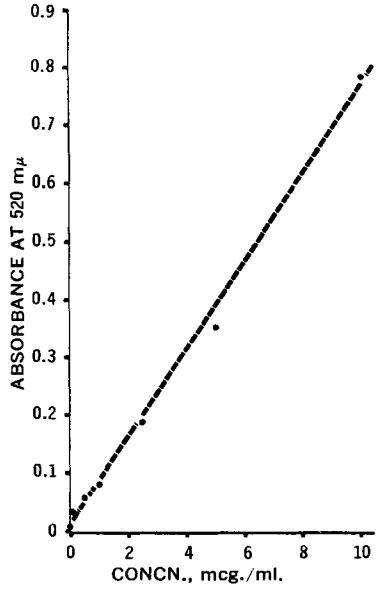


Fig. 2—Linearity between absorbance and concentration of NSC-82196, 5-[di(β-chloroethyl)triazeno-imidazole-4-carboxamide.

ml. to 5.0 mcg./ml., and 0.1 ml. of 0.2% Bratton-Marshall reagent is thoroughly shaken and placed in front of the ultraviolet light. After a 30-min. exposure, the absorbance (540 mμ for DIC and 520 mμ for NSC-82196) is measured against water. A blank is similarly run with 2.0 ml. of 0.1 *N* hydrochloric acid.

Plasma Recovery—A 3.0-ml. plasma solution of the drug is thoroughly mixed with 1.5 ml. of trichloroacetic acid. The protein precipitate is removed by centrifugation at about 2,000 r.p.m. (top speed of International clinical centrifuge, model CL) for 20 min. An aliquot of 2.0 ml. of the supernatant is withdrawn and assayed as for aqueous solutions. A blank containing no drug is likewise run. As a rule, in every experiment standards are established by adding given amounts of the drug to the pretreatment plasma collected from the subject and assayed in accordance with the above procedure. Whenever possible, three concentrations are arbitrarily chosen and run in triplicates.

The above procedure is slightly modified as follows for smaller amounts of plasma. To 1.0 ml. of a plasma solution is added 0.5 ml. of trichloroacetic acid solution. The mixture is thoroughly shaken and centrifuged. An aliquot of 0.4 ml. of the supernatant is removed and mixed with 0.02 ml. of 0.2% Bratton-Marshall reagent and assayed as before.

Urinary Recovery—All urine samples are diluted before assay because, first of all, the presence of natural pigments in urine makes direct determination impractical. Second, in actual clinical pharmacological work, the rapid excretion of the drug causes very high initial urinary concentration. For these reasons, the standard curve is constructed with urine solutions ranging from 250 mcg./ml. to 2000 mcg./ml. These are then appropriately diluted to 5.0 mcg./ml. with 0.1 *N* hydrochloric acid. Except that the Bratton-Marshall reagent used is 1% instead of 0.2%, the assay procedure is the same as described for aqueous solutions. The observed absorbance multiplied by the dilution factor gives the true value corresponding to each original concentration. Again, as in plasma recovery, urine standards are run with pretreatment urine in every experiment.

Table I—Concentration and Absorbance (± Standard Error) of NSC-45388 and NSC-82196

Concn., mcg./ml.	NSC-45388	NSC-82196
0	0.012 ± 0.0019	0.012 ± 0.0010
0.1	0.032 ± 0.0003	0.032 ± 0.0010
0.5	0.073 ± 0.0006	0.056 ± 0.0003
1	0.121 ± 0.0012	0.080 ± 0.0015
2	0.237 ± 0.0022	
5	0.569 ± 0.0063	0.353 ± 0.0041
10		0.783 ± 0.0104

RESULTS AND DISCUSSION

The excellent linearity between absorbance and concentration for both NSC-45388 and NSC-82196 in aqueous solutions is shown in Figs. 1 and 2, respectively, each point representing 10 replicates. The standard errors are illustrated in Table I. Obviously, the reproducibility is good. It appears the method is less sensitive with NSC-82196 than with NSC-45388. This is hardly surprising because the ratio of their molecular weights is about 3:2. Theoretically, the wavelength of maximal absorption should be the same for all dialkyltriazeno-imidazoles. In practice, however, there was a slight variation from drug to drug and hence the $\lambda_{max.}$ should be determined individually: it was 540 mμ for NSC-45388 and 520 mμ for NSC-82196.

After 30 min. the photodecomposition is complete and further exposure to ultraviolet light is of no

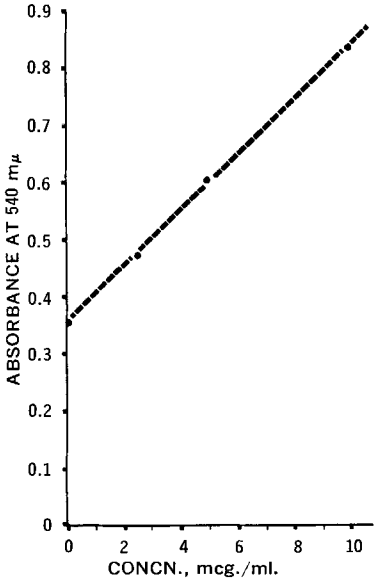


Fig. 3—Plasma standard curve of DIC, NSC-45388.

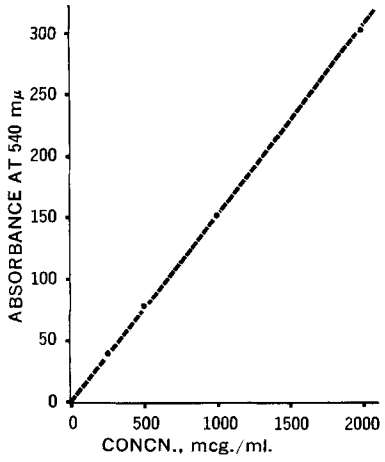


Fig. 4—Urinary standard curve of DIC (high concentration), NSC-45388.

advantage. If the Bratton-Marshall reagent is not added prior to irradiation, no color is produced, probably because the diazo compound has cyclized.

A typical plasma standard curve of DIC is represented by Fig. 3. Bratton-Marshall reagent of 1% is not used in plasma assay because it gives a higher blank than the 0.2% reagent. The results of urinary determination of the same drug are illustrated in Fig. 4. It is readily seen that the method applies well to all these determinations.

No naturally occurring material in either plasma or urine is known to give rise to a diazo compound upon illumination under ultraviolet light. Consequently, this method is specific for dialkyltriazenoimidazoles. Moreover, it is reasonable to expect the method to be applicable equally well to other dialkyltriazenoimidazoles.

The very small absorbance of the aqueous blank is accounted for by the slight brown tint produced by the Bratton-Marshall reagent after exposure to ultraviolet light. However, with plasma and especially with undiluted urine the brown tint becomes very much enhanced to afford high blanks which vary from one subject to another. This is one of the reasons why plasma and urine standards are always prepared and assayed in every experiment. The high blanks are not caused by azo dyes but by the end absorption due to certain brown pigment resulting from the interaction of Bratton-Marshall reagent and some unidentified constituents of plasma and urine after ultraviolet irradiation. Both plasma and urine blanks are assumed to remain constant throughout the experiment. This is a reasonable assumption since an experiment rarely lasts longer than 4–5 hr.

REFERENCES

(1) Shealy, Y. F., Krauth, C. A., and Montgomery, J. A., *J. Org. Chem.*, **27**, 2150(1962).

(2) Shealy, Y. F., Montgomery, J. A., and Laster, W. R., Jr., *Biochem. Pharmacol.*, **11**, 674(1962).

(3) Hano, K., Akashi, A., Yamamoto, I., Narumi, S., Horii, Z., and Ninomiya, I., *Gann*, **56**, 417(1965).

(4) Bratton, A. C., Marshall, E. K., Jr., Babbitt, D., and Hendrickson, A. R., *J. Biol. Chem.*, **128**, 537(1939).

Tumor Localizing Agents I. Synthesis and Tissue Distribution of ^{14}C-Labeled and Radioiodinated 5,6-Diacetoxyindole

By R. E. COUNSELL, T. D. SMITH, J. DOELLE, D. MEIER, and W. H. BEIERWALTES

Interest in the development of radiopharmaceuticals suitable for the diagnosis and treatment of malignant melanoma prompted the present study. One of the first approaches to this problem has involved the synthesis of suitably labeled precursors of melanin with the hope that these substances would be selectively taken up by the pigmented tumor and rapidly converted to a radiolabeled melanin within the tumor. Since 5,6-dihydroxyindole is a late precursor of melanin, the synthesis of this material labeled with ^{14}C was undertaken. This substance, however, proved to be too unstable and was therefore converted to the diacetate for animal studies. The radioiodine-labeled analog was prepared by iodine exchange of 5,6-diacetoxy-7-iodoindole with sodium iodide-^{126}I. Neither 5,6-diacetoxyindole-2-^{14}C nor the ^{126}I analog showed a predilection for melanomas in mice.

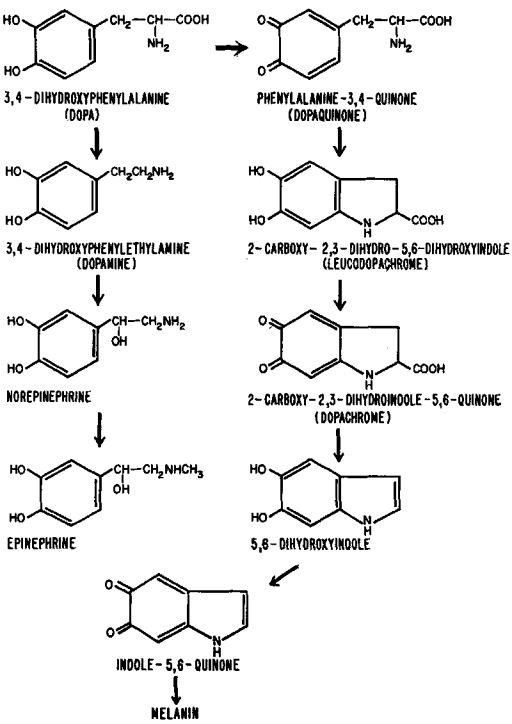
IN A RECENT REVIEW on the clinicopathological status of malignant melanoma, Raven (1) emphasized the need for new diagnostic and therapeutic agents for this disease. For several years now, the authors have been involved in a broad program aimed at the synthesis of substances which will localize in various tumors or organs. One objective of this program has been to find compounds that will selectively concentrate in melanotic tissues. Such compounds, when labeled with γ-emitting radionuclides, can be readily detected by external photoscanning techniques and thus afford a means for determining the location and metastatic nature of melanotic tumors.

One approach to the development of radiopharmaceuticals having a predilection for melanomas has involved an investigation of radio-labeled precursors of melanin. Since melanin appears to be an end product of tyrosine metabolism, it seemed possible that suitably labeled precursors of melanin could be selectively taken up by the pigmented tumor and rapidly converted to a labeled melanin which would remain within the tumor. Recent publications by several investigators have served to support the validity of this hypothesis. Blois and Kallman (2) studied the uptake of *dl*-dihydroxyphenylalanine-2-^{14}C (dopa-2-^{14}C) in mice with deeply pigmented melanomas and found the radioactivity of the different tissues to be in the order: melanoma > adrenal $\gg$ kidney > liver > spleen. Similarly, Fimiam and Dowd (3) noted that when *dl*-tyrosine-2-^{14}C was administered to melanotic mice significantly greater radioactivity was associated with melanoma than with any other tissue studied.

On the basis of this information, the authors elected to synthesize radiolabeled compounds structurally similar to biological precursors of melanin. As indicated in the proposed pathway of melanin biosynthesis (Scheme I), tyrosine and dopa serve as precursors for not only melanin but also catecholamines (4). Since a high target–nontarget ratio in melanomas compared to surrounding tissues was desired, it seemed advisable to select precursors formed in the later stages of melanogenesis for the studies. The synthesis and preliminary tissue distribution data for two radiolabeled derivatives of 5,6-dihydroxyindole are reported here.

In the preliminary experiments, 5,6-dihydroxyindole proved to be too unstable for use in the tissue distribution experiments. The protected *O*-acetylated product, however, proved to be much more stable and suitable for the animal studies.

Among the various methods previously reported for the synthesis of 5,6-dihydroxyindole and its derivatives, the approach of Beer and co-workers



Biosynthesis of Melanin and Epinephrine

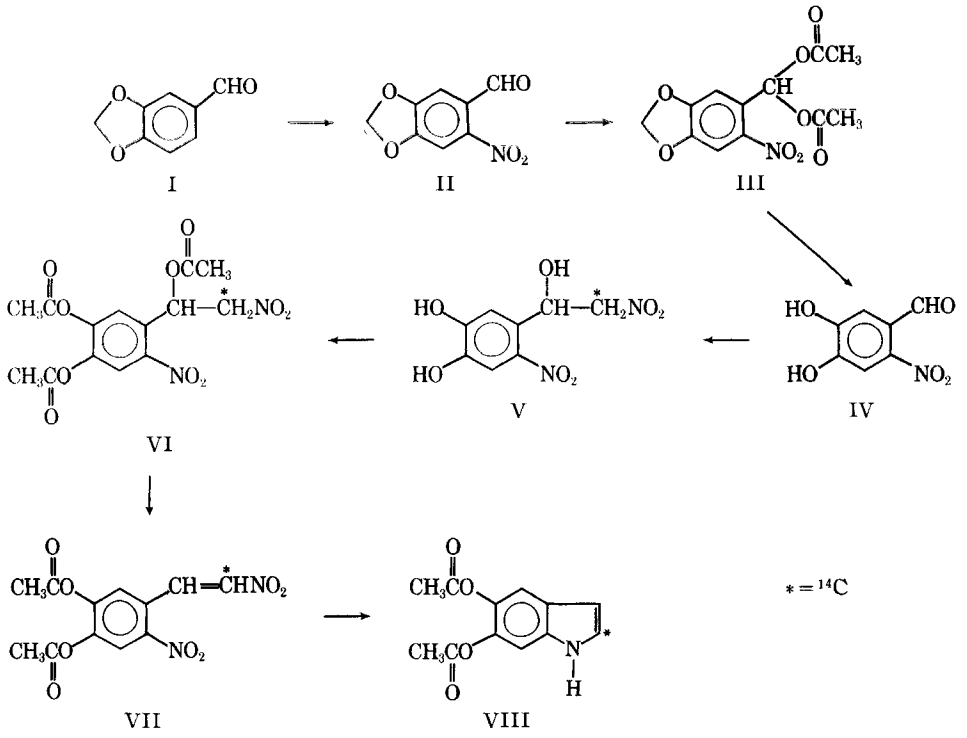
Scheme I

Received March 6, 1967, from the Laboratory of Medicinal Chemistry, College of Pharmacy and the Department of Internal Medicine (Nuclear Medicine), University of Michigan, Ann Arbor, MI 48104

Accepted for publication May 8, 1967.

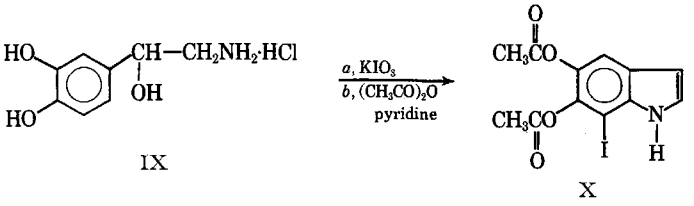
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This work was supported by training grant GM 1367-02 from the National Institutes of Health, Michigan Memorial Phoenix Project 317, Cancer Research Institute of the University of Michigan, and American Cancer Society Award PRA-18.



Synthesis of 5,6-Diacetoxyindole-2-^{14}C

Scheme II



Synthesis of 5,6-Diacetoxy-7-iodoindole

Scheme III

(5) seemed the most amenable for preparation of ^{14}C-labeled 5,6-diacetoxyindole. As outlined in Scheme II, piperonal (I) served as the starting material and was readily converted to 6-nitropiperonal (II). This product was then converted to acylal (III) and demethylenated according to the method of Parijs (6).

The radiolabel was introduced at this stage by condensation of IV with nitromethane-^{14}C. The subsequent steps were studied in detail in preliminary experiments so that the authors could proceed directly to the final product without isolating intermediates and thus minimize losses of radioactivity. Accordingly, the nitroalcohol V was immediately acetylated to give the triacetate (VI). Dehydroacetylation and reductive ring closure with iron in acetic acid afforded, after chromatography, 5,6-diacetoxyindole-2-^{14}C (VIII) in 43% over-all yield from IV.

The radioiodine-labeled product was prepared by isotope exchange of 5,6-diacetoxy-7-iodoindole (X) with sodium iodide-^{125}I. The iodinated indole was readily obtained by potassium iodate oxidation of *dl*-norepinephrine hydrochloride (IX) according to the procedure of Heacock *et al.* (7) (Scheme III). Glacial acetic acid proved to be the most suitable solvent for the iodine exchange, and the rate of exchange could be readily followed by radioscanning of thin-layer chromatograms (TLC).

Tissue Distribution Studies[1]—Table I compares the distribution of radioactivity in various tissues after administration of *dl*-dopa-2-^{14}C and the radiolabeled indole derivatives to mice with deeply pigmented melanomas. In contrast with Blois and Kallman's results (2), the *dl*-dopa-2-^{14}C showed a greater concentration of radioactivity in adrenal than in melanoma. At 24 hr., for example, the concentration in adrenal was almost 15 times that in melanoma. Similar results were obtained by Hempel and Deimel (9) with tritiated *dl*-dopa in mice with Harding-Passey melanoma.

[1] A more detailed description of these findings and related material has been reported elsewhere (8).

TABLE I—TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF RADIOLABELED MELANIN PRECURSORS IN MELANOTIC MICE[a]

Compd.	Time Sacrificed After Injection, hr.	No. of Mice[b]	Melanoma	Liver	Adrenal	Kidney	Injection Site
dl-Dopa-2-^{14}C	0.5	9	1.2	2.6	2.3	6.0	...
	2	2	2.4	2.2	9.0	3.0	...
	4	10	3.2	2.2	13.6	...	...
	24	5	4.0	2.3	59.8	7.0	...
5,6-Diacetoxyindole-2-^{14}C	0.5	2	0.1	0.5	0.9	5.0	4.5
	2	4	0.4	0.4	0.5	9.2	3.5
	4	2	0.5	0.4	0.6	14.0	12.6
	24	2	0.7	0.8	0.6	6.1	10.7
7I odo-5,6-diacetoxy-indole-^{125}I	0.5	3	0.1	0.4	0.4	4.1	22.4
	2	3	0.3	0.4	0.3	2.1	...
	4	3	0.3	0.4	0.4	2.1	15.0
	24	5	0.4	0.3	0.4	0.9	16.8

[a] Tissue–serum ratios in c.p.m./mg. [b] Male mice with transplanted deeply pigmented B16 melanomas obtained from Jackson Laboratory, Bar Harbor, Me.

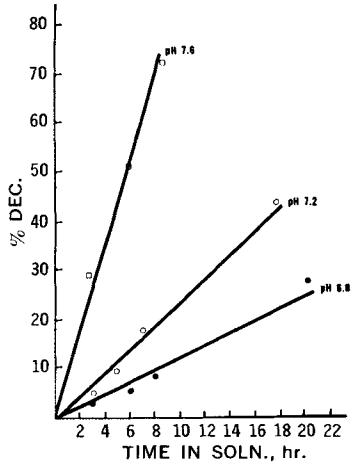


Fig. 1—Decomposition of 5,6-diacetoxyindole in aqueous solution at three pH values.

Neither 5,6-diacetoxyindole-2-^{14}C nor the ^{125}I analog showed selective uptake by the tumor. The site of injection, however, blackened rapidly after administration, suggesting that the diacetoxyindoles hydrolyzed to the dihydroxyindoles and rapidly polymerized to melanin-like materials. This undoubtedly accounted for the high levels of radioactivity at this site. The kidneys also became dark and the urine brown-black in color following administration of these compounds.[2] Presumably the concentration of radioactivity in the kidney represents renal excretion of these products or their metabolites. The rapid formation of dark melanin-like products at these sites indicates that a metabolic precursor of melanin preceding dihydroxyindole must enter the melanocyte prior to oxidative polymerization to melanin. Other potential precursors of melanin are currently under investigation.

Stability Studies—The high concentration of radioactivity at these sites prompted the authors to determine the effect of pH on the stability of 5,6-diacetoxyindole. The rate of decomposition at pH 6.8, 7.2, and 7.6 was ascertained by measurement of the absorbance of buffered aqueous solutions at 218 mμ. As shown in Fig. 1, decomposition of 5,6-diacetoxyindole increased with increasing pH. The high concentration of activity at the site of injection could therefore be related to the slightly basic pH at this site (10) or to the presence of an esterase. With regard to the latter possibility, Riley (11) recently demonstrated that 5,6-diacetoxyindole is rapidly converted to melanin in sections of human skin and presented evidence to support the presence of an indole-5,6-acetyl esterase.

EXPERIMENTAL[3]

5,6-Diacetoxyindole-2-^{14}C (VIII)—A solution of 6-nitro-protocatechuic aldehyde (5, 6) (IV, 2.5 Gm., 13.6 mmole) and nitromethane-^{14}C (0.84 Gm., 13.8 mmoles, 1.13 mc.)[4] in ethanol (25 ml.) was cooled to 0°. To this solution was added dropwise with stirring a solution of potassium hydroxide (1.8 Gm.) in 95% ethanol (25 ml.) while maintaining the reaction temperature at 0°. Following the addition period of about 1 hr., the reaction mixture was stored at 0° for 24 hr. and diluted with water (30 ml.). The mixture was acidified with 10% HCl

[2] Radiochromatogram analysis of the solutions used in the animal experiments indicated that no decomposition of the compounds had taken place prior to the tissue localization studies

[3] Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Ultraviolet spectra were recorded on a Beckman DK2A spectrophotometer in phosphate buffer system (pKa 6.8). Thin-layer chromatograms of radiolabeled materials were run with 1-in. wide Eastman chromatograms, type K301R with fluorescence indicator, and the compounds were detected with ultraviolet light. The distribution of radioactivity on the developed chromatograms was determined by scanning each strip with an Atomic Associates RCS-363 radiochromatogram scanner. The specific activities were determined with an Atomic Associates well scintillation counter model 810C and scintillation spectrometer model 530. The silicic acid used in the column chromatography was Baker and Adamson reagent grade.

[4] Purchased from New England Nuclear Corp., Boston, Mass.

and extracted with ether (6 × 25 ml.). The extract was washed with 10% sodium bisulfite solution (100 ml.) and dried over a mixture of anhydrous sodium sulfate and a decolorizing agent.[5] Removal of the solvent gave a solid (crude V) which was acetylated directly with acetic anhydride (10 ml.) containing a few drops of 70% perchloric acid. The acetylation mixture was allowed to stand at room temperature for 30 min. Water (25 ml.) was then added and the product was extracted with chloroform (5 × 25 ml.). Evaporation of the dried chloroform solution (anhydrous sodium sulfate) furnished the solid triacetate (VI).

This triacetate (VI), without further purification, was treated with acetic anhydride (12 ml.) and anhydrous sodium acetate (6.0 Gm.) at 140° for 2 min. The cooled reaction mixture was diluted with water (25 ml.) and extracted with chloroform (5 × 40 ml.). The extract was washed with 5% sodium bicarbonate solution (100 ml.), dried (anhydrous sodium sulfate), and evaporated to afford the solid, dinitrostyrene derivative (VII).

Crude VII was suspended in glacial acetic acid (65 ml.) and added dropwise over a 3-hr. period to a stirred mixture of glacial acetic acid (50 ml.) and iron powder (18.6 Gm.). No external heat was applied. The reaction mixture was stirred for an additional 3 hr. at room temperature. The solid residue was removed by filtration and washed well with hot ethanol. The resulting filtrate was diluted with water (50 ml.), neutralized with solid $KHCO_3$, and extracted with ether (7 × 50 ml.). The extract was treated with anhydrous sodium sulfate and the decolorizing agent and the solvent were removed *in vacuo*. The resulting solid brown residue was dissolved in a minimum of benzene, and adsorbed onto silica gel (25 Gm.), and the column was eluted with benzene. The solvent was removed from the combined indole-containing fractions[6] under reduced pressure to give pure VIII (1.37 Gm., 43%) m.p. 131–133° [lit. (5) m.p. 134–136°] with a specific activity of 90 μc./mmole.

Purity of the final product was checked by mixed melting point with an authentic sample, and by ascending paper chromatography using benzene as the solvent. Chemical and radiochemical purity were confirmed when the radiochromatogram scan of the developed chromatogram showed only one peak, corresponding to the same position as the indole compound.

5,6-Diacetoxy-7-iodoindole-^{125}I (X)—An aqueous solution of $Na^{125}I$ (1 ml., 350 μc.)[7] was evaporated under nitrogen (45°), and a solution of 5,6-diacetoxy-7-iodoindole (7) (101.2 mg.) in acetic acid (5 ml.) was added. The solution was refluxed for 30 min. and diluted with water (20 ml.), and solid sodium bicarbonate was added in small portions to neutralize the acetic acid. The resulting mixture was extracted with benzene (5 × 10 ml.), and the extract was washed with 5% sodium bicarbonate solution and dried (anhydrous sodium sulfate). Removal of the solvent *in vacuo* gave a residue which was purified by column chromatography over silica gel (5 Gm.) and elution with chloroform–benzene (1:1). The indole-containing fractions were combined and concentrated under nitrogen. Petroleum ether (b.p. 30–40°) was added periodically until a white crystalline material appeared. Removal of the supernatant afforded pure X (93 mg.), m.p. 125.7–127.1° [lit. (7) m.p. 125–126.5°], with a specific activity of 484 μc./mmole. Exchange occurred to the extent of 35.6%. Purity of the final product was established by TLC using ethyl ether as the eluant. The developed chromatogram was subsequently subjected to a radiochromatogram scan in which only one area of radioactivity was found which was superimposable with the developed indole spot.

Spectrophotometric Analysis of 5,6-Diacetoxyindole Solutions—Standard phosphate buffer solutions (pKa 6.8) were prepared for pH values of 6.8, 7.2, and 7.6, and their lack of absorbance in the ultraviolet region (210–360 mμ) was established. The buffered stock solutions of 5,6-diacetoxyindole were prepared by dissolving the indole in a minimum volume of methanol and diluting to volume with the respective standard phosphate buffer solutions. Subsequent dilutions of the indole stock solutions for U.V. determinations were made with the corresponding phosphate buffer. The pH of each solution was checked experimentally before and after spectrophotometric determination. Data from these experiments provided standard linear curves of concentration of indole *versus* absorbance for each pH value. The rates of decomposition for these dilute solutions of 5,6-diacetoxyindole were determined from the standard linear curves. Results are shown in Fig. 1.

[5] Norit, American Norit Co., Jacksonville, Fla.

[6] Indole-containing fractions were identified with Erlich's reagent.

[7] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.

REFERENCES

(1) Raven, R. W., *Ann. N. Y. Acad. Sci.*, **100**, 142(1963).

(2) Blois, M. S., and Kallman, R. F., *Cancer Res.*, **24**, 863(1964).

(3) Fimiam, W. J., and Dowd, G. A., *Proc. Soc. Exptl. Biol. Med.*, **102**, 95(1959).

(4) Meister, A., "Biochemistry of the Amino Acids," vol. II, 2nd ed., Academic Press Inc., New York, N. Y., 1965, pp. 909, 919.

(5) Beer, R. J. S., Clarke, K., Khorana, H. G., and Robertson, A., *J. Chem. Soc.*, **1948**, 2223.

(6) Parijs, A. H., *Rec. Trav. Chim.*, **49**, 33(1930).

(7) Heacock, R. A., Mahon, M. E., and Scott, B. D., *Can. J. Chem.*, **39**, 231(1961); Heacock, R. A., Hutzinger, O., Scott, B. D., Daly, J. W., and Witkop, B., *J. Am. Chem. Soc.*, **85**, 1825(1963).

(8) Meier, D. A., Counsell, R. E., and Beierwaltes, W. H., *Cancer Res.*, to be published.

(9) Hempel, K., and Deimel, M., *Strahlentherapie*, **121**, 22(1963).

(10) Best, C. H., and Taylor, N. B., "The Physiological Basis of Medical Practice," 7th ed., Williams and Wilkins Co., Baltimore, Md., 1961, p. 131.

(11) Riley, P. A., *Nature*, **213**, 190(1967).

Evaluation of the Stormer Viscosimeter in the Measurement of Certain Pharmaceutical Systems

By OSCAR E. ARAUJO

The performance of the Stormer viscosimeter was analyzed in detail by the study of the rheological properties of natural gums (acacia and tragacanth) and of synthetic gums (methylcellulose, sodium carboxymethylcellulose, and hydroxyethylcellulose). Two Newtonian systems (glycerin and sucrose solutions) were also investigated. Several different concentrations of each material were studied. There appeared to be an increase in viscosity with increasing rate of shear for most of the systems at the lower concentrations; a level of concentration was eventually reached where all the gum systems exhibited pseudoplastic characteristics and the Newtonian systems true linearity.

THE STORMER viscosimeter is a commercially available Searle type of viscometer described and modified for application to non-Newtonian systems by Fischer (1). The operational features and suggested useful ranges are outlined in several other references (2–5). The instrument has been widely used by investigators in the pharmaceutical sciences for well over a decade (6–12). Durability, ease of operation, and reproducibility of results are largely the strong points of the Stormer which led to its use by the above investigators.

None of the workers reported inconsistencies in the rheological data obtained. The following study was performed largely because of certain unusual results obtained in the measurement of some dilute solutions of hydrocolloids with the Stormer viscosimeter.

The initial inclination to attribute these discrepancies to experimental error was abandoned because of the recurring pattern of the results. Consequently, it was decided to carry out an extensive analysis of the performance of the Stormer viscosimeter in the measurement of various rheological systems.

EXPERIMENTAL

Preparation of Solutions—The substances used in the study, along with the range of concentration of their solutions, are given in Table I. From 7 to 10 solutions of different concentrations were prepared for each substance within the indicated range.

The solutions were prepared in the conventional manner. A Waring blender was used to disperse the gums after they had been moderately hydrated. Heat was employed to speed up solubility in the more concentrated solutions.

Rheological Measurements—A Stormer viscosimeter equipped with the modified cup and bob, as suggested by Fischer (1), was used. A weight hanger with various slotted weights provided the shearing stress. The appropriate instrumental constants, K_v, were determined from calibration data collected using standard oils, and were applied to each point on the rheograms. The apparent viscosity of the various solutions were then calculated from the expression:

$$\eta = K_v \frac{\text{Gm.}}{\text{r.p.m.}}$$

where Gm. represents the shearing stress and r.p.m. the rate of shear. All viscosity measurements were carried out at 20 ± 0.1°.

TABLE I—RANGE OF CONCENTRATION FOR EACH SUBSTANCE USED

Substance	% w/w
Acacia	7.5–45
Tragacanth	0.1–2.0
Methylcellulose	0.4–3.5
Sodium CMC	0.2–4.0
Hydroxyethylcellulose	0.5–2.0
Glycerin	35–90
Sucrose	35–70

RESULTS AND DISCUSSION

Rheograms were constructed for all the solutions by plotting r.p.m. as the ordinate and Gm. as the abscissa. Representative plots at three levels of concentration for each substance are shown in Figs. 1, 3, and 5.

The rheograms in Figs. 1 and 3 were redrawn to show the change in viscosity with rate of shear so that a more thorough analysis of the data could be performed. Figures 2 and 4 show the result of this representation.

The first series of rheograms obtained were those for the acacia solutions. As a result of the data collected, several other systems of known pseudoplastic nature were prepared as indicated in Table I; two Newtonian systems (glycerin and sucrose solutions) were also prepared to provide the basis for a complete comparison of the results.

Figure 1 shows rheograms of each solution at a low level of concentration. Each substance exhibits the same "dilatant" type curve, whereby there is an increase in viscosity with increasing rate of shear. These results can hardly be attributed to isolated experimental discrepancies, since, as can be clearly seen in Fig. 2, a definite pattern is consistent throughout. The instrument was checked as to proper alignment of the cup and bob and for any mechanical defects which might cause discrepancies in the data. No apparent mechanical anomalies were found to exist.

As the concentration of the solutions is increased, the rheograms indicate an over-all decrease and eventual disappearance of this dilatant effect. Figure 4 shows the plots for that level of concentration of each substance above which the so-called dilatant effect no longer exists.

Figure 5 shows the rheograms depicting the performance of the Stormer viscosimeter in the measure-

Received January 23, 1967, from the Department of Pharmacy, College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication May 17, 1967.

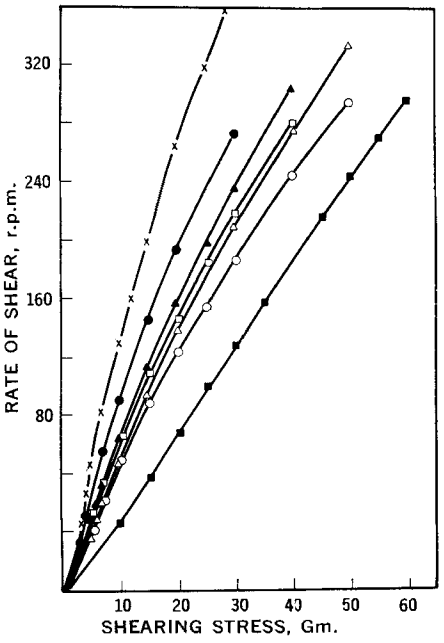


Fig. 1—Rheograms of aqueous solutions of low concentration. Key: ×, 0.15% tragacanth; ●, 10.00% acacia; ▲, 0.40% sodium carboxymethylcellulose; □, 63.50% glycerin; △, 0.70% methylcellulose; ○, 51.50% sucrose; ■, 0.50% hydroxyethylcellulose.

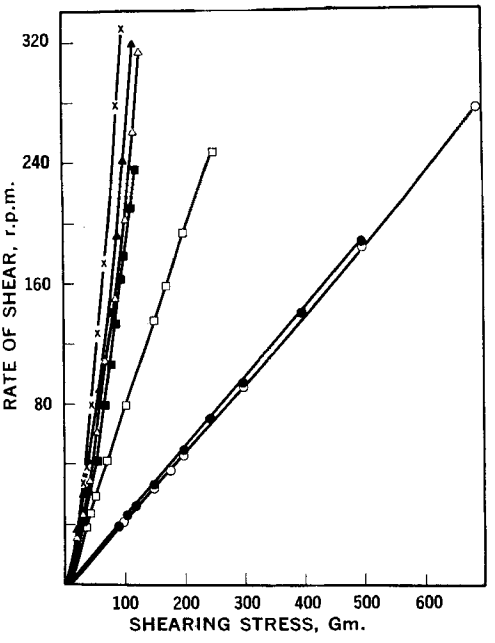


Fig. 3—Rheograms of aqueous solutions of concentration above which no dilatant effect exists. Key: ×, 0.95% tragacanth; ●, 35.00% acacia; ▲, 1.25% sodium carboxymethylcellulose; □, 88.00% glycerin; △, 1.30% methylcellulose; ○, 70.00% sucrose; ■, 0.75% hydroxyethylcellulose.

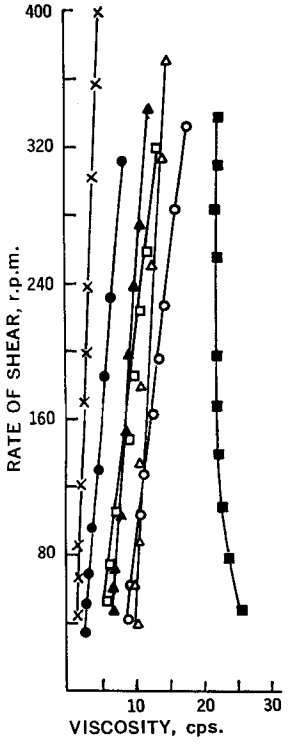


Fig. 2—Viscosity–shear rate dependence of aqueous solutions. Key: ×, 0.15% tragacanth; ●, 10.00% acacia; ▲, 0.40% sodium carboxymethylcellulose; □, 63.50% glycerin; △, 0.70% methylcellulose; ○, 51.50% sucrose; ■, 0.50% hydroxyethylcellulose.

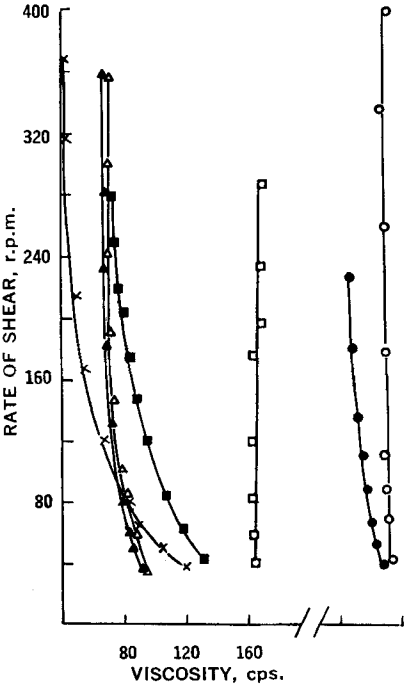


Fig. 4—Viscosity–shear rate dependence of aqueous solutions. Key: ×, 0.95% tragacanth; ●, 35.00% acacia; ▲, 1.25% sodium carboxymethylcellulose; □, 88.00% glycerin; △, 1.30% methylcellulose; ○, 70.00% sucrose; ■, 0.75% hydroxyethylcellulose.

ment of the gum systems at the highest level of concentration of each, as indicated in Table I. All the

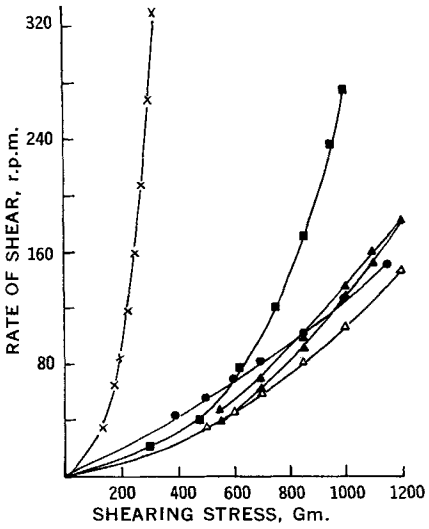


Fig. 5—Rheograms of aqueous gum solutions of high concentration. Key: ×, 1.8% tragacanth; ●, 45% acacia; ▲, 4.0% sodium carboxymethylcellulose; △, 3.5% methylcellulose; ■, 2.0% hydroxyethylcellulose

systems are cleary pseudoplastic in nature, except for sodium carboxymethylcellulose which exhibits a slight degree of thixotropy. No apparent problems are evident at this level of concentration with the instrument used.

From all of the results obtained, unless one cares to argue the case for actual dilatancy of all the systems at low concentrations, there are some interesting inferences which can be made.

It is conceivable that all gums used are pseudoplastic throughout the entire range of shear at all concentrations, and that there is an inherent "dilatant" producing effect with the Stormer which shifts all systems in that direction at lower concentrations. This effect then becomes less pronounced as the concentrations are increased and the true pseudoplastic nature of all the hydrocolloids eventually becomes predominant rendering the dilatant appearance imperceptible.

The other possibility is that these hydrocolloids are Newtonian in nature at the lower concentrations and the dilatant behavior simply indicates that the Stormer cannot be used effectively for systems having a viscosity lower than 20 cps. As the concentration is increased the systems become truly pseudoplastic and the Stormer performance is satisfactory at all rates of shear. This approach would explain the glycerin rheograms, since they exhibit Newtonian behavior only after a certain concentration is reached. Glycerin solutions, incidentally, should not be used to calibrate the Stormer viscosimeter in order to obtain the instrumental constants, K_v, because of their hygroscopic nature.

SUMMARY

1. Various hydrocolloids at several concentrations were studied using the Stormer viscosimeter.

2. Two Newtonian systems (glycerin and sucrose solutions) at various concentrations were also studied rheologically.

3. Results showed some interesting features not usually attributed to these systems: (*a*) there appeared to be a dilatant effect for all systems at low concentrations; (*b*) there seemed to be a minimum concentration for all gum systems, above which pseudoplastic behavior is observed throughout the entire range of shear rate.

4. The Stormer viscosimeter appeared to perform well in the rheological measurements of the more concentrated gum systems.

REFERENCES

(1) Fischer, E. K., "Colloidal Dispersions," John Wiley & Sons, Inc., New York, N. Y., 1950, pp. 159–168.

(2) "Directions for Use of Stormer Viscosimeter," Arthur H. Thomas Co., Philadelphia, Pa., 1948.

(3) Martin, A. N., "Physical Pharmacy," Lea and Febiger Publishing Co., Philadelphia, Pa., 1960.

(4) Van Wazer, J. R., Lyons, J. W., Kim, K. Y., and Colwell, R. E., "Viscosity and Flow Measurement," John Wiley & Sons, Inc., New York, N. Y., 1963, p. 150.

(5) Bean, H. S., Beckett, A. H., and Carless, J. E., "Advances in Pharmaceutical Sciences," vol. I, Academic Press, London, England, 1964, p. 39.

(6) Araujo, O. E., *J. Pharm. Sci.*, **55**, 636(1966).

(7) Kabre, S. P., DeKay, H. G., and Banker, G. S., *ibid.*, **53**, 492(1964).

(8) Boylan, J. E., DeKay, H. G., and Banker, G. S., *ibid.*, **51**, 742(1962).

(9) Chong, C. W., Eriksen, S. P., and Swintosky, J. V., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 547(1960).

(10) Foernzler, E. C., Martin, A. N., and Banker, G. S., *ibid.*, **49**, 249(1960).

(11) Joslin, R. S., and Sperandio, G. J., *Drug Std.*, **25**, 72 (1957).

(12) Kostenbauder, H. B., and Martin, A. N., *J. Am. Pharm. Assoc. Sci. Ed.*, **43**, 401(1954).

Acetylsalicylic Acid Hydrolysis in Aqueous Solutions of Polysorbate 80

By K. S. MURTHY and E. G. RIPPIE

The hydrolysis of acetylsalicylic acid (ASA) has been shown to occur at a measurable rate within polysorbate 80 micelles in an aqueous environment at pH 1.8. An estimate of the apparent pseudo first-order rate constant for the micellar degradation is made on the basis of the distribution of the undissociated ASA between the aqueous and the micellar pseudophase.

HYDROLYTIC SUSCEPTIBILITY of various drug species, in aqueous systems containing surfactants at concentrations above their CMC, has been the subject of several studies. Generally, it has been found that hydrolysis occurs within the micellar pseudophase as well as in the aqueous phase. Riegelman (1) demonstrated that benzocaine, solubilized in the polyoxyethylene palisade layer of certain cetyl alcohol polyoxyethylene ethers, is subject to hydroxyl-ion catalyzed hydrolysis. However, recent studies (2) indicate that no hydroxyl-ion catalyzed hydrolysis of chlorobutanol occurs within micelles formed by lauromacrogal or polysorbate 20. It has been suggested by Nogami (3) that the observed suppression by surfactants of the hydrolysis, below pH 5, of undissociated acetylsalicylic acid is due to partitioning of a part of the ASA into the micelles, where it becomes less hydrolyzable. The present study was undertaken to determine quantitatively the degree of protection afforded by polysorbate 80 to aqueous solutions of ASA.

EXPERIMENTAL

Reagents—Acetylsalicylic acid U.S.P. (Mallinckrodt) and polysorbate 80 (Atlas Chemical Industries, Inc.) were used as received. All other chemicals used were reagent grade.

Apparatus—All studies were performed using a Sargent constant-temperature water bath held at 30° ± 0.01°, Burrell wrist action shaker, Beckman model GS pH meter, and Beckman model DU spectrophotometer.

Experimental Procedure—Since solutions of relatively high buffer capacity were required in many cases, all solutions were adjusted to an ionic strength of 1.00. Analysis of salicylic acid and ASA was accomplished spectrophotometrically by the method of Edwards (4).

Solubility studies were conducted in vials of 20-ml. capacity. Excess solid ASA was added to vials containing 20 ml. of 0, 1, 2.5, and 4% solutions of polysorbate 80 at various pH's in the range 2.63–4.43. The solutions were agitated until successive assays indicated a state of equilibration. In general, three separate equilibrations were run at each combination of pH and polysorbate 80 concentration. Samples were forced through Millipore G.S. 0.22-μ filters prior to assay and the pH of the filtrate noted at this time. Buffers were as follows: pH 2.63, phosphate; pH 3.63, 4.10, 4.21, and 4.43, citrate.

Kinetic studies were carried out with saturated suspensions as well as with saturated and half-saturated solutions of ASA at a pH of 1.8. The suspensions and solutions were sampled periodically, and the samples filtered and analyzed as before. Kinetics were followed at 0, 2, and 4% polysorbate 80 levels for periods of 48 hr. Phosphate buffer (1.69 M) was used, and duplicate runs were conducted at each polysorbate concentration.

RESULTS AND DISCUSSION

The apparent pKa of acetylsalicylic acid under the conditions of these studies was obtained from

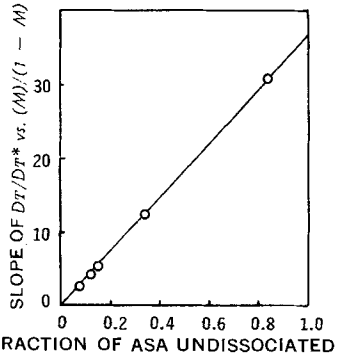


Fig. 1—Graph showing the slopes of $(D)_t/(D)_t^*$ vs. $(M)/(1 - M)$ *plotted* vs. *the fraction of ASA undissociated. The intercepts are the partition coefficients of the undissociated and dissociated acid.*

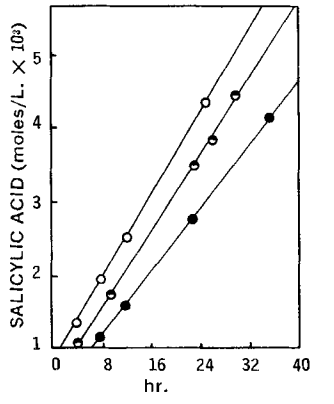


Fig. 2—Graph showing the rate of appearance of salicylic acid in aqueous suspensions of ASA at pH = 1.8 and 30°. Polysorbate 80 concentrations: ○, *4%;* ◓, *2%;* ●, *0%.*

Received March 16, 1967, from the Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

Accepted for publication May 19, 1967.

Abstracted in part from a dissertation presented by K. S. Murthy to the Graduate School, University of Minnesota, Minneapolis, in partial fulfillment of Master of Science degree requirements.

TABLE I—ZERO-ORDER RATE CONSTANTS OF ASA HYDROLYSIS IN AQUEOUS SUSPENSIONS[a]

Polysorbate 80, % w/v	Over-All Zero-Order Rate Constants (moles-L.$^{-1}$ hr.$^{-1}$ × 10^4)
0	1.10
2	1.23
4	1.40

[a] Conditions: pH = 1.80; ionic strength = 1.00; temperature = 30° C.

TABLE II—FIRST-ORDER RATE CONSTANTS OF ASA HYDROLYSIS IN AQUEOUS SOLUTIONS[a]

Polysorbate 80, % w/v	Over-All First-Order Rate Constants[b] (hr.$^{-1}$ × 10^3)	Micellar First-Order Rate Constants[b] (hr.$^{-1}$ × 10^3)
0[c]	9.2	...
2[c]	5.4	1.8
4[c]	4.5	2.2
0[d]	9.6	...
2[d]	5.7	1.9
4[d]	4.4	1.8

[a] Conditions: pH = 1.80; ionic strength = 1.00; temperature = 30° C. [b] Constants are calculated on the basis of the micellar pseudophase, assuming its volume in ml. is equal to the weight of polysorbate 80 in Gm., and assuming a partition coefficient of 51.9. [c] Saturated solutions of ASA. [d] Half-saturated solutions of ASA.

solubility data and found to be 3.35. The usual literature value cited is 3.49.

Hydrolysis of polysorbate 80 at pH 1.8 and unit ionic strength was found to be approximately 3% after an elapsed time of 48 hr. This degree of degradation did not have a measurable effect on either the solubility or the stability of ASA.

Partitioning of ASA into the Micellar Pseudophase—Results of the solubility determinations were interpreted assuming a linear partition isotherm, as described previously (5). In the present studies, however, the volume occupied by the micelles was not assumed to be negligible. Figure 1 represents a plot of the slope of the ratio $(D)_t/(D)_t^*$ *versus* $(M)/(1 - M)$, against the mole fraction of undissociated ASA. $(D)_t$ and $(D)_t^*$ are the total concentration of ASA in solution, and the concentration of ASA exclusive of that present in the micelles, respectively; (M) is the volume fraction of micellar pseudophase in the system, assuming the micellar volume in ml. is equal to the weight of surfactant in Gm. The curve passes through the origin, indicating the absence of dissociated ASA in the micellar pseudophase of the polysorbate 80 solutions. These findings are in agreement with those of Nogami (3).

The apparent partition coefficient of undissociated ASA, under the conditions of the kinetics studies, was found to be 52. This differs from that (about 37) predicted by Fig. 1. In this case, the high percentage of phosphoric acid (6.7%) present in the medium would change the polarity of the solvent so as to increase the activity coefficient of the undissociated ASA. This was confirmed by solubility determinations of ASA at pH 1.55 and unit ionic strength in aqueous solutions at various phosphoric acid concentrations.

Kinetic Studies—Since from the solubility studies it is inferred that the dissociated form of ASA exists only in the aqueous phase, the observed increase in degradation rate constants in suspensions (pseudo zero-order rate constants) with added polysorbate 80 is due to instability of undissociated ASA in the micellar phase. These constants were calculated from the rate of appearance of salicylic acid in the suspensions as illustrated in Fig. 2. Results are given in Table I.

Rates of hydrolysis were also determined in homogeneous solutions at saturation and half-saturation under conditions similar to the suspensions. The results are presented in Table II, where it can be seen that the rate of hydrolysis of ASA in polysorbate 80 micelles, while lower than in the aqueous phase, is not negligible. The reaction occurring within the micellar pseudophase is presumably hydrogen-ion catalyzed since the hydroxyl and water catalyzed reactions do not occur to a measurable extent at this pH (4).

Salicylic acid, produced during the hydrolysis, has been shown (6) to have no significant effect on the apparent solubility of ASA in aqueous systems.

SUMMARY AND CONCLUSIONS

Hydrolysis of ASA occurs to a significant extent within polysorbate 80 micelles in an aqueous medium at a pH of 1.8. An estimate of the pseudo first-order rate constants for the micellar degradation indicates a rate approximately one fifth that in the corresponding aqueous phase. For this reason, the addition of polysorbate 80 to aqueous suspensions of ASA can be expected to accelerate hydrolytic degradation at pH's below approximately 5.

REFERENCES

(1) Riegelman, S., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 339(1960).
(2) Anderson, R. A., and Slade, A. H., *J. Pharm. Pharmacol.*, **18**, 640(1966).
(3) Nogami, H., Awazu, S., and Nakojima, N., *Chem. Pharm. Bull. (Tokyo)*, **10**, 503(1962).
(4) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723(1950).
(5) Rippie, E. G., Lamb, D. J., and Romig, P. W., *J. Pharm. Sci.*, **53**, 1346(1964).
(6) Garrett, E. R., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 584(1957).

Phase Transitions I.

Preliminary Study of Theophylline Hydrate–Anhydrate System

By ELI SHEFTER and GERALD KMACK*

The phase transformation of theophylline hydrate to an anhydrous form was measured at a variety of temperatures using X-ray powder diffractometry. The conversion rate appeared to be first order with respect to the amount of hydrate present.

NUMEROUS MEDICINAL AGENTS are capable of existing in the solid state as hydrates and nonhydrated polymorphs. Since the physical chemical properties of many of these modifications of a drug can be markedly different, this may in turn influence its pharmaceutical formulation. In many instances it would therefore be important to understand what factors might influence a solid state transition from a hydrate to an anhydrous form, or *vice versa*, and at what rate this transformation occurs.

The primary purpose of this preliminary study on phase transitions of pharmaceuticals is to demonstrate that by the use of X-ray powder diffractometry, kinetic data for the conversion of a hydrate to its anhydrous state can easily be obtained. The method has the advantage over other techniques in that one is measuring directly the crystal structure transformation. For this study the theophylline system was chosen, since the crystal structure of the hydrate has already been reported (1) and the thermodynamics of dissolution for the system has been investigated (2).

EXPERIMENTAL

Theophylline (U.S.P. grade) was recrystallized from water and then allowed to dry on filter paper at room temperature for a week. The material was then ground in a glass mortar until a fine powder was obtained. Since the particle size was not measured in these experiments, it was necessary to grind all the experimental material at once, so that the particle size distribution from run to run would be fairly constant.

A series of samples weighing approximately 4 Gm. each was distributed evenly on the bottom of Petri dishes by shaking the glass vessels. The dishes were placed in an oven with the temperature previously set. During the experiment, a thermometer recorded the air temperature directly above the sample. Samples were taken from the oven at hourly intervals, without altering the temperature more than a degree or two, and then added to tightly-stoppered vials.

Some of the material was heated at a temperature of 100° for a period of 1 week. Though conversion of the hydrate to the anhydrous form occurred at an extremely rapid rate at this temperature, the week-long heating ensured that all the material was converted to the latter form. X-Ray diffraction powder patterns of the two modifications were measured on a GE XRD-6 diffractometer. There were many diffraction regions in which the two forms had intense peaks which were separated sufficiently to permit quantitative analysis of mixtures of the two, in a manner similar to that described by Azaroff and Buerger (3) and Shell (4). The diffraction peaks utilized were those at 13.2° (2θ) for the hydrate and at 12.6° (2θ) for the anhydrous crystals.

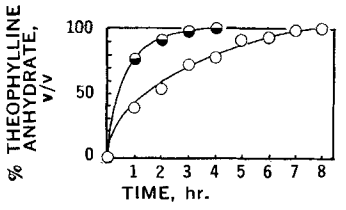


Fig. 1—Anhydrate formation from theophylline hydrate at 40° and 50°. Key: ◒, 50°; O, 40°.

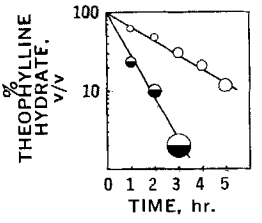


Fig. 2—Conversion of theophylline hydrate to the anhydrate–transformation rates at 40° and 50°. Key: ◒, 50°; O, 40°.

RESULTS AND DISCUSSION

Studies carried out at room temperature (approximately 25°) showed that there was no detectable transformation of the hydrate to the anhydrous form, over a period of 2 months. Above 60° the rate of conversion was so rapid that it was not possible to measure it by the present technique. At 60° the rate was measureable but appeared to be suffering from the lag time necessary for the material to reach the oven temperature. The conversion curves (Figs. 1 and 2) at 40° and 50° gave apparent first-order transformation rates. It is felt that at these temperatures the time necessary for the solid material to reach the oven temperature was probably negligible compared to the initial rate of transformation. Since particle size and humidity were not controlled, it is felt that a detailed analysis of the rate data would be presumptuous. It is interesting to note, however, that the transition temperature for this phase transformation probably lies not too far above room temperature but below 40°. This temperature is considerably lower than that obtained by the solubility method of Shefter and Higuchi (2); where 73° was obtained for the transition temperature in water. This is a direct indication that hu-

Received March 8, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, NY 14214, and Center for Crystallographic Research, Roswell Park Memorial Institute, Buffalo, NY 14203

Accepted for publication May 8, 1967.

The authors thank Professor David Harker, Crystallographic Center at R.P.M.I., for kindly permitting them to use his facilities.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

* Participant in NSF Summer Program at Roswell Park Memorial Institute.

midity probably plays a major role in this phase transformation.

The transformation of caffeine hydrate to an anhydrous state was also carried out in the same manner. The results obtained for the two purine systems are quite different. Hydrated caffeine had a fairly rapid rate of transformation at room temperature, and the conversion appeared to go through various hydrate intermediates, as evidenced through certain changes in the X-ray powder patterns with time. This latter phenomenon is in agreement with the findings of Waters and Beal (5). The general differences in the transformation processes for the two purine hydrates are consistent with structural studies on the hydrates of the two compounds (1, 6, 7). The hydrogen bonding schemes in the two hydrate crystal structures are markedly different with respect to the waters. It would be tempting to correlate the semiquantitative rate data with the crystal structures of the hydrates; however, such speculations would be premature at this time in view of the lack of structural data on the anhydrous crystals and more detailed phase transformation data.

REFERENCES

(1) Sutor, D. J., *Acta Cryst.*, **11**, 83(1958).
(2) Shefter, E., and Higuchi, T., *J. Pharm. Sci.*, **52**, 781 (1963).
(3) Azaroff, L. V., and Buerger, M. J., "The Powder Method," McGraw-Hill Book Co., New York, N. Y., 1958, p. 200.
(4) Shell, J. W., *J. Pharm. Sci.*, **52**, 24(1963).
(5) Waters, K. L., and Beal, G. D., *J. Am. Pharm. Assoc., Sci. Ed.*, **35**, 12(1946).
(6) Sutor, D. J., *Acta Cryst.*, **11**, 453(1958).
(7) Gerdil, R., and Marsh, R. E., *ibid.*, **13**, 166(1960).

Stereospecific Hydrogenations Using Palladium-on-Poly-L-leucine

By ROBERT L. BEAMER, CAROLYN S. FICKLING, and JAMES H. EWING

Studies have been conducted concerning the stereospecific nature of catalysts prepared by depositing palladium upon a poly-L-amino acid. The poly-L-amino acid chosen for this study was poly-L-leucine which was prepared from *N*-carboxy-L-leucine anhydride. The substrates employed in this work were α-methylcinnamic acid and α-acetamidocinnamic acid. Hydrogenation of α-methylcinnamic acid catalyzed by palladium-on-poly-L-leucine produced predominately R(−)-dihydro-α-methylcinnamic acid and hydrogenation of α-acetamidocinnamic acid using the same catalyst produced, after hydrolysis, S(−)-phenylalanine.

THE STEREOSPECIFICITY of catalysts prepared from silica gels precipitated in the presence of cinchona alkaloids has been shown by Padgett and Beamer (1). Beamer and Lawson have presented evidence of substrate specificity with similarly prepared gels (2). The silica gel work was based on prior evidence indicating the existence of stereospecific and substrate-specific centers in palladium-on-charcoal catalysts (3). Also, Akabori *et al.* had demonstrated the stereospecificity of palladium-on-silk fibroin (4) and Beckett *et al.* had shown the stereospecificity of adsorption in specially prepared silica gels (5–7).

The present work concerns the observation of stereospecificity in palladium-on-poly-L-amino acids. The poly-L-amino acid chosen for this study was poly-L-leucine which was prepared by polymerization of the *N*-carboxy-α-amino acid anhydride *in vacuo*.

Stereoselection in these catalysts should occur from asymmetric induction arising from stereoselective adsorption to active sites on the catalyst surface followed by *cis*-addition of hydrogen (3).

Received January 16, 1967, from the School of Pharmacy, University of South Carolina, Columbia, SC 29208
Accepted for publication May 16, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This work was supported in part by grant GM-12109-03 from the U.S. Public Health Service, Bethesda, Md.
The authors express their appreciation to Mrs. Annie Leeper and Miss Lucy Harvie, Department of Chemistry and Pharmaceutical Chemistry, Medical College of Virginia, for the carbon, hydrogen, and nitrogen analyses reported in this study.

EXPERIMENTAL

Reagents—α-Methylcinnamic acid (Aldrich), carbobenzyloxy chloride (Nutritional Biochemicals), L-leucine (Mann Biochemicals).

Poly-L-leucine—This polypeptide was prepared from the *N*-carboxy-α-amino acid anhydride by the Bergman procedure (8) which consisted of preparation of the carbobenzyloxy amino acid (CBZ amino acid) from CBZ chloride and L-leucine. The anhydride was prepared by Leuch's procedure from the CBZ amino acid and thionyl chloride (8). By melting the anhydride under high vacuum (10^{-3} mm. Hg) the polyamino acid was obtained. The yield was 23% of theory (based on L-leucine). Infrared spectra (Nujol mull) of the resulting polyamino acid were identical with those given by Bamford *et al.* (9). Carbon, hydrogen, and nitrogen analyses indicate the product is the hemihydrate and this has been confirmed by drying in an oven. Molecular weight determinations are currently being carried out and will be reported at a later date.

Anal.—Calcd. for $C_6H_{11}NO \cdot {}^1/_2H_2O$: C, 58.99; H, 9.90; N, 11.47. Found: C, 59.66; H, 9.51; N, 11.55.

α-Acetamidocinnamic Acid—This compound was prepared by hydrolysis of the azlactone, 2-methyl-4-benzilideneoxazolin-5-one (10). The product melted from 190° to 191.5° (uncorrected). [Lit. (10) m.p. 191°–192°.]

Preparation of Catalysts—Two methods were used to prepare the catalysts.

Method A—Sixteen milliliters of a 2.5% palladous

midity probably plays a major role in this phase transformation.

The transformation of caffeine hydrate to an anhydrous state was also carried out in the same manner. The results obtained for the two purine systems are quite different. Hydrated caffeine had a fairly rapid rate of transformation at room temperature, and the conversion appeared to go through various hydrate intermediates, as evidenced through certain changes in the X-ray powder patterns with time. This latter phenomenon is in agreement with the findings of Waters and Beal (5). The general differences in the transformation processes for the two purine hydrates are consistent with structural studies on the hydrates of the two compounds (1, 6, 7). The hydrogen bonding schemes in the two hydrate crystal structures are markedly different with respect to the waters. It would be tempting to correlate the semiquantitative rate data with the crystal structures of the hydrates; however, such speculations would be premature at this time in view of the lack of structural data on the anhydrous crystals and more detailed phase transformation data.

REFERENCES

(1) Sutor, D. J., *Acta Cryst.*, **11**, 83(1958).
(2) Shefter, E., and Higuchi, T., *J. Pharm. Sci.*, **52**, 781 (1963).
(3) Azaroff, L. V., and Buerger, M. J., "The Powder Method," McGraw-Hill Book Co., New York, N. Y., 1958, p. 200.
(4) Shell, J. W., *J. Pharm. Sci.*, **52**, 24(1963).
(5) Waters, K. L., and Beal, G. D., *J. Am. Pharm. Assoc., Sci. Ed.*, **35**, 12(1946).
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Stereospecific Hydrogenations Using Palladium-on-Poly-L-leucine

By ROBERT L. BEAMER, CAROLYN S. FICKLING, and JAMES H. EWING

Studies have been conducted concerning the stereospecific nature of catalysts prepared by depositing palladium upon a poly-L-amino acid. The poly-L-amino acid chosen for this study was poly-L-leucine which was prepared from *N*-carboxy-L-leucine anhydride. The substrates employed in this work were α-methylcinnamic acid and α-acetamidocinnamic acid. Hydrogenation of α-methylcinnamic acid catalyzed by palladium-on-poly-L-leucine produced predominately R(−)-dihydro-α-methylcinnamic acid and hydrogenation of α-acetamidocinnamic acid using the same catalyst produced, after hydrolysis, S(−)-phenylalanine.

THE STEREOSPECIFICITY of catalysts prepared from silica gels precipitated in the presence of cinchona alkaloids has been shown by Padgett and Beamer (1). Beamer and Lawson have presented evidence of substrate specificity with similarly prepared gels (2). The silica gel work was based on prior evidence indicating the existence of stereospecific and substrate-specific centers in palladium-on-charcoal catalysts (3). Also, Akabori *et al.* had demonstrated the stereospecificity of palladium-on-silk fibroin (4) and Beckett *et al.* had shown the stereospecificity of adsorption in specially prepared silica gels (5–7).

The present work concerns the observation of stereospecificity in palladium-on-poly-L-amino acids. The poly-L-amino acid chosen for this study was poly-L-leucine which was prepared by polymerization of the *N*-carboxy-α-amino acid anhydride *in vacuo*.

Stereoselection in these catalysts should occur from asymmetric induction arising from stereoselective adsorption to active sites on the catalyst surface followed by *cis*-addition of hydrogen (3).

Received January 16, 1967, from the School of Pharmacy, University of South Carolina, Columbia, SC 29208
Accepted for publication May 16, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This work was supported in part by grant GM-12109-03 from the U.S. Public Health Service, Bethesda, Md.
The authors express their appreciation to Mrs. Annie Leeper and Miss Lucy Harvie, Department of Chemistry and Pharmaceutical Chemistry, Medical College of Virginia, for the carbon, hydrogen, and nitrogen analyses reported in this study.

EXPERIMENTAL

Reagents—α-Methylcinnamic acid (Aldrich), carbobenzyloxy chloride (Nutritional Biochemicals), L-leucine (Mann Biochemicals).

Poly-L-leucine—This polypeptide was prepared from the *N*-carboxy-α-amino acid anhydride by the Bergman procedure (8) which consisted of preparation of the carbobenzyloxy amino acid (CBZ amino acid) from CBZ chloride and L-leucine. The anhydride was prepared by Leuch's procedure from the CBZ amino acid and thionyl chloride (8). By melting the anhydride under high vacuum (10^{-3} mm. Hg) the polyamino acid was obtained. The yield was 23% of theory (based on L-leucine). Infrared spectra (Nujol mull) of the resulting polyamino acid were identical with those given by Bamford *et al.* (9). Carbon, hydrogen, and nitrogen analyses indicate the product is the hemihydrate and this has been confirmed by drying in an oven. Molecular weight determinations are currently being carried out and will be reported at a later date.

Anal.—Calcd. for $C_6H_{11}NO \cdot {}^1/_2H_2O$: C, 58.99; H, 9.90; N, 11.47. Found: C, 59.66; H, 9.51; N, 11.55.

α-Acetamidocinnamic Acid—This compound was prepared by hydrolysis of the azlactone, 2-methyl-4-benzilideneoxazolin-5-one (10). The product melted from 190° to 191.5° (uncorrected). [Lit. (10) m.p. 191°–192°.]

Preparation of Catalysts—Two methods were used to prepare the catalysts.

Method A—Sixteen milliliters of a 2.5% palladous

chloride solution (representing 200 mg. of $PdCl_2$ per Gm. of polyamino acid carrier) was added to 2.0 Gm. of poly-L-leucine suspended in 200 ml. of distilled water. The mixture was shaken 1 hr. on a Parr low-pressure hydrogenator prior to the introduction of hydrogen. Following the shaking period, the system was evacuated and hydrogen was introduced. The mixture was shaken for 1 hr. in an atmosphere of hydrogen. The hydrogen pressure was 2.8 Kg./cm.^{2}. The catalyst was collected by suction filtration, washed with a total of 300 ml. of distilled water, and dried for 48 hr. in a vacuum desiccator over sulfuric acid.

Method B—Two grams of poly-L-leucine was suspended in 200 ml. of distilled water in a 500 ml., two-necked, round-bottomed flask equipped with a dropping funnel and a motor-driven stirrer. While the mixture was stirred vigorously, 16 ml. of a 2.5% palladous chloride solution was added dropwise. After the addition of the palladous chloride solution was complete, the mixture was stirred vigorously for 1 hr. The mixture was then transferred to a Parr hydrogenation bottle and hydrogenated for 1 hr. at a hydrogen pressure of 2.8 Kg./cm.^{2}. Following hydrogenation, the catalyst was treated as has been described under *Method A*.

Elutriation Studies—The catalyst prepared by method *A* produced nonreproducible results when used to hydrogenate α-methylcinnamic acid according to the procedure of Padgett and Beamer (1).

Since the lack of reproducibility apparently stemmed from inhomogeneity within the catalyst preparation, an elutriation technique was employed to separate the catalyst into five arbitrarily assigned layers numbered 1 through 5 with 1 representing the bottom layer in the elutriation column. Each layer was then used to catalyze the hydrogenation of α-methylcinnamic acid according to the method of Padgett and Beamer (1) using 2.9 Gm. of the substrate and 0.36 Gm. of catalyst.

Hydrogenation Studies—Two substrates were used for the hydrogenation studies. These substrates were α-methylcinnamic acid and α-acetamidocinnamic acid.

α-Methylcinnamic Acid—Except for the hydrogenations using the catalysts obtained by the elutriation technique noted above, 4.05 Gm. (0.025 mole) of α-methylcinnamic acid and 0.5 Gm. of catalyst (method *B*) were employed. The hydrogenations were carried out in 50 ml. of absolute ethanol with a Parr low-pressure hydrogenation apparatus. The initial pressure was 4.2 Kg./cm.^{2} and a fall in pressure of 2.8×10^{-1} Kg./cm.^{2} was observed (theoretical: 2.9×10^{-1} Kg./cm.^{2}).

Following hydrogenation, the catalyst was removed by filtration and the solvent evaporated. The residue was vacuum distilled. The yield was 2.6 Gm. representing 63.4% of theory. The boiling point was 106°/0.35 mm. Hg (uncorrected). [Lit. (11) m.p. 160°/13 mm. Hg.] The $[\alpha]_D^{25}$ of the distilled product was −0.406 (c = 25 in benzene) was determined using a Rhudolph 200S polarimeter. The specific rotation represents 1.50% of the literature value of −27.06° (11). Infrared spectra of the product were identical with those given by Beamer and Lawson (2). The neutralization equivalent of the product agreed with that calculated for dihydro-α-methylcinnamic acid.

α-Acetamidocinnamic Acid—Hydrogenation of this substrate was carried out in 100 ml. of absolute ethanol with a Parr low-pressure hydrogenator. The weight of α-acetamidocinnamic acid was 4.8 Gm. (0.023 mole). The initial pressure of hydrogen was 4.2 Kg./cm.^{2}. One gram of catalyst was used. The observed fall in pressure was the same as that expected from theory (1.3×10^{-1} Kg./cm.^{2}).

Following hydrogenation, the catalyst was removed by suction filtration and the solvent was evaporated leaving a white, crystalline residue. The residue was treated according to the directions of Akabori (4) by boiling under reflux with 10% hydrochloric acid. Following the acid hydrolysis, the solution was concentrated under vacuum to approximately one-half its volume and treated with acetone to precipitate the amino acid hydrochloride. The phenylalanine was obtained by adjustment of a 50% v/v hydromethanolic solution to the isoelectric point of phenylalanine (pH 5.4) and collecting the resulting precipitate by suction filtration. The product weighed 3.5 Gm. representing a yield which was 92.3% of theory. The product melted with decomposition from 269° to 270° (uncorrected). [Lit. (12) m.p. 271°–273° dec.] Descending filter paper chromatography using butanol–acetic acid showed the R_f value of the product to be identical with that of known phenylalanine determined under identical conditions. The infrared spectra of the product and known phenylalanine were identical.

The phenylalanine was dissolved in 6.4% sodium hydroxide solution, placed in a 100 mm. polarimeter tube, and read in a Rhudolph 200S spectropolarimeter giving an observed rotation of −0.26° representing an $[\alpha]_D^{25}$ of −2.6° (c = 10). The literature value for the specific rotation of phenylalanine is −48.4° (13). From these data an optical yield of 5.4% may be calculated.

Table I—Hydrogenation of α-Methylcinnamic Acid Using Palladium-on-Poly-L-leucine Separated by Elutriation[a]

Layer	HMCA[b] Yield, Gm.	Yield,[c] %	Reduction Time, hr.	$[\alpha]_D^{25}$ [d]	Pressure Drop, Kg./cm.^{2} [e]
1	2.6	90	1	−0.146 ± .01°	7×10^{-2} [f]
2	2.48	85.5	1	−0.536 ± .01°	8.4×10^{-2}
3	2.45	84.5	1	−0.250 ± .01°	5.6×10^{-2}
4	0.00	0.00	1	...	1.4×10^{-2}
5	0.00	0.00	1	...	7×10^{-3}

[a] Weight of catalyst was 0.36 Gm. [b] HMCA = dihydro-α-methlcinnamic acid. [c] Based on 2.9 Gm. of α-methylcinnamic acid. [d] Determined in benzene using a 100-mm. tube (c = 25). The instrument used was the Rhudolph 200S spectropolarimeter. [e] Initial pressure was 4.2 Kg./cm.^{2} (gauge). [f] Hydrogen uptake for complete hydrogenation of 2.9 Gm. of substrate was calculated to be 1.04×10^{-1} Kg./cm.^{2}

RESULTS

The results of the hydrogenation experiments using the elutriated catalyst prepared by method *A* are given in Table I.

The catalyst prepared by method *B* appeared more homogeneous to the eye and in divided catalysts produced uniformly reproducible results. However, the product of the hydrogenation using the method *B* catalyst was not so optically active as that obtained from the second layer of the elu-

triated method *A* catalyst (see Table I). The hydrogenation product using method *B* catalyst was more optically active than the hydrogenation product using layers one and three of the elutriated method *A* catalyst.

The results of the other hydrogenations are given under *Experimental*. Hydrogenation of α-acetamidocinnamic acid, followed by hydrolysis, produced predominately (−)-phenylalanine.

The results of the hydrogenations using the elutriated catalyst are reproducible.

DISCUSSION

While it is too early in the studies involving these catalysts to form definite conclusions regarding the experimental evidence already gathered, one can form some preliminary ideas concerning these catalysts.

The similarities between proteins and polypeptides and the catalytic action of certain proteins (enzymes) prompted the investigation in this area. Does the stereospecificity observed from enzymes stem from the asymmetry of their individual amino acid residues or from the secondary, tertiary, or even quaternary structures of the proteins involved? Recent reports (14–16) indicate that synthetic polyamino acids, far from being random, may coil in either a righthanded or a lefthanded manner, and the direction of coiling is dependent in part on the amino acid involved. These reports are in sharp contrast to earlier data showing a more random arrangement (17).

Since these hydrogenation catalysts have been developed as models of enzymes, it might be well to critically examine our studies in the light of current concepts of enzyme specificity. The well-known theory of Koshland (18), which has been applied by Belleau (19) to include drug receptors, involves the assumption of a conformational perturbation occurring in the protein as a result of the forces of interaction between the substrate (or drug) and the enzyme (or drug-receptor). One particular interaction involved stems from the hydrophobic nature of the substrate molecule and the hydration of the protein. Other forces can also contribute to the induced perturbation (20).

It would be speculation to conceive of such a mechanism operating in the catalysts described in this paper. However, the similarities involved between synthetic polypeptides and proteins and the recent observations of Scheraga *et al.* (15) indicate something in this regard. Certain groups within the peptide chain do influence the synthetic peptide's secondary structure.

More definite conclusions may be obtained from a consideration of the absolute configurations of the products of the hydrogenations using palladium-on-polyleucine. As noted above, hydrogenations of both α-methylcinnamic acid and α-acetamidocinnamic acid yield levorotatory products. The absolute configurations of (−)-dihydro-α-methylcinnamic acid and (−)-phenylalanine have been determined (21–23). (−)-Dihydro-α-methylcinnamic acid has been shown to have the R configuration as shown in Fig. 1. (−)-Phenylalanine, however, possesses the S configuration.

Obviously the two substrates must be interacting—at least, in part—at two different sites on the catalyst surface. Analysis of the structure of α-methylcinnamic acid indicates four points which could bind with the catalytic surface: the olefinic group (C=C) at which reduction takes place, the carboxyl group which could form a salt bond with a cationic group or could form a hydrogen bond with a suitable group on the catalyst, a phenyl group which could bind by van der Waal's forces, and a methyl group which also could bind by van der Waal's forces. The methyl group is replaced by an acetamido group in α-acetamidocinnamic acid, but all the other groups are common to the two substrates. The methyl group is nonpolar but can bind to the catalyst by van der Waal's forces. The acetamido group can bind to the catalyst by hydrogen bonds or by induced dipole interaction. If the respective points of attachment are suitably situated and if two active centers or sites of reaction are envisioned, the production of the configurations described above can be explained. Schemes I and II illustrate possible mechanisms explaining the observed results. The hydrogen in these figures is shown enter-

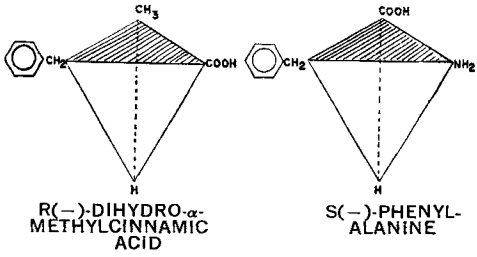
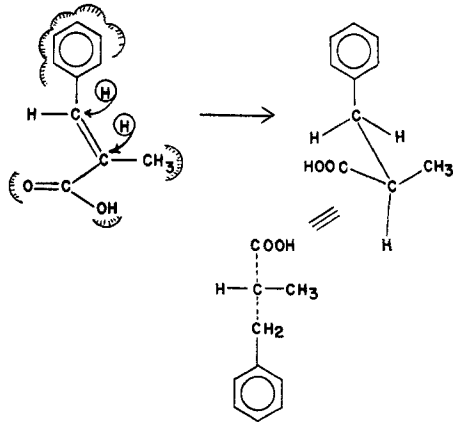
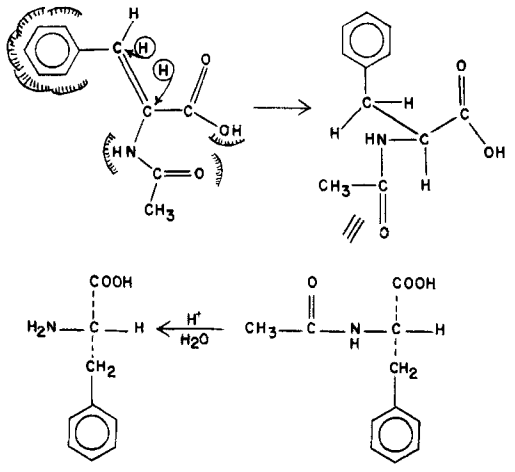


Fig. 1—Absolute configurations of (—)-dihydro-α-methylcinnamic acid as reported by Schrecker (21) and (—)-phenylalanine as reported by Karrer et al. *(22, 23).*



Proposed Scheme for the Stereospecific Hydrogenation of α-Methylcinnamic Acid Catalyzed by Palladium-on-Poly-L-leucine. The Hydrogen Should Be Viewed as Entering the Molecule Cis *and from the Catalytic Surface,* i.e., *the Hydrogen Attack is in a Direction from Out of the Page and Toward the Side Away from the Viewer*

Scheme I



Proposed Scheme for the Stereospecific Hydrogenation of α-Acetamidocinnamic Acid Catalyzed by Palladium-on-Poly-L-leucine. The Hydrogen Should be Viewed as Entering the Molecule Cis *and from the Catalytic Surface,* i.e., *the Hydrogen Attack is in a Direction from Out of the Page and Toward the Side Away from the Viewer*

Scheme II

ing the α-methylcinnamic and acid *cis* and from the catalytic side, *i.e.*, beneath the molecule or toward the side of the molecule away from the viewer. Stereoselective adsorption apparently occurs prior to addition of hydrogen and stereospecific hydrogenation is the result.

Later work with palladium-on-synthetic polypeptides may provide answers to the questions raised in this paper and indicate the fidelity of this model in explaining the complex phenomena of enzymatic catalysis.

SUMMARY

Hydrogenations using palladium-on-poly-L-leucine with α-methylcinnamic acid and α-acetamidocinnamic acid as substrates gave optically active products. The absolute configurations of the hydrogenation products indicate the two substrates fit—at least, in part—different active sites.

REFERENCES

(1) Padgett, R. E., Jr., and Beamer, R. L., *J. Pharm. Sci.*, **53**, 689(1964).

(2) Beamer, R. L., and Lawson, W. W., *ibid.*, **55**, 53 (1966).

(3) Beamer, R. L., Smith, J. D., Andrako, J., and Hartung, W. H., *J. Org. Chem.*, **25**, 798(1960).

(4) Akabori, S., Izumi, Y., Tujii, Y., and Sakurai, S., *J. Chem. Soc. Japan*, **77**, 1374(1956).

(5) Beckett, A. H., and Anderson, P., *Nature*, **179**, 1074 (1957).

(6) Beckett, A. H., and Anderson, P., *J. Pharm. Pharmacol. Suppl.*, **12**, 228T(1960).

(7) Beckett, A. H., and Youssef, H. Z., *ibid.*, **15**, 253T (1963).

(8) Bamford, C. H., Elliott, A., and Hanby, W. E., "Synthetic Polypeptides," Academic Press, Inc., New York, N. Y., 1956, pp. 45–46.

(9) *Ibid.*, pp. 143–212.

(10) Herbst, R. M., and Shemin, D., "Organic Syntheses," vol. II, John Wiley & Sons, New York, N. Y., 1943, p. 1.

(11) Heilbron, I. M., "Dictionary of Organic Compounds," vol. II, Oxford University Press, New York, N. Y., 1936, p. 738.

(12) Lange, N. A., "Handbook of Chemistry," Handbook Publishers, Inc., Sandusky, Ohio, 1952, p. 634.

(13) Weast, R. C., Selby, S. M., and Hodgman, C. D., "Handbook of Chemistry and Physics," Chemical Rubber Co., Cleveland, Ohio, 1965, p. C472.

(14) Vanderkooi, G., Leach, S. J., Némethy, G., Scott, R. A., Scheraga, H. A., *Biochemistry*, **5**, 2991(1966).

(15) *Chem. Eng. News*, **44**, No. 43, 70(1966).

(16) Perlmann, G. E., and Diringer, R., *Ann. Rev. Biochem.*, **29**, 151(1960).

(17) Bamford, C. H., Elliott, A., and Hanby, W. E., *op. cit.*, pp. 113–140.

(18) Koshland, D. E., Jr., *Advan. Enzymol.*, **22**, 45(1960).

(19) Belleau, B., *J. Med. Chem.*, **7**, 776(1964).

(20) Ariens, E. J., "Molecular Pharmacology," Academic Press Inc., New York, N. Y., 1964, pp. 179–183.

(21) Schrecker, A. W., *J. Org. Chem.*, **22**, 33(1957).

(22) Karrer, P., and Ehrhardt, K., *Helv. Chim. Acta*, **34**, 2202(1951).

(23) Karrer, P., Portmann, P., and Suter, M., *ibid.*, **31**, 1617(1948).

Isolation of a Potential Antitumor Fraction from *Rumex hymenosepalus* (*Polygonaceae*) II. Identification of the Active Fraction

By LEONARD BUCHALTER and JACK R. COLE

Continued investigation into the character of the antitumor fraction of *Rumex hymenosepalus* has resulted in the identification of leucodelphinidin and leucopelargonidin as components of this fraction. The activity was shown to reside in the polymeric fraction. The monomeric fraction was shown to be inactive.

In a previous communication (1) the authors have reported that the roots and tubers of *Rumex hymenosepalus* yielded a tannin extract which demonstrated antitumor activity. The active extract was subsequently fractionated into two components, A and B. The presence of leucoanthocyanidin units in fraction B was confirmed by the conversion of leucocyanidin to cyanidin.

Further investigation of fraction B has led to the characterization of this fraction as consisting of polymeric leucoanthocyanidin units consisting of leucopelargonidin, leucodelphinidin, as well as the leucocyanidin previously reported. In subsequent tests for antitumor activity, it was found that the activity resided in the polymeric fraction, and attempts at purification of the polymeric material did not enhance the antitumor activity.

The identification of the polymeric nature of fraction B was carried out by paper chromatographic analysis, visible spectra analysis, and degradation by potassium fusion of the anthocyanidins produced from the polymeric leucoanthocyanidins.

Subsequent investigation of fraction A has shown that this fraction consisted of monomeric leucoanthocyanidin units of the same type as was found in the polymeric condensed unit. The leucoanthocyanidins—leucocyanidin, leucodelphinidin, and leucopelargonidin—were identified by conversion to their corresponding anthocyanidins and identification was again made by means of paper chromatography, visible spectra analysis, and degradation by potassium fusion. The results of the antitumor testing of this fraction indicated that the monomeric leucoanthocyanidins were inactive.

EXPERIMENTAL

Conversion of Fractions A and B from Leucoanthocyanidins to Anthocyanidins—Both fractions A (water-soluble ethyl acetate extractable fraction of the tannin extract) and B (water-soluble ethyl acetate nonextractable fraction of the tannin extract), when boiled for 15 to 30 min. in ethanolic 10% hydrochloric acid, gave red solutions, indicating that both fractions consisted of leucoanthocyanidins which were convertible to anthocyanidins.

Paper Chromatography of Anthocyanidins—The anthocyanidins were applied to Whatman No. 1 filter paper for chromatographic analysis. Descending chromatography was used. The chromatogram was developed for 6 hr. and travelled 18 in. The following three solvent systems were utilized as they have proved effective for identifying anthocyanidins on paper (2, 3): (*a*) formic acid–3 *N* HCl (1:1), (*b*) water–acetic acid–concentrated HCl (10:30:3), (*c*) acetic acid–concentrated HCl–water (5:1:5).

All three solvent systems indicated the presence of three anthocyanidin pigments. The R_f values in solvent system (*a*) matched those described in references for delphinidin, cyanidin, and pelargonidin. The R_f values obtained in solvent systems (*b*) and (*c*) verified the presence of these three anthocyanidins. The same anthocyanidins were produced from both fractions A and B. (See Table I.)

Visible Spectra Analysis of Anthocyanidins—Although anthocyanidins characteristically exhibit intense absorption in the 500–550 mμ, addition of a few drops of aluminum chloride solution produces a bathochromic shift (15–50 mμ) of the principal $\lambda_{max.}$ of those anthocyanidin derivatives which contain adjacent hydroxyl groups.

Papers containing the three pigments corresponding to the anthocyanidins were extracted with methanol–0.1% HCl and subjected to visible light analysis in a Beckman DB spectrophotometer. The results of this visible analysis were compared with the values obtained by Harborne (4). Each material was also analyzed after the addition of 3 drops of aluminum chloride solution to the methanolic 0.1% HCl in which they were originally extracted.

The results of this analysis (Table II) verified the pigments that had been previously identified on paper. Delphinidin showed a maximum absorption at 546 mμ and a bathochromic shift of 23 mμ when aluminum chloride solution was added. Pelargonidin showed a maximum absorption in the visible region at 520 mμ and no bathochromic shift occurred on the addition of the aluminum chloride. Cyanidin showed a maximum absorption of 535 mμ, and a bathochromic shift of 20 mμ when aluminum chloride was added.

Degradation by Potassium Fusion of Anthocyanidins—In order to substantiate further chemically the structure of the anthocyanidins, the anthocyanidins were degradated by fusion with potassium hydroxide (5). This procedure resulted in phenolic and acidic portions which were then subjected to paper chromatography.

Paper Chromatography Identification of Fragments—Commercial samples of gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and phloroglucinol were obtained, and chromatography of the degradation products of potassium fusion and commercial samples was carried out in the follow-

Received February 23, 1967, from the Division of Pharmaceutical Chemistry, College of Pharmacy, University of Arizona, Tucson, AZ 85721

Accepted for publication April 5, 1967.

This investigation was supported in part by contract PH 43-63-1136, Cancer Chemotherapy National Service Center, research grant CA 05076-MCHB from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., the American Cancer Society institutional grant, University of Arizona, and the Smith Kline & French Foundation.

Previous paper: Cole, J. R., and Buchalter, L., *J. Pharm. Sci.*, **54**, 1376(1965).

TABLE I—R_f VALUES OF ANTHOCYANIDINS DEVELOPED FROM FRACTIONS A AND B[a]

Solvent System	R_f Value	Color	Ref.	R_f Value (2, 3)
(*a*)	0.11	Violet brown	Delphinidin	0.11
	0.22	Pink red	Cyanidin	0.22
	0.50	Light orange	Pelargonidin	0.55
(*b*)	0.29	Brown	Delphinidin	0.29
	0.50	Pink red	Cyanidin	0.50
	0.71	Orange	Pelargonidin	0.71
(*c*)	0.20	Choc. brown	Delphinidin	0.22
	0.32	Red	Cyanidin	0.34
	0.55	Light orange	Pelargonidin	0.55

[a] All spots on paper responded to ammonia fumes by becoming blue or blue-gray, and upon exposure to concentrated hydrochloric acid fumes reverted to their original colors. A commercial sample of cyanidin obtained from K & K Laboratories, Inc., was used to verify R_f values with those of the literature.

TABLE II—VISIBLE SPECTRA ANALYSIS OF ANTHOCYANIDINS FROM FRACTIONS A AND B[a]

Pigment	$\lambda_{max.}$ Methanolic 0.1% HCl, mμ	$\lambda_{max.}$ with Aluminum Chloride, mμ	$\lambda_{max.}$ (4) Methanolic 0.1% HCl, mμ	$\lambda_{max.}$ (4) Aluminum Chloride, mμ
Delphinidin	546	569	546	569
Cyanidin	535	555	535	553
Pelargonidin	520	520	520	520

[a] Measurement made on a Beckman DB spectrophotometer.

TABLE III—R_f VALUES OF COMMERCIAL SAMPLES AND FUSION FRAGMENTS OF ANTHOCYANIDINS FROM FRACTIONS A AND B

Sample	Solvent System (*1*) R_f Spot Color	Solvent System (*2*) R_f Spot Color	Solvent System (*3*) R_f Spot Color
Gallic acid	0.73 Blue gray	0.13 Blue gray	0.48 Blue gray
Fusion fragment	0.73 Blue gray	0.13 Blue gray	0.48 Blue gray
Protocatechuic acid	0.89 Green	0.455 Green	0.58 Green
Fusion fragment	0.89 Green	0.455 Green	0.58 Green
p-Hydroxybenzoic acid	0.773 Yellow	0.773 Yellow	0.685 Yellow
Fusion fragment	0.95 Yellow	0.773 Yellow	0.685 Yellow
Phloroglucinol	0.77 Light gray	0.28 Light gray	0.65 Light gray
Fusion fragment	0.77 Light gray	0.28 Light gray	0.65 Light gray

ing three solvent systems: (*1*) butanol–acetic acid–water (4:1:5) (upper phase only), (*2*) phenol–water (9:1), (*3*) 5% glacial acetic acid.

Five per cent ferric chloride solution was used as the chromogenic spray to identify the fragments produced on Whatman No. 1 filter paper. The results of the paper chromatography of the products of potassium fusion are shown in Table III. These results further verified the identity of the anthocyanidins produced from fractions A and B.

SUMMARY AND CONCLUSIONS

It has been determined that the active antitumor fraction of ground roots and tubers of *R. hymenosepalus* consists of polymeric leucoanthocyanidin units. These units are leucocyanidin, leucodelphinidin, and leucopelargonidin. The exact method of polymerization of these units in the active fraction has not yet been determined.

Monomeric flavanoidal units of the same type contained in the polymer were investigated and found to be devoid of antitumor activity.

REFERENCES

(1) Cole, J. R., and Buchalter, L., *J. Pharm. Sci.*, **54**, 1376(1965).
(2) Venkataraman, K., in "Chemistry of Flavanoid Compounds," Geissman, T. A., ed., The Macmillan Co., New York, N. Y., 1962, p. 76.
(3) Hayashi, K., in *ibid.*, p. 275.
(4) Harborne, J. B., *Biochem. J.*, **70**, 22(1958).
(5) Willstätter, R., and Mallison, H., *Ann.*, **408**, 40(1915).

Cation Radius and Deoxycholic Acid Polymer-Like Structure Stability

By C. BOTRÉ, P. A. CICCONETTI, G. LIONETTI, and M. MARCHETTI

The stability of the polymer-like structure displayed by deoxycholic acid gels under certain conditions has been investigated. The influence of cation dimensions, hydration, and hydrogen bonds is shown. A peculiar behavior of deoxycholic acid quaternary ammonium salts is discussed. Some biological implications are mentioned briefly.

Deoxycholic acid (DCA) is a unique molecule from its physical, chemical, and biological aspects. The physicochemical properties of DCA have been reported by Giacomello (1, 2), and more recently by Rich (3, 4). It has been found that under appropriate conditions sodium deoxycholate aggregates in solution to form a gelatinous complex of macromolecular dimensions. X-Ray diffraction studies of fibers of this complex showed that the molecules had assumed an elongated helical configuration 36 Å. in diameter (3, 4). The formation of a helical complex of macromolecular dimension from sodium deoxycholate is highly specific since closely related compounds do not exhibit this effect.

In previous work the influence of counterions on the conformation of either synthetic or natural polyelectrolytes (5, 6) has been shown. Therefore, it seemed worthwhile to study DCA gels in order to acquire further information on the many factors which influence the stability of these structures. It was the objective of the present study to determine if the formation of gels of macromolecular dimensions was in some way affected when deoxycholic acid salts other than sodium deoxycholate were used. The effects of cation dimensions, hydration, and hydrogen bonds on the stability of the structure were also investigated.

The importance of DCA and related structures to the physicochemical properties of biological membranes, as well as the interactions between these polymer-like structures and drugs, must not be overlooked. The study of the variation of the ionic activity of quaternary ammonium ions in a polyelectrolyte solution and the resulting influence on the conformation of the structure is of basic importance in order to interpret, on physicochemical grounds, biological problems such as nerve transmission.

EXPERIMENTAL

Material—DCA (Merck) was recrystallized from alcohol before use.

Solutions of different DCA salts were prepared by neutralizing DCA solution with the hydroxides of different cations. The DCA salt solutions thus obtained were then lyophilized.

Stock solutions of Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+, and quaternary ammonium bases were freshly prepared and standardized with a solution of 0.1 *N* HCl.

Lithium hydroxide solution was prepared from Li_2SO_4 and $Ba(OH)_2$. A small excess of Li_2SO_4 was employed so as to have a minimum of Ba^{2+} in the LiOH solution. The $BaSO_4$ was separated by filtration, and the LiOH solution free of sulfate and carbonate was obtained by passing the solution through an anion exchange column.[1]

Rubidium hydroxide and CsOH solutions were prepared by exchanging RbCl and CsCl solutions through the above-mentioned ion exchanger.

In a similar way quaternary ammonium bases were prepared by exchanging the corresponding iodides. The absence of halogens (Cl^- or I^-) from these solutions was checked.

All reagents used were of analytical grade.

PROCEDURE

The different DCA salts were prepared by dissolving DCA with an amount, slightly less than that required, of hydroxide solutions of the corresponding cations. The excess of acid was removed by filtration. The solution was evaporated and the DCA salt recovered.

DCA gels were obtained by dissolving known amounts of the DCA salts in water and then adjusting the pH to a value of 6.8–6.9 by adding very small amounts of HCl under continuous and constant stirring. All gels obtained were stored at a temperature of about 5°.

Conductivity measurements were carried out in a constant-temperature bath regulated within 0.01° for temperatures from 20° to 30° and within 0.3° for higher or lower temperatures. The complete filling and closing of the cell prevented evaporation of the solution. A WTW bridge (model LF_2) was employed for such measurements.

RESULTS AND DISCUSSION

In Fig. 1 specific conductivity data recorded in a solution of NaDCA are plotted against temperatures. The break in the trend, corresponding to a particular value of temperature, indicates a transition which is a function of the concentration of DCA in solution and of the radius of the cation which neutralizes DCA. As shown in Fig. 1, graphs of different monovalent cations (Li, Na, K, Rb, Cs, and NH_4) have the same general shape but are shifted toward higher or lower temperatures.

In Fig. 2 data obtained from solutions of different concentrations (0.05 and 0.10 *M*) of the above-mentioned DCA salts are reported. The transitional temperatures are plotted against the crystallographic radius of the different cations. The thermal stability of the helical complex decreases with the increasing crystallographic radius following the cation sequence.

In the case of NaDCA the influence of concentration on the transitional temperatures is shown in Fig. 3. The trend of the plot shows that the stability of the complex is temperature-dependent below a concentration of about 6×10^{-2} *M*. Above

Received January 24, 1967, from the Centro Nazionale di Chimica del Farmaco del C. N. R. I. Sez. I Rep., Istituto Chimico Farmaceutico, Universitá di Roma, Rome, Italy.

Accepted for publication May 8, 1967.

The authors are indebted to Professor G. Giacomello for is suggestions and assistance.

[1] Ionenaustauscher III, Merck.

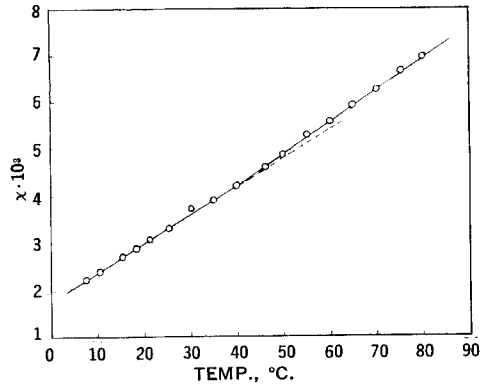


Fig. 1—The specific conductivity χ of a NaDCA solution (3×10^{-2} M) as a function of temperature. The break in the trend indicates a transition. Graphs of different monovalent cations have the same general shape.

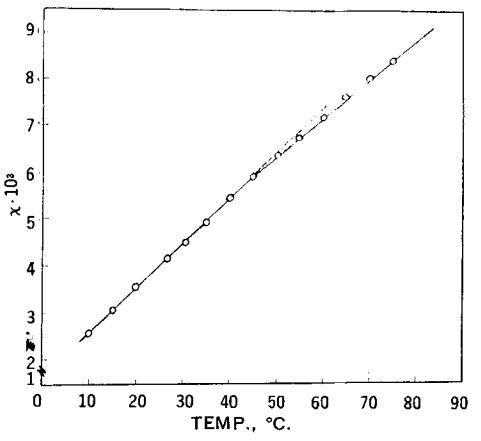


Fig. 4—The specific conductivity χ of an NH_4DCA solution (5×10^{-2} M) as a function of temperature. The plot differs in trend from that of Fig. 1.

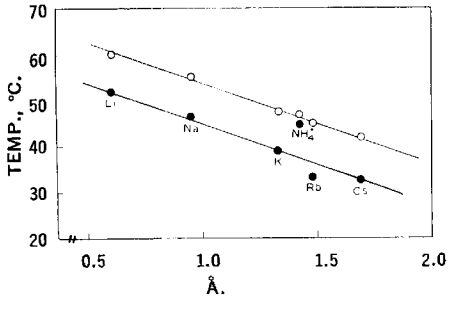


Fig. 2—The thermal stability of the helical complexes decreases with the increasing crystallographic radius following the cation sequence. Deoxycholate concentrations: ●, 0.05 M; ○, 0.10 M.

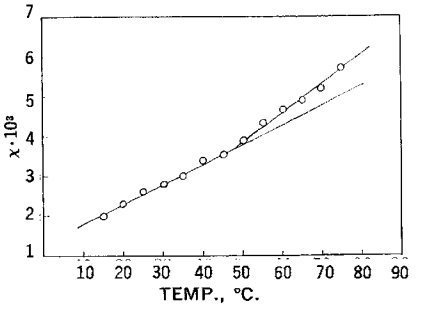


Fig. 5—The specific conductivity χ of an NH_4DCA solution (7.5×10^{-2} M) added of urea 6 M as a function of temperature. The plot shows the same trend of all the other deoxycholates.

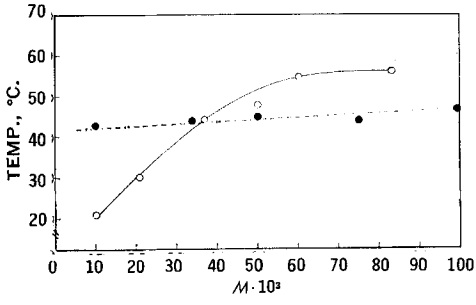


Fig. 3—Transitional temperatures dependence upon concentration of NaDCA (○) and NH_4DCA (●) solutions.

this value, the transitional temperatures are no longer affected by DCA concentration in solution.

In Fig. 2 the anomalous position in the plot of NH_4^+ which seems to have almost the same transitional temperature for the two different concentrations, 0.05 and 0.10 *M*, is evident. This is due to the fact that, in the case of NH_4DCA, the trend of the plot of transitional temperature *versus* concentration differs from those of the other cations. In a range of concentration between 0.01 and 0.10 *M*, the transitional temperatures of NH_4DCA go from 43° to 47°, while in the same range of concentration of NaDCA, as well as the other monovalent cations, there is a shift of about 35° (see Fig. 3).

Furthermore, the plot of specific conductivity against temperature of an NH_4DCA solution shows a different trend. Below and above the transitional temperature the plot differs in slope (see Fig. 4). In addition, viscosity measurements in an NH_4DCA solution show a deviation toward higher values than those obtained from solutions of other salts. This suggests that the presence of ammonium ions, through hydrogen bond formation, affects the complexity of these structures, which will grow in all directions forming a matted matrix of high viscosity. In fact, addition of urea to a solution of NH_4DCA brings the plot back to the shape common to the other cations (see Fig. 5).

To further investigate the role played by ionic dimensions in these polymer-like structures, solutions of some organic quaternary nitrogen salts have been studied. They are monomethylammonium (MMA), dimethylammonium (DMA), tetramethylammonium (TMA), tetraethylammonium (TEA), and tetrabutylammonium (TBA) deoxycholate.

In Fig. 6 the transitional temperatures of the above-mentioned DCA salts are plotted against the

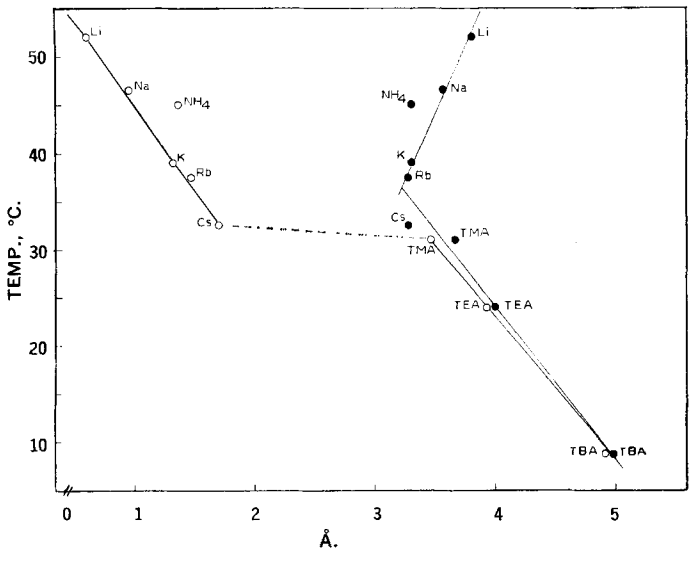


Fig. 6—Transitional temperatures of different DCA salts as a function of the crystallographic radius (○) and the hydrated radius (●).

crystallographic radius and against the hydrated radius (7). If the increment of crystallographic radius were the only reason for the increase of transitional temperatures, we should find much higher values in that part of the plot where Li, Na, K, Rb, and Cs are placed. In other words, the plot of transitional temperature as a function of crystallographic radius should be represented by the same straight line. When the hydrated radii are taken into consideration, the resulting plot shows a completely different trend. In fact, whenever there is a slight difference between crystallographic and ionic radii, that is, whenever the amount of water bound to cations is negligible, as in the case of organic cations, the resulting plots are almost identical. Instead, when the amount of structuralized or iceberg-like water bound to cations begins to be relevant, that part of the plot referring to inorganic cations exhibits a marked change in the slope and shows a discontinuity.

The experimental data summarized in Fig. 6 prove that structuralized or iceberg-like water bound to cations increases the thermal stability of the helical complex.

Water, therefore, proves to be of fundamental importance to the structural stability of such polymer-like compounds.

In conclusion, three factors were found to influence the stability of the structure: cation dimension, hydration, and hydrogen bonds.

REFERENCES

(1) Kratky, O., and Giacomello, G., *Monatsh.*, **69**, 427 (1936).
(2) Giacomello, G., and Romeo, M., *Gazz. Chim. Ital.*, **73**, 285(1943).
(3) Rich, A., and Blow, D. M., *J. Am. Chem. Soc.*, **82**, 3566(1960).
(4) Blow, D. M., and Rich, A., *Nature*, **182**, 423(1958).
(5) Botré, C., Solinas, M., and Ascoli, F., *Biopolymers*, **3**, 545(1965).
(6) Botré, C., Borghi, S., Marchetti, M., and Baumann, M., *ibid.*, to be published.
(7) Nightingale, E. R., Jr., *J. Phys. Chem.*, **63**, 1381(1959).

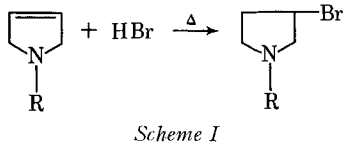
Synthesis of 1-Substituted-3-bromopyrrolidines

By D. C. RUOPP, F. A. E. SCHILLING, and B. B. BROWN

Considerable interest has been shown in 3-substituted pyrrolidines because of their physiological activity on the central nervous system. The preparation of several 1-substituted-3-bromopyrrolidines is described. In addition, a unique technique of halopyrrolidine synthesis, virtually eliminating self-quaternization, is presented.

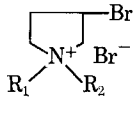
INTEREST in 1-alkyl-3-halopyrrolidines has been stimulated by their usefulness as intermediates for the preparation of central nervous system stimulants, antispasmodics, ganglionic stimulants, anticholinergics, and parasympathetic depressants (1–5). Many theoretical routes to 1-alkyl-3-halopyrrolidines are possible; however, most are quite costly and involve several steps. Preparation of 1-alkyl-3-halopyrrolidines by hydrohalogenation of 1-alkyl-Δ^3-pyrrolines offers an inexpensive and direct approach to this type of compound. Extensive information regarding the addition of hydrogen halides to straight chain olefins is present in the literature (6–8). Some work has been reported using cyclic olefins (9, 10); however, no reference was found disclosing the addition of hydrogen halides to Δ^3-pyrrolines.

1-Alkyl-Δ^3-pyrrolines were prepared by cyclization of *cis*-1,4-dichlorobutene-2 with the appropriate primary amines (11, 12). Low reaction temperature conditions favorable for hydrobrominating butene-1 (8) and pentene-1 (6) were not successful with the 1-substituted-Δ^3-pyrrolines in aqueous or nonaqueous media. Successful hydrobromination was achieved using 60% aqueous HBr at 120–125°. No reaction was obtained with the commercially available 48% HBr at the same conditions. Although hydrobromination of 1-substituted-Δ^3-pyrrolines obviously does not occur with the facility of the olefin systems, here too, the rate of HBr addition was found to far exceed hydrochlorination under the necessary temperature conditions. (Scheme I.)



Scheme I

The 1-substituted-3-bromopyrrolidines were subsequently converted to their amine and quaternary salts. (I–VI.)



R_1	R_2
I, H	CH_3
II, CH_3	CH_3
III, H	C_2H_5
IV, CH_3	C_2H_5
V, H	$C_6H_5CH_2$—
VI, CH_3	$C_6H_5CH_2$—

The preparation of the above pyrrolidine salts, with the exception of V, afforded no difficulty. Some dissociation of the pyrroline salt occurs during the hydrobromination reaction as shown by the resultant formation of a mixture of 1-ethyl-3-chloropyrrolidine and 1-ethyl-3-bromopyrrolidine when starting with 1-ethyl-Δ^3-pyrroline hydrochloride. Self-quaternization occurs to a significant degree also during the hydrobromination reaction, especially with I, forming presumably 1-alkyl-1-(1′-alkyl-3′-pyrrolidyl)-3-bromopyrrolidinium bromides. The authors have found that by first forming the quaternary salt of the 1-substituted-Δ^3-pyrrolines, self-quaternization during the hydrobromination reaction is minimized. For example, hydrobromination of 1-methyl-Δ^3-pyrroline methobromide produced 57% of II and a 32% recovery of the starting quaternary, whereas hydrobromination of 1-methyl-Δ^3-pyrroline gave only 22% of I and a 10% recovery of the starting amine.

EXPERIMENTAL

The hydrobromination reaction was conducted in a 500-ml. pressure bottle mounted on a Parr shaker apparatus equipped for heating. Since bromopyrrolidines tend to self-quaternize even under refrigeration, the products were converted immediately after isolation to their amine or quaternary salts with hydrogen bromide or methyl bromide. Benzene was used as the reaction medium and afforded a quantitative recovery of the respective pyrrolidine salts.

1-Methyl-3-bromopyrrolidine—A solution of 16.39 Gm. (0.1 mole) of 1-methyl-Δ^3-pyrroline hydrobromide in 67.5 Gm. (0.5 mole) of 60% HBr was heated at 120–125° for 24 hr. After cooling to 25°, the reaction mixture was adjusted to pH 11 by the addition of 25% NaOH (100 Gm.). The oil layer was extracted with benzene (2 × 25 ml.), washed with water (10 ml.), and azeotroped dry at atmospheric pressure while charcoaling for 15 min. Filtration of the charcoal resulted in a light yellow benzene solution which was analyzed by vapor phase chromatography; yield 3.6 Gm. of 1-methyl-3-bromopyrrolidine (22% of theory). This benzene solution was carefully gassed with dry hydrogen bromide to pH 7. The amine salt was filtered and recrystallized from acetone to yield I, m.p. 92–93°.

Anal.—Calcd. for $C_5H_{11}Br_2N$: N, 5.72; Br^-, 32.6; Br, 65.2. Found: N, 5.79; Br^-, 33.4; Br, 64.6.

1-Ethyl-3-bromopyrrolidine—This compound was prepared as described above in 60% of theory yield and was converted to III, m.p. 89–90°.

Anal.—Calcd. for $C_6H_{13}Br_2N$: N, 5.4; Br^-, 30.9; Br, 61.8. Found: N, 5.5; Br^-, 31.8; Br, 62.1.

1-Benzyl-3-bromopyrrolidine—This substance was prepared in the same manner in 52% of theory yield and converted by the addition of methyl bromide gas to VI, m.p. 134–135°.

Received March 22, 1967, from the S. B. Penick Research Laboratory, Orange, N J 07050

Accepted for publication May 19, 1967.

The authors thank J. Swidinsky for synthesizing the substituted pyrrolines and Mr. Russo and his staff, S. B. Penick and Company Control Laboratory, for the analyses reported in this paper.

Anal.—Calcd. for $C_{12}H_{17}Br_2N$: N, 4.2; Br^-, 23.9; Br, 47.8. Found: N, 4.9; Br^-, 24.8; Br, 48.9.

1-Methyl-Δ^3-pyrroline Methobromide—A solution of 8.3 Gm. (0.1 mole) of 1-methyl-Δ^3-pyrroline in 100 ml. of methyl ethyl ketone was treated with 9.5 Gm. of methyl bromide gas. The resulting solid was filtered to yield 17.79 Gm., 100% of theory, 1-methyl-Δ^3-pyrroline methobromide, m.p. 303–304°.

Anal.—Calcd. for $C_6H_{12}BrN$: N, 7.88; Br^-, 44.9; Br, 44.9. Found: N, 8.58; Br^-, 45.37; Br, 45.40.

1-Methyl-3-bromopyrrolidine Methobromide (II)—A solution of 17.79 Gm. (0.1 mole) of 1-methyl-Δ^3-pyrroline methobromide in 67.5 Gm. of 60% HBr was reacted as described above. The excess hydrobromic acid was distilled and the residue crystallized from 2 B ethyl alcohol (90 ml.) to yield 57% of theory II, m.p. 190–191°.

Anal.—Calcd. for $C_6H_{13}Br_2N$: N, 5.4; Br^-, 30.9; Br, 61.8. Found: N, 5.3; Br^-, 32.1; Br, 62.9.

1-Ethyl-3-bromopyrrolidine Methobromide (IV)—This compound, similarly prepared from 1-ethyl-Δ^3-pyrroline methobromide, m.p. 265–266°, was extremely hygroscopic and required rapid crystallization from a mixture of methyl ethyl ketone (50 ml.) and 2 B ethyl alcohol (10 ml.), m.p. 134–135°.

REFERENCES

(1) Lunsford, C. D., Cale, A. D., Jr., Ward, J. W., Franko, B. V., and Jenkins, H., *J. Med. Chem.*, **7**, 302(1964).
(2) Wu, Y. H., Feldkamp, R. F., Corrigan, J. R., and Rhodes, H. J., *J. Org. Chem.*, **26**, 1524(1961).
(3) Jones, A., *J. Pharmacol. Exptl. Therap.*, **141**, 195 (1963).
(4) Parke Davis and Co., Brit. pat. 831,934(1960).
(5) Biel, J. H., U. S. pat. 3,091,570(1963).
(6) Sherrill, M. L., Mayer, K. E., and Walter, G. F., *J. Am. Chem. Soc.*, **56**, 926(1934).
(7) Whitmore, F. C., and Johnston, F., *ibid.*, **55**, 5020 (1933).
(8) Lucas, H. J., Dillon, R. T., and Young, W. G., *ibid.*, **52**, 1952(1930).
(9) Grosse, A. V., and Linn, C. B., *J. Org. Chem.*, **3**, 27 (1938).
(10) Noller, C. R., and Adams, R., *J. Am. Chem. Soc.*, **48**, 2444(1926).
(11) Bobbitt, J. M., Amundsen, L. H., and Steiner, R. I., *J. Org. Chem.*, **25**, 2230(1960).
(12) Amundsen, L. H., Mayer, R. H., Pitts, L. S., and Malentacchi, L. A., *J. Am. Chem. Soc.*, **73**, 2118(1951).

Communications

Synthesis of the *Veratrum* Alkaloid Verazine from Tomatid-5-en-3β-ol

Sir:

Recently, several new minor alkaloids have been isolated from *Veratrum album* subsp. *lobelianum* (Bernh.) Suessenguth (1). In a preceding communication (2) we described the structural elucidation of one of these new alkaloids, verazine, which according to chemical transformations and physical measurements possesses the structure 25*S*-22,26-epimino-cholesta-5,22(*N*)-dien-3β-ol (I). In this paper the establishment of this structure by a nine-step synthesis of compound I starting from the spirosolane alkaloid tomatid-5-en-3β-ol (II) (3) is reported.

Reduction of II with sodium borohydride in methanol afforded (22*S*:25*S*)-22,26-epimino-cholest-5-ene-3β,16β-diol (III), m.p. 165–167°, 189–191°, and $[\alpha]_D$ −69.1°[1] in 70% yield. Acetylation of III with acetic anhydride/pyridine at 20° for 14 hr. gave nearly quantitatively the amorphous *O,O,N*-triacetate (IV) with $[\alpha]_D$ −10.3°. Hydrolysis of this compound by refluxing with 5% potassium hydroxide in methanol for 2 hr. furnished (22*S*:25*S*)-22,26-acetylepimino-cholest-5-ene-3β, 16β-diol (V), m.p. 248–250°, $[\alpha]_D$ −35° (yield 81%). The *N*-acetyl-diol (V) through partial oxidation with 1 equivalent chromium trioxide in acetic acid–sodium acetate yields 80% (22*S*:25*S*)-22,26-acetylepimino-3β-hydroxy-cholest-5-en-16-one (VI), m.p. 213–215°, $[\alpha]_D$ −121.1°. Treatment of this ketone (VI) with ethanedithiol–hydrochloric acid followed by desulfurization of the obtained thioketal (VII) with Raney nickel in ethanol gave (22*S*:25*S*)-22,26-acetylepimino-cholest-5-en-3β-ol (VIII), m.p. 256–258°, and $[\alpha]_D$ −19.4° (74%).

[1] Melting points are corrected. All rotations in chloroform (c = 0.5). The infrared and molecular mass spectra of all new compounds described in this communication are in agreement with the proposed structure. The authors are indebted to Dr. R. Tummler, Dresden, for the molecular mass spectra.

Anal.—Calcd. for $C_{12}H_{17}Br_2N$: N, 4.2; Br^-, 23.9; Br, 47.8. Found: N, 4.9; Br^-, 24.8; Br, 48.9.

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(1) Lunsford, C. D., Cale, A. D., Jr., Ward, J. W., Franko, B. V., and Jenkins, H., *J. Med. Chem.*, **7**, 302(1964).
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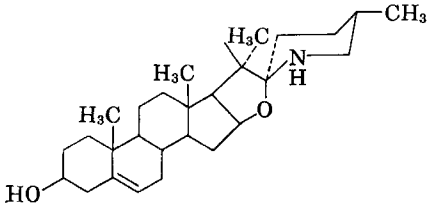
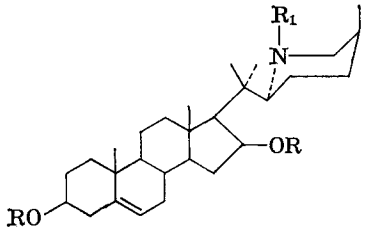
Recently, several new minor alkaloids have been isolated from *Veratrum album* subsp. *lobelianum* (Bernh.) Suessenguth (1). In a preceding communication (2) we described the structural elucidation of one of these new alkaloids, verazine, which according to chemical transformations and physical measurements possesses the structure 25*S*-22,26-epimino-cholesta-5,22(*N*)-dien-3β-ol (I). In this paper the establishment of this structure by a nine-step synthesis of compound I starting from the spirosolane alkaloid tomatid-5-en-3β-ol (II) (3) is reported.

Reduction of II with sodium borohydride in methanol afforded (22*S*:25*S*)-22,26-epimino-cholest-5-ene-3β,16β-diol (III), m.p. 165–167°, 189–191°, and $[\alpha]_D$ −69.1°[1] in 70% yield. Acetylation of III with acetic anhydride/pyridine at 20° for 14 hr. gave nearly quantitatively the amorphous *O,O,N*-triacetate (IV) with $[\alpha]_D$ −10.3°. Hydrolysis of this compound by refluxing with 5% potassium hydroxide in methanol for 2 hr. furnished (22*S*:25*S*)-22,26-acetylepimino-cholest-5-ene-3β, 16β-diol (V), m.p. 248–250°, $[\alpha]_D$ −35° (yield 81%). The *N*-acetyl-diol (V) through partial oxidation with 1 equivalent chromium trioxide in acetic acid–sodium acetate yields 80% (22*S*:25*S*)-22,26-acetylepimino-3β-hydroxy-cholest-5-en-16-one (VI), m.p. 213–215°, $[\alpha]_D$ −121.1°. Treatment of this ketone (VI) with ethanedithiol–hydrochloric acid followed by desulfurization of the obtained thioketal (VII) with Raney nickel in ethanol gave (22*S*:25*S*)-22,26-acetylepimino-cholest-5-en-3β-ol (VIII), m.p. 256–258°, and $[\alpha]_D$ −19.4° (74%).

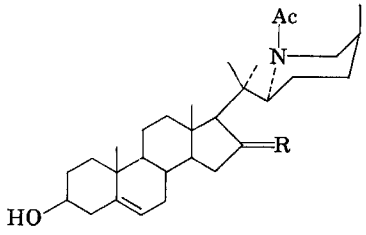
[1] Melting points are corrected. All rotations in chloroform (c = 0.5). The infrared and molecular mass spectra of all new compounds described in this communication are in agreement with the proposed structure. The authors are indebted to Dr. R. Tummler, Dresden, for the molecular mass spectra.



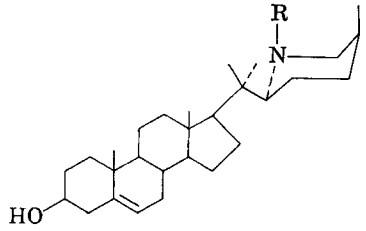
II



III, R, R_1 = H
IV, R, R_1 = Ac
V, R = H, R_1 = Ac

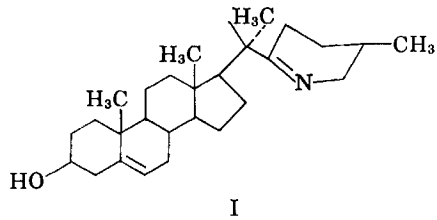


VI, R = O
VII, R = S
S



VIII, R = Ac
IX, R = H
X, R = Cl

Saponification of the amide (VIII) furnished the amine (IX) in only 11% yield even after employing the drastic Schmidt-Thome conditions [refluxing with potassium hydroxide in ethylene glycol for 8 hr. (4)]. The amine (IX) was separated from unchanged starting amide (VIII) by preparative thin-layer chromatography on Silica Gel G (development five times with chloroform) and has m.p. 167–171°, $[\alpha]_D$ −39.0°. *N*-Chlorination of IX with *N*-chloro succinimide in methylene dichloride at −5° and subsequent treatment of the unisolated *N*-chloro derivative (X) with sodium methoxide in methanol led to compound I with 24% yield, m.p. 173–176°, and $[\alpha]_D$ −83.4°, which was identical in every respect (mixed m.p., infrared spectrum, thin-layer chromatography) with authentic verazine, isolated from *V. album*. Since the starting tomatid-5-en-3β-ol (II) is already total synthetically obtainable (3,5) the above reported sequence of reactions also represents the total synthesis of verazine (I).



I

(1) Tomko, J., and Vassova, A., *Chem. Zvesti*, **18**, 266 (1964).
(2) Adam, G., Schreiber, K., Tomko, J., and Vassova, A., *Tetrahedron*, **23**, 167(1967).
(3) Schreiber, K., and Ronsch, H., *Tetrahedron Letters*, **1963**, 329; Schreiber, K., and Ronsch, H., *Liebigs Ann. Chem.*, **681**, 187, 196(1965).
(4) Schmidt-Thome, J., *Chem. Ber.*, **88**, 895(1955).
(5) Schreiber, K., and Adam, G., *Tetrahedron Letters*, **1960**, 5; Schreiber, K., and Adam, G., *Experientia*, **17**, 13(1961); Schreiber, K., and Adam, G., *Liebigs Ann. Chem.*, **666**, 155(1963).

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Received April 14, 1967.
Accepted for publication June 12, 1967.
Presented to the Pharmacognosy and Natural Products Section, A.Ph.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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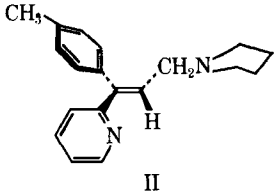
Assignment of Absolute Configurations to Receptor Sites and Drugs

Sir:

Based on the facts that racemic 9-(1′-[2′-dimethylamino]propyl) - 10 - thia - 1,9 - diazaanthracene, isothipendyl (I) is antihistaminically active and that the antihistaminically more active geometric isomer of 1-(2′-pyridyl)-1-(*p*-methylphenyl)-3-pyrrolidinoprop-1-ene, triprolidine (II) has the *trans*-configuration and exhibits an ultraviolet spectrum characteristic of vinyl pyridine and not styrene, Barlow (1) proposed *A* as the hypothetical antihistaminic receptor to which II, in the conformation written (1) presents its lower face. However, the equally probable mirror image receptor configuration (*B*) may be deduced upon adsorption by the opposite face of II.

Nevertheless, assuming that *A* is the receptor configuration, Barlow's conclusion (1) that (+)-1 - (2′ - pyridyl) - 1 - (*p* - chlorophenyl) - 3 - dimethylaminopropane, (+)-chlorpheniramine, the antihistaminically more active antipode, has the (R)-configuration (III) is unwarranted since III



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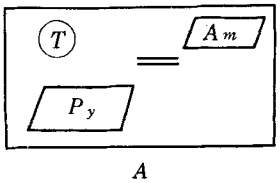
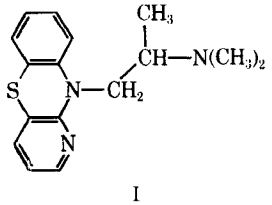
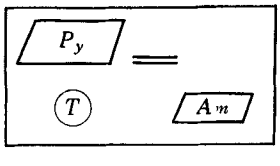


A

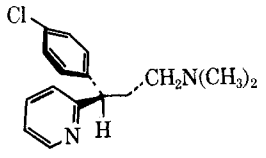


I



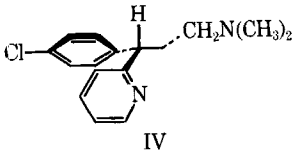
B

(R)



III

(S)



IV

(complementary to *A*) with the methine hydrogen toward the receptor surface, presents the same receptor pattern (*A*) as IV, the (S)-configuration with the hydrogen away from the receptor surface. The adsorption of (+)-chlorpheniramine with the hydrogen toward the receptor is clearly arbitrary. Thus, even if one knew the absolute configuration of the (+)-pheniramines to be (R) there would be no justification for assigning *A* as the absolute configuration of the hypothetical antihistaminic receptor since presentation of the upper face of III (methine hydrogen away from the receptor surface) affords the receptor pattern *B*.

Any argument based upon steric considerations, for example, that the receptor should adsorb to the bottom face of III since this represents the sterically less hindered approach to the molecule, is equally arbitrary and is not in keeping with current concepts regarding the conformational mobility and adaptability of proteins (2, 3) or with the related duality of conformational (4) and configurational (5) requirements of analogs of model drugs presumably acting on the same receptors.

The topography and configuration of the receptor, or more likely, the drug-bound receptor, may be deduced only for the selected case where one face of a conformationally rigid molecule, presenting the requisite configuration and pharmacophoric array, is accessible for binding. Indeed, conclusions based upon such models need not be valid for conformationally less restrained systems or for systems exhibiting chemical and spatial differences in peripheral groups. Assuming involvement of the same receptor and possible accessory binding or hindering moieties, these peripheral groups may modify the receptor–drug interaction and result not only in different modes of attachment to the receptor (4) but also in inversion of optical specificity (5). Since the

topography of the hypothetical antihistaminic receptor and/or drug-bound receptor is unknown, *A* and *B* should only be looked upon as highly schematized two-dimensional representations of a three-dimensional pattern for the bound receptor. In addition, the structures presented here are not necessarily uniformly representative of the conformations of the bound drug. There is no evidence to suggest that the conformationally mobile antihistamines are bound in their most stable conformations nor is there any evidence to suggest that I, II, and IV bind to the hypothetical receptor in the same way.

Finally, it is clear that Barlow's (1) rationalization leading to assignment of the (S)-configuration for the antihistaminically more active antipode of isothipendyl (I) is subject to the same objections already discussed. Accordingly, this assignment of configuration, if correct, is only fortuitously so. While the α-methyl may sterically inhibit the drug receptor binding as apparently suggested (1), it may instead contribute to and thus re-enforce the other drug–receptor interactions through hydrophobic and more highly distant specific van der Waals interactions with the receptor. The latter possibility would lead to selection of the (R)-configuration as the more active antipode. Neither observation is necessarily productive of sound configuration-activity relationships without data on the desmethyl analog as well.

The finding (6) that the absolute configuration of all the (+)-pheniramines is (S) and not (R) as proposed by Barlow (1) does not affect this critique, and we obviously defer assignment of configuration to the hypothetical antihistaminic receptor until more definitive experiments, now in progress, are completed.

(1) Barlow, R. B., "Introduction to Chemical Pharmacology," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1964, pp. 371–374, and references cited therein.
(2) Koshland, D. E., Jr., "Proceedings of the First International Pharmacological Meeting," Stockholm, 1961, vol. 7, Pergamon Press Ltd., London, 1963, p. 161, and reference cited.
(3) Belleau, B., and Lacasse, *J. Med. Chem.*, **7**, 768(1964); Belleau, B., *ibid.*, **7**, 776(1964).
(4) Smissman, E. E., and Steinman, M., *ibid.*, **9**, 455 (1966); Portoghese, P. S., Abstracts of the 153rd Meeting of the American Chemical Society, Medicinal Chemist Section, April 1967, Miami Beach, Fla., M/14.
(5) Portoghese, P. S., *J. Med. Chem.*, **8**, 609(1965); Belleau, B., and Puranen, J., *ibid.*, **6**, 325(1963), and references cited therein.
(6) Shafi'ee, A., and Hite, G., Abstracts of the 153rd Meeting of the American Chemical Society, Medicinal Chemistry Section, April 1967, Miami Beach, Fla., M/48.

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Received January 26, 1967.
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Tumor Localizing Agents III. Radioiodinated Quinoline Derivatives

Sir:

For several years we have been interested in developing an agent which would be useful for the diagnostic localization and treatment of melanotic tumors. Our approach to this problem has involved the synthesis of radiolabeled compounds which would selectively localize in these tumors much like radioiodine localizes in the thyroid. In order to achieve this tumor selectivity, the initial selection of compounds for radiolabeling has fallen into two categories, namely: (*a*) precursors of melanin and (*b*) compounds which are known to interact with melanin. Previous publications (1, 2) from this laboratory described the results with several radiolabeled melanin precursors.

In recent years, several reports have appeared indicating that chloroquine has a marked affinity for melanin and is only slowly released from pigmented tissues (3–5). This information prompted us to synthesize a number of radioiodinated analogs of chloroquine and study their distribution in mice with transplanted melanomas. This report describes initial results with one of these radioiodinated analogs.

The general procedure of Price and Roberts (6) was employed to obtain the key intermediate 4-chloro-7-iodoquinoline (V). (Scheme I.)

Ethoxymethylenemalonic ester was condensed with 3-iodoaniline to give ethyl α-carbethoxy-β-(3-iodophenylamino)acrylate (I) in 77% yield, m.p. 88–89°.

Anal.—Calcd. for $C_{14}H_{16}INO_4$: C, 43.21; H, 4.14. Found: C, 43.34; H, 4.11.

The addition of I to refluxing diphenylether afforded 3-carbethoxy-4-hydroxy-7-iodoquinoline (II) in 94% yield. Recrystallization from pyridine gave an analytical sample which starts to sublime at 255°.

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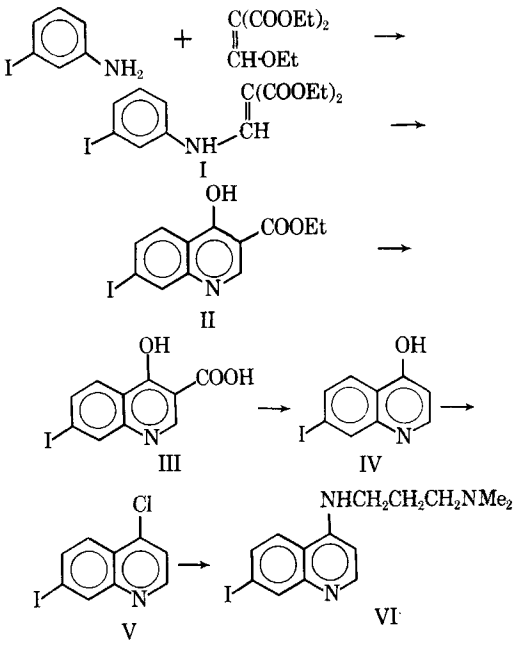

Scheme I

Anal.—Calcd. for $C_{12}H_{10}INO_3$: C, 42.02; H, 2.94. Found: C, 42.11; H, 2.83.

Saponification in aqueous ethanol followed by acidification furnished the carboxylic acid (III) in 98% yield, melting with decarboxylation at 278°.

Anal.—Calcd. for $C_{10}H_6INO_3$: C, 38.13; H, 1.92. Found: C, 38.18; H, 1.88.

When III was recrystallized from dimethysulfoxide a crystalline solvate was obtained which sublimed at 280–290°, $\gamma_{max.}$ 1720 (C=O) and 1030–1010 cm.$^{-1}$ (S=O).

Anal.—Calcd. for $C_{10}H_6INO_3 \cdot C_2H_6SO$: C, 36.66; H, 3.08. Found: C, 36.58; H, 2.96.

Decarboxylation of III in refluxing diphenylether afforded crude 4-hydroxy-7-iodoquinoline (IV) which, without further purification, was treated with phosphorous oxychloride. Recrystallization of the resulting product from chloroform furnished 4-chloro-7-iodoquinoline (V) in 70% yield, m.p. 97–98°. [Reported m.p. 95.5–97° (7).] Treatment of V with refluxing 3-dimethylaminopropylamine gave the desired chloroquine analog, 4-(3-dimethylaminopropylamino)-7-iodoquinoline (VI) in 65% yield, m.p. 101–103°. The NMR spectrum ($CDCl_3$) showed a singlet at 7.64 δ (NCH_3), a triplet at 7.43 δ ($-CH_2N$) (J = 6.0 c.p.s.), and a multiplet centered at 6.67 δ (CH_2NH—) which became a triplet upon deuteration (J = 6.0 c.p.s.).

Anal.—Calcd. for $C_{14}H_{18}IN_3$: C, 47.35; H, 5.11. Found: C, 47.36; H, 5.17.

Iodine-125 or ^{131}I was introduced into VI by isotope exchange with radioactive sodium iodide in ethylene glycol at 170–175°. By this procedure the ^{125}I and ^{131}I-labeled compounds were obtained with specific activities of 2.03 and 1.42 mc./mmoles, respectively. The radiolabeled compounds were purified by recrystallization and analyzed by thin-layer chromatography (TLC). In both cases, a radiochromatogram scan showed a single radioactive area coincident with the single TLC spot seen under ultraviolet light.

Four to five-week-old male, black mice of the BL6J strain were injected intraperitoneally with 10 μc. of 4-(3-dimethylaminopropylamino)-7-iodoquinoline-^{125}I and sacrificed at 12, 24, and 48 hr. Control mice were injected *via* the same route with 10 μc. of sodium iodide-^{125}I and sacrificed at the same time intervals. Counting was done in a commercial well counter. Table I shows the concentrations in six tissues, two of

TABLE I—TISSUE DISTRIBUTION OF SODIUM IODIDE-^{125}I AND 4-(3-DIMETHYLAMINOPROPYLAMINO)-7-IODOQUINOLINE-^{125}I IN c.p.m./mg. ± S.E. AFTER INTRAPERITONEAL INJECTION IN MICE

No. of Mice	Av. Wt. of Group, Gm.	Dose, μc.	Time of Sacrifice After Injection, hr.	Tissues					
				Eyes	Skin[b]	Liver	Adrenal	Kidney	Thyroid
Sodium Iodide-^{125}I									
4	23.4	10	12	14.6 ± 2.1	30.9 ± 4.5	26.5 ± 2.9	32.6 ± 7.7	43.9 ± 7.4	3.18×10^5 ± 1.78×10^{5a}
3	20.6	10	24	11.0 ± 1.1	23.1 ± 1.2	36.3 ± 3.6	26.7 ± 15.2	79.6 ± 8.5	8×10^5 ± 3.17×10^{5a}
	21.2	10	48	13.2 ± 2.5	21.8 ± 5.0	70.7 ± 20.4	28.3 ± 7.7	249.6 ± 58.8	5.4×10^5 ± 1.24×10^{5a}
4-(3-Dimethylaminopropylamino)-7-iodoquinoline-^{125}I									
4	22.6	10	12	259.1 ± 14.7[a]	90.5 ± 3.1[a]	170.0 ± 11.0[a]	415.6 ± 32.4[a]	133.4 ± 8.6[a]	1120.5 ± 385.1
4	21.8	10	24	338.9 ± 26.3[a]	97.8 ± 13.6[a]	224.9 ± 85.4	460.6 ± 108.2[a]	234.4 ± 133.0	2047.6 ± 1247.3
3	22.8	10	48	461.0 ± 69.4[a]	88.5 ± 5.6[a]	83.7 ± 12.4	221.3 ± 13.3[a]	81.0 ± 20.0	1645.6 ± 795.4

[a] $p < 0.05$. [b] Skin radioactivity was obtained by counting the ear lobe. Therefore, the concentration in skin *per se* is probably at least twice that shown.

which are known to contain large amounts of melanin (eye, skin). The distribution of activity of the radioiodinated analog of chloroquine was similar to that seen with chloroquine in rats (8) and chloroquine-^{14}C in mice (9). Moreover, the low thyroid activity observed for the animals receiving the radioiodinated compound *versus* those given radioiodide, indicates that significant deiodination did not occur. Studies using mice with transplanted melanomas are now in progress.

(1) Counsell, R. E., Smith, T. D., Doelle, J. Meier, D. A., and Beierwaltes, W. H., *J. Pharm. Sci.*, **56,** 1019(1967).
(2) Meier, D. A., Beierwaltes, W. H., and Counsell R. E., *Cancer Res.*, to be published.
(3) Bernstein, H., Zvaifler, N., Rubin, M., and Mansour, A. M., *Invest. Ophthal.*, **2,** 384(1963).
(4) Sams, W. M., and Epstein, J. H., *J. Invest. Dermatol.*, **45,** 482(1965).
(5) Potts, A. M., *Invest. Ophthal.*, **3,** 399(1964).
(6) Price, C. C., and Roberts, R. M., *J. Am. Chem. Soc.*, **68,** 1204(1946).
(7) Surrey, A. R., and Hammer, H. F., *ibid.*, **68,** 113(1946).
(8) McChesney, E. W., Banks, W. F., and Sullivan, D. J., *Toxicol. Appl. Pharmacol.*, **7,** 627(1965).
(9) Morales, J. O., unpublished data.

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Received April 21, 1967.
Accepted for publication June 6, 1967.

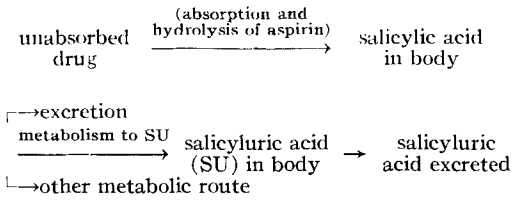
This work was supported by research grants CA-08349-02 and CA-08429-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and PRA-18 from the American Cancer Society, New York, N. Y.

Evidence for Nonfirst-Order Kinetics of Salicylate Elimination—A Rebuttal

Sir:

Wagner's recent article (1) relating to the pharmacokinetics of salicylate elimination contains unwarranted conclusions and therefore requires an early rebuttal.

He claims that our pharmacokinetic model for salicylate elimination in man, which assumes capacity-limited formation of salicyluric acid at doses of 1 Gm. aspirin and above (2), is unjustified, and that all processes are likely to be apparent first-order. He bases his reasoning on a model involving a catenary chain of the type shown in Scheme I.



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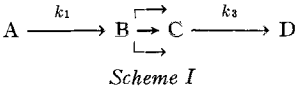

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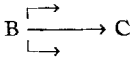

Scheme II

This is accomplished by injecting B intravenously and following its concentration in the plasma as a function of time.[1]

Wagner's model requires that the half-life for the decline of salicylate concentrations in the plasma after intravenous injection of sodium salicylate be independent of dose. In fact, the average half-life [or the apparent half-life, since we do not consider the kinetics at higher doses to be first-order (2)] is 2.4 hr. with 0.25-Gm. doses (4), 6.1 hr. with 1.3-Gm. doses (5), and 19 hr. with 10–20-Gm. doses (6). In all instances, the drug was injected intravenously as salicylate.[2] These data, which were cited in our original paper, provide direct evidence that Wagner's contention is incorrect.

Wagner's pharmacokinetic model consists entirely of first-order rate processes. As a consequence (and in addition to the requirement stated in the preceding paragraph), the time course of urinary excretion of drug in all forms,

[1] Salicylate distribution is so rapid relative to elimination (3) that blood level data are not distorted by the distributive process beyond a very short time following intravenous injection.

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which are known to contain large amounts of melanin (eye, skin). The distribution of activity of the radioiodinated analog of chloroquine was similar to that seen with chloroquine in rats (8) and chloroquine-^{14}C in mice (9). Moreover, the low thyroid activity observed for the animals receiving the radioiodinated compound *versus* those given radioiodide, indicates that significant deiodination did not occur. Studies using mice with transplanted melanomas are now in progress.

(1) Counsell, R. E., Smith, T. D., Doelle, J. Meier, D. A., and Beierwaltes, W. H., *J. Pharm. Sci.*, **56,** 1019(1967).
(2) Meier, D. A., Beierwaltes, W. H., and Counsell R. E., *Cancer Res.*, to be published.
(3) Bernstein, H., Zvaifler, N., Rubin, M., and Mansour, A. M., *Invest. Ophthal.*, **2,** 384(1963).
(4) Sams, W. M., and Epstein, J. H., *J. Invest. Dermatol.*, **45,** 482(1965).
(5) Potts, A. M., *Invest. Ophthal.*, **3,** 399(1964).
(6) Price, C. C., and Roberts, R. M., *J. Am. Chem. Soc.*, **68,** 1204(1946).
(7) Surrey, A. R., and Hammer, H. F., *ibid.*, **68,** 113(1946).
(8) McChesney, E. W., Banks, W. F., and Sullivan, D. J., *Toxicol. Appl. Pharmacol.*, **7,** 627(1965).
(9) Morales, J. O., unpublished data.

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Received April 21, 1967.
Accepted for publication June 6, 1967.

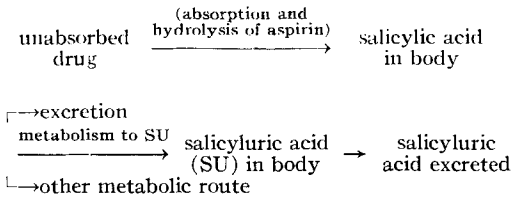
This work was supported by research grants CA-08349-02 and CA-08429-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and PRA-18 from the American Cancer Society, New York, N. Y.

Evidence for Nonfirst-Order Kinetics of Salicylate Elimination—A Rebuttal

Sir:

Wagner's recent article (1) relating to the pharmacokinetics of salicylate elimination contains unwarranted conclusions and therefore requires an early rebuttal.

He claims that our pharmacokinetic model for salicylate elimination in man, which assumes capacity-limited formation of salicyluric acid at doses of 1 Gm. aspirin and above (2), is unjustified, and that all processes are likely to be apparent first-order. He bases his reasoning on a model involving a catenary chain of the type shown in Scheme I.



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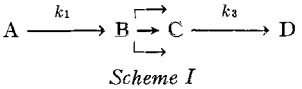

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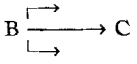

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the shape of a cumulative plot of salicylurate excretion *versus* time, and the composition of urinary metabolites must be independent of dose (*i.e.*, superimposable) when expressed in per cent (or fraction of dose) terms.[3] Here are the actual facts:

(*a*) *All* published data on the urinary excretion of salicylates of which this author is aware show that the urinary excretion rate of total salicylates (*i.e.*, fraction of dose excreted after t hours, where t is less than the time required for total excretion) decreases with increasing dose. Appropriate data may be found in *References 2, 7, 8,* and *9*.

(*b*) The composition of urinary metabolites of salicylate changes with dose when the doses exceed about 0.5 Gm. in adults. Specifically, the fraction excreted as salicylurate decreases with increasing dose (2,10).

(*c*) Cumulative plots of salicylurate excretion as a function of time after salicylate administration are curved with low doses (10, 11) and linear over an appreciable period of time after higher doses (10, 12). An example of this is shown in Fig. 1. Wagner's kinetic model predicts that the two curves in the figure should be superimposable, *i.e.*, that all data points (regardless of dose) should fall on the same line!

(*d*) Semilogarithmic plots of total drug remaining to be excreted *versus* time after drug administration become linear, independent of dose, when the drug in the body has declined to a certain *amount* (2) rather than to a certain *fraction* of the dose, as would be the case with Wagner's model.

(*e*) Figure 2, based on the work of Salassa *et al.* (13), shows that a semilogarithmic plot of salicylate concentration in the plasma following a 3-Gm. dose of sodium salicylate *curves downward* for almost 30 hr. It is practically a straight line when plotted on linear coordinates, reflecting the constant rate of formation of salicylurate and the fact that this process is still the predominant one in the elimination of salicylate in the range of drug levels encountered in the study. When the salicylurate formation process is effectively blocked by co-administration of *para*-aminobenzoate, the decline of salicylate concentration in the plasma becomes exponential. This evidence is discussed in greater detail in previous publications from this laboratory (2, 14).

All of the evidence cited so far (except for the specific data in Fig. 1) is taken from papers which

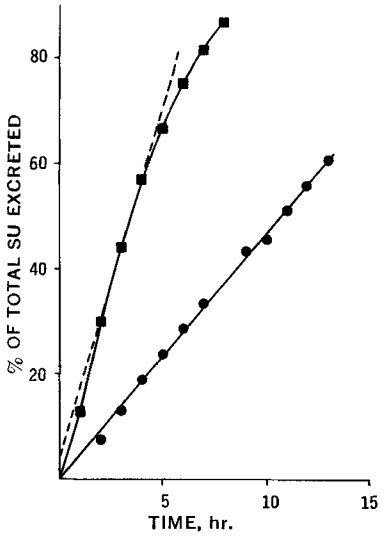

Fig 1—Cumulative excretion of salicyluric acid after 300 mg. (■) and 2 Gm. sodium salicylate (●) given orally in solution to a healthy adult. More than 98% of the dose was recovered in each case. Seventy-five per cent of the low dose and 62% of the high dose were transformed to salicyluric acid. The stippled line is straight and shows that the experimental points for the low dose do not fit a straight line. First-order kinetic models predict that the two curves would be superimposable.

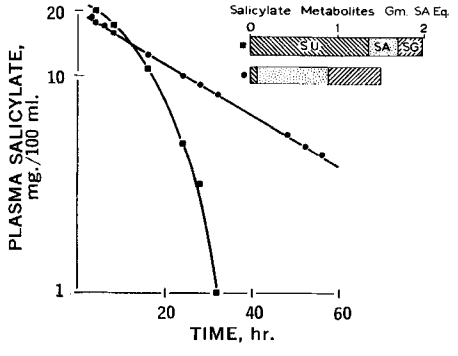

Fig. 2—Plasma concentration of salicylate following a 3-Gm. oral dose of sodium salicylate alone (■) and with para*-aminobenzoate (●) which blocks the salicylurate formation process. Bar graph shows output of salicylate (SA), salicylic glucuronides (SG), and salicyluric acid (SU) in the urine during the time when plasma concentrations were measured. Data from* Reference 13. *The curvature of the plot for salicylate alone reflects the capacity-limited formation of salicylurate and the nonfirst-order elimination of salicylate.*

appeared in the literature prior to the submission of Wagner's article but none of it was mentioned in that article. The impression was given (1) that our own conclusions concerning the elimination kinetics of salicylate in man (2) were based entirely on the time course of urinary excretion data. A review of our paper will show that our

[3] This is shown readily by dividing Wagner's Eq. 8 by X_A°, thus yielding X_D/X_A° (*i.e.*, fraction of dose) on one side and only k's and t on the other.

conclusions were based on much more extensive evidence. While additional reports from our laboratory concerning the elimination kinetics of salicylate as a function of age, dose, route of administration, co-administration of other drugs, *etc.*, will be forthcoming, this rebuttal is limited intentionally to presently published data to show that Wagner's "fallacy" is the result of his disregard for the totality of the evidence available and of his sole reliance on hypothetical models.

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(11) Levy, G., and Amsel, L. P., *Biochem. Pharmacol.*, **15**, 1033(1966).
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Books

REVIEWS

Organisch-Chemische Arzneimittel und Ihre Synonyma (Organic Chemical Medicaments and Their Synonyms). By MARTIN NEGWER. Published by the Akademie-Verlag, Berlin, Germany. Distributed by Pergamon Press, Inc. 44–01 21st Street, Long Island City, New York, N. Y. 11101, 1966. vii + 1224 pp. 17.5 × 24 cm. Price $35.00.

In tabular fashion, this book lists approximately 3,500 compounds with more than 26,000 synonyms. The book is written in German, but because of the organization, it can be used by those not able to read German. Compounds are presented by their molecular formulas in order of the number of carbon atoms and are numbered individually. For example compound No. *1* is CCl_4, No. *2* is $CHBr_3$, No. *3* is $CHCl_3$, *etc.*, through No. *3567* which is $C_{254}H_{377}N_{65}O_{75}S_6$ (insulin). The information given for each compound includes its structural formula, chemical name, salt form, its synonyms including trade names from many countries, and therapeutic use or pharmacologic category. All of the synonyms and names appearing throughout the book are listed in the index, with cross-references to the compound number. Completeness of coverage and accuracy can be judged only upon continued use.

Staff review

A Systematic Approach to the Interpretation of Infrared Spectra. By HERMAN A. SZYMANSKI. Hertillon Press, Box 1677, Buffalo, NY 14216, 1967. vi + 150 pp. 22 × 28 cm. Price $2.00. Paperbound.

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Advances in Chromatography. Volumes II and III. Edited by J. C. GIDDINGS and R. A. KELLER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1966. 15.5 × 23 cm. Vol. II, xvi + 377 pp. Price $14.50. Vol. III, xii + 271 pp. Price $11.50.

Those who are actively interested in a particular phase of chromatography will maintain a continuous acquaintanceship with the developments in that realm. This series, as noted in the review of Vol. I [*J. Pharm. Sci.*, **55**, 863(1966)] is intended to enable the reader who cannot keep up with the explosive growth in the entire field to maintain an over-all view of the progress of chromatography.

Since the reader will have a greater interest in the fields allied to his own, it is inevitable that any one chapter of the series will be regarded with varying degrees of interest by an individual reader. From the point of view of the pharmaceutical chemist, Volumes II and III succeed in fulfilling the aims of the Editors to about the same extent as did Volume I. Two chapters in each volume, devoted primarily to highly technical aspects of a limited area of chromatography, are perhaps too narrow and deep to provide the desired broad picture. It is recognized that without rigorous theoretical investigation of each aspect of chromatography we would be dependent upon empirical findings alone. Many readers whose interest lie in the applications of chromatography would, however, prefer less detailed, but broader, expositions of this theoretical groundwork as background for helping to evaluate the potentialities of a particular technique. At the other extreme are two chapters, one in each volume, which may be considered to be too shallow for a series of this type.

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Reviewed by Joseph Levine
Division of Pharmaceutical Chemistry
Food and Drug Administration
Washington, D. C.

Atomic Absorption Spectroscopy. By JAMES W. ROBINSON. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1966. xi + 204 pp. 15 × 23 cm. Price $9.75.

As has been true of so many new analytical techniques, the development of atomic absorption spectroscopy into a practical laboratory tool has largely awaited the development of simple, reliable, and relatively inexpensive instrumentation. To a large extent this has now been accomplished. There are now a number of well-designed instruments on the market that meet these requirements rather adequately. So it is only fitting that a simplified text be made available to explain the theory and practice of atomic absorption spectroscopy in terms that the average analytical or pharmaceutical chemist, too rushed to delve deeply into the subject, can understand and use. Dr. Robinson has written just such a text.

Atomic absorption spectroscopy is an efficient procedure for assaying large numbers of samples for small amounts of metals. While it cannot entirely replace polarography, colorimetry, X-ray fluorescence, or arc, spark, or flame emission spectroscopy, it can, under appropriate conditions, be more rapid, more accurate, more reproducible, and more convenient than any of these. Selecting the appropriate conditions can, however, require considerable background, understanding, and experience. Dr. Robinson's book would certainly be an excellent place for a person unacquainted with the subject to start.

Addressed, according to the author, to the graduate student, to the research worker not well-versed in analytical chemistry, and to the analytical chemist interested in rapid and accurate analyses for research and industry, the book was apparently not designed to provide comprehensive coverage of the subject. Indeed, a more apt title for the book might be "An Introduction to Atomic Absorption Spectroscopy," for that is what it is. It is a short book, consisting of little more than 200 pages. Written in a simple straightforward style, it can be read in a few hours without unduly taxing the eye or the mind.

This is not to indicate that it is a superficial book. In fact, it covers the subject matter quite well for a book of this size. Among the more important subjects included are a brief description of the sources of atomic absorption spectra, a short discussion of advantages and disadvantages of atomic absorption, an extensive discussion of instrument components and parameters, a fairly comprehensive discussion of analytical parameters such as atomizer effects, effects of anions and other cations, solvents, sensitivity,

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Those who are actively interested in a particular phase of chromatography will maintain a continuous acquaintanceship with the developments in that realm. This series, as noted in the review of Vol. I [*J. Pharm. Sci.*, **55**, 863(1966)] is intended to enable the reader who cannot keep up with the explosive growth in the entire field to maintain an over-all view of the progress of chromatography.

Since the reader will have a greater interest in the fields allied to his own, it is inevitable that any one chapter of the series will be regarded with varying degrees of interest by an individual reader. From the point of view of the pharmaceutical chemist, Volumes II and III succeed in fulfilling the aims of the Editors to about the same extent as did Volume I. Two chapters in each volume, devoted primarily to highly technical aspects of a limited area of chromatography, are perhaps too narrow and deep to provide the desired broad picture. It is recognized that without rigorous theoretical investigation of each aspect of chromatography we would be dependent upon empirical findings alone. Many readers whose interest lie in the applications of chromatography would, however, prefer less detailed, but broader, expositions of this theoretical groundwork as background for helping to evaluate the potentialities of a particular technique. At the other extreme are two chapters, one in each volume, which may be considered to be too shallow for a series of this type.

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

September 1967 volume 56, number 9

Review Article

Applications of Nuclear Magnetic Resonance Spectroscopy in Medicinal and Pharmaceutical Chemistry

By A. F. CASY

NUCLEAR MAGNETIC RESONANCE (NMR) spectroscopy is a physical method which enables chemists to study certain atomic nuclei within molecules. One phase of this area, proton magnetic resonance (PMR) spectroscopy, is of particular interest to the organic chemist. The spectroscopic technique was first applied to organic compounds soon after 1950 and its use has grown enormously over the last decade. It is probable that most medicinal and pharmaceutical chemists have encountered the method and are aware of its physical basis. Several textbooks written with the requirements of the organic chemist in mind have been published (1–5) since the pioneer account of Jackman (6). One of the more recent publications is a comprehensive two-volume work by Emsley, Feeney, and Sutcliffe (7).

Numerous reviews are available dealing with the application of NMR spectroscopy to specific topics, such as the structure elucidation of natural products (8, 9) and biochemistry (10). (Other reviews are mentioned throughout this paper.) However, no particular emphasis has been given to the potentialities for application of the NMR technique to problems of interest to medicinal chemists, and the present review has been written to this end. A comprehensive account is not intended—rather, a selection of the literature has been made, and the topics therein outlined and discussed in some detail so that the value and versatility of NMR spectroscopy in the field of medicinal chemistry may be demonstrated.

A general outline of the basic principles of NMR spectroscopy is not included here because of space limitations and the large number of introductory accounts now available. Apart from the textbooks referred to previously, several texts describing the use of physical methods in organic chemistry include useful chapters on NMR spectroscopy (11–13).

The interpretation of NMR spectra is based on three sets of parameters which characterize the absorption of radiofrequency radiation by atomic nuclei placed in a magnetic field (H): (a) the frequencies of the absorbed radiation (ν) expressed as the *chemical shift* relative to an arbitrary standard absorption line; (b) the multiplicity of the lines originating from a given group of nuclei and described by the appropriate *coupling constants* (J values); and (c) the decay times characterizing the return of the nuclei excited by the absorption of radiation to a lower energy state referred to as *relaxation times*.

Additional information is derived from the integration curve which enables the relative number of protons within each absorption band to be determined. Of the above parameters, the first two are the most widely applied to chemical

Received from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.

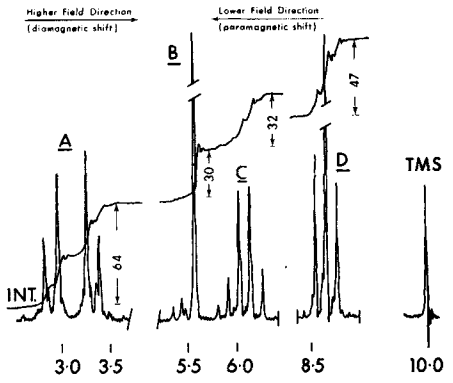


Fig. 1—PMR spectrum of 4-ethoxybenzyl chloride ($CH_3CH_2OC_6H_4$p-CH_2Cl) in $CDCl_3$ (60 Mc./sec.). Key: A, *aryl protons* (A_2B_2 *system*), *symmetrical about 413 c.p.s.;* B, $ArCH_2Cl$ *singlets*, ν *262 c.p.s.;* δ *4.37 p.p.m.;* τ *5.63 p.p.m.;* C, CH_3CH_2O *quartet* (J *7 c.p.s.*), ν *231 c.p.s.;* δ *3.85 p.p.m.;* τ *6.15 p.p.m.;* D, CH_3CH_2O *triplet* (J *7 c.p.s.*), ν *79 c.p.s.;* δ *1.32 p.p.m.;* τ *8.68 p.p.m.*

problems, although more attention is now being given to relaxation times because of their value in the study of molecular interactions (14). NMR spectra are displayed as plots of a detector signal (ordinate) against a magnetic field (abscissas) calibrated in cycles per second (c.p.s.) since H is proportional to ν. In many cases chemical shifts and coupling constants may be read directly from a spectrum (first-order treatment);[1] an example is the spectrum of 4-ethoxybenzyl chloride (Fig. 1). In this spectrum chemical shifts are measured relative to tetramethylsilane (TMS), the most commonly employed internal standard [in deuterium oxide solutions the water-soluble analog, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) may be employed (16)]. Three scales are used to express the chemical shift: (*a*) a frequency scale (ν) given in c.p.s. for TMS set at zero (coupling constants are also measured in these units); (*b*) parts per million (δ), obtained from the expression:

$$\delta = \frac{\text{c.p.s.} \times 10^6}{\text{oscillator frequency in c.p.s.}}$$

TMS at zero; and (*c*) the τ (or tor) scale where:

$$\tau = 10 - \delta(\text{TMS at } 10)$$

The last two scales are dimensionless, while the first depends on the operating frequency and must always be used in conjunction with the frequency value. (This is not the serious drawback it first appears because the great majority of reported spectra are recorded at a frequency of 60 Mc./sec.) All three systems are in common use [their relative merits have been discussed by Bible (4)]; therefore, in studying NMR literature, care must be taken to ascertain the chemical shift scale and the standard employed. In this review, no attempt has been made to standardize chemical shift values, and examples of all three scales are included.

[1] Sometimes nonmagnetically equivalent nuclei give rise to spectra that appear to be susceptible to first-order treatment, but are really not; such "deceptively simple" spectra are considered in a review by Becker (15).

USE IN ANALYSIS

Integral data may be used for the analysis of mixtures when overlap between signals due to individual components does not occur (or may be corrected for), and NMR methods for the quantitative assay of mixtures of aspirin, phenacetin, and caffeine (17) and of barbiturates (18) have been described. The spectrum of an APC tablet in $CDCl_3$ (binders such as starch and lactose are insoluble and do not interfere) is shown in Fig. 2. The signal at 2.3 p.p.m. is due to OCO*Me* of aspirin, and its integral value (compared with that of Me in a standard solution of caffeine) enables the aspirin content of the mixture to be determined. The signals at 3.4 and 3.6 p.p.m. (both due to Me) give the caffeine content, and the integral of the quartet centered near 4 p.p.m., due to OCH_2Me (and corrected for the overlapping N^7-Me signal of caffeine), gives the phenacetin content. The method is claimed to have the advantage of speed and to be of an accuracy sufficient for quality control. An example in the field of organic synthesis is the determination of isomeric ratios obtained in alkylations employing 2-chloro-1-dimethylaminopropane (these proceed *via* ethyleniminium ions and hence lead to mixtures of products). Thus, it is found that alkylation of diphenylacetonitrile leads to an approximately 1:1 mixture of the cyanide precursors of methadone and isomethadone (Fig. 3), while that of 2-benzylbenzimidazole gives a mixture in which one isomer shows a marked preponderance (19) (Fig. 4). A molecular weight

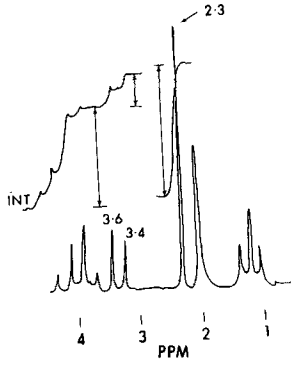


Fig. 2—Part of the PMR spectrum of an aspirin, phenacetin, and caffeine mixture in $CDCl_3$. [*After Hollis (17) and reproduced with permission from* Anal. Chem., **35,** *1682 (1963).*]

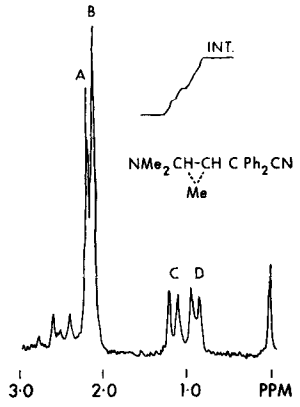


Fig. 3—Part of the PMR spectrum of mixed methadone-isomethadone cyanides in $CDCl_3$. A (N-Me) and C(sec Me) are due to $NMe_2CH_2CHMeCPh_2CN$(iso), and B (N-Me) and D (sec Me) to $NMe_2CHMeCH_2CPh_2CN$.

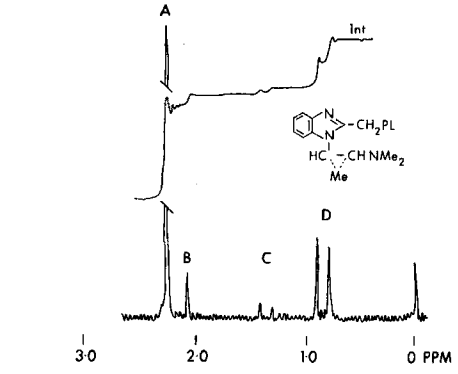


Fig. 4—Part of the PMR spectrum of product of alkylating 2-benzylbenzimidazole with 2-chloro-1-dimethylaminopropane (solvent $CDCl_3$); A(NMe_2) and D (sec Me) are due to isomer with $CH_2CHMeNMe_2$ N-1 side chain, and B (NMe_2) and C (sec Me) are due to isomer with $CHMeCH_2NMe_2$ side chain.

determination method based on PMR integrals has been described (20). It consists of comparing the integrated intensities of an added standard and a recognizable peak or group of peaks of the unknown in a solution containing known weights of standard and unknown. Molyneux, Rhodes, and Swarbrick (21) used a PMR method to check the homogeneity of the alkyl chain in some betaines (I). With D_2O as solvent, distinct signals for the proton groups a–e were observed and, using the integral of the Me signal (a) as standard, the number of protons in the other groups were calculated.

$$\underset{a}{Me}\ \underset{b}{(CH_2)_{n-2}}\underset{c}{CH_2}\overset{+}{N}\underset{d}{Me_2}\underset{e}{CH_2}C\bar{O}_2$$

I

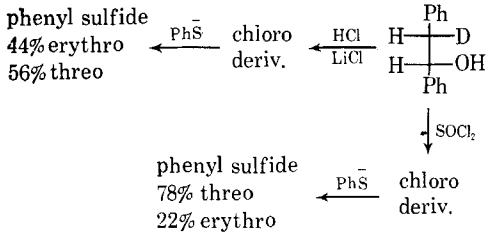
Diastereoisomeric mixtures may be analyzed by NMR spectroscopy provided the spectrum exhibits one or more signals, due to each isomer, which do not overlap. Raban and Mislow (22) have pointed out the principles involved but give, as an example, a case in which a clear separation of isomer signals is not observed. We have used this method to analyze isomeric 4-phenyl-3-methyl-4-piperidinols obtained by treating a 3-methyl-4-piperidone with lithium phenyl, and find that mixtures of two parts of the *trans* (3-Me/4-Ph) and one part of the *cis* isomer result (23); the analyses were based on the secondary Me signals which do not overlap and are particularly well separated in the base hydrochlorides, as discussed later. The isomeric mixture of alcohols (II)—the propionate ester of the α-isomer is propoxyphene—may be analyzed similarly.[2]

$$Me_2NCH_2CHMeCPh(OH)CH_2Ph$$

II

Chemical shifts of CH*Me* (c.p.s. from TMS in $CDCl_3$) are: α-(base) 50, β-(base) 66, α-(HCl) 75, and β-(HCl) 97.

Kingsbury and Thornton (25) have obtained evidence of the stereochemistry of displacement reactions using diastereoisomeric substrates, reaction mixtures being analyzed by measurement of NMR integrals (Scheme I). The advantage of the method is that the amount of retention and displacement product can be directly observed in favorable cases.



Scheme I

Mixtures of structurally isomeric alkenes are also amenable to assay by the integration technique. Figure 5 shows the spectrum of the product of dehydration of 3-methyl-4-phenyl-4-piperidinol. The 3-methyltetrahydropyridine content of the mixture is obtained from the 3-Me integral (broad singlet at 92 c.p.s.), and the 5-methyl isomer from integrals of the secondary Me (doublet at 59 c.p.s.) and vinylic proton (triplet at 357 c.p.s.) signals (26). By this analytical procedure, a study of the relative stabilities of isomeric 3- and 5-methyltetrahydropyridines and of the influence of structure and stereochemistry upon the direction of elimina-

[2] Treatment of $NMe_2CH_2CHMeCOPh$ with benzyl magnesium bromide gives the alcohols (II) in the ratio of 5(α) to 1(β) from integral data derived from the spectrum of the total base (24).

tion of 4-aryl-3-methyl-4-piperidinols has been made (27).

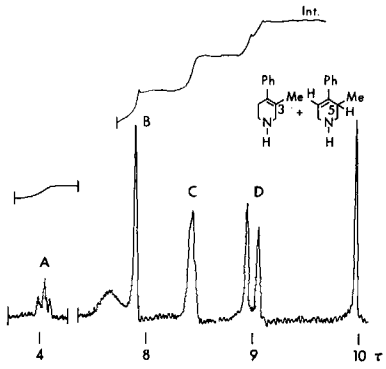
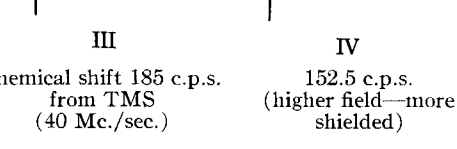


Fig. 5—Part of the PMR spectrum of the dehydration product of 3-methyl-4-phenyl-4-piperidinol in $CDCl_3$. C (tert Me) is due to 3-methyl-4-phenyl-1,2,5,6-tetrahydropyridine, and A (vinylic) and D (sec Me) to the 5-methyl isomer; B (N-H) is due to both isomers.

CONFIGURATIONAL AND CONFORMATIONAL STUDIES IN CYCLIC MOLECULES

In cyclohexane derivatives, an equatorial proton is generally less shielded than the corresponding axial one [*e.g.*, 4-H in *cis*(III) and *trans*(IV) 4-bromo-*tert*-butylcyclohexane (28)] and much use has been made of this observation



III — chemical shift 185 c.p.s. from TMS (40 Mc./sec.)

IV — 152.5 c.p.s. (higher field—more shielded)

in calculating the conformational preferences of substituent groups (29). A valuable relationship for assigning configuration and conformation in rigid systems is the dependence of coupling constants between protons on adajcent carbon atoms (vicinal coupling) upon the dihedral angle (ϕ) between the protons. A number of formulas have been developed to express this relationship (30, 31), and the plot of J_{vic} against ϕ for Williamson and Johnson's formula (31) is shown in Fig. 6. From this, J_{vic} should have a value of about 16 c.p.s. when $\phi = 180°$ (as in axially related protons) and 2.5 c.p.s. when $\phi = 60°$ (as in a/e or e/e related protons). In practice, J_{aa}, J_{ae}, and J_{ee} values deviate from these values as a result of distortion of the ideal dihedral angles

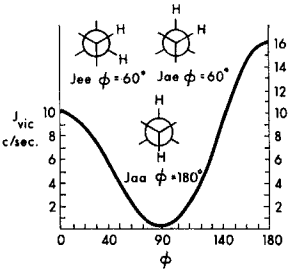


Fig. 6—Plot showing the dihedral angle dependence of vicinal coupling constants from Williamson and Johnson (31).

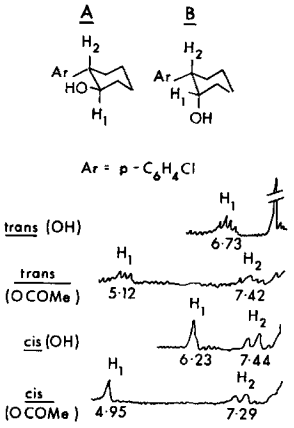
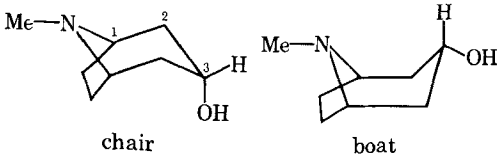


Fig. 7—Part of the PMR spectra of trans *and* cis *2-(p-chlorophenyl) cyclohexanol and corresponding acetate esters; chemical shifts in τ values from TMS, solvent CCl_4. [Adapted with permission from* J. Org. Chem., **27**, *715(1962).]*

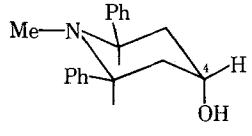
and the influence of substituent electronegativities upon J values, but it is clear that J_{aa} coupling constants exceed J_{ae} and J_{ee} values. Thus, large J_{vic} coupling constants between protons in a cyclic structure may be associated with an approximately diaxial orientation of atoms, and smaller separations with a–e or e–e interactions. Much use has been made of these principles in establishing the stereochemistry of biologically active cyclic compounds. A typical example, taken from Huitric's extensive study of aryl substituted cyclohexanols (32), is the investigation of *cis*- and *trans*-2-(chlorophenyl)cyclohexanol (33). Signals of the 1- and 2-protons are shown in Fig. 7; these are readily differentiated by the large downfield shift of the 1-hydrogen upon acetylation (6, 34). [Use of the upfield shift of the methine proton which follows methylation of *sec* alcohols has recently been proposed as an alternative method of identifying protons on carbon bearing a hydroxyl group (35).] The broad unresolved multiplets for the 1 and 2-hydrogens in A (OH and acetate) are consistent with their *trans* configuration; in the geometry depicted in A, both are axial and each is adjacent to two nonidentical axial hydrogens. In the *cis* geometry, B, the 1-hydrogen is equatorial and has little spin–spin coupling with adjacent hydrogens (hence an approximate singlet results), while the

2-hydrogen is axial and adjacent to only one other axial hydrogen giving rise to what is essentially a doublet with $J_{2,3}$ of about 11.5 c.p.s. PMR evidence of the same nature has been used to settle the controversial problem of whether the piperidine ring of tropane derivatives exists in a chair or a boat conformation (36, 37). The most valuable information in this respect derives from the approximate triplet due to the 3-proton of tropine (V)—the small value of its base width shows that it cannot involve a *trans* (or near *trans*) coupling (as in the boat form) and establishes that the 3-proton must be attached equatorially to a six-membered ring. Furthermore, the characteristics of this signal are very similar to those of the 4-proton in the β-isomer of 1-methyl-2,6-diphenylpiperidin-4-ol (VI) of known conformation (38).



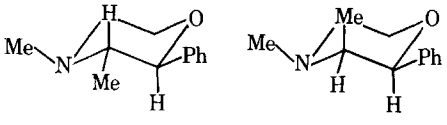
V

base-width 3-H, 16.8 c.p.s.



VI

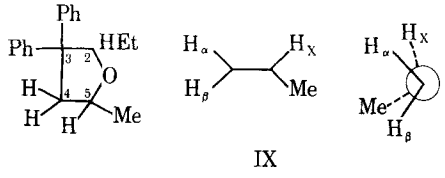
4-H, 16.0 c.p.s.



VII VIII

Acid-catalyzed cyclization of *N*-(β-hydroxyethyl)-(−)-ephedrine (erythro configuration) yields the appetite depressing drug (+)-3,4-dimethyl-2-phenylmorpholine (VII, phendimetrazine) while cyclization of *N*-chloroacetyl ephedrine and reduction of the product with lithium aluminium hydride gives the corresponding diastereoisomer (VIII) (without influence upon appetite). From the $J_{2,3}$ coupling constants, phendimetrazine is assigned a threo ($J_{2,3}$ 8.8 c.p.s., a value indicative of *trans* coupling) and its isomer an erythro ($J_{2,3}$ 2.7 c.p.s.) configuration (39). Hence, the acid-catalyzed process must proceed with inversion of configuration.

Knowledge of the stereochemistry of the α- and β-2-ethyl-5-methyl-3,3-diphenyltetrahydrofurans (IX) derived from the diastereoisomeric analgesics α- and β-methadol, respectively (40, 41), is of importance in that it provides evidence of the configurations of the precursor amino alcohols themselves (41). The PMR spectra of the isomeric cyclic ethers differ markedly in the respect to the 4-methylene signals (40), which form the AB part of an ABX system (Fig. 8). Assignments to H_α and H_β (*cis* and *trans* to the methine proton, H_x) may be made by use of the dihedral angle dependence of coupling constants [the reasonable assumption is made that the C-4 and C-5 substituents are staggered (29) with the $H_\alpha H_x$ and $H_\beta H_x$ dihedral angles intermediate between 0° and 60°, and 120° and 180°, respectively, leading to the conclusion that $J_{\beta x}$ should exceed $J_{\alpha x}$], and it is apparent, from comparisons with the AB signals of a series of related 5-methyl-3,3-diphenyltetrahydrofurans, that the H_α proton has an abnormally high field position in the α-isomer (IX). This is a result to be expected if the same isomer has *cis*-2-Et/5-Me geometry, since a consideration of the probable conformations of the *gem* diphenyl groups in this molecule (that of phenyl *trans* to 2-Et will be determined largely by the orientation of the *cis*-phenyl group, itself influenced by the bulky 2-Et substituent) leads to a conformer in which the α-C-4 methylene proton falls within the screening zone of the adjacent phenyl group (B in Fig. 9). In the β-isomer (*trans*-2-Et/5-Me) interactions between 2-Et and a flanking phenyl group will likewise be an important factor in determining the preferred conformation of phenyl *trans* to Et. Here, however, the methylene proton *cis* to 5-methyl (H_β) will be screened and, as a result, the H_α-H_β chemical shift difference will increase because H has the higher field position in the absence of differential screening effects; these arguments account for the well separated H_α and H_β signals and the unusually high field position of H_β in the β-isomer,



IX

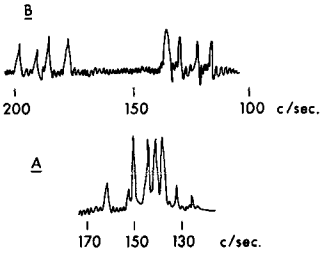


Fig. 8—C-4 Methylene protons PMR signal of A, *α-2-ethyl-5-methyl-3,3-diphenyltetrahydrofuran and* B, *the β-isomer. Chemical shifts in c.p.s. from TMS.*

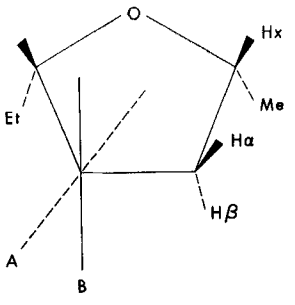


Fig. 9—Diagram of Dreiding model of cis-*2-ethyl-5-methyl-3,3-diphenyltetrahydrofuran viewed from above. Heavy lines lie above, and dotted lines below the plane of the tetrahydrofuran ring;* A *and* B *denote the planes of the aromatic rings* cis *and* trans *to 2-Et, respectively.*

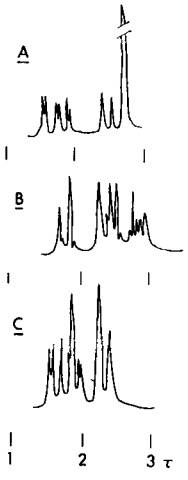
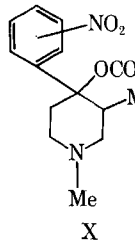


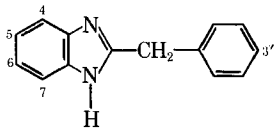
Fig. 10—Aromatic PMR signals of some nitro-2-benzylbenzimidazoles in dimethylsulfoxide. Key: A, *5-nitro;* B, *3′-nitro;* C, *3′5-dinitro. Chemical shifts in τ values from TMS.*

PROBLEMS OF AROMATIC SUBSTITUTION

The position taken by an aromatic substituent can often be identified by PMR spectroscopy from considerations based upon a combination of chemical shift, spin–spin couplings (*ortho* 5–9, *meta* 2–3, and *para* coupling 0–1 c.p.s.), and integration data. A simple example is provided by the nitration product of alphaprodine (X) (42). If the nitro group enters the *para* position,

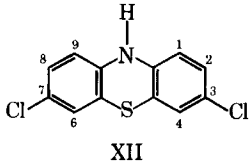


X



XI

the aromatic protons will comprise an A_2X_2 (or A_2B_2) system, and their signal will be a pair of superimposed doublets with J values typical of *ortho* coupling. If the nitro group enters the *ortho* or *meta* positions, the aromatic protons will comprise nonsymmetrical groups, and their resonance signal will be correspondingly more complex. In fact, the aromatic signal of nitro alphaprodine consists of two doublets that both integrate for two protons and have a J value of 9 c.p.s.; hence X is a *p*-nitro derivative. Orientation in the case of the dinitro product obtained from 2-benzylbenzimidazole (XI) was assisted by comparison of its PMR spectrum with that of 3′- and 5-nitro-2-benzylbenzimidazole (Fig. 10) (43). The A_2B_2-4 proton quartet of the 3′-nitro derivative and signals due to protons of the benzimidazole nucleus of the 5-nitro isomer may both be discerned (in spite of the signal overlap) in the spectrum of the dinitro derivative. In the 5-nitro isomer, assignment of the lowest field doublet (511 c.p.s.) to the 4-proton (on the basis of its being flanked by electronegative, deshielding groups) is confirmed by the fact that it exhibits a coupling constant (2 c.p.s.) typical of *meta* placed protons. The doublet of doublets (center 490 c.p.s. J 2 and 9) must arise from the 6-proton since this is *meta* coupled to the 4- and *ortho* coupled to the 7-proton, the latter giving a signal of the expected multiplicity and J value (doublet at 464.5 c.p.s. J 9 c.p.s.), *ortho* coupling alone being involved in this case. Craig and others (44) have reported chemical shift data for a series of mono- and di- derivatives (mostly chloro and methoxy) of phenothiazine and have demonstrated the feasibility of deducing orientation in substituted phenothiazines by analysis of the aromatic proton resonance signals. When substitution is 3,7, for example, two doublets, both having J = 9 and one further split by *meta* coupling [due to the 1 (9) and 2 (8) protons, respectively], should be differentiated —as in the spectrum of 3,7-dichlorophenothiazine



XII

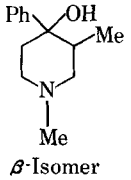
(XII). The problem of orientation has particular significance in characterizing hydroxylated phenothiazines (formed in the metabolism of pharmacologically active derivatives) since the necessary synthetic reference compounds are not yet available. Warren *et al.* (45) have published the PMR spectra of a collection of commercially available phenothiazines for use in their identification.

HYDROGEN BONDING STUDIES

The resonance position of a hydroxylic proton moves to lower fields when it is hydrogen bonded

(2, 6, 7, 46), a phenomenon which has made PMR spectroscopy a valuable tool for the study of hydrogen bond formation. The dependence of the OH resonance position (a time average of a weighted average of the various hydrogen-bonded species present) upon the extent of hydrogen bonding accounts for the changes in the OH chemical shift which result when the concentration of the hydroxyl compound or the temperature are varied. Concentration independent (or largely so) OH resonance signals are obtained when solvents capable of forming strong hydrogen bonds such as dimethylsulfoxide and pyridine (47, 48) are employed; solvent–substrate bonding (unaffected by substrate concentration) then becomes the predominant hydrogen-bonded species because of the large excess of solvent over solute molecules. $CDCl_3$ is also a solvent of this nature as seen from the OH chemical shifts of β-1,3-dimethyl-4-phenylpiperidin-4-ol, which are far more sensitive to concentration changes in CCl_4 than in $CDCl_3$ (49) as the data in Table I demonstrate.

TABLE I—CHEMICAL SHIFTS OF



β-Isomer

Solvent	Concn., mg./ml.	Chemical Shift (from TMS), c.p.s.
CCl_4	70	122
CCl_4	23	95.5
$CDCl_3$	70	133
$CDCl_3$	23	122

The chemical shift of the hydroxyl proton has been shown to be linearly related to concentration in CCl_4 in the 0.015 to 0.002 mole fraction range and, by extrapolating to infinite dilution, the chemical shift of the monomeric OH proton can be determined (48). Comparisons of such values in triads of compounds such as those shown in Fig. 11 have enabled the conformation preferences of cyclohexane substituents to be calculated (48). It is probable (on steric grounds) that an axial OH group is less hydrogen bonded than one in the equatorial positon; hence, the OH chemical shift of an axial alcohol should be less concentration-dependent than that of the epimer. From these arguments evidence of stereochemistry may be derived from the limiting slopes of chemical shift–concentration plots (48). Thus, from the data of Fig. 11, the slope for *cis*-4-*tert*-butylcyclo-

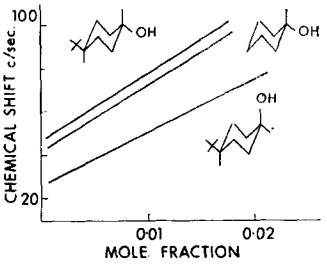


Fig. 11—Chemical shift dependence of hydroxyl proton with concentration at 40°. Solvent CCl_4. [Adapted with permission from J. Am. Chem. Soc., **86**, *4378 (1964).]*

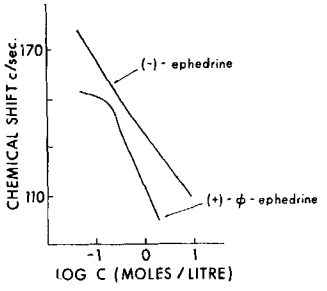


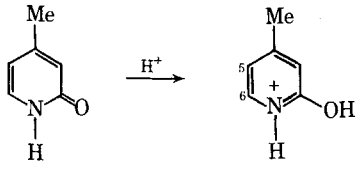
Fig. 12—Chemical shift dependence of NH/OH protons with concentration in $CHCl_3$. [Adapted with permission from Can. J. Chem., **38**, *125 (1960).]*

hexanol (axial OH) is less than that of the *trans* isomer (equatorial OH). A more subtle use of PMR spectroscopy in these studies is the detection of intra- in distinction from intermolecular hydrogen bonding, as may occur in diols and amino alcohols. Inter- is more sensitive than intramolecular hydrogen bonding to dilution, and this difference should be reflected in OH chemical shift–concentration plots. It is found, for example, that the concentration dependence of the *o*-chlorophenol OH resonance frequency reaches a maximum at low concentrations, while that of the *m*- and *p*-isomers continues to increase throughout the dilution range studied (intramolecular hydrogen bonding is only possible in the *o*-chloroisomer) (50). Figure 12 shows the concentration dependence of resonance frequencies of the NH and OH protons of ephedrine and pseudoephedrine (these appear as a single line because of rapid exchange). At low concentrations the pseudoephedrine signals are less sensitive to concentration changes and Hyne (51) advances this fact as evidence for intramolecular hydrogen bonding (N---H—O—) in the pseudoisomer. The position of the OH resonance lines in chloramphenicol and its isomers in acetone at low fields compared with those of the OH of simple monohydric alcohols at infinite dilution in CCl_4, indicates that both OH groups of the antibiotic are involved in hydrogen bonds. These bonds are considered to be of an intra- rather than intermolecular nature because of the comparative concentration independence of the OH chemical shifts, while bonding to the solvent

was thought unlikely on account of evidence that inter- and intramolecular hydrogen bonds are stronger than those formed between hydroxyl groups and acetone (the chemical shifts of monohydric alcohols at infinite dilution in acetone are at higher fields than those of OH in dihydric alcohols capable of intramolecular bonding) (52).

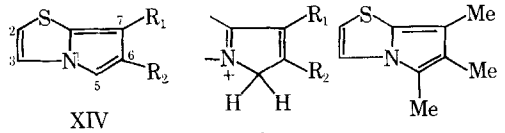
SITE OF PROTONATION

The problem of identifying the site of protonation in a molecule containing more than one basic center, often encountered in medicinal chemistry, may frequently be solved by studying the effect of proton uptake on chemical shifts and the spin–spin coupling patterns of protons in the vicinity of the various possible sites. Some examples follow. The C-6 proton peak of 4-methyl-2-pyridone (XIII) in sulfuric acid appears as a triplet indicating approximately equal coupling with the C-5 proton and *one* proton on the nitrogen atom (53). This result demonstrates the



XIII

O-protonation of the pyridone (XIII); if *N*-protonation had occurred, the C-6 proton signal would have been a quartet (two protons on nitrogen). Similar evidence supports sulfur and ring nitrogen as protonation sites in thiopyridones and aminopyridines, respectively (54). Protonation of the pyrrolo[2,1-*b*]thiazoles (XIV*a–c*) is considered to occur at a carbon atom because



XIV salt XV

a, $R_1 = R_2 = H$
b, $R_1 = H$, $R_2 = Me$
c, $R_1 = R_2 = Me$

PMR spectra of the salts display a two proton singlet that has a position characteristic of a methylene group (55). A low field AB system (two doublets), which is common to spectra of all the salts of XIV*a–c*, and XV can only arise from the C-2 and 3 protons; hence protonation must occur in the pyrrole ring. C-5 must be the site since, in XIV*c*, a methylene group can only develop at this position; the same site is postulated for the other derivatives since the methylene signals of the spectra (XIV*a–c*), all have similar chemical shifts. In the salt of XV, the C-5 proton signal is the anticipated quartet. In the next example evidence for the protonation site is obtained by identifying the proton most deshielded when a base is converted to its salt. The aromatic proton signals of the 2-benzylbenzimidazole shown in Fig. 13 are readily analyzed. The lowest field signal is assigned to the C-4 proton (it is flanked by electronegative groups and exhibits *meta* coupling only), the quartet (center τ 1.8) to the C-5 proton (it exhibits *ortho* and *meta* coupling), and the high field doublet (*ortho* coupled) to the C-6 proton. In the monohydrochloride, this pattern of signals, although disturbed, is maintained, and the downfield shifts may be measured. These are C-4 (7.2 c.p.s.), C-5 (13.2 c.p.s.), and C-6 (24.5 c.p.s.). The fact that the C-6 proton suffers the greatest shift indicates that N-1, rather than N-3, is the protonation site (56).

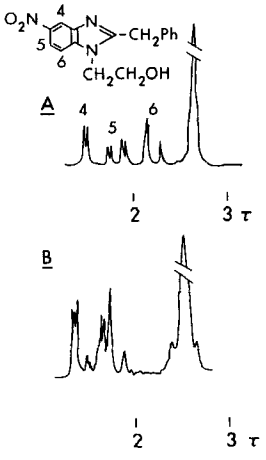


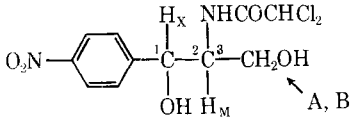
Fig. 13—Aromatic PMR signals of 2-benzyl-1-(2-hydroxyethyl)-5-nitrobenzimidazole in dimethylsulfoxide. Key: A, free base; B, hydrochloride. Chemical shifts in τ values from TMS.

Appropriate to this section is mention of the potentiality of PMR spectroscopy in determining pKa values of acids and bases. When an acid ionizes, the chemical shifts of protons near the acidic function change and if the PMR spectra of the unionized acid (HA) alone, and the anion (A^-) alone are known, the ratio HA/A^- for mixtures may be derived from chemical shift values [the spectral characteristics resulting from two species undergoing rapid chemical exchange depend only upon the PMR parameters of the individual species and their relative amounts (2)]. The same arguments apply in the case of a base and its conjugate acid. These principles have been applied by Birchall and Jolly (57) to the determination of the relative acidities of weak acids in liquid ammonia.

CONFORMATION OF PHARMACOLOGICALLY ACTIVE MOLECULES

Knowledge of the probable conformations of pharmacologically active molecules in solution, particularly as solutes in water, is of great importance to medicinal chemists because of the value of such information in extending the understanding of receptor sites. Some of the relatively few examples in which NMR spectroscopy has been employed in this connection are discussed below.

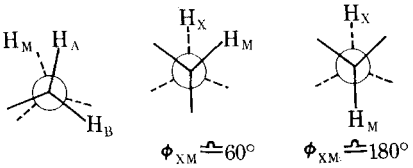
Jardetzky (52) has made a detailed analysis of the PMR spectra of chloramphenicol (XVI) and its erythro isomer. Signals arising from the



J_{AB} = 10–11 c.p.s
$J_{AM} = J_{BM}$ = 5–6 c.p.s
J_{MX} = 2–3 c.p.s

XVI

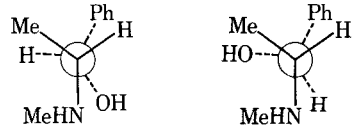
proton group attached to carbons 1, 2, and 3 of chloramphenicol could be analyzed by the ABMX procedure, and it was concluded (*a*) from the smallness of the J_{MX} value (2.6 c.p.s.) and (*b*) the nonequivalence of the two hydrogens on C-3, that rotation about the single C—C bonds of the propanol side chain is restricted. In principle, methylene protons (AB) adjacent to an asymmetric center must be nonequivalent because of differences in the averaging of the A and B shifts by rapid rotation (rotational nonequivalence); however, nonequivalence of this type is not observed in the PMR spectra of many asymmetrically substituted molecules in which "free" rotation can be inferred from the magnitude of the coupling constants [*e.g.*, catecholamines (58)], and for it to be observed, differences in conformation populations are an additional requirement. The preferred conformation (XVII) of the C_2 and C_3 portion of the molecule (with



XVII $\phi_{XM} \simeq 60°$ XVIII $\phi_{XM} \simeq 180°$ XIX

the AM and BM dihedral angles close to 0° and 180°, respectively) is based upon the values and equality of the J_{AM} and J_{BM} coupling constants (see plot of J_{vic}/dihedral angle, Fig. 6), while the low value of the J_{MX} constant (2–3 c.p.s.) shows the X and M protons to have a gauche conformation as in XVIII. In the erythro isomer the larger J_{MX} value (6 c.p.s.) shows the X and M protons to be *trans* as in XIX. A choice of the most likely conformations, influenced also by the assumption of an intramolecular hydrogen bond (evidence discussed elsewhere in this review) led to a structure which bore a striking resemblance to a pyrimidine ribonucleotide. Any alteration of the geometry of the propanol side chain (as in erythro isomers) destroys the similarity to the ribose moiety; hence this relationship is considered to have a possible bearing upon the stereochemical features of structure-activity relationships in the chloramphenicol series (52).

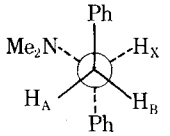
Hyne (59) has studied the conformation of a related diastereoisomeric pair, ephedrine and pseudoephedrine, in chloroform. The preferred "off staggered" conformations he proposes for these molecules (XX and XXI) are based upon



pseudoephedrine XX ephedrine XXI

$Ph^1CH(OH)^2CH(NHMe)Me$

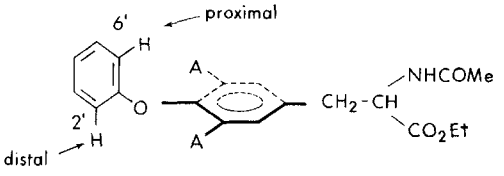
PMR evidence for similar methyl environments in the two isomers and for a greater C_1-H/C_2-H dihedral angle in the case of pseudoephedrine (J_{vic} 8.2 for pseudo- and 4.0 c.p.s. for ephedrine). The occurrence of intramolecular hydrogen bonding in the pseudoisomer (see previous discussion) is also accounted for by conformation XX; in the ephedrine conformation (XXI) the tendency for hydrogen bonding should be less because of the great OH-NH distance. Sasaki *et al.* (60), using similar arguments, have advanced a favored conformation for (−)-*N*,*N*-dimethyl-1,2-diphenylethylamine (XXII), an analgesic one-half to one-third as potent as morphine (61).



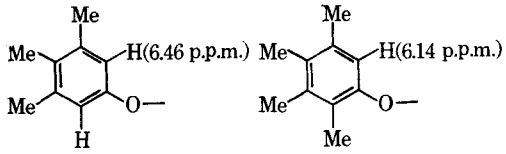
J_{AB} 13.9
J_{BX} 6.4
J_{AX} 9.3
(hydrochloride in D_2O)

XXII

Lehman and Jorgensen (62) have presented PMR evidence in support of a preferred skewed conformation for *O*,*O*-disubstituted diphenyl ethers (XXIII) related to thyroxine; this work is

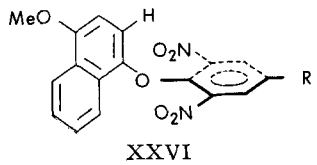


XXIII



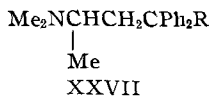
XXIV XXV

related to Jorgensen's proposed receptor for the thyroid hormone (63). Study of models shows that the proximal 6′-proton (but not the distal 2′-proton) lies well within the region in which its magnetic resonance would be shielded by the π-cloud as estimated by Johnson and Bovey (64). In compounds substituted at the 3- and 5-positions (A in XXIII) by iodine or nitro but lacking a 2′-substituent, signals due to the aromatic protons in the outer ring ranged from 6.36 to 8.2 p.p.m. However, in trisubstituted derivatives the 6′-protons resonated at unusually high fields (6.22 p.p.m. in the dinitro- and 6.07 p.p.m. in the diiodo series) as should be the case if the skewed conformation (XXIII) is preferred in these compounds. A consistent difference between the chemical shifts of the 6′-proton and other protons in the same ring was seen throughout the series which included a variety of substituents attached at various points; hence, the possibility of the observed shifts being due to substituent effects is unlikely. Furthermore, the chemical shifts of the 6′-protons in XXIV and XXV may be compared; the upfield shift suffered by this proton when the 2′-proton is replaced by methyl is unlikely to be a substituent effect (the 2′-methyl group is too far removed) and is more reasonably attributed to a steric positioning of the outer ring as in XXIII. Evidence for positioning was found for 2,6-dinitro and diiodo ethers with cyclohexyl, isopropyl, allyl, trifluoromethyl, and methyl C-2′ substituents and also in the case of α-naphthyl derivatives such as XXVI.



XXVI

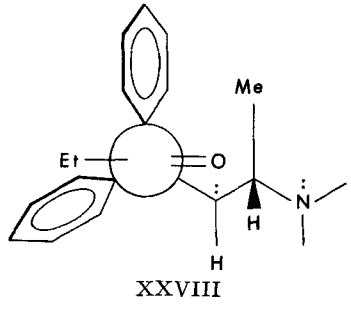
In the case of methadone (XXVII*a*), evidence for probable conformations has been derived by comparing the PMR spectrum of this analgesic with those of related 1-amino-3,3-diphenyl-



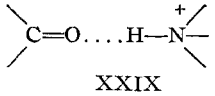
XXVII

R = *a*, COMe; *b*, H; *c*, OH; *d*, CN; *e*, C(NH)Et, *f*, CO_2Et; *g*, CH(OCOMe)Et

propane derivatives (65). It is found that the chemical shift of the secondary methyl group in methadone (XXVII*a*) (29 c.p.s.) is at an abnormally high field position compared with those of the derivatives (XXVII*b*, *c*, and *d*) (all near 60 c.p.s.). Unusually high field *sec* Me signals are also observed in the ketimine (XXVII*e*, 20 c.p.s.) and analogs of methadone bearing different basic groups. When the basic derivatives (XXVII) are protonated, the *sec* Me signals move downfield as a result of the greater deshielding influence of positively charged nitrogen; however, downfield shifts are low (13 compared with 20–25 c.p.s.) in the amino ketones. These results are taken to indicate that in favored conformations of methadone and related ketones, the *sec* Me group lies close to and above the plane of one of the aromatic rings in the molecule, as is the case in crystalline methadone hydrobromide whose conformation has been established by X-ray crystallography (66). The model (XXVIII), based upon the structure of the crystalline salt, is therefore taken to represent a favored conformation for methadone hydrochloride as solute in $CDCl_3$ or water (an unusually high field *sec* Me signal is also observed when D_2O is solvent). The conformation (XXVIII) could be stabilized by an interaction of the type XXIX and the PMR demonstration of the nonequivalence of the two *N*-methyl groups

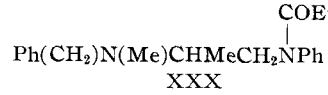


XXVIII



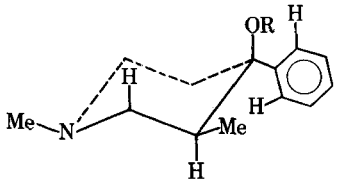
XXIX

(in $CDCl_3$, the NMe_2 signal is a doublet of doublets collapsing to a singlet when D_2O is added) provides evidence for such an interaction; an association involving the protonated basic group may be expected, if sufficiently strong, to lead to restricted rotation about the nitrogen atom (65, 67). High field *sec* Me chemical shifts are also observed in nonketonic analgesically active 3-amino-1,1-diphenylpropane derivatives such as XXVII*f* and *g* (68). In diampromide (XXX,



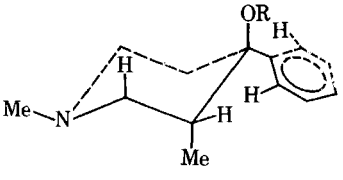
XXX

regarded as a methadone analog in which the quaternary carbon and one phenyl group of the parent are replaced by nitrogen), the *sec* Me group is not especially shielded by the aromatic groups, this and other spectroscopic evidence (69) indicating that the phenyl-*sec* Me-basic groups orientations in the two analgesics differ—a possible reason for their differing stereochemical and basic group features.



XXXI

trans 3-Me/4-Ph



XXXII

cis 3-Me/4-Ph

R = H, COMe, or COEt

The configuration of the two diastereoisomeric propionate esters of 1,3-dimethyl-4-phenylpiperidin-4-ol (α- and β-prodine) has been the subject of several papers (70) and is now firmly established as *trans* (3-Me/4-Ph) for α- and *cis* for β-prodine by X-ray crystallography (71). Differences in the PMR spectra of the two esters and of the alcohols from which they are derived provide evidence of the conformation of these molecules as solutes in $CDCl_3$ and support structures XXXI and XXXII as the most probable conformations for the α- and β-isomers, respectively (49). Signals of greatest value in this

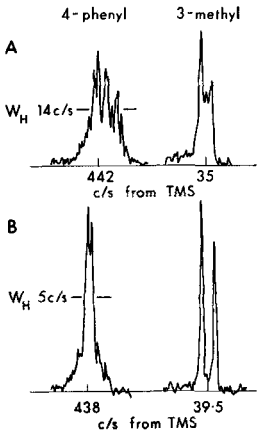
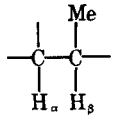


Fig. 14—Part of the PMR spectra of α-1,3-dimethyl-4-phenyl-4-piperidinol (A) *and of the corresponding β-isomer* (B) *in CCl_4. [Reproduced with permission from* Tetrahedron, **22,** *2711 (1966).]*

interpretation are those of the 4-aryl and 3-methyl groups. It is postulated that chemical shift differences among the aryl protons should be greater in the α-diastereoisomer with the result that the *trans*- aromatic signal should be more complex than that of the *cis* isomer; this is confirmed experimentally (Fig. 14). Dreiding models of XXXI and XXXII show that both *sec* Me groups fall within the diamagnetic screening zone of the benzene nucleus; this fact accounts for their high field resonance positions (relative to *sec* Me in compounds such as 3,4-dimethyl-1-phenethyl-4-piperidinol, *sec* Me chemical shift 54.5 c.p.s. in $CDCl_3$) but does now allow a stereochemical differentiation to be made (the α/β-*sec* Me chemical shift difference is small with the α-signal at higher field by 5–6 c.p.s.). However, axial Me (in XXXII) is closer than equatorial Me (in XXXI) to the ring nitrogen atom; hence, when the deshielding influence of this atom is enhanced by protonation the β-Me signal should suffer the greater downfield shift. Again this is found to be the case, shifts being 2 c.p.s. for α-Me and 16 c.p.s. for β-Me. The nature of the α- and β-Me signals also provides evidence of conformation. The distorted appearance of the α-signal (Fig. 14) is a typical result of virtual coupling which occurs in the system:



when the coupling constant between H_α and H_β is large and of the same order as the chemical shift difference between the two protons (72). Such conditions are more likely to prevail in XXXI than in XXXII since the former contains an axial proton at C-3 which will be strongly coupled to the C-2 axial proton. In propionate

esters of the piperidinols the $COCH_2Me$ and $CO\mathit{CH_2}Me$ signals are at higher field in the β-isomer. These results are also consistent with the *cis* ester having a preferred conformation akin to XXXII because the acyl portion of the ester group will spend some of its time above the plane of the phenyl group (*i.e.*, well within the aromatic shielding zone) if the planes of the C—O bond and the aromatic ring are approximately perpendicular. The conformation of drug molecules in water is of greater importance from a biological point of view and PMR studies of the prodine esters and alcohols (as hydrochlorides) in D_2O show that replacement of $CDCl_3$ by D_2O brings about significant changes in conformational equilibria. Thus, while the downfield shifts of the aromatic and *N*-methyl signals (resulting from this solvent change) are similar in α- and β-1,3-dimethyl-4-phenylpiperidin-4-ol hydrochlorides, that of the β-*sec*-Me group is almost 11 c.p.s. less than that observed for the corresponding α-signal. In addition, the chemical shift difference between the α- and β-*sec* Me groups is much less in D_2O than in $CDCl_3$. These results indicate an increase in the population of equatorial *sec* Me (chair) and/or skew-boat conformations at the expense of axial-*sec* Me conformers in the β-isomer when $CDCl_3$ is replaced by D_2O. Such an increase would reduce the deshielding influence of protonated nitrogen upon *sec* Me, the chemical shift of this group moving nearer to that of *sec* Me in the α-isomer in consequence. Radical conformational changes through solvent variation must also occur in esters of the piperidinols as is evident from the *sec* Me chemical shifts recorded in Table II. The zero or near-zero chemical shift differences between the α- and β-*sec* Me signals observed with D_2O as solvent are particularly noteworthy in this respect (24). When $CDCl_3$ is replaced by D_2O as solvent, a considerable increase in the degree of solvation of both the protonated basic center and the oxygen function of the piperidinol derivatives is probable and the larger destabilizing Me/H and OR/H 1,3-diaxial interactions obtaining in the solvated molecules are considered responsible for the decrease in the conformational preference for axial 3-Me conformers (49).

TABLE II—SOLVENT EFFECTS UPON *sec* ME CHEMICAL SHIFTS IN ESTERS OF α- AND β-1,3-DIMETHYL-4-PHENYLPIPERIDIN-4-OL HYDROCHLORIDES

4-Function	Solvent	Chemical Shift (c.p.s.) [a] α-Me	β-Me	Difference (β − α)
OCOMe	$CDCl_3$	44	61	+17
	D_2O	44	41	− 3
OCOEt	$CDCl_3$	44	62	+18
	D_2O	43.5	43.5	zero
$OCOC_3H_7$-*n*	$CDCl_3$	44	61	+17
	D_2O	40	42.5	− 2.5

[a] From TMS, internal in $CDCl_3$ and external in D_2O (60 Mc./sec. spectra).

RELAXATION TIME STUDIES

The extreme sensitivity of spin–spin (T_2) and spin–lattice (T_1) relaxation processes to variations in the molecular environment of the proton species concerned provide an elegant method for the study of the interactions of drugs with macromolecules of a type liable to be encountered in the body (14). In this technique, relaxation constants are measured for the drug alone and for the drug in the presence of a macromolecule. (T_2 is obtained from measurements of line widths at half the maximum peak height, and T_1 by measuring the recovery time of a signal which has been saturated by the application of a strong R.F. field.) Relative changes in T_1 and T_2 for each proton group that occur in the presence of the added substance are then derived from the data. Differing values indicate that a specific binding mechanism is involved (a non-specific mechanism such as an increase in viscosity would shorten all relaxation times by the same factor), the groups implicated being identified as those which exhibit the greatest decreases in relaxation times. In a study of the binding of sulfonamides to albumin (73), it was found that addition of this protein to the sulfonamide solution generally produced a larger increment in the relaxation rate of the *p*-aminobenzenesulfonamide aromatic protons than for any protons of the N-1 substituent. Results for sulfacetamide (Fig. 15), for example, showed that the aromatic moiety is preferentially stabilized by the interaction of the drug molecule with albumin, whereas the acetyl group retains much greater freedom of motion; in this molecule it is probable, therefore, that the aromatic ring is the primary binding site. A similar study of the binding of benzyl penicillin to serum albumin identifies the benzyl group as the binding site (74), while, in the case of the interaction between epinephrine and adenosine triphosphate (a complex of this nature is thought to be the storage form of catecholamines in the chromaffin granules of the adrenal medulla), results indicate that the propanol side-chain, but not the aromatic ring, is involved in complex formation (14). Chapman and Penkett (75) have studied the interaction of phospholipids with cholesterol by relaxation studies and have obtained evidence for the direct interaction of a portion of the phospholipid (particularly the hydrocarbon chains, the major

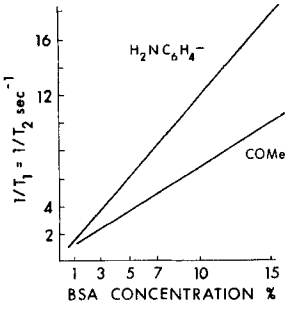
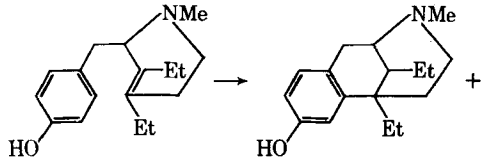


Fig. 15—Relaxation rates of 0.1 M sulfacetamide peaks, pH 8.8, as a function of bovine serum albumin (BSA) concentration. [Adapted with permission from Mol. Pharmacol., **1**, *214 (1965).]*

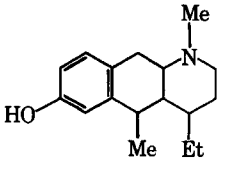
PMR peak arising from protons associated with the hydrocarbon chain of lecithin virtually disappears when an equimolar proportion of cholesterol is present) with the cholesterol molecule. It is obvious that work of this nature has great potentialities in the study of drug–enzyme and drug–receptor interactions.

MISCELLANEOUS

The routine use of PMR data as an aid to structure determination in natural products and in organic synthesis is self-evident. To quote but one example, cyclization of the tetrahydropyridine (XXXIII) by aluminum bromide gives an analgesically active benzo[*g*]quinoline (XXXV) as well as the expected benzomorphan derivatives (XXXIV), the former being characterized



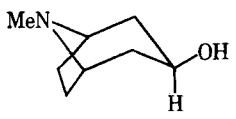
XXXIII *cis* and *trans*-Et/Et XXXIV



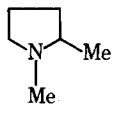
XXXV

by a *sec* Me doublet and a single CH_2Me triplet in its PMR spectrum (76).

Systems in which protons may exchange their environment within the same, or in a different, molecule are also studied conveniently by PMR spectroscopy. If the exchange rate is slow, absorption lines for each environment are seen, fast rates yield single, sharp, lines (corresponding to a weighted average of the separate environmental chemical shifts) while intermediate rates give rise to broad signals. [See Delpuech (77) for a recent review of this topic.] The kinetics of proton exchange in salts of organic bases may be studied in this way. In cyclic bases such as pseudotropine (XXXVI) and 1,2-dimethylpyrrolidine (XXXVII), the separate existence of isomers formed as a result of the protonated basic



XXXVI



XXXVII

HCl N—Me doublets ($J5$)	
130.5 c.p.s. (minor)	6.97 (major)
138.5 c.p.s. (major)	7.27 (minor)
from reference in H_2O, pH 1.0	from TMS in $CHCl_3$

center having two possible configurations is made evident by the appearance of two N-Me signals in the PMR spectra of their salts (78, 79) when solvents unfavorable to proton exchange are employed. The signals differ in intensity, and the more pronounced is assumed to be due to the more stable configuration (equatorial N-Me for XXXVI, *trans* 1,2-diMe for XXXVII). When the rate of proton exchange is accelerated, single N-Me signals are obtained. Protonated N-epimers are also observed in 1,5-dimethyl-4-phenyl-1,2,5,6-tetrahydropyridine (XXXVIII) hydrochloride and related compounds (80).

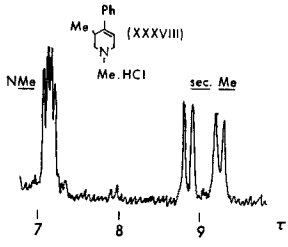
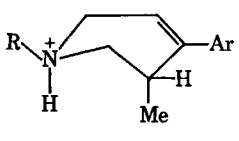
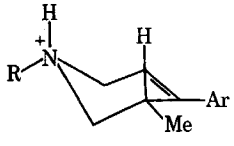


Fig. 16—Part of the PMR spectrum of 1,5-dimethyl-4-phenyl-1, 2, 5, 6-tetrahydropyridine hydrochloride in $CDCl_3$.

Thus, the spectrum of XXXVIII hydrochloride in $CDCl_3$ displays a doublet of doublets N-Me signal and two well-separated *sec* Me doublets (Fig. 16) which collapse to a singlet and a doublet, respectively, when D_2O is added. In these cases, there is evidence that the epimers differ in their 5-methyl rather than N-methyl configurations, XXXIX and XL being the proposed preferred conformations.



XXXIX



XL

Interesting developments in the differentiation of optical enantiomorphs by PMR spectroscopy

PhCH(OH)CF_3 PhCH(OH)$CHMe_2$
XLI XLII

have recently been reported. Magnetic equivalence of the two methyl groups of isopropanol no longer obtains when the alcohol is esterified with the acid chloride derived from (R)-*O*-methylmandelic acid; the now diastereoisomeric methyl groups in the resultant ester give rise to two doublets which differ in chemical shift by 5 c.p.s. at 60 Mc./sec. (81). The optical purity of propan-2-ol-1-d_3 may then be checked by conversion to the *O*-methylmandelate and examination of the PMR spectrum of the ester; if the material is a single enantiomorph, only one of the *sec* Me doublets of the nondeuterated ester should appear. It has now been shown that optical enantiomorphs themselves may have different PMR characteristics when examined in an optically active solvent as a result of diastereoisomeric interactions (82). Thus, the fluorine resonance signal of racemic 2,2,2-trifluoro-1-phenylethanol (XLI) is a doublet in racemic α-phenethylamine, but a doublet of doublets (of equal intensity) in the levorotatory base. (Each doublet is further split by long-range coupling with aromatic protons.) Partially resolved XLI in the latter solvent gives a doublet pair of unequal intensity. Similarly, the signal of the carbinyl proton of racemic phenylisopropylcarbinol (XLII), a doublet in racemic α-(1-naphthyl)ethylamine, forms two equally intense doublets in the dextrorotatory base (separation 1.6 c.p.s. at 60 Mc. and 2.5 c.p.s. at 100 Mc./sec.).

In conclusion, attention is drawn to some useful reviews. Surveys of the whole field are published periodically in *Analytical Chemistry* (83) and *Annual Reports of the Chemical Society* (for last review, see *Reference 84*). Kinetic studies by NMR (77), NMR of fluorine (85), of ^{13}C (86), of organometallic compounds (87), spin–spin coupling and double resonance (88) [see also Baldeschweiler and Randall (89) for the latter topic], and NMR and electronegativity (90), have all been recently reviewed. Useful accounts of long-range spin–spin coupling (91), geminal coupling constants in methylene groups (92), and conformational analysis of cyclohexane derivatives by NMR spectroscopy (93) are also available.

REFERENCES

(1) Roberts, J. D., "Nuclear Magnetic Resonance Applications to Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1959.

(2) Pople, J. A., Schneider, W. G., and Bernstein, H. J., "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959.

(3) Bhacca, N. S., and Williams, D. H., "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964.

(4) Bible, R. H., "Interpretations of NMR Spectra," Plenum Press, New York, N. Y., 1965.

(5) "Nuclear Magnetic Resonance for Organic Chemists," Mathieson, D. W., ed., Academic Press, London, England, 1966.

(6) Jackman, L. M., "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, New York, N. Y., 1959.

(7) Emsley, J. W., Feeney, J., and Sutcliffe, L. H., "High Resolution Nuclear Magnetic Resonance Spectroscopy," vols. 1 and 2, Pergamon Press, Oxford, England, 1965–1966.

(8) Conroy, H., Nuclear Magnetic Resonance in Organic Structural Elucidation, in "Advances in Organic Chemistry," Raphael, R. A., Taylor, H. C., and Wynberg, H., eds., vol. II, Interscience Publishers, Inc., New York, N. Y., 1960.

(9) Stothers, J. B., Applications of Nuclear Magnetic Resonance Spectroscopy, in "Techniques of Organic Chemistry," vol. XI, part I, Interscience Publishers, Inc., New York, N.Y., 1960.

(10) Kowalsky, A., and Cohn, M., *Ann. Rev. Biochem.*, 33, 481(1964).

(11) Dyer, J. R., "Applications of Absorption Spectroscopy of Organic Compounds," Foundations of Modern Organic Chemistry Series, Rinehart, K. L., ed., Prentice Hall, Englewood Cliffs, N.J., 1965.

(12) Brand, J. C. D., and Eglinton, G., "Applications of Spectroscopy to Organic Chemistry," Oldbourne, London, England, 1965.

(13) Fleming, I., and Williams, D. H., "Spectroscopic Methods in Organic Chemistry," McGraw-Hill, London, England, 1966.

(14) Jardetzky, O., "Advances in Chemical Physics," vol. 7, John Wiley & Sons, London, England, 1964, p. 499.

(15) Becker, E. D., *J. Chem. Educ.*, **42**, 591(1965).

(16) Tiers, G. V. D., and Cook, R. I., *J. Org. Chem.*, **26**, 2097(1961).

(17) Hollis, D. P., *Anal. Chem.*, **35**, 1682(1963).

(18) Philipsborn, W. D. V., *Arch. Pharm.*, **34**, 58(1964).

(19) Casy, A. F., and Wright, J., *J. Chem. Soc. (C)*, **1966**, 1167.

(20) Barcza, S., *J. Org. Chem.*, **28**, 1914(1963).

(21) Molyneux, P., Rhodes, C. T., and Swarbrick, J., *Trans. Faraday Soc.*, **61**, 1042(1965).

(22) Raban, M., and Mislow, K., *Tetrahedron Letters*, (No. 48), 4249(1965).

(23) Casy, A. F., Iorio, M. A., and Pocha, P., *J. Chem. Soc. (C)*, **1967**, 942.

(24) Casy, A. F., and Pocha, P., unpublished data.

(25) Kingsbury, C. A., and Thornton, W. B., *J. Am. Chem. Soc.*, **88**, 3159(1966).

(26) Casy, A. F., Beckett, A. H., Iorio, M. A., and Youssef, H. Z., *Tetrahedron*, **21**, 3387(1965).

(27) Casy, A. F., Beckett, A. H., and Iorio, M. A., *Tetrahedron*, **23**, 1405(1967).

(28) Eliel, E. L., *Chem. Ind.*, **1959**, 568.

(29) Eliel, E. L., Allinger, N. L., Angyal, S. J., and Morrison, G. A., "Conformational Analysis," John Wiley & Sons, Inc., New York, N.Y., 1965.

(30) Karplus, M., *J. Chem. Phys.*, **30**, 11(1959); Karplus, M., *J. Am. Chem. Soc.*, **85**, 2870(1963).

(31) Williamson, K. L., and Johnson, W. S., *J. Am. Chem. Soc.*, **83**, 4623(1961).

(32) Staiff, D. C., and Huitric, A. C., *J. Org. Chem.*, **29**, 3106(1964), and references cited therein.

(33) Huitric, A. C., Clarke, W. G., Jr., Leigh, K., and Staiff, D. C., *ibid.*, **27**, 715(1962).

(34) Culvenor, C. C. J., *Tetrahedron Letters*, (No. 10), 1091(1966).

(35) Narayanan, C. R., and Iyer, K. N., *ibid.*, (No. 42), 3741(1965).

(36) Chen, C.-Y., and Le Fèvre, R. J. W., *J. Chem. Soc.*, **1965**, 3473.

(37) Bishop, R. J., Fodor, G., Katritzky, A. R., Soti, F., Sutton, L. E., and Swinbourne, F. J., *ibid.*, (C), **1966**, 74.

(38) Chen, C.-Y., and Le Fèvre, R. J. W., *ibid.*, **1965**, 3467.

(39) Dvornik, D., and Schilling, G., *J. Med. Chem.*, **8**, 466(1965).

(40) Casy, A. F., and Hassan, M. M. A., *Tetrahedron*, to be published.

(41) Portoghese, P. S., and Williams, D. A., *J. Pharm. Sci.*, **55**, 990(1966).

(42) Casy, A. F., and Armstrong, N. A., *J. Med. Chem.*, **8**, 57(1965).

(43) Casy, A. F., and Wright, J., *J. Chem. Soc. (C)*, **1966**, 1511.

(44) Craig, J. C., Green, D. E., Roy, S. K., Piette, L. H., and Loeffler, K. O., *J. Med. Chem.*, **8**, 392(1965).

(45) Warren, R. J., Eisdorfer, I. B., Thompson, W. E., and Zarembo, J. E., *J. Pharm. Sci.*, **55**, 144(1966).

(46) Pimental, G. C., and McClellan, A. L., "The Hydrogen Bond," Freeman, San Francisco, Calif., 1960.

(47) Chapman, O. L., and King, R. W., *J. Am. Chem. Soc.*, **86**, 1256(1964).

(48) Ouellette, R. J., *ibid.*, **86**, 3089(1964); *ibid.*, **86**, 4378(1964); Ouellette, R. J., Booth, G. E., and Liptak, K., *ibid.*, **87**, 3436(1965); Ouellette, R. J., and Booth, G. E., *J. Org. Chem.*, **30**, 423(1965).

(49) Casy, A. F., *Tetrahedron*, **22**, 2711(1966).

(50) Huggins, C., Pimental, G., and Shoolery, J., *J. Phys. Chem.*, **60**, 1311(1956).

(51) Hyne, J. B., *Can. J. Chem.*, **38**, 125(1960).

(52) Jardetzky, O., *J. Biol. Chem.*, **238**, 2498(1963).
(53) Katritzky, A. R., and Reavill, R. E., *J. Chem. Soc.*, **1963**, 753.
(54) *Ibid.*, **1965**, 3825.
(55) Molloy, B. B., Reid, D. H., and McKenzie, S., *ibid.*, **1965**, 4368.
(56) Casy, A. F., and Wright, J., unpublished data.
(57) Birchall, T., and Jolly, W. L., *J. Am. Chem. Soc.*, **88**, 5439(1966).
(58) Weiner, N., and Jardetzky, O., *Biochem. Pharmacol.*, **8**, 115(1961).
(59) Hyne, J. B., *Can. J. Chem.*, **39**, 2536(1961).
(60) Sasaki, T., Kanematsu, K., Tsuzuki, Y., and Tanaka, K., *J. Med. Chem.*, **9**, 847(1966).
(61) Nakazaki, M., Mita, I., and Toshioka, N., *Bull. Chem. Soc., Japan*, **36**, 161(1963).
(62) Lehman, P. A., and Jorgensen, E. C., *Tetrahedron*, **21**, 363(1965).
(63) Jorgensen, E. C., Lehman, P. A., Greenburg, C., and Zenker, N., *J. Biol. Chem.*, **237**, 3832(1962).
(64) Johnson, C. E., and Bovey, F. A., *J. Chem. Phys.*, **29**, 1012(1958).
(65) Casy, A. F., *J. Chem. Soc. (B)*, **1966**, 1157.
(66) Hanson, A. W., and Ahmed, F. R., *Acta Cryst.*, **11**, 724(1958).
(67) Smith, L. I., *J. Pharm. Sci.*, **55**, 101(1966).
(68) Casy, A. F., and Hassan, M. M. A., unpublished data.
(69) Casy, A. F., and Hassan, M. M. A., *J. Pharm. Pharmacol.*, **19**, 114(1967).
(70) Beckett, A. H., Casy, A. F., and Harper, N. J., *Chem. Ind.*, **1959**, 19, and references cited therein.
(71) Kartha, G., Ahmed, F. R., and Barnes, W. H., *Acta Cryst.*, **13**, 525(1960); Ahmed, F. R., Barnes, W. H., and Masironi, L. D., *ibid.*, **16**, 237(1963).
(72) Musher, J. I., and Corey, E. J., *Tetrahedron*, **18**, 791 (1962).
(73) Jardetzky, O., and Wade-Jardetzky, N. G., *Mol. Pharmacol.*, **1**, 214(1965).
(74) Fischer, J. J., and Jardetzky, O., *J. Am. Chem. Soc.*, **87**, 3237(1965).
(75) Chapman, D., and Penkett, S. A., *Nature*, **211**, 1304 (1966).
(76) Joshi, B. C., May, E. L., Fales, H. M., Daly, J. W., and Jacobson, A. E., *J. Med. Chem.*, **8**, 559(1965).
(77) Delpuech, J. J., *Bull. Soc. Chim. France*, **1964**, 2695.
(78) Closs, G. L., *J. Am. Chem. Soc.*, **81**, 5456(1959).
(79) McKenna, J., McKenna, J. M., Tulley, A., and White, J., *J. Chem. Soc.*, **1965**, 1711.
(80) Casy, A. F., Beckett, A. H., Iorio, M. A., and Youssef, H. Z., *Tetrahedron*, **21**, 3387(1965).
(81) Raban, M., and Mislow, K., *Tetrahedron Letters*, (No. 33), 3961(1966).
(82) Pirkle, W. H., *J. Am. Chem. Soc.*, **88**, 1837(1966); Burlingame, T. G., and Pirkle, W. H., *ibid.*, **88**, 4294(1966).
(83) Foster, H., *Anal. Chem.*, **36**, 266R(1964); Lustig, E., *ibid.*, **38**, 331R(1966).
(84) Williams, D. H., in *Ann. Rept.*, **62**, 221(1965).
(85) Béguin, C., *Bull. Soc. Chim. France*, **1964**, 2711.
(86) Stothers, J. B., *Quart. Rev.*, **19**, 144(1965).
(87) Hayes, S., *Bull. Soc. Chim. France*, **1964**, 2715.
(88) Parello, J., *ibid.*, **1964**, 2033.
(89) Baldeschweiler, J. D., and Randall, E. W., *Chem. Rev.*, **63**, 81(1963).
(90) Muller, J. C., *Bull. Soc. Chim. France*, **1964**, 1815.
(91) Sternhell, S., *Rev. Pure Appl. Chem.*, **14**, 15(1964).
(92) Cookson, R. C., Crabb, T. A., Frankel, J. J., and Hudec, J., *Tetrahedron*, (Suppl. 7), 355(1966).
(93) Franklin, N. C., and Feltkamp, H., *Angew. Chem., Intern. Ed.*, **4**, 774(1965).

Research Articles

Effect of Deuterium Oxide on Culturing of *Penicillium janczewskii* I

Growth, Nutritional Factors, and Antibiotic Production

By D. A. NONA, M. I. BLAKE, and J. J. KATZ*

Penicillium janczewskii, **a mold which produces the antifungal antibiotic griseofulvin, was cultured in media containing 50, 75, and 99.6 per cent deuterium oxide. Protio- and deuterio-carbohydrates were used as carbon sources. Griseofulvin production decreased markedly with increase in deuterium content of the medium. There was also a marked reduction in mycelial dry weights with increase in deuterium oxide in the nutrient. The surface culture technique proved more effective than the shake method for both tissue growth and antibiotic formation.**

A VARIETY OF organisms, including bacteria, fungi, and algae, have been successfully cultured in media containing pure heavy water. The growth of the heterotroph *Euglena gracilis* in a fully deuteriated form has also been recently reported (1). Higher plants, including peppermint (2, 3), belladonna (4, 5), and duckweed (6), have been grown in nutrient solutions containing as high as 70% deuterium oxide. These studies have been summarized by Flaumenhaft (7) and by Katz (8), who have reviewed extensively the biological effects of deuterium.

Deuteriated organisms provide a practical source of metabolites containing deuterium in all

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Accepted for publication May 25, 1967.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by grant AI 06825-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and was performed in part under the auspices of the U. S. Atomic Energy Commission, Washington, D.C.

(52) Jardetzky, O., *J. Biol. Chem.*, **238**, 2498(1963).
(53) Katritzky, A. R., and Reavill, R. E., *J. Chem. Soc.*, **1963**, 753.
(54) *Ibid.*, **1965**, 3825.
(55) Molloy, B. B., Reid, D. H., and McKenzie, S., *ibid.*, **1965**, 4368.
(56) Casy, A. F., and Wright, J., unpublished data.
(57) Birchall, T., and Jolly, W. L., *J. Am. Chem. Soc.*, **88**, 5439(1966).
(58) Weiner, N., and Jardetzky, O., *Biochem. Pharmacol.*, **8**, 115(1961).
(59) Hyne, J. B., *Can. J. Chem.*, **39**, 2536(1961).
(60) Sasaki, T., Kanematsu, K., Tsuzuki, Y., and Tanaka, K., *J. Med. Chem.*, **9**, 847(1966).
(61) Nakazaki, M., Mita, I., and Toshioka, N., *Bull. Chem. Soc., Japan*, **36**, 161(1963).
(62) Lehman, P. A., and Jorgensen, E. C., *Tetrahedron*, **21**, 363(1965).
(63) Jorgensen, E. C., Lehman, P. A., Greenburg, C., and Zenker, N., *J. Biol. Chem.*, **237**, 3832(1962).
(64) Johnson, C. E., and Bovey, F. A., *J. Chem. Phys.*, **29**, 1012(1958).
(65) Casy, A. F., *J. Chem. Soc. (B)*, **1966**, 1157.
(66) Hanson, A. W., and Ahmed, F. R., *Acta Cryst.*, **11**, 724(1958).
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(87) Hayes, S., *Bull. Soc. Chim. France*, **1964**, 2715.
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(90) Muller, J. C., *Bull. Soc. Chim. France*, **1964**, 1815.
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Accepted for publication May 25, 1967.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by grant AI 06825-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and was performed in part under the auspices of the U. S. Atomic Energy Commission, Washington, D.C.

molecular positions where hydrogen is normally present. Many compounds of biological importance are not readily prepared by conventional synthetic methods, and for these, biosynthetic procedures are essential. Thus, Blake *et al.* (9) isolated deuteriated mannose, glucose, and several amino acids from *Scenedesmus obliquus* grown in 99% deuterium oxide, and Crespi *et al.* (10) obtained deuterio-DNA from deuteriated algae. Organisms which elaborate pharmacologically active compounds are of particular interest. A clavine alkaloid-yielding strain of *Claviceps purpurea* has been cultured (11) in pure D_2O and a highly deuteriated alkaloid has been prepared (12) by a replacement culture technique. Other molds have also attracted interest. Shaffer *et al.* (13) cultured *Penicillium notatum* and *Aspergillus fonseaceus* in heavy water some years ago, but antibiotic production and isolation were not investigated. The present study concerns the nutritional requirements for culturing the griseofulvin-producing mold, *P. janczewskii*, in a deuterium environment, and reports the effects of deuterium oxide on the production of the antibiotic griseofulvin. Griseofulvin, a systemically active antifungal agent, was originally isolated by Oxford *et al.* (14) and is produced biosynthetically by surface or submerged culture techniques (15–17) with various species of *Penicillia* (14, 18, 19). This constitutes the first report on the detection and isolation of an antibiotic obtained from a deuteriated organism.

EXPERIMENTAL

Preparation of Slant Cultures—The griseofulvin-producing strain, *P. janczewskii* (NRRL 2301), was used throughout this investigation. Slopes of malt extract agar in cylindrical glass tubes were streaked with a wire loopful of mold tissue. The tubes were plugged with cotton and incubated for 7 days at 25 ± 1°. Slants were stored in a refrigerator at 5° where they remained viable for at least 3 months.

Water Culture Techniques—A Czapek-Dox medium modified by addition of 0.5% potassium chloride (Table I) provided a well-defined synthetic medium for this investigation. The pH was adjusted, if necessary, to 4.8. The H_2O culture medium served as a control. The medium was placed in suitable culture flasks and plugged with nonabsorbent cotton. The flasks were sterilized by autoclaving for 15 min. at 15 p.s.i. in a Thermomatic-60 autoclave (Wilmot-Castle). Sugars and nutrient salt solutions were sterilized separately and combined aseptically. Carbohydrates were sterilized either by autoclaving, or by passing a solution through a Swinney adapter equipped with a Millipore filter disk of 0.22 μ porosity. Upon cooling, the medium was inoculated with 1 to 5 ml. (depending upon the medium volume) of a stock spore suspension.

TABLE I—COMPOSITION OF BASIC CULTURE MEDIUM FOR *P. janczewskii* (NRRL 2301)

Culture Medium	
Sodium nitrate	2.3
Potassium dihydrogen phosphate	1.0
Magnesium sulfate · $7H_2O$	0.5
Potassium chloride	0.5
Minor element concentrate	1.0
Dextrose	75.0
Distilled water, dilute to	1000.0
Minor Element Concentrate	
Ferrous sulfate	0.1
Copper sulfate	0.015
Zinc sulfate	0.1
Potassium molybdate	0.01
Distilled water, dilute to	100.00

Fermentation was carried out by employing one of two techniques.

Shake Method—Sixty milliliters of culture medium was placed in 250-ml. conical flasks which provided a liquid depth of approximately 2 cm. The inoculated culture flasks were agitated on an Eberbach rotary shaker at 200 r.p.m., and incubation was allowed to proceed in the dark at 25 ± 2°.

Surface Method—For small scale fermentation studies, 60 ml. of nutrient medium was placed in a 250-ml. conical flask. For preparative cultures, 500 ml. of medium was placed in 3-L. conical flasks, providing a liquid depth of about 2 cm. After inoculation, the cultures were incubated in darkness at 25 ± 2° without agitation.

Preparation of Deuteriated Media—Exchangeable hydrogen of inorganic salts and protio-sugars was replaced with deuterium by dissolution in pure deuterium oxide. The solutions then were evaporated to dryness under reduced pressure. This procedure was repeated to ensure complete exchange. A concentrate of minor elements of the culture medium was prepared with pure deuterium oxide (Table I). Partially deuteriated media composed of the basic nutrients and protio-glucose (7%) were prepared using 50, 75, and 99.6% deuterium oxide. A partially deuteriated medium was also prepared with fully deuteriated sugars (7%) and ordinary water. Fully deuteriated media were prepared using deuterio-sugars (7%) and pure deuterium oxide. The deuteriated monosaccharides were obtained from deuteriated algae according to the procedure of Blake *et al.* (9). Sugars obtained by this method contain 40% deuterio-glucose, 50% deuterio-mannose, and 10% of other hexoses and pentoses. Flasks containing deuteriated media were plugged with cotton and covered with aluminum foil to minimize isotopic dilution by exchange with atmospheric moisture.

Organic Substrates and Additives—A variety of carbon sources were tested for assimilation by the mold in media containing deuterium oxide. Glucose, glycerin, mannose, sodium acetate, and succinic acid were studied in concentrations of 3, 5, 7, and 9%, and dihydroxyacetone was tested in concentrations of 7%. All carbon sources were exchanged with D_2O prior to sterilization. For these experiments 60 ml. of the Czapek-Dox medium was prepared with pure deuterium oxide. Each of the substrates to be evaluated was sterilized separately and then added

TABLE II—COMPOSITION OF VITAMIN SUPPLEMENT ADDED TO FULLY DEUTERIATED MEDIA

Vitamin	Concn., mg./100 ml. D_2O
Biotin	0.5
Calcium pantothenate	0.5
Pyridoxine HCl	5.0
Riboflavin	0.5
Thiamine HCl	5.0

to the sterilized nutrients contained in 250-ml. conical flasks. An analogous series of controls was prepared with H_2O. Culture flasks were inoculated with 1 ml. of a stock spore suspension prepared with D_2O, whereas an H_2O spore suspension was used for the control cultures. The cultures were then incubated as static cultures at room temperature in the dark. Mycelial growth in each case was observed and recorded as a graded response during a 60-day period.

An additive of vitamin B-complex (Table II) was evaluated as a medium supplement in cultures grown in pure deuterium oxide. Concentrations of 1 to 2% were added to the culture medium. Algae extract (1%), bacto-peptone (2%), and corn steep solids (1–2%) were also studied as additives.

Mycelial Dry Weights—Two series of cultures were prepared, each consisting of ten 250-ml. conical flasks. Sixty milliliters of culture medium was prepared with protio-glucose and pure D_2O and placed into each flask of the first series. The same volume of an H_2O medium was placed into a second series of flasks to serve as controls. Each culture was inoculated with 1 ml. of a stock spore suspension in D_2O (H_2O for controls). At varying time intervals, up to a total of 64 days, the mycelium from a culture of each series was separated from its fermentor broth by filtration. The mycelium was dried to a constant weight in a vacuum oven at 100°. Both series were run in duplicate and the dry weights obtained were averaged and plotted as weight *versus* time in days.

Determination of Griseofulvin in Fermentor Broth—A Beckman DU spectrophotometer, model 2400 (Beckman Instruments, Inc.) equipped with matched silica cells of 10 mm. light path was employed for estimating griseofulvin in fermentor broth.

The analytical procedure employed in this study was a slight modification of the technique for the estimation of griseofulvin in fermentor broth developed by Holbrook *et al.* (20). The method is based on a process for the isomerization of griseofulvin to isogriseofulvin by acid treatment in methanol, as described by Grove *et al.* (21). Since untreated fermentor broth is unsuitable for spectrophotometric measurement, preliminary extraction of griseofulvin is necessary. Ethyl acetate was selected as the extractant because it does not absorb light between 260 mμ and 280 mμ and griseofulvin is reasonably soluble in it. The applicability of the procedure to a deuterium oxide medium was established by assaying fermentor broth both before and after additions of known quantities of standard griseofulvin.

Deuterium Analysis—At the conclusion of the growth period an aliquot of the culture medium was analyzed spectrophotometrically for D_2O content according to the method of Crespi and Katz (22). Harvested mycelium was analyzed for fixed deuterium which included both exchangeable and non-exchangeable deuterium. Five grams of dried tissue in a combustion boat was placed in a microfurnace and burned in a stream of oxygen at 700°. The water of combustion was collected in a −78° trap and transferred to an ampul which was then analyzed for deuterium content.

RESULTS AND DISCUSSION

Growth Observations—*P. janczewskii* grows well in a shake culture of protio-sugars and water. Growth is observed after 1 or 2 days of incubation. Tan pellicles are formed which reach a maximum diameter of about 70 to 75 mm. after 15 to 20 days. The medium becomes highly pigmented, imparting an amber color to the culture. When the mold is grown in a nutrient consisting of protio-sugars in pure D_2O, a lag period of about 10 days is observed before growth begins. Typically, the maximum pellicle size ranges between 10 and 20 mm. in diameter after 30 to 40 days of incubation (Fig. 1). Pigmentation of the fermentor broth is also delayed until approximately the fifteenth day, at which time the medium becomes tinged with yellow.

The mold also shows excellent growth in surface culture. In the H_2O controls, growth is observed 1 day after inoculation and by the seventh day a floating mycelial mat is formed which covers the entire surface of the culture medium. After about 10 days the mycelial mat begins to change color from white to gray-green. The fermentor broth becomes a deep amber by the eleventh day of incubation. In a culture medium containing 50% deuterium oxide, the appearance of the mycelial mat formed is much like that of the H_2O controls. The growth rate is delayed, but not drastically. A thick mycelial felt covers the entire surface by the fifteenth day of incubation as shown in Fig. 2. Pigmentation imparts a yellow color to the culture liquid early in the fermentation. As the D_2O content of the culture medium is increased to 75%, a further increase in lag period is observed. Little growth is noted before the seventh day of incubation, and by the fifteenth day the mycelial mat does not quite cover the entire surface of the culture medium. The gross morphology of the mycelium was similar to that cultured in 50% D_2O or the water controls. The mycelium differs, however, in that the mat becomes yellow by the fifteenth day. The mold can grow directly in a culture medium containing pure deuterium oxide; subculturing is not required. Only scant growth is observed before the tenth day, and isolated colonies begin to connect, forming a white lattice-type of growth after 15 days of incubation, as shown in Fig. 3. After 35 days, the surface of the culture medium becomes essentially fully covered. At the completion of the fermentation period the mycelium is not as mature as is the H_2O controls (Fig. 4). Figure 5 shows a marked reduction in the mycelial dry weights of the D_2O cultures when compared to the controls. The fermentation period necessary to reach the maximum weight was more than twice that of the water cultures. Dry weights obtained from both the water and deuterium cultures somewhat decrease, after reaching a maximum, because

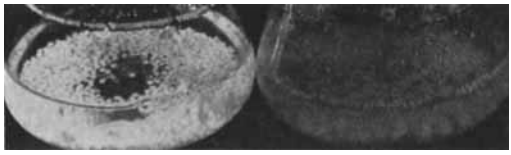

Fig. 1—Shake cultures 30 days after inoculation in 99% D_2O (left) and H_2O (right). Both cultures contain 7% protio-glucose.

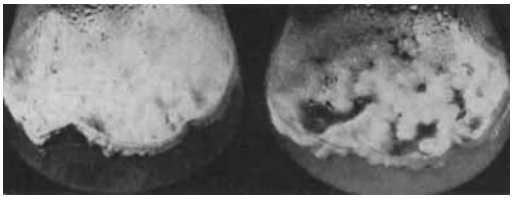

Fig. 2—Surface cultures 15 days after inoculation in 50% D_2O (left) and H_2O (right). Both cultures contain 7% protio-glucose.

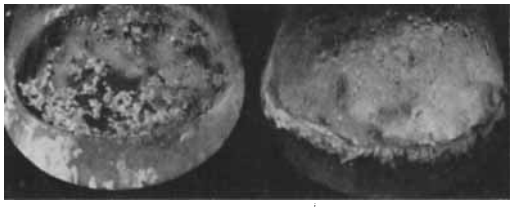

Fig. 3—Surface cultures 15 days after inoculation in 99% D_2O (left) and H_2O (right). The cultures contain 7% deuterio-glucose and 7% protio-glucose, respectively.

Fig. 4—Surface culture after 60 days' growth in medium containing 99% D_2O and 7% deuterio-glucose.

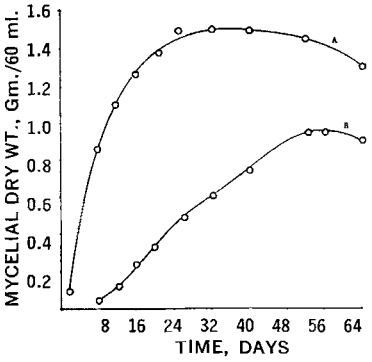

Fig. 5—Comparison of mycelial dry weights of surface cultures grown in (A) water and (B) 99% D_2O. Both cultures contained 7% protio-glucose.

of autolysis. Mature mycelium produced at the 99.6% deuterium level maintains a felt-like appearance. However, the color becomes yellowish-tan and the liquid portion of the medium shows a yellow pigmentation. At the conclusion of each fermentation period, a sample of the broth was analyzed for deuterium content. In no case was the final deuterium oxide concentration found more than 2% below the initial concentration.

Nutritional Factors—An organic substrate is essential to the growth of the mold. Protio-substrates were chosen for study which were also available as deuterio-moieties, and their ability to support growth in pure deuterium oxide was determined. Several concentrations (3, 5, 7, and 9% w/v) of each protio-substrate were prepared with 99.6% D_2O culture media. Growth was evaluated visually by assigning graded responses. The results are recorded in Table III. It is apparent that the mold can efficiently use glucose, mannose, glycerin, and, less successfully, succinic acid. Sodium acetate and dihydroxyacetone were considered as possible substrates since griseofulvin may be considered a condensation of acetic acid units, but the experimental results did not substantiate this hypothesis. Glucose or a deuterio-glucose and deuterio-mannose mixture in a concentration of 7 to 7.5% was employed in all D_2O culture media.

The major difficulty that the mold experiences in growing in a deuteriated environment appears to be in adapting to the solvent D_2O. The incorporation of deuteriated substrates into a culture medium seems to cause less of a problem for the fungus, no matter what the level of solvent deuteriation. Growth response of the organism in a nutrient solution composed of deuterio-sugars (7%) in ordinary water was not unlike that observed in culture media composed of protio-sugars in H_2O. The fixed deuterium of the dried mycelium in this experiment (obtained by combustion analysis) was found to be 18.8%. Growth characteristics observed in a fully deuteriated culture medium composed of deuterio-sugars and D_2O resembled closely those shown in media composed of protio-sugars in D_2O. A greater time period was required before initial growth, but thereafter no significant difference could be detected in the growth pattern. Combustion analysis of the dried mycelium showed the deuterium level to be slightly above 94% in the fully deuteriated mold.

Earlier studies have established that deuteriated fungi often show an increased nutritional fastidiousness. For instance, the yeasts *Torulopsis utilis* and *Saccharomyces cerevisiae* developed additional requirements for vitamins, particularly those of the B-complex group (23). Vitamins as well as algae extracts were necessary for the growth of fully deuteriated *Euglena gracilis* (1). The effect of medium fortification by vitamins was, therefore, studied. The carbohydrate concentration of the culture medium was 7%, and the response to various medium supplements was noted. The vitamin additive appeared to have little effect

TABLE III—UTILIZATION OF VARIOUS CARBON SUBSTRATES IN D_2O CULTURES

Substrate	% w/v	Incubation Period, Days[a] 6	10	12	14	16	18	20	24	28	32	38	60
Glycerin	3	0	±	±	±	+	+	+	+	+	+	+	+
	5	±	±	+	+	+	++	++	++	++	++	++	++
	7	±	±	+	+	++	++	++	+++	+++	+++	+++	+++
	9	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
Glucose	3	±	±	±	±	+	+	+	++	++	++	++	++
	5	±	±	±	+	++	++	++	++	++	+++	+++	+++
	7	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
	9	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
Mannose	3	0	0	±	±	±	±	+	+	++	++	++	++
	5	0	±	±	±	±	+	++	++	++	++	+++	+++
	7	0	±	±	+	+	+	++	+++	+++	+++	+++	+++
	9	0	±	±	+	+	+	+++	+++	+++	+++	+++	+++
Sodium acetate	3	0	0	0	0	0	±	±	±	±	±	±	±
	5	0	0	0	0	±	±	±	±	±	±	±	±
	7	0	0	0	0	±	±	±	±	+	+	+	+
	9	0	0	0	0	±	±	±	+	+	+	+	+
Succinic acid	3	0	0	0	0	±	±	±	±	±	±	±	+
	5	0	0	0	0	±	±	±	±	+	+	+	+
	7	0	0	0	±	±	±	+	+	+	++	+++	+++
	9	0	0	0	±	±	±	+	+	+	++	+++	+++

[a] 0, No growth; ±, marginal growth; +, ++, +++, graded response.

on the rate of mold growth. However, the mycelial mat formed was decidedly thicker and obviously superior to the nonvitamin control. Algae extract and bacto-peptone supplements exhibited no marked advantage. Corn steep solids showed a dramatic increase in growth in both water controls and in pure deuterium oxide media. The indefinite composition and the introduction of protio-atoms from such a supplement may interfere with deuteriation of the antibiotic biosynthesized, and was therefore not employed in further studies.

Antibiotic Production—The extent of griseofulvin production in surface cultures is noted in Fig. 6. Each point on the curve represents an average of values obtained from 5 separate batches. Antibiotic production in both surface and shake culture is observed to reach a plateau, which was followed by a gradual reduction in concentration. The effect of D_2O concentrations on peak antibiotic levels and fermentation periods are summarized in Table IV. Shake fermentation methods resulted in lower antibiotic titers than static culture conditions (Fig. 7). Griseofulvin titers in shake cultures of 99% D_2O exhibited a marked depression. Although there was good growth at this D_2O level,

TABLE IV—EFFECT OF D_2O CONCENTRATION ON PEAK GRISEOFULVIN PRODUCTION LEVELS

D_2O, %	Griseofulvin Titer, mcg./ml.	Time, Days
	Surface Culture	
0	115	17
50	90	24
75	52	40
99.6[a]	50	46
	Shake Culture	
0	55	17
99.6[a]	25	45

[a] Vitamin supplementation.

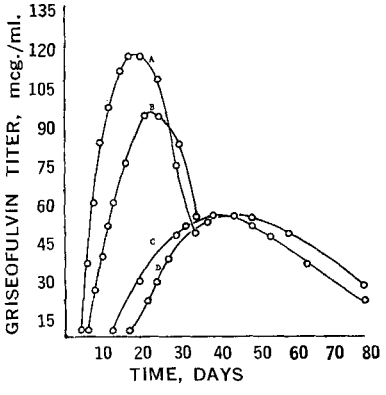

Fig. 6—Effect of D_2O content of the medium on griseofulvin production in surface culture. Key: A, *0% D_2O;* B, *50% D_2O;* C, *75% D_2O;* D, *99% D_2O plus vitamin supplement. All cultures contained 7% protio-glucose.*

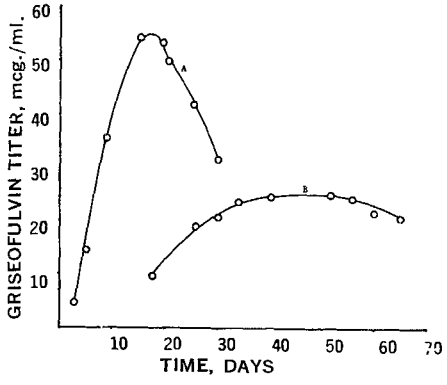

Fig. 7—Production of griseofulvin in shake cultures containing 7% protio-glucose and grown in (A) H_2O *and* (B) *99% D_2O plus vitamin supplement.*

it became apparent during the course of this study that good growth is not synonymous with good griseofulvin production. The fermentation method of choice proved to be surface culture, and was therefore used exclusively throughout the investigation after early fermentation trials.

In surface cultures the culture media, consisting of protio-sugars in 50% D_2O, exhibited about a 20% reduction in griseofulvin titer when compared with H_2O controls. When the concentration of deuterium oxide was increased to 75%, the griseofulvin titer of the fermentor broth was decreased by approximately one-half. At a D_2O concentration of 99.6%, griseofulvin production was severely impaired, and only small amounts of griseofulvin were produced. By supplementing the 99.6% D_2O culture medium with vitamin B-complex, a griseofulvin titer could be attained which approached that observed in the 75% D_2O cultures.

CONCLUSIONS

The deuterium isotope effect on the growth of the mold *P. janczewskii* is clearly a repressive one. Growth, griseofulvin production, and pigmentation of the mold are all inhibited with increasing concentrations of deuterium oxide. Corn steep solids and vitamin B-complex additives have demonstrated an enhancement of both mycelial growth and griseofulvin production in highly deuteriated media. It is tempting to infer from these experimental observations that the isotope effect in the mold is due to an incomplete medium. However, the deuterium effect in biological systems is extremely complex and no doubt many factors are involved. A normally endogenous metabolite may become a required exogenous factor. A maximum enhancement level is reached with such additives; a 5% concentration of vitamin supplement offers no advantage over a 2% concentration. Similar trends were noted with corn steep solids. High deuterium concentrations result in the utilization of available metabolites to maintain the nutrition of the isotopically altered mold. The fungus apparently becomes frugal, meeting only those needs required for survival. Griseofulvin production and elaboration of pigment can be considered to be nonessential for mold survival, and can therefore be depressed without damage to the organism. Deuterium seems to interfere with the basic integrity of the mold enzymes which are necessary to antibiotic and pigment production. The morphological changes in the deuteriated fungi appear to be due to a general disruption of the cell enzyme physiology.

It is the objective of subsequent studies to isolate and characterize the partially and fully deuteriated griseofulvin produced by the organism grown under the conditions described here. Such compounds may prove useful in studying the biogenesis of antibiotics and may contribute to the elucidation of the mechanism by which these compounds function in the body. The effect of deuterium on the antibiotic activity must also be examined. It is expected that since carbon-to-deuterium bonds are more stable than carbon-to-hydrogen bonds, and if detoxification requires the rupture of such bonds, then the deuterio form of the drug should possess a more prolonged antifungal activity. Preliminary results appear to show that this is the case.

REFERENCES

(1) Mandeville, S. E., Crespi, H. L., and Katz, J. J., *Science*, **146**, 769(1964).
(2) Blake, M. I., Crane, F. A., Uphaus, R. A., and Katz, J. J., *J. Pharm. Sci.*, **53**, 79(1964).
(3) Crane, F. A., Blake, M. I., Uphaus, R. A., and Katz, J. J., *ibid.*, **53**, 612(1964).
(4) Uphaus, R. A., Crane, F. A., Blake, M. I., and Katz, J. J., *ibid.*, **54**, 202(1965).
(5) Blake, M. I., Crane, F. A., Uphaus, R. A., and Katz, J. J., *Lloydia*, **30**, 111(1967).
(6) Cope, B. T., Bose, S., Crespi, H. L., and Katz, J. J., *Botan. Gazz.*, **126**, 214(1965).
(7) Flaumenhaft, E., Bose, S., Crespi, H. L., and Katz, J. J., *Intern. Rev. Cytol.*, **18**, 313(1965).
(8) Katz, J. J., "Chemical and Biological Studies with Deuterium," 39th Annual Priestley Lecture Series, Pennsylvania State University, State College, Pa.
(9) Blake, M. I., Crespi, H. L., Mohan, V., and Katz, J. J., *J. Pharm. Sci.*, **50**, 425(1961).
(10) Crespi, H. L., Marmur, J., and Katz, J. J., *J. Am. Chem. Soc.*, **84**, 3489(1962).
(11) Mrtek, R. G., Crespi, H. L., Blake, M. I., and Katz, J. J., *J. Pharm. Sci.*, **54**, 1450(1965).
(12) *Ibid.*, to be published.
(13) Shaffer, R. L., Crespi, H. L., and Katz, J. J., *Botan. Gazz.*, **119**, 24(1957).
(14) Oxford, A. E., Raistrick, H., and Simonart, P., *Biochem. J.*, **33**, 240(1939).
(15) Rhodes, A., and McGonagle, M., U. S. pat. 3,095,360 (1963).
(16) Rhodes, A., Crosse, R., Ferguson, T. P., and Fletcher, D. L., U. S. pat. 2,843,527(1957).
(17) Hockenhull, D. J. D., U. S. pat. 3,038,839(1962).
(18) Jefferys, E. C., Brian, F., Hemming, H. G., and Lowe, D., *J. Gen. Microbiol.*, **9**, 314(1953).
(19) MacMillan, J., *Chem. Ind.*, **1951**, 719.
(20) Holbrook, A., Bailey, F., and Bailey, G. M., *J. Pharm. Pharmacol.*, **15**, 270(1963).
(21) Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Rogers, M. A. T., *J. Chem. Soc.*, **1952**, 3957.
(22) Crespi, H. L., and Katz, J. J., *Anal. Biochem.*, **2**, 274 (1961).
(23) Mohan, V. S., Crespi, H. L., and Katz, J. J., *Nature*, **193**, 189(1962).

Qualitation and Quantitation of Some Terphenylquinones

By M. C. GAYLORD, J. R. DEBOER, and L. R. BRADY

Microchemical and photochemical procedures which would permit rapid, sensitive detection of atromentin, aurantiacin, and thelephoric acid were examined. Photochemically induced fluorescence and chromophore formation with a ceric ammonium sulfate (CAS) reagent were useful for this purpose, especially when the terphenylquinones were adsorbed on polyamide powder. Quantitation of atromentin was possible with a CAS procedure, and all three of the terphenylquinones could be quantitated with pyridine–water (Py–W) and combined Py–W–CAS procedures. The relative sensitivities for the quantitative methods for atromentin were CAS > Py–W–CAS > Py–W. The procedures permitted the determination of atromentin and aurantiacin in concentrations of 2 to 120 mcg./ml. and 4 to 120 mcg./ml., respectively, and of thelephoric acid in concentrations of 2 to 40 mcg./ml. Precise standardization of analytical conditions was critical.

QUINONES comprise a highly reactive, heterogeneous group of compounds which are attracting increasing interest as basic research tools in biochemistry. Quinones and compounds capable of forming quinoid-like structures are strongly conjugated chemical systems which have the capacity for rapid electronic delocalization. This important molecular feature is of potential biochemical significance in energy transfer, long-range transmission of electronic excitation, and resonance stabilization of activated complexes (1). Molecular biology and pharmacology (2, 3), the molecular orbital approach to quantum chemistry (1, 4), photochemistry of organic compounds (5–7), and oxidation-reduction mechanisms in polymers (8) are examples of other areas where study of quinoid systems is useful.

When information about the quinoid compounds is more complete, application of this knowledge will permit elucidation of the mechanisms of action of many drugs and will improve their utilization in therapy. Webb (3) has emphasized the medicinal potential for biologically active quinones by reviewing their use as antitumor or antimitotic agents, as enzyme inhibitors, as possible acceptors in electron transport systems, as uncouplers of oxidative phosphorylation, and as antimicrobial agents. Other interesting functions of quinones in biologic systems include radiosensitizers in tumor therapy (9), antioxidants (10), and storage of solar energy (11).

Terphenylquinones are a group of naturally occurring pigments about which very little is known. The available knowledge of this group of quinones is restricted to elucidation of their chemical structures, distribution of certain of the constituents in a limited sampling of fungi and lichens (12, 13), and gross observations of the antitumor activity of polyporic acid (14, 15) and the anticoagulant action of atromentin (16, 17). There is a paucity of information on many of the chemical properties of these compounds, and information on the biologic involvement of these constituents at the molecular level is totally lacking for both plant and animal systems.

Studies were undertaken to develop sensitive procedures for qualitative and quantitative determination of atromentin, aurantiacin, and thelephoric acid. The availability of such analytical techniques would facilitate investigations on the role of these quinones in biologic systems. A secondary objective of the study was accumulation of data on the chemical properties of the three terphenylquinones.

EXPERIMENTAL

Materials and Reagents—Atromentin, aurantiacin, and thelephoric acid were isolated from suitable fungi using procedures which had been tested in this laboratory (13, 16, 18). Purity of the isolated compounds was verified using melting points, elemental analyses, and absorption spectroscopy (infrared and ultraviolet). Eastman spectrograde methanol and pyridine were used routinely when spectroscopic manipulations were involved. The 1% ceric ammonium sulfate (CAS) reagent in syrupy *o*-phosphoric acid was prepared as described by Farnsworth *et al.* (19) and was stored in a tightly stoppered actinic glass flask. The undiluted CAS reagent was used as a spray for detection of the terphenylquinones on the thin-layer chromatograms, but the reagent was diluted with an equal volume of distilled water immediately prior to its use in quantitative procedures.

General Quantitative Methodology—Preliminary results indicated that daily preparations of standard solutions of the terphenylquinones and storage

Received April 14, 1967, from the Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105

Accepted for publication June 13, 1967.

Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by grant GM 07515-07 from the U. S. Public Health Service, Bethesda, Md.

TABLE I—ABSORPTION MAXIMA OF THELEPHORIC ACID IN AQUEOUS PYRIDINE MIXTURES

Vol. of Pyridine, ml.	Vol. of Water, ml.	Absorption Max., mμ
3	0.5	525,340
3	0.75	540,338
3	1.0	552,338
3	2.0	640,338

of the solutions in tightly stoppered actinic glass containers were essential for reproducibility. Standard stock solutions were prepared by dissolving 120 mcg./ml. of atromentin or aurantiacin or 40 mcg./ml. of thelephoric acid in pyridine or 10 mcg./ml. of atromentin in an absolute methanol–glacial acetic acid mixture (99:1) using actinic glass volumetric flasks. These concentrations were selected when preliminary evidence indicated that they were near the upper limits which gave reproducible results. Five dilutions were made from each stock solution, and samples of these solutions were utilized in developing the quantitative procedures. The visible spectra of the colored solutions were scanned using a Beckman recording spectrophotometer, model DB, to determine absorption maxima. A corresponding reagent blank was routinely employed in all spectrophotometric examinations. Studies were also conducted to determine the time which was required for maximum color formation and to show the relative stability of the colored complex. After suitable absorption maxima were selected and optimal experimental conditions were determined, standard curves were prepared and molecular extinction coefficients were calculated using average data from triplicate quantitations. The standard curves were plotted on the bases of quinone concentrations in the pyridine or methanol solutions, but allowances were made for the volume of additives in calculating the molecular extinction coefficients of the colored complexes.

Pyridine–Water (Py–W) Colorimetric Procedure—Sawada (20) qualitatively utilized the color change from a grape-red to blue when water was added to a pyridine solution of thelephoric acid. Variations of this approach were found to give quantitative results with atromentin, aurantiacin, and thelephoric acid. The quantity of distilled water which was added to the pyridine solutions of atromentin and aurantiacin had no significant qualitative influence on the color, but the absorption maxima of the colored complex varied when different amounts of water were added to the thelephoric acid solutions (Table I). A standard technique utilizing 3 ml. of a terphenylquinone-containing pyridine solution and 0.75 ml. of water was selected as being feasible. The absorbance of the atromentin solution was measured at 560 mμ 15 to 30 min. after thorough mixing. Absorbance of the thelephoric acid colored complex was measured within 2 to 35 min. at 540 mμ. (Fig. 1.)

Color formation with aurantiacin was very slow to develop at room temperature, and difficulty was noted in obtaining reproducible color intensities. This was presumably caused by the need for hydrolysis of the benzoate esters of aurantiacin to yield atromentin prior to the formation of the colored complex. Satisfactory quantitative results were obtained when the aurantiacin mixture was heated initially. The aqueous pyridine solution was placed in an actinic glass test tube which was covered with a marble to prevent evaporation, the solution was heated in a steam bath for 10 min., and the mixture was cooled rapidly by immersing the tube in water at room temperature. Absorbance was measured at 560 mμ within 30 min. (Fig. 1).

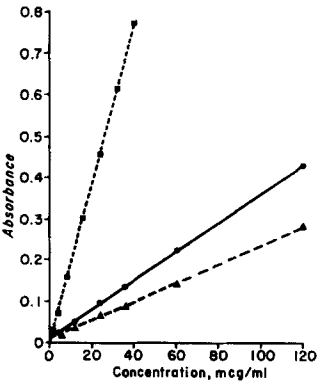

Fig. 1—Quantitation of atromentin, aurantiacin, and thelephoric acid with the Py–W procedure. Key: ●, atromentin, 560 mμ; ▲, aurantiacin, 560 mμ; ■, thelephoric acid, 560 mμ.

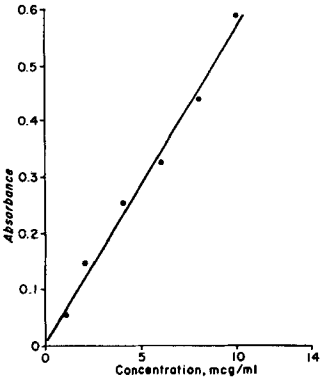

Fig. 2—Quantitation of atromentin at 418 mμ with the CAS procedure.

Reproducibility among the triplicate determinations was excellent, varying no more than ±0.005 absorbance units for any tested concentrations of the terphenylquinones.

CAS Colorimetric Procedure—CAS has been used to measure oxidation-reduction potentials of some quinones (21), and the reagent has been shown to have unusual utility in the chromatographic determination of *Catharanthus* alkaloids (19). These applications suggested the possibility of this reagent for the qualitative or quantitative evaluation of the terphenylquinones. A ceric hydroxide precipitate forms in alkaline solutions, and it was considered desirable to use methanol rather than pyridine to dissolve the pigments. A methanol–glacial acetic acid mixture (99:1) was selected after preliminary results with atromentin revealed that the small quantity of acetic acid did not effect quantitation but did greatly facilitate elution from thin-layer chromatograms. Atromentin could be quantitated by adding 0.04 ml. of the 0.5% CAS reagent to 4 ml. of the methanolic solutions using a 0.1-ml. pipet

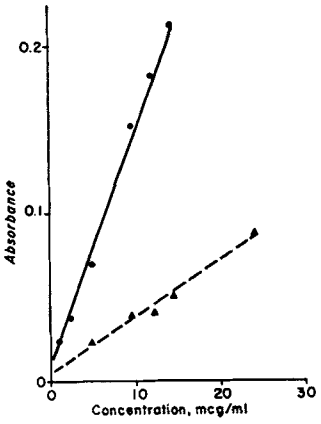

Fig. 3—Quantitation of atromentin and aurantiacin with the Py – W – CAS procedure. Key: ●, atromentin, 525 mμ; ▲, aurantiacin, 500 mμ.

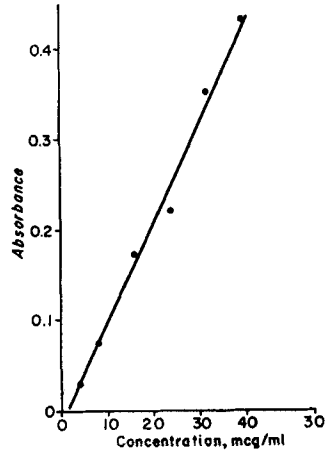

Fig. 4—Quantitation of thelephoric acid at 500 mμ with the Py–W–CAS procedure.

calibrated in 0.002-ml. increments, mixing vigorously several times during a 30-min. reaction period, and measuring the absorbance of the bright yellow color at 418 mμ (Fig. 2). Background absorption with the CAS reagent necessitated particularly careful preparation of the blank.

Reproducibility among the triplicate determinations was approximately ±3%. The procedure had a sensitivity for atromentin that was more than 15 times that of the Py–W procedure. However, the CAS reagent could not be used to quantitate aurantiacin and thelephoric acid. Thelephoric acid is not appreciably soluble in nonbasic organic solvents, and no suitable method was found to hydrolyze aurantiacin without interfering with chromophore formation.

Pyridine–Water–Ceric Ammonium Sulfate (Py–W–CAS) Colorimetric Procedure—The Py–W procedure had the advantage of applicability to all three of the terphenylquinones which were studied, but the advantageous feature of the CAS procedure was increased sensitivity. The possibility of combining these two advantages was explored, and preliminary results revealed that quantitation was possible with dilute solutions of the quinones. Several different combinations of conditions and reagents were observed to give reproducible quantitative results, but the procedure which was selected as being most useful involved the initial measurement of the terphenylquinones using the previously described Py–W procedure. After reading the absorbance of an aqueous pyridine solution, it was transferred quantitatively from the cell to an actinic glass test tube with the aid of 3.75 ml. of methanol, a component which was found to be essential for the subsequent quantitation, and 0.04 ml. of 0.5% CAS reagent was added. The solution was mixed thoroughly, and the color was allowed to develop for 1 hr. Absorbance of the colored complex was measured at 525 mμ for atromentin and at 500 mμ for aurantiacin and thelephoric acid (Figs. 3 and 4).

Reproducibility among the triplicate determinations was equivalent to that obtained with the Py–W procedure. The combined procedure was more sensitive than the Py–W method for atromentin and aurantiacin, but when allowance was made for volume change, the sensitivities of the two procedures were comparable for thelephoric acid. The combined method was approximately half as sensitive as the CAS procedure for atromentin.

Qualitative Determinations—Characteristic absorption maxima (Table II) and other individualistic properties which were observed during development of the quantitative procedures were useful in the qualitative evaluation of the three terphenylquinones. It was also noted that these terphenylquinones were susceptible to oxidative and photochemical changes, properties which are well documented for other quinones. This instability limited the types of manipulations which could be utilized in qualitative verification of small quantities of these pigments, and studies were undertaken to discover additional methods which would contribute to rapid identification. It was considered desirable to include a chromatographic procedure in the qualitative evaluation to permit examination of mixtures and to provide a possible basis for subsequent quantitation of impure materials or components of mixtures.

TABLE II—ABSORPTION MAXIMA AND MOLECULAR EXTINCTION COEFFICIENTS OF ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID AND THEIR COLORED COMPLEXES

Compd.	Solvent	Untreated Soln. mμ	Untreated Soln. log ε	Complex with Py–W mμ	Complex with Py–W log ε	Complex with CAS mμ	Complex with CAS log ε	Complex with Py–W–CAS mμ	Complex with Py–W–CAS log ε
Atromentin	Dioxane	268	4.58	340	3.96	418	4.30	525	4.09
		385	3.75	560	3.16				
Aurantiacin	Dioxane	240	4.63	330	3.98	...	...	352	3.87
		405	3.92	560	3.20			500	3.61
Thelephoric acid	Pyridine	311	4.51	338	4.35	...	...	500	3.53
		495	4.11	540	3.95				

TABLE III—QUALITATIVE EVALUATION OF ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID USING POLYAMIDE THIN-LAYER CHROMATOGRAPHY[a]

Compd.	R_f	Color[b]	Color with CAS Spray[b]
Atromentin	0.13–0.25[c]	10dc, orchid haze	2 min.: 23ic, apple green 15 min.: 1kb, light citron yellow 30 min.: 2kb, golden yellow
Aurantiacin	0.80	2fb, straw wheat	2fb, straw wheat
Thelephoric acid	0.05	8ec, rose mist	9ic, raspberry rose

[a] Methanol–chloroform–water–glacial acetic acid (54:36:6:1) solvent system. [b] Color chip number and trivial name from "Color Harmony Manual." [c] Migration of atromentin was observed to be concentration dependent. Larger quantities of the quinone, up to the point where the system became overloaded, showed greater movement.

Preliminary results revealed that columnar and paper partition chromatographic separations were too slow for any evaluations of these compounds involving ultimate quantitation. Elution of the terphenylquinones following their separation with an established thin-layer chromatographic method (13) was inefficient, and the solvent interfered with quantitative attempts. Thin-layer chromatography with polyamide was found to be useful qualitatively and to offer quantitative possibilities. When the polyamide chromatograms were prepared without a binder, extreme care was necessary to avoid mechanical loss in removing spots of the brittle film from the chromatograms for quantitative purposes. The other extreme was noted with a modified formulation of the adsorbent (22) where adherence to the plates made quantitative applications impractical. The most satisfactory results were obtained with a modification employing one-fourth the rice-starch binder recommended by Nordby *et al.* (22). Rice starch (0.2 Gm.), silica gel without binder (Woelm, 0.1 Gm.), and water (9 ml.) were heated in a covered beaker on a steam bath for 40 min. with frequent stirring. The binder was transferred, with the aid of 3 ml. of water, to a Waring blender containing 5.5 Gm. of Woelm polyamide TLP and 30 ml. of methanol, and the mixture was blended for 5 min. at high speed. The slurry was applied to glass plates with a chromatographic spreader; the plates were allowed to air-dry for 15 min. and were placed in a desiccator over anhydrous $CaCl_2$ for a minimum of 2 hr. before use. Several chromatographic solvent systems effected separation, but some tailing was always noted with atromentin, presumably due to a pronounced tendency for hydrogen bonding. Methanol–chloroform–water–glacial acetic acid (54:36:6:1) was selected as the best solvent for combined qualitative and quantitative objectives; higher proportions of water or acetic acid enhanced the instability of the terphenylquinones. The chromatograms were placed in the dark at room temperature for 15 min. to permit the solvent to evaporate prior to qualitative or quantitative evaluation.

The distinctive colors of the terphenylquinones were recorded by direct comparison with chips from a "Color Harmony Manual" (23, 24), and the chromatograms were subjected to further evaluations. Particularly characteristic results were observed with atromentin when chromatograms were sprayed lightly with 1% CAS reagent. The chromatograms became transparent when sprayed, but the complexes could be visualized by briefly layering the adsorbent with a film of water to make the background opaque. The initial colors were relatively stable with aurantiacin and thelephoric acid, whereas a characteristic color sequence developed with atromentin (Table III).

None of the terphenylquinones fluoresced upon initial examination of the chromatograms under U.V. light, but fluorescence was observed in solutions of the compounds which had undergone photochemical changes while stored in tightly stoppered Pyrex glass containers. Unsprayed chromatograms were exposed to a Hanovia high pressure quartz mercury vapor lamp, model 30600, at approximately 15 cm. for 24 hr.; this radiation produced no detectable changes with thelephoric acid, but a yellow fluorescence was noted with atromentin and aurantiacin.

The qualitative fluorescent properties of the photoproducts of the terphenylquinones were examined further by radiating methanolic solutions of the compounds and determining their excitation and emission peaks with a Baird Atomic Fluorispec, model SF-1. Solutions containing 20 mcg./ml. of atromentin or aurantiacin or 10 mcg./ml. of thelephoric acid were prepared and radiated in Pyrex glass volumetric flasks with the mercury vapor lamp. The fluorescent properties of the photoproducts were determined after radiating atromentin and aurantiacin for 5 days and thelephoric acid for 10 days (Table IV).

Quantitation of Chromatographically Separated Terphenylquinones—Studies with pure terphenylquinones revealed that semiquantitative to quantitative results could be achieved by applying the various colorimetric procedures following thin-layer chromatographic separation. Appropriate zones of the polyamide adsorbent were transferred to actinic glass test tubes as rapidly as possible, and the colorimetric reagents were added to the tubes. The colors were allowed to develop for the

TABLE IV—QUALITATIVE FLUORESCENT PROPERTIES OF PHOTOPRODUCTS FROM ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID

Compd.	Excitation, mμ	Emission, mμ
Atromentin	365	415 525
	395	525
Aurantiacin	365	485
	400	515
Thelephoric acid	370	415 495
	397	502

previously indicated periods of time with frequent agitation, the polyamide powder was removed by filtration, and the absorbances were measured spectrophotometrically at the appropriate wavelength.

The Py–W–CAS procedure was found to be the method of choice for atromentin and aurantiacin. In the case of aurantiacin, the aqueous pyridine solution was subjected to the hydrolytic conditions prior to addition of the methanol and CAS reagent. Since the total volume of liquid with this procedure was greater than that required to fill the cell, the sensitivity could be doubled by utilizing only half the specified volume of each component in the reaction mixture. The more complex Py–W–CAS procedure offered no sensitivity advantage over the Py–W method for thelephoric acid, so the latter was utilized. Acceptable quantitation of atromentin was possible upon application of the CAS colorimetric procedure, but the method had little apparent practicality.

Combination of any of the colorimetric procedures with chromatography resulted in the expected losses due to transfer and elution of the adsorbent, but the most critical factor was found to be losses through oxidative and photochemical changes if the manipulations were not handled rapidly. The best results were obtained with the relatively stable thelephoric acid. When chromatograms were exposed to air and light for 2 hr. before evaluation, losses of atromentin were 30% or more. Tailing of atromentin on the chromatograms sometimes further complicated quantitations, and occasionally inexplicable results were obtained following hydrolysis of aurantiacin in the presence of the polyamide powder. However, under optimal conditions the colorimetric results were consistently ±5–10% of the theoretical values.

DISCUSSION

Three rapid and sensitive colorimetric procedures were developed for the quantitation of atromentin, aurantiacin, and thelephoric acid (Figs. 1–4). Reproducibility, both within replicate determinations and with analyses conducted at different times, required strict adherence to precautionary measures to minimize oxidative and photochemical changes. Photochemical changes could be controlled during some of the manipulations by utilizing actinic glassware. However, oxidative interference still necessitated completion of the analyses in the minimum possible time.

Quinones are known to be susceptible to such photochemically induced reactions as fragmentation, polymerization or addition of other available substituents, rearrangement, and reduction (5, 7). The exact nature of the photochemical reaction(s) with the terphenylquinones was not determined due to the difficulty in distinguishing between oxidative and photochemical changes. The shift in fluorescent characteristics when solutions were radiated for varying periods of time suggested the solutions contained mixtures of transformation products. Atromentin was the least stable of the three terphenylquinones, and observations on the changes with this pigment were of the greatest indicative value.

The transformation products were observed to cause two types of problems in the quantitative procedures. These substances did not form the characteristic colored complexes, an observation which was substantiated by adding water to a radiated pyridine solution of atromentin and noting that the absorption maximum at 560 mμ was missing (the absorption peak at 340 mμ remained). The second problem suggested that polymerization was at least one of the photochemical changes. After cells had been used for several analyses, fluorescent interference was noted in the ultraviolet region. It was determined that the interference was caused by the deposition of minute amounts of insoluble photoproducts on the walls of the cells. The problem was noted initially during studies where the absorbance of a solution was read periodically to determine the time interval which gave quantitative results, but subsequent studies showed that the effect was cumulative when a succession of freshly prepared solutions were allowed to remain in the cell for a few minutes each. The deposit could be removed either with alcoholic KOH or dichromate cleaning solution, but it was insoluble in the normal organic solvents. The insolubility of the product suggested it was polymeric, thus making this problem similar to that reported by Pitts *et al.* (25).

Finite characterization of the chromophores in the various colorimetric procedures was not possible with the available data, but observations suggested some of the apparent prerequisite features of the terphenylquinones. The most important molecular feature appeared to be the 3,6-dihydroxy substituents. Aurantiacin was presumably hydrolyzed to atromentin prior to formation of the chromophore in the Py–W quantitation. This presumption was supported by the failure of aurantiacin to form a colored complex immediately and by the observation that the ratio of absorbances of the colored complexes formed with equal quantities of atromentin and aurantiacin was 3:5, a ratio approximately that of their molecular weights. The predicted involvement of the 3,6-dihydroxy groups was consistent with the observed failure of 2,5-diphenyl-1,4-benzoquinone to form a chromophore in the Py–W system. Consideration of the importance of the hydroxyl substituents and the conjugated nature of these terphenylquinones suggested the chromophore in Py–W was probably a resonance hybrid of the parent quinone.

Resonance hybridization also appeared to explain the chromophores which were formed when the terphenylquinones were chromatographed on polyamide powder. The chromophores which were formed on the polyamide chromatograms did not interfere with subsequent chromophore formation in the colorimetric procedures when the quinones were eluted, thus indicating that chemical change was unlikely. The possibility that the nitrogen atoms in the adsorbent stabilize the resonance hybrid, presumably by sharing protons, was further suggested when comparable chromophores were noted upon the addition of the terphenylquinones to solutions of gelatin. It should be noted that the chromophore with thelephoric acid and polyamide or gelatin was greenish turning to pink upon addition of small quantities of acetic acid.

CAS is an oxidizing agent, and chromophores developed with the terphenylquinones and this

reagent appeared to involve irreversible chemical change. Attempts to determine the oxidation-reduction potentials of the terphenylquinones with CAS were unsuccessful, but the availability of this reagent for reaction with the quinones was found to be the limiting factor in the CAS and Py–W–CAS colorimetric procedures. CAS had limited solubility in the solvents which were employed in these methods, and quantitative chromophore formation was limited by this factor. The solubility of CAS in the solvent mixture of the Py–W–CAS procedure was greater than in the methanolic solvent of the CAS method. However, attempts to determine solvent mixtures which permitted higher concentrations of CAS encountered problems with the solubilities of the terphenylquinones or with the fundamental involvement of methanol in the chromophore-yielding reactions. Addition of CAS to an aqueous pyridine solution of atromentin resulted in a loss of the absorption maximum at 560 mμ, but formation of a characteristic new chromophore with peak absorption at 525 mμ was observed only when methanol was in the reaction mixture.

Qualitative data suggested that thelephoric acid did not react with CAS. Addition of methanol and the CAS reagent to an aqueous pyridine solution appeared merely to dilute the colored complex; the color intensity could be predicted by correcting for volume change. Absorption maximum of the thelephoric acid complex with the Py–W–CAS method was 500 mμ, but this shift was attributed to the known qualitative influence of additives on the chromophore of this quinone in pyridine solutions (Table I). The observation that methanol was not an essential component of reaction mixtures containing CAS and thelephoric acid, in contrast to mixtures involving atromentin and aurantiacin, was consistent with the presumed lack of chromophore formation with thelephoric acid, the most stable of the three terphenylquinones.

Preliminary results revealed that the colorimetric methods were suitable for proximate quantitations of pure terphenylquinones following thin-layer chromatography. This suggests the possibility of devising conditions which are suitable for quantitative evaluation of the terphenylquinones in fungi or other biologic systems. Application of some type of rapid analysis appears mandatory to obtain reasonable quantitation of these reactive compounds, but it will be necessary to check each new condition or system to exclude possible interference which may be caused by the new condition or by other components in the biologic system.

REFERENCES

(1) Pullman, A., and Pullman, B., in "Horizons in Biochemistry," Kasha, M., and Pullman, B., eds., Academic Press Inc., New York, N. Y., 1962, pp. 553–580.
(2) Szent-Györgyi, A., "Introduction to Submolecular Biology," Academic Press Inc., New York, N. Y., 1960.
(3) Webb, J. L., "Enzyme and Metabolic Inhibitors," vol. 3, Academic Press Inc., New York, N. Y., 1966, pp. 421–594.
(4) Zimmerman, H. E., *Science*, **153**, 837(1966).
(5) Turro, N. J., "Molecular Photochemistry," W. A. Benjamin, Inc., New York, N. Y., 1965.
(6) Bruce, J. M., and Knowles, P., *J. Chem. Soc. (C)*, **1966**, 1627.
(7) Calvert, J. G., and Pitts, J. N., Jr., "Photochemistry," John Wiley & Sons, Inc., New York, N. Y., 1966.
(8) Cassidy, H. G., *Am. Scientist*, **54**, 184(1966).
(9) Mitchell, J. S., and Marrian, D. H., in "Biochemistry of Quinones," Morton, R. A., ed., Academic Press Inc., New York, N. Y., 1965, pp. 503–537.
(10) Bennett, G. J., and Uri, N., *Nature*, **192**, 354(1961).
(11) Marcus, R. J., Hatchett, J. L., and Sancier, K. M., in "Photochemistry in the Liquid and Solid States," Daniels, F., ed., John Wiley & Sons, Inc., New York, N. Y., 1963, pp. 122–125.
(12) Thomson, R. H., in "Comparative Biochemistry," vol. 3, Florkin, M., and Mason, H. S., eds., Academic Press Inc., New York, N. Y., 1962, pp. 648–667.
(13) Sullivan, G., Brady, L. R., and Tyler, V. E., Jr., *Lloydia*, **30**, 84(1967).
(14) Cain, B. F., *J. Chem. Soc.*, **1961**, 936.
(15) *Ibid. (C)*, **1966**, 1041.
(16) Euler, K. L., Tyler, V. E., Jr., Brady, L. R., and Malone, M. H., *Lloydia*, **28**, 203(1965).
(17) Khanna, J. M., Malone, M. H., Euler, K. L., and Brady, L. R., *J. Pharm. Sci.*, **54**, 1016(1965).
(18) Montfort, M. L., Tyler, V. E., Jr., and Brady, L. R., *ibid.*, **55**, 1300(1966).
(19) Farnsworth, N. R., Blomster, R. N., Damratoski, D., Meer, W. A., and Cammarato, L. V., *Lloydia*, **27**, 302 (1964).
(20) Sawada, M., *Nippon Ringaku Kaishi*, **34**, 110(1952).
(21) Cassidy, H. G., and Kun, K. A., "Oxidation-Reduction Polymers (Redox Polymers)," Interscience Publishers, New York, N. Y., 1965.
(22) Nordby, H. E., Kew, T. J., and Fisher, J. F., *J. Chromatog.*, **24**, 257(1966).
(23) Jacobson, E., "Color Harmony Manual," 4th ed., Container Corporation of America, Chicago, Ill., 1958.
(24) Taylor, H. D., Knoche, L., and Granville, W. C., "Descriptive Color Names Dictionary," suppl. to "Color Harmony Manual," 3rd ed., Container Corporation of America, Chicago, Ill., 1950.
(25) Pitts, J. N., Foote, J. K., and Wan, J. K. S., *Photochem. Photobiol.*, **4**, 323(1965).

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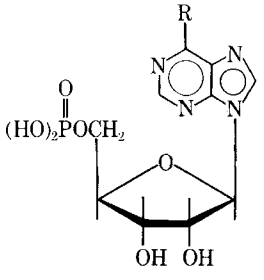
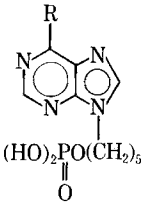
Hydrophobic Interactions with Succinoadenylate Kinosynthetase and 9-Substituted Adenines

By B. R. BAKER and EDWARD H. ERICKSON

Adenines substituted at the 9-position with *n*-butyl or benzyl groups were complexed about tenfold better to succinoadenylate kinosynthetase than 9-methyladenine. The same *n*-butyl or benzyl substituents on 6-mercaptopurine failed to show this hydrophobic interaction even though 5′-adenylate and thioinosinate are complexed equally well to the enzyme. It is proposed that either (*a*) 6-mercaptopurine can complex in only one way to the inosinate binding locus regardless of the 9-substituent, whereas adenine can complex to the inosinate locus as one of two rotomers dependent upon whether the 9-substituent is hydrophilic or hydrophobic, or (*b*) the adenines complex at the inosinate binding locus, but the 6-mercaptopurines complex at the cofactor (guanosine-5′-triphosphate) binding locus.

SUCCINOADENYLATE kinosynthetase is an anabolic enzyme that condenses inosinate (I) with L-aspartate to form succinoadenylate (II) where



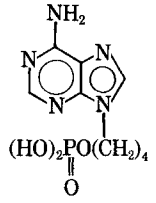


I, R = OH
II, R = L-NHCH(COOH)CH_2COOH
III, R = NH_2
IV, R = SH

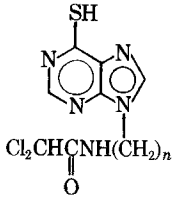
V, R = NH_2
VI, R = SH

guanosine-5′-triphosphate is used as the cofactor (1–4). The enzyme can be inhibited by 5′-adenylate (III) (3, 4) and by thioinosinate (IV) (4–7). The relative contribution of the functional groups of the ribofuranose moiety of III and IV to binding to the enzyme has been previously reported by Baker and Tanna (4, 8). The phosphate group contributed more than all the other oxygen functions combined; only a thirteenfold loss in binding occurred with the pentanol phosphates V and VI compared to III and IV, while the corresponding nucleosides were less than one-sixtieth as effective as III and IV.

In a further study (4, 9), the conformational aspects of binding of V and VI were considered. It was predicted that the butanol phosphate (VII) should bind better than the pentanol phosphate (V); the staggered ground state conformation of VII should position its phosphate group closer to the binding point for the phosphate of the nucleotide III than does the pentanol phosphate (V). If the dichloroacetamido group was simulating the binding of the phosphate group (10), it was predicted that IX should have a better ground state conformation for optimum binding than VIII (4, 9). The emergence of hydrophobic bonding (11) to such enzymes as adenosine deaminase (12), dihydrofolic reductase (13), thymidine phosphorylase (14), guanine deaminase (15), and others (11) suggested the possibility that V, VI, and VIII might have additional binding to succinoadenylate due to a hydrophobic interaction by part of the pentyl group. Therefore additional studies were performed on the mode of binding of the pentyl group of V, VI, and VIII; the results are reported in this paper, along with inhibition studies on VII and IX.



VII



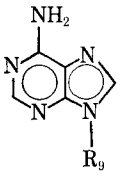
VIII, $n = 5$
IX, $n = 3$

DISCUSSION

Enzyme Results—In Table I are listed the inhibition results with adenine-9-pentanol phosphate (V) and adenine-9-butanol phosphate (VII); note that the binding of the two compounds is essentially the same. If the phosphate group of V and VII is indeed being complexed to the same locus on the enzyme that complexes the 5′-phosphate of 5′-adenylate (III), then it is apparent that there is no

Received April 17, 1967, from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, CA 93106
Accepted for publication June 13, 1967.
This work was supported in part by grant CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Previous paper: Baker, B. R., *J. Med. Chem.*, **10**, September(1967). Previous paper on this enzyme: see *Reference 9*.
The assistance of Ann Jaqua with the assays in Tables I–III is acknowledged.

TABLE I—INHIBITION OF SUCCINOADENYLATE KINOSYNTHETASE[a] BY



Compd.	R_9	m*M* Concn.	% Inhibition	Estimated Concn. for 50% Inhibition	Source
III	RP[b]	0.068	50	0.068[c]	NBC
V	$-(CH_2)_5OPO(OH)_2$	0.97	50	0.97[c]	(8)
VII	$-(CH_2)_4OPO(OH)_2$	1.05	50	1.05	Exptl.
X	$-CH_3$	5.8	50	5.8	(30)[d]
XI	$-C_4H_9$-*n*	0.75	50	0.75	CCNSC
XII	$-C_5H_{11}$-*i*	3.5	50	3.5	Exptl.
XIII	Cyclopentyl	3.0	33	6.0	Schaeffer
XIV	Cyclohexyl	0.25[e]	0	>1.0[f]	Montgomery
XV	$-C_6H_5$	0.5[e]	0	>2.0[f]	(31)[d]
XVI	$-CH_2C_6H_5$	0.51	50	0.51	Schaeffer
XVII	$-(CH_2)_2C_6H_5$	1.1	50	1.1	Exptl.
XVIII	$-(CH_2)_3C_6H_5$	1.7	50	1.7	Exptl.

[a] Succinoadenylate kinosynthetase was isolated from *E. coli* B and assayed with 30.6 μ*M* IMP, 50 μ*M* GTP, and 3.76 μ*M* L-aspartate in pH 7.4 Tris buffer in the presence of 10% *N*,*N*-dimethylformamide as previously described (8). [b] RP = 5-phospho-β,D-ribofuranosyl. [c] Data from *Reference 8*. [d] Prepared in this laboratory by W. F. Wood for another project by the method of Montgomery and Temple (25, 26). [e] Near maximum solubility. [f] Since 25% inhibition is readily detectable, the concentration for 50% inhibition is greater than four times that measured.

difference in the energy required for the butyl and pentyl group to assume the proper conformation; the ground state conformation of the alkylene group of V and VII is not necessarily staggered in aqueous solution as it is in nonaqueous solution (16), but may be slightly coiled due to internal hydrophobic bonding. Since it was found that 9-butyladenine (XI) can bind slightly better than the phosphate (V and VII) by a hydrophobic interaction of the *n*-butyl group with the enzyme, it is possible that V and VII show no phosphate binding of the 9-side chain, but only hydrophobic bonding of the alkyl portion.

The nature of the hydrophobic interaction of the enzyme with adenines bearing a hydrocarbon moiety at the 9-position was then investigated. Note that the 9-butyl group of XI gives an eightfold increment in binding over the 9-methyl group of X. Since the isoamyl (XII), cyclopentyl (XIII), and cyclohexyl (XIV) groups were complexed considerably poorer than *n*-butyl (XI), it appeared that chain branching of these types does not give a good fit to the hydrophobic region of this enzyme; these results are different from those observed with hydrophobic interactions with dihydrofolic reductase (13), where isoamyl is complexed better than *n*-butyl, cyclohexyl is equal to *n*-butyl, and cyclopentyl is poorer than *n*-butyl (17).

Possible hydrophobic interaction with succinoadenylate kinosynthetase by phenylalkyl groups on the 9-position of adenine was then investigated (Table I). The in-plane phenyl group of XV gave no useful amount of hydrophobic bonding. In contrast, the 9-benzyl group of XVI gave the best hydrophobic bonding in Table I, the increment in binding being elevenfold better than the 9-methyl of X. That maximum hydrophobic bonding by an aralkyl group was obtained with the 9-benzyl group of XVI was indicated by the poorer binding of the higher homologs, phenethyl (XVII) and phenylpropyl (XVIII). A further study that might lead to better inhibitors of this enzyme should be performed by substitution on the benzene ring of 9-benzyladenine (24).

Hydrophobic interaction was then investigated with 6-mercaptopurine bearing hydrocarbon groups at the 9-position (Table II). In contrast to the adenine series in Table I, the 9-butyl (XX) and 9-benzyl-6-mercaptopurine (XXIV) did not show detectable hydrophobic interaction. These results are indeed surprising in view of the facts that adenine and 6-mercaptopurine substituted by the 9-ribosyl phosphate moiety (III, IV) bind nearly identically—as do the two purines substituted by the pentanol phosphate moiety (V, VI).

These discrepancies indicated that 9-alkyladenines and 9-alkyl-6-mercaptopurines did not complex to the enzyme in the same way. Three possibilities were considered as follows.

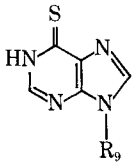
(*a*) Adenines bearing a 9-hydrocarbon group might be complexed to the enzyme locus that normally complexes the cofactor, guanosine-5′-triphosphate, whereas 6-mercaptopurines bearing a 9-hydrocarbon group might bind at the inosinate binding locus.

(*b*) The converse of (*a*) might be possible where the adenines bind at the inosinate locus and the mercaptopurines at the guanosine-5′-triphosphate locus.

(*c*) Both the adenines and the 6-mercaptopurines bind at the inosinate locus, but in different rotomeric configurations depending upon the 9-substituent.

To help differentiate between these explanations, the inhibition by guanines bearing a hydrocarbon group at the 9-position was investigated and the results are shown in Table III. Note that guanine substituted by 9-methyl (XXVI), 9-phenyl (XXIX),

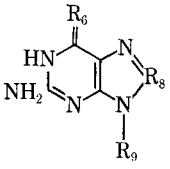
TABLE II—INHIBITION OF SUCCINOADENYLATE KINOSYNTHETASE[a] BY



Compd.	R_9	mM Concn.	% Inhibition	Estimated Concn. for 50% Inhibition	Source
IV	RP[b]	0.070	50	0.070[c]	Montgomery
VI	$-(CH_2)_5OPO(OH)_2$	0.83	50	0.83[c]	(8)
VIII	$-(CH_2)_5NHCOCHCl_2$	0.96[d]	19	4.0[e]	(9)
XIX	$-(CH_2)_5OH$	1.2[d]	30	2.8[e]	(9)
IX	$-(CH_2)_3NHCOCHCl_2$	1.0[d]	0	>4.0[e]	Exptl.
XX	$-C_4H_9$-*n*	1.0[d]	0	>4.0[e]	CCNSC
XXI	$-C_5H_{11}$-*i*	1.0[d]	0	>4.0[e]	CCNSC
XXII	Cyclohexyl	0.25[f]	0	>1.0[e]	Montgomery
XXIII	$-C_6H_5$	0.33[f]	0	>1.3[e]	CCNSC
XXIV	$-CH_2C_6H_5$	0.25[f]	0	>1.0[e]	CCNSC
XXV	$-CH_2C_6H_4COOH$-*p*	0.83	50	0.83[c]	(9)

[a] See Table I for assay conditions. [b] RP = 5-phospho-β,D-ribofuranosyl. [c] Data from *Reference 9*. [d] Maximum concentration allowing full light transmission. [e] Since 25% inhibition is readily detectable, the concentration for 50% inhibition is greater than four times that measured. [f] Near maximum solubility.

TABLE III—INHIBITION OF SUCCINOADENYLATE KINOSYNTHETASE[a] BY

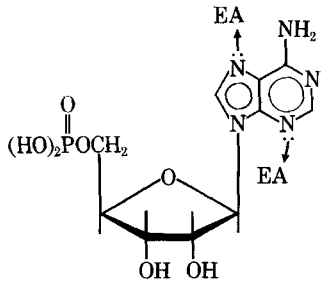


Compd.	R_6	R_8	R_9	mM Concn.	% Inhibition	Estimated Concn. for 50% Inhibition	Source
XXVI	O	CH	$-CH_3$	0.40	50	0.40	Robins
XXVII	O	CH	$-C_5H_{11}$-*n*	0.30[b]	0	>1.2[c]	Robins
XXVIII	O	CH	Cyclohexyl	0.50[b]	0	>2.0[c]	Robins
XXIX	O	CH	$-C_6H_5$	0.33	50	0.33	Robins
XXX	S	CH	$-C_6H_5$	0.30[b]	39	0.75	Robins
XXXI	O	CH	$-C_6H_4Cl$-*p*	0.38	50	0.38	Robins
XXXII	O	N	$-C_6H_4Cl$-*p*	0.05[b]	0	>0.20[c]	Robins
XXXIII	O	CH	$-CH_2C_6H_5$	0.39	50	0.39	Robins
XXXIV	O	N	$-CH_2C_6H_5$	0.35	50	0.35	Robins
XXXV	O	CH	$-CH_2C_6H_4Cl$-*p*	0.30[b]	29	0.63	Robins
XXXVI	O	CH	$-CH_2C_6H_4Cl$-*o*	0.32	50	0.32	Robins
XXXVII	O	CH	2-Furfuryl	0.4[b]	0	>1.6[c]	Robins
XXXVIII	O	C-C_6H_5	H	0.23	50	0.23	(13)
XXXIX	O	C-$(CH_2)_2C_6H_5$	H	0.25[b]	0	>1.0[c]	(13)
XL	O	C-$(CH_2)_3C_6H_5$	H	0.25[b]	0	>1.0[c]	(13)

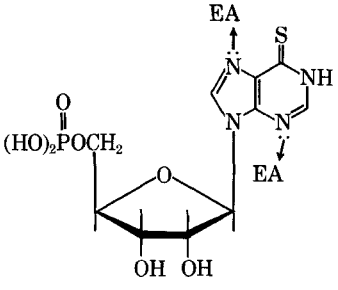
[a] The enzyme from *E. coli* B was prepared and assayed as previously described (8), except that 33 μM GTP and 122 μM IMP were employed. [b] Near maximum solubility. [c] Since 25% inhibition is readily detectable, the concentration for 50% inhibition is at least four times greater than that measured.

and 9-benzyl (XXXIII) have essentially the same binding, thus indicating that there is no hydrophobic bonding by these 9-substituted guanines and that guanines do not bind the same way as the adenines substituted by 9-methyl (X), 9-phenyl (XV), or 9-benzyl (XVI) groups (Table I). Furthermore, a variety of other related compounds such as 8-substituted guanines and 9-substituted 8-azaguanines failed to show hydrophobic bonding. Thus possibility (*a*) is eliminated, but (*b*) and (*c*) remain definite possibilities.

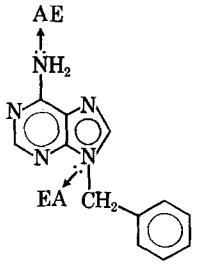
Partial support for possibility (*b*) can be gleaned by noting that 5′-adenylate (III) is a competitive inhibitor of inosinate (I) and a noncompetitive inhibitor of guanosine-5′-triphosphate (3); contrariwise, thioinosinate (IV) is a noncompetitive inhibitor of inosinate (I) but whether it is competitive with guanosine-5′-triphosphate was not determined (37). Although it would not be too difficult to determine whether thioinosinate (IV) is competitive with guanosine-5′-triphosphate, this has not been done, since the interpretation of binding based on competitive or noncompetitive observations is subject to numerous interpretations (38) besides a competitive inhibitor being one that resides on the throne of the active site.



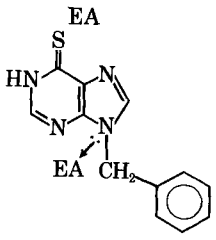
IIIa



IVa



XVIa



XXIVa

Possibility (*c*) merits explanation. Suppose 9-substituted adenines can complex to the inosinate (I) locus in more than one conformation; the particular binding rotomer could be dependent upon whether the 9-substituent is hydrophobic or hydrophilic. Such rotomeric binding conformations have been invoked to account for the binding of unnatural D-isomers to chymotrypsin (18, 19), to dihydrofolic reductase by 5- and 6-hydrophobically substituted pyrimidines (20–22), and for the binding of isoadenosine and isoadenylic acid to certain enzymes (22, 23).

Possibility (*c*) suggests that both the adenines and the 6-mercaptopurines complex with the inosinate (I) locus. If such were the case, then the NHC=S moiety of thioinosinate (IV) and the N=C—NH_2 moiety of 5′-adenylate (III) cannot be binding points to the enzyme, since the latter moiety is an electron donor at the 1-position and the former is an electron acceptor (7, 24); therefore different rotomer configurations for binding adenine would have to consider this difference in binding at the 1- and 6-positions of the purine. For example, suppose the purine ring of 5′-adenylate is complexed as rotomer III*a* where the N-7 and N-3 groups are electron donors to acceptor groups on the enzyme, AE. Thioinosinate could complex similarly through N-7 and N-3 of the purine as rotomer IV*a*. Suppose the hydrophobic bonding region of the enzyme can complex the benzyl group of 9-benzyladenine in conformation XVI*a*; the 6-amino group and 9-nitrogen could now be electron donors to the two acceptor groups on the enzyme in place of N-7 and N-3 of rotomer III*a*. In order for the 9-benzyl group attached to 6-mercaptopurine to bind in this hydrophobic region as depicted in XVI*a*, 9-benzyl-6-mercaptopurine would have to assume the XXIV*a* conformation. But rotomer XXIV*a* will bind poorly since the 6-thione group is a poor electron donor (24); hence, the binding point normally complexing N-3 in configuration III*a* will be lost. Other rotomers to account for the relative binding of 9-benzyladenine (XVI) and 9-benzyl-6-mercaptopurine (XXIV) can probably be visualized, but further experiments would be necessary to differentiate between the possibilities.

Since hydrophobic bonding cannot be detected with 9-benzyl-6-mercaptopurine, possibility (*c*) suggests that all the compounds in Table II complex with the purine as the same rotomer as thioinosinate, such as IV*a*. It follows that VI, VIII, XIX, and XXV should have their extra functional group complexed in the locus for binding the 5-phospho-D-ribosyl moiety of IV*a*. Since the dichloroacetamido group of IX (Table II) binds less effectively than VIII, it also follows that the dichloroacetamido group is not simulating phosphate binding with either possibility (*b*) or (*c*).

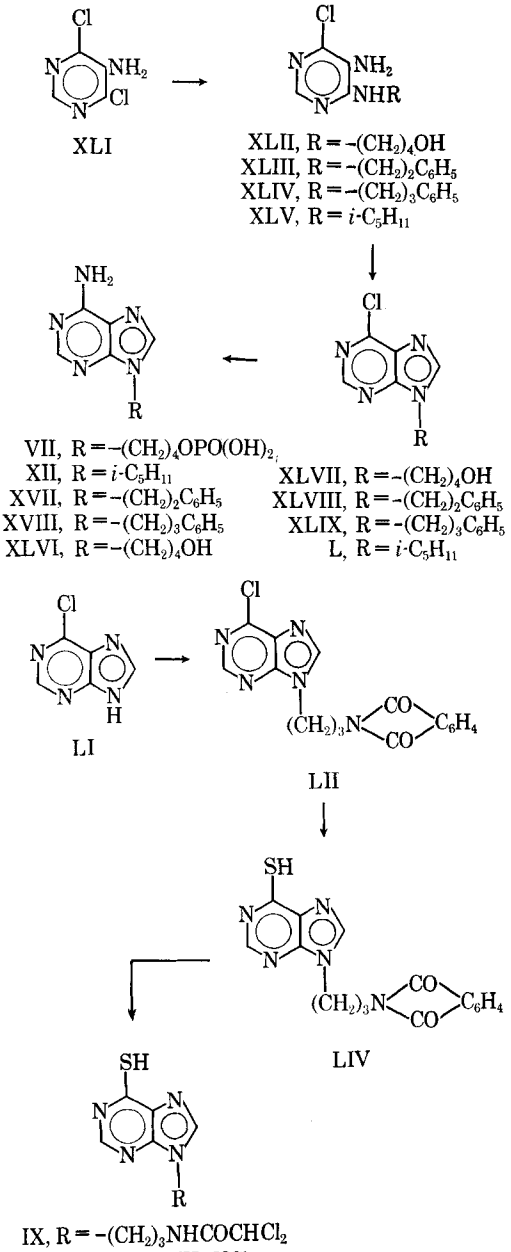
Chemistry[1]—Some of the compounds were available in this laboratory from other enzyme studies, as indicated by the references in the tables. The remainder of the compounds were synthesized as follows (Scheme I).

Reaction of 5-amino-4,6-dichloropyrimidine (XLI) with the appropriate amine in *n*-butanol containing triethylamine afforded the 6-alkylaminopyrimidines (XLII–XLV) by the elegant method of Montgomery and Temple (25, 26). Ring closure of the 6-alkylaminopyrimidines (XLII–XLV) with ethyl orthoformate in the presence of one equivalent of hydrogen chloride (27) afforded the corresponding 6-chloropurines (XLVII–L), which were not purified, but were directly converted to the adenines (XII, XVII, XVIII, XLVI) with ethanolic ammonia at 90° (25). The synthesis of XLVI has been previously described (28), as has the synthesis of XVII (32).

Treatment of 9-(4′-hydroxybutyl)adenine (XLVI) with polyphosphoric acid at 70°, as previously described for the higher homolog (V) (8), gave the butanol phosphate (VII) isolated as its barium salt; the barium salt of VII was converted to the zwitterion of VII with 0.1 *N* aqueous hydrochloric acid.

Alkylation of 6-chloropurine (LI) with *N*-(3-bromopropyl)phthalimide in dimethylsulfoxide in the presence of potassium carbonate (25, 29) afforded a 41% yield of LII; that alkylation had occurred on the 9-position was verified by conversion of LII to the 9-substituted-6-diethylaminopurine with its characteristic U.V. spectra (34–36). Reaction of LII with thiourea (25) smoothly proceeded to the 6-mercaptopurine derivative (LIV). The phthalyl group was cleaved with hydrazine in 2-methoxyethanol (9) and the resultant amine was

[1] The authors thank Drs. J. A. Montgomery, R. K. Robins, H. J. Schaeffer, and H. B. Wood, Jr., CCNSC, for some of the compounds mentioned in the tables.



Scheme I

isolated as its hydrochloride (LIII). Reaction of the free base of LIII with dichloroacetyl chloride in *N,N*-dimethylformamide in the presence of potassium carbonate afforded the desired dichloroacetamide (IX).

EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% ethanol, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF (unless otherwise indicated), and spots were detected by visual examination under ultraviolet light, except in the case of 6-mercaptopurines where iodine vapor was employed.

9H-Adenine-9-yl-butanol Phosphate (VII)—9-(4′-Hydroxybutyl)adenine (XLVI) (28) was prepared from XLI by the general methods described in the literature (25–27). To a solution of 1.54 Gm. of P_2O_5 in 2.06 Gm. of 85% H_3PO_4 at 66° was added 292 mg. (1.4 mmoles) of XLVI. After being heated in a bath at 66–73° for 18 hr., the solution was diluted with 15 ml. of water and heated on a steam bath for 30 min. The pH was carefully adjusted to pH 6.5 with hot saturated aqueous $Ba(OH)_2$. The barium phosphate was removed by filtration through a pad of diatomaceous earth.[2] The combined filtrate and aqueous washings were spin evaporated *in vacuo;* yield, 91 mg. of crude barium salt that moved as single spot on TLC with Brinkmann MN–polyamide–U.V. 254 in *n*-butanol–water–acetic acid (5:3:2). The barium salt was dissolved in 10 ml. of hot 0.1 *N* aqueous hydrochloric acid; the filtered solution was allowed to stand at 5° for about 18 hr. when crystallization of the zwitterion of VII was complete; yield, 50 mg. (14%) of white crystals that moved as a single spot on polyamide as above. For analysis the zwitterion was recrystallized once more from 0.1 *N* aqueous hydrochloric acid to give white crystals, m.p. 244–245°; $\nu_{max.}$ 3420 (NH); 3200–2600 (NH+); 1700 (C=NH+); 1225, 1050, 940 cm.$^{-1}$ (P—O); $\lambda_{max.}$ (pH 1): 263 mμ; (pH 13); 265 mμ.

Anal.—Calcd. for $C_9H_{14}N_5O_4P$: C, 37.6; H, 4.91; N, 24.4. Found: C, 38.1, 37.4; H, 5.38, 5.05; N, 23.8, 24.0.

9-(i-Amyl)adenine (XII)—A solution of 500 mg. (3.05 mmoles) of XLI, 280 mg. (3.22 mmoles) of isoamylamine, 15 ml. of *n*-butanol, and 1 ml. of triethylamine was refluxed for 17 hr. The solution was spin evaporated *in vacuo.* The residue was extracted with hot ethyl acetate, then the solution filtered to remove the insoluble triethylamine hydrochloride. Spin evaporation of the solution *in vacuo* left crude XLV as a brown oil which was dissolved in 12 ml. of ethyl orthoformate and treated with 0.4 ml. of 12 *N* aqueous HCl. After 18 hr. at ambient temperature, at which time the ultraviolet maximum at 297 mμ had shifted to 263 mμ, the solution was evaporated *in vacuo.* The residual L was dissolved in 15 ml. of ethanol previously saturated with ammonia at 0°, then the solution was heated in a Parr bomb at 90° for 18 hr. The solution was kept at −15° overnight, then the product was collected on a filter; yield, 201 mg. (33%), m.p. 136–141°, that gave one spot on TLC in *n*-butanol–acetic acid–water (10:5:1); additional material could be isolated from the filtrate. Recrystallization from ethanol gave white crystals, m.p. 140–142°; $\lambda_{max.}$ (1 *N* HCl): 252 mμ; (1 *N* NaOH): 264 mμ; $\nu_{max.}$ 3400, 3100 (NH); 1660, 1500 cm.$^{-1}$ (C=C, C=N, NH).

Anal.—Calcd. for $C_{10}H_{15}N_5$: C, 58.5; H, 7.37; N, 34.1. Found: C, 58.4; H, 7.50; N, 34.0.

[2] Marketed as Celite by Johns-Manville Corp., New York, N.Y.

9-(3-Phenylpropyl)adenine (XVIII)—This was prepared in 24% over-all yield from XLI as described for the preparation of XII, white crystals, m.p. 184–186°; $\lambda_{max.}$ (1 *N* HCl): 262 mμ; (1 *N* NaOH): 266 mμ.

Anal.—Calcd. for $C_{14}H_{15}N_5$: C, 66.4; H, 5.97; N, 27.7. Found: C, 66.5; H, 6.01; N, 27.6.

Similarly, 9-(4-phenylbutyl)adenine, m.p. 183–185°, was prepared from XLI in 33% over-all yield.

Anal.—Calcd. for $C_{15}H_{17}N_5$: C, 67.4; H, 6.41; N, 26.2. Found: C, 67.4; H, 6.55; N, 26.2.

9-Phenethyladenine (XVII)—This compound was prepared in 15% over-all yield from XLI; white crystals, m.p. 185–188°. [Lit. (32) m.p. 179–180°.] This compound was also prepared by direct alkylation of the sodium salt of adenine in *N,N*-dimethylformamide (33) to give a 36% yield, m.p. 183–186°; $\lambda_{max.}$ (1 *N* HCl): 262 mμ; (1 *N* NaOH): 266 mμ.

6-Chloro-9-(3′-phthalimidopropyl)purine (LII)—A mixture of 3.30 Gm. (21.3 mmoles) of LI, 130 ml. of dimethylsulfoxide, 2.9 Gm. (21 mmoles) of anhydrous potassium carbonate, and 5.40 Gm. (20.2 mmoles) of *N*-(3-bromopropyl)phthalimide was magnetically stirred at ambient temperature for 17 hr. The mixture was filtered. The filtrate was diluted with water, then the product was collected on a filter; yield, 3.02 Gm. (41%) that moved as a single spot on TLC in chloroform–ethanol (1:1) that was different from each starting material. Recrystallization of a sample from ethanol gave an analytical sample, m.p. 185–188°; $\nu_{max.}$ 1770, 1690 (phthalyl C=O); 1580, 1540 (C=C, C=N); 725, 712 cm.$^{-1}$ (C_6H_4); $\lambda_{max.}$ (EtOH): 267, 299 mμ.

Anal.—Calcd. for $C_{16}H_{12}ClN_5O_2$: C, 56.4; H, 3.72; N, 20.5. Found: C, 56.1; H, 3.52; N, 20.4.

The structure of LII was verified by reaction with diethylamine. A solution of 71 mg. (0.21 mmole) of LII, and 0.21 Gm. (3 mmoles) of diethylamine in 5 ml. of ethanol was refluxed for 90 min.; at this time TLC with petroleum ether–chloroform–ethanol (1:1:1) gave only a single spot which moved differently from starting material. That this was a 9-substituted 6-diethylaminopurine was shown by its $\lambda_{max.}$ (pH 1): 275 mμ; (pH 13): 280 mμ.

6-Mercapto-9-(3′-phthalimidopropyl)purine (LIV)—A mixture of 0.48 Gm. (6.3 mmoles) of thiourea, 1.09 Gm. (3.2 mmoles) of LII, and 50 ml. of ethanol was refluxed with magnetic stirring for 12 hr., during which time the product separated. The cooled mixture was filtered and the product was washed with ethanol; yield, 0.59 Gm. (53%), which was suitable for further transformation. Recrystallization of a sample from 2-methoxyethanol gave nearly white crystals, m.p. 310–315°; $\lambda_{max.}$ (pH 1): 327 mμ; $\nu_{max.}$ 2710 (acidic H); 1760, 1710 (phthalyl C=O); 1540, 1575, 1490 (C=C, C=N); 725, 718 cm.$^{-1}$ (C_6H_4). The compound moved as a single spot on TLC in chloroform–ethanol (1:1).

Anal.—Calcd. for $C_{16}H_{13}N_5O_2S$: C, 56.1; H, 3.41; N, 20.5. Found: C, 56.4; H, 3.62; N, 20.4.

6-Mercapto-9H-purine-9-ylpropylamine Hydrochloride (LIII)—A mixture of 2.51 Gm. (7.3 mmoles of LIV, 60 ml. of 2-methoxyethanol, and 1.38 ml. of 85% hydrazine hydrate was refluxed with magnetic stirring for 20 min., then diluted with 160 ml. of ethyl acetate and stored at 5° for 2 hr. The intermediate phthalamide was collected on a filter and washed with ethyl acetate. The solid was stirred with 80 ml. of 1 *N* aqueous hydrochloric acid at about 80° for 20 min. The phthalhydrazide containing considerable LIV was removed by filtration and washed with water. The phthalhydrazide containing LIV (1.47 Gm.) was retreated with hydrazine and aqueous hydrochloric acid as above. The two hydrochloric acid solutions were combined and spin evaporated *in vacuo*. Recrystallization from aqueous acetone gave 1.78 Gm. (86%) of nearly white crystals, m.p. 274–276°. A second recrystallization of a portion from aqueous acetone gave the analytical sample, m.p. 274–276°; $\lambda_{max.}$ (H_2O): 327 mμ; $\nu_{max.}$ 3100–2400 (NH, NH+); 1600, 1540 (C=C, C=N); no C=O near 1700 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_{11}N_5S \cdot HCl \cdot H_2O$: C, 36.4; H, 5.35; N, 26.5. Found: C, 36.5; H, 5.22; N, 26.5.

9-(3′-Dichloroacetamidopropyl)-6-mercaptopurine (IX)—A solution of 442 mg. (1.8 mmoles) of LIII in 3.5 ml. of 1 *N* aqueous sodium hydroxide was spin evaporated *in vacuo*. The dry residue was suspended in 3.4 ml. of *N,N*-dimethylformamide, then cooled in an ice bath. To the magnetically stirred mixture was added 519 mg. of anhydrous K_2CO_3 followed by 0.70 ml. of dichloroacetyl chloride. After being stirred in the ice bath for 1 hr., the mixture was diluted with 50 ml. of water. The product was collected on a filter and washed with water; yield, 120 mg. (21%), m.p. 180–190°, which gave a single spot on TLC in *n*-butanol–acetic acid–H_2O (10:5:1). Recrystallization from aqueous acetone gave nearly white crystals, m.p. 203–205°; $\nu_{max.}$ 3400 (NH); 2600–2500 (acidic H); 1690 (amide C=O); 1590, 1575 cm.$^{-1}$ (C=C, C=N); $\lambda_{max.}$ (pH 1): 326 mμ; (pH 13): 312 mμ.

Anal.—Calcd. for $C_{10}H_{11}Cl_2N_5OS$: C, 37.5; H, 3.47; N, 21.8. Found: C, 37.2; H, 3.60; N, 21.8.

REFERENCES

(1) Lieberman, I., *J. Biol. Chem.*, **223**, 327(1956).
(2) Fromm, H. J., *Biochim. Biophys. Acta*, **29**, 255(1958).
(3) Wyngaarden, J. B., and Greenland, R. A., *J. Biol. Chem.*, **238**, 1054(1963).
(4) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, New York, N.Y., 1967, chap. V.
(5) Salser, J. S., Hutchison, D. J., and Balis, M. E., *J. Biol. Chem.*, **235**, 429(1960).
(6) Davidson, J. D., *Cancer Res.*, **20**, 225(1960).
(7) *Reference 4*, chap. I.
(8) Baker, B. R., and Tanna, P. M., *J. Pharm. Sci.*, **54**, 845(1965).
(9) *Ibid.*, **54**, 1774(1965).
(10) Jardetzky, O., *J. Biol. Chem.*, **238**, 2498(1963).
(11) *Reference 4*, chap. II–XII.
(12) Schaeffer, H. J., and Vogel, D., *J. Med. Chem.*, **8**, 507(1965).
(13) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(14) Baker, B. R., and Kawazu, M., *J. Med. Chem.*, **10**, 311 (1967).
(15) Baker, B. R., and Santi, D. V., *ibid.*, **10**, 62(1967).
(16) Baker, B. R., Kawazu, M., Santi, D. V., and Schwan, T. J., *ibid.*, **10**, 304(1967).
(17) Baker, B. R., and Lourens, G. J., *J. Heterocyclic Chem.*, **2**, 344(1965).
(18) Hein, G. E., and Niemann, C., *J. Am. Chem. Soc.*, **84**, 4495(1962).
(19) *Reference 4*, chap. III.
(20) Baker, B. R., and Shapiro, H. S., *J. Pharm. Sci.*, **55**, 308(1966).
(21) Baker, B. R., and Jordaan, J. H., *ibid.*, **54**, 1740 (1965).
(22) *Reference 4*, chap. X.
(23) Leonard, N. J., and Laursen, R. A., *Biochemistry*, **4**, 354, 365(1965).
(24) *Reference 4*, chap. II.
(25) Montgomery, J. A., and Temple, C., Jr., *J. Am. Chem. Soc.*, **83**, 630(1961).
(26) *Ibid.*, **79**, 5239(1957).
(27) Temple, C., Jr., Kussner, C. L., and Montgomery, J. A., *J. Med. Pharm. Chem.*, **5**, 866(1962).

(28) Ikehara, M., Ohtsuka, E., Kitagawa, S., Yagi, K., and Tonomura, Y., *J. Am. Chem. Soc.*, **83**, 2679(1961).
(29) Schaeffer, H. J., and Weimar, R. D., Jr., *ibid.*, **81**, 197(1959).
(30) Robins, R. K., and Lin, H. H., *ibid.*, **79**, 490(1957).
(31) Daly, J. W., and Christensen, B. E., *J. Org. Chem.*, **21**, 177(1956).
(32) Leese, C. L., and Timmis, G. M., *J. Chem. Soc.*, **1958**, 4107.
(33) Leonard, N. J., Carraway, K. L., and Helgeson, J. P., *J. Heterocyclic Chem.*, **2**, 291(1965).
(34) Baker, B. R., Schaub, R. E., and Joseph, J. P., *J. Org. Chem.*, **19**, 638(1954).
(35) Baker, B. R., and Sachdev, H. S., *J. Pharm. Sci.*, **52**, 933(1963).
(36) Townsend, L. B., Robins, R. K., Loeppky, R. N., and Leonard, N. J., *J. Am. Chem. Soc.*, **86**, 5320(1964).
(37) Atkinson, M. R., Morton, R. K., and Murray, A. W., *Biochem. J.*, **92**, 398(1964).
(38) Reiner, J. M., "Behavior of Enzyme Systems," Burgess Publishing Co., Minneapolis, Minn., 1959, pp. 151–152.

Irreversible Enzyme Inhibitors 99

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THYMIDINE PHOSPHORYLASE is an enzyme that catalyzes phosphorolysis of thymidine and related 2′-deoxynucleosides to thymine (1) or the reverse conversion of thymine to thymidine (2), depending upon the stress of genetic or dietary deficiencies upon the cell line (3). The enzyme can also convert the anticancer agent, 5-fluorouracil, to its 2′-deoxyriboside (FUDR) or *vice versa* (3, 4). The chemotherapeutic utility for a tissue-specific inhibitor of thymidine phosphorylase has been previously discussed (5), and initial studies from this laboratory on the mode of binding of inhibitors to this enzyme have been reported (5–10).

It was previously noted that hydrophobic bonding to the enzyme could take place with alkyl or aralkyl groups on uracil at the 1-position (6) or the 5- and 6-positions (7, 8), for example, 6-benzyluracil (XV) was eighteenfold more effective than uracil (7) (Table I). Furthermore, binding of uracils could be made more effective when substituted with electron-withdrawing groups at the 5- or 6-position which increased the acidity of the uracil (9); for example, 5-bromouracil (III) was ninefold more effective than uracil (I). Finally, the best inhibitors were obtained by combining both phenomena, that is, hydrophobic bonding and increased acidity (10); for example, 5-bromo-6-benzyluracil (XVIII) was complexed to the enzyme fortyfold more effectively than the substrate, FUDR, and 150-fold more effectively than uracil (I) (Table I). Therefore, further studies on combinations of hydrophobic groups and electron-withdrawing groups attached to the 5- and 6-positions of uracil were initiated and are the subject of this paper.

Received May 1, 1967, from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, CA 93106
Accepted for publication June 14, 1967.
This work was generously supported by grant CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
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(28) Ikehara, M., Ohtsuka, E., Kitagawa, S., Yagi, K., and Tonomura, Y., *J. Am. Chem. Soc.*, **83**, 2679(1961).
(29) Schaeffer, H. J., and Weimar, R. D., Jr., *ibid.*, **81**, 197(1959).
(30) Robins, R. K., and Lin, H. H., *ibid.*, **79**, 490(1957).
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binding over 6-methyluracil (II); similarly, it has now been observed that the electron-withdrawing methylsulfonyl group at the 6-position (V) gave fifteenfold better binding than the 6-methyl group (II). Therefore, a search for hydrophobic bonding by hydrocarbon groups at the 5-position of 6-(trifluoromethyl)uracil was undertaken.

The first two compounds studied were the 5-(*p*-chlorophenyl) (VII) and 5-phenylbutyl (XI) uracils since they were readily synthesized by deamination of available 2-aminopyrimidines (XIX, XXII) previously prepared in this laboratory (11). The *p*-chlorophenyl substituent of VII give a fourfold increment in binding over 6-(trifluoromethyl)uracil (IV). The 5-phenylbutyl substituent of XI gave a tenfold increment in binding over IV. The intermediate phenylalkyl derivatives (VIII–X) were then synthesized and evaluated; none were as effective as the phenylbutyl substituent (XI). The *n*-amyl substituent at the 5-position (VI) was then investigated; it showed only a twofold increment in binding due to its hydrocarbon interaction with the enzyme. From these studies emerge two problems worthy of pursuit: (*a*) a similar hydrophobic bonding study of 5-substituents on 6-(methylsulfonyl)uracil (V) and (*b*) placement of leaving groups and the benzene ring of VII–XI to convert them to potential active-site-directed irreversible inhibitors (12, 13) of thymidine phosphorylase that could be further modified for isozyme specificity (14).

Since 5-(phenylazo)uracils are readily synthesized by one of two routes and since the phenylazo group should increase acidity of the uracil by its electron-withdrawing power, a series of 5-(phenylazo)uracils with hydrocarbon substituents were investigated. Note that the 5-phenylazo group (XII) on 6-methyluracil (II) gives a threefold increment in binding, which is not as effective an increment as observed with the 5-bromo (III), 6-methylsulfonyl (V), or 6-trifluoromethyl (IV) groups. Replacement of the 6-methyl group of XII by 6-phenyl (XIII) resulted in a loss of binding—a not unexpected result based on the binding ability of 6-phenyluracil previously observed (8). Better hydrophobic interaction was observed with the 6-benzyl (XIV) and 6-(*n*-amyl) (XVI) groups.

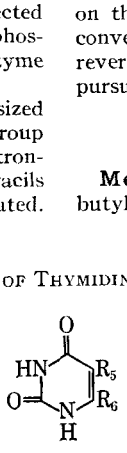
The 6-benzyl group (XIV) on 5-(phenylazo)-uracil gave a fivefold increment in binding due to a hydrophobic interaction; this increment is considerably less than the 37-fold increment between 6-benzyl (XV) and 6-methyluracil (II). Thus it would appear that the large phenylazo group hinders the ability of the benzyl group to assume the proper conformation for optimum binding, as previously observed with 1,5,6-trisubstituted uracils (7). The more flexible *n*-amyl group (XVI) on 5-(phenylazo)uracil gave a 23-fold increment in binding over the 6-methyl group of XII; compared another way, the 5-phenylazo group of XVI gave an eightfold increment in binding compared to the 6-(*n*-amyl)uracil (XVII).

Further studies on placement of a leaving group on the 5-phenyl substituent of XIV and XVI, to convert them to potential active-site-directed irreversible inhibitors (12, 13), would be worthy of pursuit.

CHEMISTRY

Methods—Treatment of the 2-amino-5-phenylbutyl-4-pyrimidinol (XXII) (11) with sodium

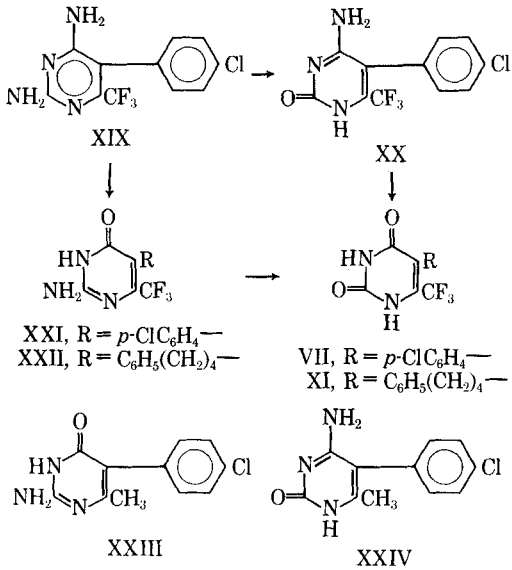
Table I—Inhibition of Thymidine Phosphorylase[a] by



Compd.	R_5	R_6	m*M* Concn. for 50% Inhibition	$[I/S]_{0.5}$[b]
I	H	H	1.5	3.9[c]
II	H	CH_3	3.2	8.0[d]
III	Br	H	0.18	0.45[d]
IV	H	CF_3	0.47	1.2[d]
V[e]	H	SO_2CH_3	0.21	0.52
VI	*n*-C_5H_{11}—	CF_3	0.21	0.52
VII	*p*-ClC_6H_4—	CF_3	0.13	0.32
VIII	$C_6H_5CH_2$—	CF_3	0.30	0.75
IX	$C_6H_5(CH_2)_2$—	CF_3	0.10	0.25
X	$C_6H_5(CH_2)_3$—	CF_3	0.20	0.50
XI	$C_6H_5(CH_2)_4$—	CF_3	0.049	0.12
XII	$C_6H_5N{=}N$—	CH_3	1.3	3.2
XIII	$C_6H_5N{=}N$—	C_6H_5—	~3.[f]	~8.
XIV	$C_6H_5N{=}N$—	$C_6H_5CH_2$—	0.25	0.63
XV	H	$C_6H_5CH_2$—	0.090	0.22[c]
XVI	$C_6H_5N{=}N$—	*n*-C_5H_{11}—	0.055	0.14
XVII	H	*n*-C_5H_{11}—	0.46	1.1[e]
XVIII	Br	$C_6H_5CH_2$—	0.010	0.025[g]

[a] Thymidine phosphorylase was a 45–90% saturated ammonium sulfate fraction from *E. coli* B that was prepared and assayed with 0.4 m*M* 5-fluoro-2′-deoxyuridine (FUDR) in arsenate–succinate buffer (pH 5.9) diluted with 10% dimethylsulfoxide as previously described (5). [b] Ratio of concentration of inhibitor to 0.4 m*M* FUDR giving 50% inhibition. [c] Data from *Reference 8*. [d] Data from *Reference 9*. [e] The authors thank Professor William Prusoff for a sample of this compound. [f] Estimated from 18% inhibition at 0.5 m*M*, the maximum concentration allowing full light transmission. [g] Data from *Reference 10*.

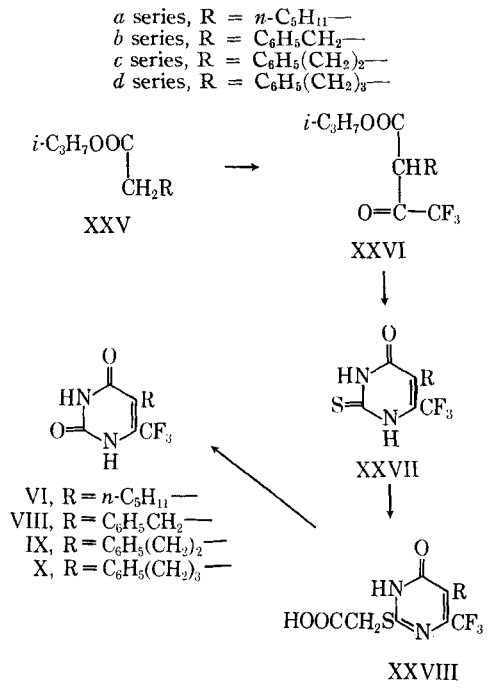
nitrite in glacial acetic acid at room temperature gave a 76% yield of the 5-phenylbutyluracil (XI). Similar treatment of the 5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (XIX) (11) at room temperature did not give the desired uracil derivative (VII), but gave instead an aminopyrimidinol which would have structure XX or XXI. According to Trattner *et al.* (15) treatment of the 6-ethyl analog of XIX with nitrous acid deaminated the 2-amino group; this result was based on comparative ultraviolet data where the 2-amino-4-hydroxypyrimidine showed no shifts between acid and base solution. Later work in this laboratory (16) did not agree with these ultraviolet values—XXIII, synthesized in an unequivocal manner, had an ultraviolet maximum at 15 mμ longer wavelength in alkali than in acid solution, but the 2-hydroxy-4-aminopyrimidine XXIV, also synthesized by an unequivocal method, showed no difference in absorption maximum in acid or base solution. Since the product of deamination of XIX showed a shift of 35 mμ to longer wavelengths in alkali, the 2-amino-4-hydroxypyrimidine structure (XXI) was assigned to this product. These results conflict with those of Trattner *et al.* (15); repetition of their experiments indicated that the product of both hydrochloric acid hydrolysis or nitrous acid deamination was the 2-amino-4-pyrimidinol resulting from attack at the 4-position (17), in agreement with the results of deamination of XIX to XXI. (Scheme I.)



Scheme I

When the reaction of the diaminopyrimidine (XIX) with sodium nitrite was performed at 50° in aqueous acetic–hydrochloric acid, deamination of both amino groups took place to give the desired phenyluracil (VII).

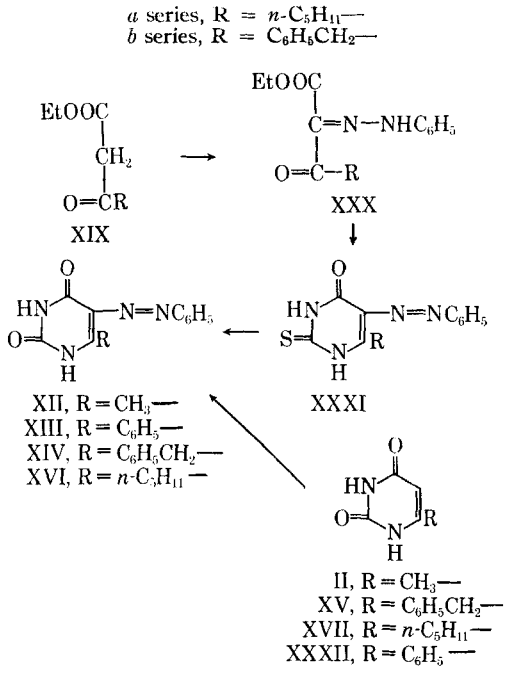
The remaining 5-substituted-6-(trifluoromethyl)-uracils (VI, VIII–X) (Scheme II) were synthesized by a variant of the earlier synthesis (11). The appropriate isopropyl esters (XXV) were condensed with ethyl trifluoroacetate in the presence of sodium hydride; the resultant β-keto esters (XXVI) were then condensed with thiourea to give the 2-mercapto-4-pyrimidinols (XXVII), rather than condensed with guanidine (11). Treatment of XXVII with boiling 5% aqueous chloroacetic acid converted the mercapto group to the carboxymethylthio group (XXVIII), but the thio group did not hydrolyze in the reaction mixture to give the desired uracils (VI, VIII–X), as previously observed with other 5- or 6-substituted-2-thiouracils (8). However, the thioglycolate residue was readily hydrolyzed with aqueous hydrochloric acid. Apparently, the 6-trifluoromethyl group of XXVIII deactivated attack by acid. If it is logically assumed that the pyrimidine ring of XXVIII must be protonated before attack by water occurs, then an acid stronger than 5% aqueous chloroacetic acid would have to be used for protonation since the 6-trifluoromethyl group decreases the basicity of the pyrimidine by about 5 pKa units compared to 6-methyl (11).



Scheme II

6-Methyl-5-(phenylazo)uracil (XII) has been previously synthesized (18) by coupling 6-methyluracil (II) with phenyldiazonium chloride; this reaction was successfully repeated to give a 48% yield of pure material. The 6-phenyl analog (XIII) was also synthesized in this manner in 31% yield of pure material; XIII has been prepared previously by an alternate method (19). The 6-benzyl analog (XIV) was prepared similarly from 6-benzyluracil (XV) in 63% yield. (Scheme III.)

The identical 5-phenylazo-6-benzyluracil (XIV) was also synthesized by an alternate route previously used (19) for the 6-phenyl derivative (XIII) which gave a structurally unequivocal product. Ethyl γ-phenylacetoacetate (XIX*b*) was coupled with phenyldiazonium chloride in aqueous alcohol buffered with sodium acetate; the resultant phenyl-



Scheme III

azo keto ester (XXX*b*) was condensed with thiourea to give the 2-*thio*uracil derivative (XXXI*b*) in good over-all yield. Since hydrolysis of the 2-mercapto group of XXXI*b* with aqueous chloroacetic acid to XIV was unsuccessful, the group was removed by oxidative cleavage with hydrogen peroxide (19) to give XIV in 58% yield that was identical with XIV prepared *via* XV. The remaining 6-(*n*-amyl)-5-phenylazouracil (XVI) was synthesized *via* XXXI*a* since the route starting with 6-(*n*-amyl)uracil (XVII) gave a mixture which was difficult to purify.

EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% alcohol with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF, and spots were detected by visual examination under ultraviolet light.

2 - Amino - 5 - (p - chlorophenyl) - 6 - (trifluoromethyl) - 4 - pyrimidinol (XXI)—*Method C*—To a stirred solution of 58 mg. (2 mmoles) of XIX (11) in 20 ml. of glacial acetic acid cooled in an ice bath was added a solution of 560 mg. (8 mmoles) of $NaNO_2$ in 20 ml. of water at such a rate that the temperature was 2–5°. The solution was then allowed to stand at ambient temperature for about 18 hr. during which time the product separated. The product was collected on a filter and recrystallized from 50% aqueous acetic acid; yield, 300 mg. (52%) of slightly yellow plates, m.p. 326–328°; $\nu_{max.}$ 3470, 3300, 3180 (NH); 1650–1600, 1550 (NH, C=O, C=C, C=N); 1020 (CF_3); 840 cm.$^{-1}$ (p-C_6H_4). See Table II for additional data.

5 - (p - Chlorophenyl) - 6 - (trifluoromethyl)-uracil (VII)—To a stirred solution of 350 mg. (1.2 mmoles) of XIX (11) in 10 ml. of glacial acetic acid and 5 ml. of 10% aqueous hydrochloric acid maintained at 50° was added dropwise a solution of 480 mg. (7 mmoles) of $NaNO_2$ in 10 ml. of water. After standing 3 days at ambient temperature, the mixture was filtered and the product was recrystallized from aqueous ethanol; yield, 120 mg. (34%) of white plates, m.p. 250–251°; $\nu_{max.}$ 3180, 3130, 3030 (NH); 1710, 1680 (uracil C=O); 1600 (C=C); 1000 (CF_3); 830 cm.$^{-1}$ (p-C_6H_4). See Table II for additional data.

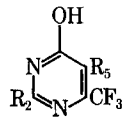
5 - Substituted - 6 - (trifluoromethyl) - 2 - thiouracils (XXVII)—*Method A*—The appropriate α-substituted isopropyl γ,γ,γ-trifluoroacetoacetate (XXVI) was prepared by condensation of XXV with ethyl trifluoroacetate as previously described for XXVI, R = $C_6H_5(CH_2)_4$ (11). A mixture of 30 mmoles of XXVI, 50 ml. of *tert*-butyl alcohol, 30 mmoles of thiourea, and 30 mmoles of solid sodium methoxide was refluxed with magnetic stirring for about 18 hr., then spin evaporated *in vacuo*. The residue was dissolved in water, the solution was clarified by filtration, then acidified with acetic acid. The product was collected on a filter, washed with water, then recrystallized from aqueous ethanol. See Table II for additional data.

5 - Substituted - 6 - (trifluoromethyl)uracils—*Method B*—A mixture of 3 mmoles of XXVII and 20 ml. of 5% aqueous chloroacetic acid was refluxed for 5 hr.; the intermediate (XXVIII) which could be isolated at this point, was further hydrolyzed by addition of 10 ml. of 10% aqueous hydrochloric acid and by reflux for another 5 hr. The mixture was cooled, the product was collected on a filter, washed with water, then crystallized from aqueous ethanol. See Table II for additional data.

6 - Substituted - 5 - (phenylazo)uracils—*Method D*—A solution of 0.93 Gm. (10 mmoles) of aniline in 5.0 ml. of 12 *N* aqueous HCl was diazotized at −5 to 0° in the usual fashion with a solution of 0.69 Gm. (10 mmoles) of $NaNO_2$ in 5 ml. of water. This diazonium solution was added to a stirred and recooled solution of 10 mmoles of XXXII or XVII in 150 ml. of 2 *N* aqueous NaOH at such a rate that the temperature was 0–5° (in some cases, such as with XXXII, it was necessary to add about 50 ml. more of water to dissolve the uracil prior to addition). After being stirred for an additional 3 hr. in the ice bath, the solution was acidified with acetic acid. The product was collected on a filter and washed thoroughly with water, then recrystallized from aqueous dioxane. See Table III for additional data.

Ethyl α - Phenylazo - γ - phenylacetoacetate (XXXb)—To a stirred solution of 4.12 Gm. (20 mmoles) of XXIX*b* (20) in 72 ml. of ethanol cooled in an ice bath was added a solution of 10.7 Gm. (0.13 mole) of anhydrous sodium acetate in 25 ml. of water; water was then added dropwise until the separated sodium acetate dissolved. Then the diazonium solution from 1.86 Gm. (20 mmoles) of aniline (see under *Method D*) was added dropwise with vigorous stirring, maintaining the temperature at 0–5°. After the addition of 25 ml. more of water, the mixture was stirred in the ice bath for another hour. The product was collected on a filter and washed with cold 50% alcohol; yield, 5.4 Gm.

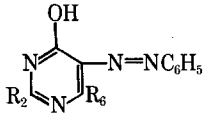
TABLE II—PHYSICAL CONSTANTS OF



Compd.[a]	R_2	R_5	Method	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found	λmax. (mμ) pH 6	λmax. (mμ) pH 13
VI	OH	*n*-C_5H_{11}—	*B*	80	157–158	C, 48.0 H, 5.24 N, 11.2	C, 47.9 H, 5.27 N, 11.3	280	303
VII	OH	*p*-ClC_6H_4—		34	250–251	C, 45.6 H, 2.08 N, 9.68	C, 45.5 H, 2.00 N, 9.42	272[c]	302
VIII	OH	$C_6H_5CH_2$—	*B*	40	207–208	C, 53.4 H, 3.36 N, 10.4	C, 53.5 H, 3.33 N, 10.2	270	304
IX	OH	$C_6H_5(CH_2)_2$—	*B*	47	233–234	C, 54.9 H, 3.89 N, 9.85	C, 54.7 H, 3.99 N, 10.1	270	304
X	OH	$C_6H_5(CH_2)_3$—	*B*	53	148–149	C, 56.3 H, 4.39 N, 9.39	C, 56.3 H, 4.40 N, 9.23	273	305
XI	OH	$C_6H_5(CH_2)_4$—	*C*	76	123–124	C, 57.7 H, 4.84 N, 8.98	C, 57.6 H, 4.91 N, 9.15	270	303
XXI	NH_2	*p*-ClC_6H_4—	*C*	52	326–328	C, 45.5 H, 2.77 N, 14.2	C, 45.7 H, 2.61 N, 14.2	280[c]	315
XXVII*a*	SH	*n*-C_5H_{11}—	*A*	49	168–169	C, 45.4 H, 4.93 N, 10.6	C, 45.4 H, 4.96 N, 10.5	263 330	263 308
XXVII*b*	SH	$C_6H_5CH_2$—	*A*	25	205–206	C, 50.3 H, 3.16 N, 9.79	C, 50.0 H, 2.99 N, 9.69	270 315	265 308
XXVII*c*	SH	$C_6H_5(CH_2)_2$—	*A*	10[d]	204–205	C, 52.0 H, 3.66 N, 9.33	C, 52.0 H, 3.74 N, 9.41	262 328	262 323
XXVII*d*	SH	$C_6H_5(CH_2)_3$—	*A*	6[d]	147–149	C, 53.5 H, 4.17 N, 8.93	C, 55.3 H, 4.39 N, 8.65	273 315	263 320

[a] All compounds had infrared spectra in agreement with their assigned structures and moved as a single spot on TLC. [b] See under *Experimental*. [c] At pH 1. [d] Over-all yield from XXV.

TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	R_2	R_6	Method	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found	λmax. (mμ) pH 6	λmax. (mμ) pH 13
XII	OH	CH_3—	*D*	48	256–257[b]			338	355
XIII	OH	C_6H_5—	*D*	31[d]	230–233[c,d]			277 348	279 368
XIV	OH	$C_6H_5CH_2$—	*D*	63	221–223	C, 65.8[e] H, 4.93 N, 17.1	C, 65.8 H, 4.92 N, 17.2	288 343	295 364
XIV	OH	$C_6H_5CH_2$—	*F*	58	221–223			288 343	295 364
XVI	OH	*n*-C_5H_{11}—	*F*	52	148–149	C, 60.4[f] H, 6.48 N, 18.9	C, 60.3 H, 6.52 N, 18.8	258 338	290 358
XXXI*a*	SH	*n*-C_5H_{11}—	*E*	96	169–170	C, 58.5[g] H, 6.05 N, 18.1	C, 58.6 H, 6.06 N, 18.1	290 370	395
XXXI*b*	SH	$C_6H_5CH_2$—	*E*	60	189–190	C, 63.4 H, 4.35 N, 17.4	C, 63.2 H, 4.27 N, 17.1	287 380	395

[a] All compounds had infrared spectra in agreement with their assigned structures and moved as a single spot on TLC. [b] Lit. (18) m.p. 253–255°. [c] Lit. (19) m.p. 218–225°. [d] Recrystallized from 80% ethanol, then ethyl acetate. [e] Calcd. with 0.25 mole dioxane. [f] Calcd. with 2/3 mole water. [g] Calcd. with 1/3 mole water.

(87%), m.p. 58–59°. Recrystallization from aqueous ethanol gave yellow crystals, m.p. 58–59°; $\lambda_{max.}$ (EtOH): 242, 362 mμ; (pH 13): 242, 370 mμ.

Anal.—Calcd. for $C_{18}H_{18}N_2O_3$: C, 69.7; H, 5.81; N, 9.03. Found: C, 69.5; H, 5.84; N, 8.91.

Similarly, XXX*a* was obtained as an oil that was isolated by ether extraction in 37% yield.

6 - Benzyl - 5 - phenylazo - 2 - thiouracil (XXXIb)—*Method E*—A mixture of 3.10 Gm. (10 mmoles) of XXX*b*, 1.10 Gm. (14 mmoles) of thiourea, and 1.08 Gm. (20 mmoles) of solid sodium methoxide in 20 ml. of methanol was refluxed with magnetic stirring for about 18 hr., then processed as under *Method A*. Recrystallization from ethanol gave 1.9 Gm. (60%) of yellow crystals, m.p. 189–190°. See Table III for additional data.

6 - Benzyl - 5 - (phenylazo)uracil (XIV)—*Method F*—To a stirred solution of 1.61 Gm. (5 mmoles) of XXXI*b* in 20 ml. of 2 *N* aqueous NaOH cooled in an ice bath was added dropwise 20 mmoles of dilute aqueous hydrogen peroxide; the deep red solution became bright yellow during the addition. The solution was then stirred for 2 hr. with the ice bath removed; occasionally the solution was cooled to keep the temperature at 27–30°. The solution was acidified with aqueous HCl, then the product was collected on a filter. After being washed with water and ice cold alcohol, the product was recrystallized from aqueous dioxane; yield, 0.90 Gm. (58%) of red needles. See Table III for additional data.

REFERENCES

(1) Friedkin, M., and Roberts, D., *J. Biol. Chem.*, **207**, 245(1954).
(2) *Ibid.*, **207**, 257(1954).
(3) Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J., *Proc. Natl. Acad. Sci. U. S.*, **44**, 1004(1958).
(4) Birnie, G. D., Kroeger, H., and Heidelberger, C., *Biochemistry*, **2**, 566(1963).
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(7) Baker, B. R., Kawazu, M., Santi, D. V., and Schwan, T. J., *ibid.*, **10**, 304(1967).
(8) Baker, B. R., and Kawazu, M., *ibid.*, **10**, 311(1967).
(9) *Ibid.*, **10**, 313(1967).
(10) *Ibid.*, **10**, 316(1967).
(11) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).
(12) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(13) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N. Y., 1967.
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(15) Trattner, R. B., Elion, G. B., Hitchings, G. B., and Sharefkin, D. M., *J. Org. Chem.*, **29**, 2674(1964).
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(19) Bamberg, P., Hemmerich, P., and Erlenmeyer, H., *Helv. Chim. Acta*, **43**, 395(1960).
(20) Baker, B. R., Santi, D. V., and Shapiro, H. S., *J. Pharm. Sci.*, **53**, 1317(1964).

Irreversible Enzyme Inhibitors 100

Inhibitors of Thymidine Phosphorylase 8. 6-(*p*-Bromoacetamidobenzyl)uracil, an Active-Site-Directed Irreversible Inhibitor

By B. R. BAKER and MITSUTAKA KAWAZU

Four candidate active-site-directed irreversible inhibitors for thymidine phosphorylase have been synthesized from 6-benzyluracil (I). Of these four, only 6-(*p*-bromoacetamidobenzyl)uracil (III) inactivated the enzyme; since neither iodoacetamide nor the *m*-isomer (VI) of III inactivated the enzyme under the conditions used for III, the inactivation by III most probably proceeds by a neighboring group reaction within the enzyme–inhibitor complex—the so-called active-site-directed mechanism.

PREVIOUS PAPERS in this series have described the mode of uracil binding (1, 2) to thymidine phosphorylase. Hydrophobic bonding to this enzyme by uracil derivatives has also been detected (2–6) and the mode of binding has been summarized (7). An effective inhibitor of this enzyme was 6-benzyluracil (I) (5), which was complexed 4.5-times better than the substrate, 5-fluoro-2′deoxyuridine; increasing the acidity of the uracil system by introduction of a 5-bromo group (II) gave a further ninefold increment in binding (6), II being complexed fortyfold better than the substrate. Therefore, 6-benzyluracil (I) and its 5-bromo derivative (II) were considered logical structures to be further modified for con-

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Accepted for publication June 13, 1967.
This work was generously supported by grant CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
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Irreversible Enzyme Inhibitors 100

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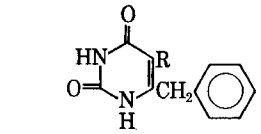

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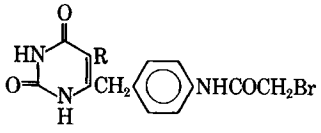
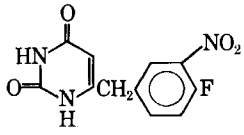
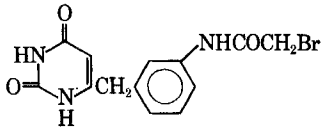
II, R = Br



III, R = H
IV, R = Br



V



VI

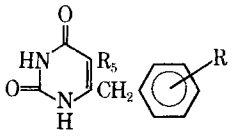
version to potential active-site-directed irreversible inhibitors (8, 9) of thymidine phosphorylase. The *p*-bromoacetamido derivatives (III and IV) of I and II were selected for initial study; also selected were the 3′-nitro-4′-fluoro (V) and the *m*-bromoacetamido (VI) derivatives of 6-benzyluracil (I). The enzyme results with these compounds and their intermediates are the subject of this paper.

DISCUSSION

Since a number of new intermediates were available from this study, they were investigated for reversible inhibition in order to shed further light on the anomalous binding of 5-bromo-6-(*p*-nitrobenzyl) uracil (VIII) (6). It was previously noted that the 4.5-fold enhancement in binding by 5-bromo group of II and the sevenfold enhancement of the *p*-nitro group of VII were not additive (Table I); in fact, VIII was considerably less effective than II or VII (6).

It was previously proposed (6) that either the nitro group of VII had an electronic effect on the binding of the benzyl moiety or that the nitro group was itself complexed directly to the enzyme. Since the 3-nitro group of IX has even a more beneficial effect on binding than the 4-nitro group of VII, it is clear that the nitro group of VII cannot be binding directly to the enzyme—else it would be position sensitive. Therefore, an electronic effect is much more likely. The evidence previously cited against an electronic effect by the nitro group of VII was the fact that a *p*-sulfonamide group was detrimental to binding (6). This difference can now be attributed to a difference in the hydrophilic character of

TABLE I—REVERSIBLE INHIBITION OF THYMIDINE PHOSPHORYLASE[a] BY



Compd.	R_5	R	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$
I	H	H	90	0.22[c]
II	Br	H	10	0.025[d]
III	H	4-$BrCH_2CONH$-	26	0.065
IV	Br	4-$BrCH_2CONH$-	950[e]	2.4
V	H	3-NO_2-4-F	35	0.088
VI	H	3-$BrCH_2CONH$-	60	0.15
VII	H	4-NO_2	13	0.033[c]
VIII	Br	4-NO_2	67	0.17[d]
IX	H	3-NO_2	3.4	0.0085
X	H	4-CH_3CONH-	670	1.7
XI	H	3-NO_2-4-CH_3CONH-	30	0.075
XII	H	3-NO_2-4-NH_2	2.5	0.0063
XIII	H	F	51	0.13
XIV	Br	3-NO_2-4-CH_3CONH-	420	1.1
XV	Br	4-CH_3CONH-	1200[f]	3.0
XVI	H	4-NH_2	150	0.37[c]
XVII	Br	4-NH_2	11	0.028[d]
XVIII	Br	3-NO_2-4-NH_2	3.2	0.0080

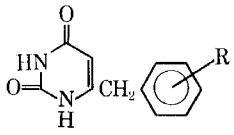
[a] Thymidine phosphorylase was a 45–90% ammonium sulfate fraction prepared from *E. coli* B and assayed with 400 μM FUDR in arsenate–succinate buffer (pH 5.9) in the presence of 10% dimethylsulfoxide as previously described (15). [b] The ratio of the concentration of inhibitor to 400 μM FUDR giving 50% inhibition. [c] Data previously reported (5). [d] Data previously reported (6). [e] Estimated from 30% inhibition at 400 μM, the maximum concentration still allowing light transmission. [f] Estimated from 31% inhibition at 500 μM, the maximum concentration still allowing light transmission.

the nitro group and sulfonamide groups with Hansch π-constants of +0.24 (to −0.28) and −1.82, respectively (10). That a polar group on the benzene is detrimental to binding is indicated by the seven- to eightfold loss in binding when the polar 4-acetamido group is placed on I or IX to give X or XI, respectively. Conversely, if a polar group on the benzyl group is detrimental to binding, then a hydrocarbon group could enhance binding; the latter observation has now been made (11).

That nitration of the benzyl group could enhance binding by 16–60-fold was noted in four out of five cases; compare I and VII, I and IX, XVI and XII, and X and XI where enhancement was good, but in contrast 6-(4-fluorobenzyl)uracil (XIII) showed little change in binding on nitration to V.

The introduction of a 5-bromo group had one of three effects in the seven pairs of compounds that were studied, a loss in binding, a gain in binding, or little change. A 9–14 gain in binding with II and XVII was observed only with the parent benzyluracil (I) or when the phenyl ring was substituted by an electron-donating amino group (XVI). The opposite effect—a loss in binding—was noted when the benzene ring was substituted by electron withdrawing groups; compare the pairs VII and VIII, XI and XIV, and III and IV. Little change in binding was noted with the electronically neutral acetamido group and with a benzene ring containing

TABLE II—IRREVERSIBLE INHIBITION OF THYMIDINE PHOSPHORYLASE[a] BY



Compd.	R	μM Concn. for 50% Reversible Inhibition[b]	μM Incubation Concn.	Time, hr.	% Inactivation
III	4-$BrCH_2CONH$-	26	100	1	24
			100	2	36
			26	2	25
			26	2	25
V	3-NO_2-4-F	35	75	1	0
VI	3-$BrCH_2CONH$-	60	120	2	0
Iodoacetamide		Large	100	2	0

[a] See *Experimental* for inactivation procedure performed at 37°. [b] Data from Table I.

both the electron withdrawing 3-nitro and electron-donating 4-amino substituents; compare the pairs X and XV, and XII and XVIII.

Since 5-bromo-(6-*p*-bromoacetamidobenzyl)uracil (IV) was such a poor reversible inhibitor (Table I), it was not investigated as an irreversible inhibitor. Of the other three candidate irreversible inhibitors, III, V, and VI, only 6-(*p*-bromoacetamidobenzyl)-uracil (III) showed inactivation of the enzyme (Table II). At a concentration of 100 μM, III showed 24% inactivation of the enzyme in 1 hr. and 36% inactivation in 2 hr. In contrast, an equal concentration of iodoacetamide run simultaneously showed no inactivation of the enzyme in 2 hr.; this latter result clearly demonstrates that III does not inactivate the enzyme by a random bimolecular process with no specificity, but supports the suggestion that III inactivates the enzyme within a reversible enzyme–inhibitor complex by the active-site-directed mechanism (12, 13). That the active-site-directed mechanism was operable for irreversible inhibition of thymidine phosphorylase by III was further supported by the observation that the *m*-bromoacetamido isomer (VI) showed no inactivation of the enzyme.

Further studies on inhibition of thymidine phosphorylase by 6-substituted uracils are continuing (11) to determine if additional hydrophobic bonding can be obtained by substitution of hydrocarbon groups on the 6-benzyl group of I or related compounds and to determine if faster active-site-directed irreversible inhibitors can be found before tissue specificity (9) studies are started.

CHEMISTRY

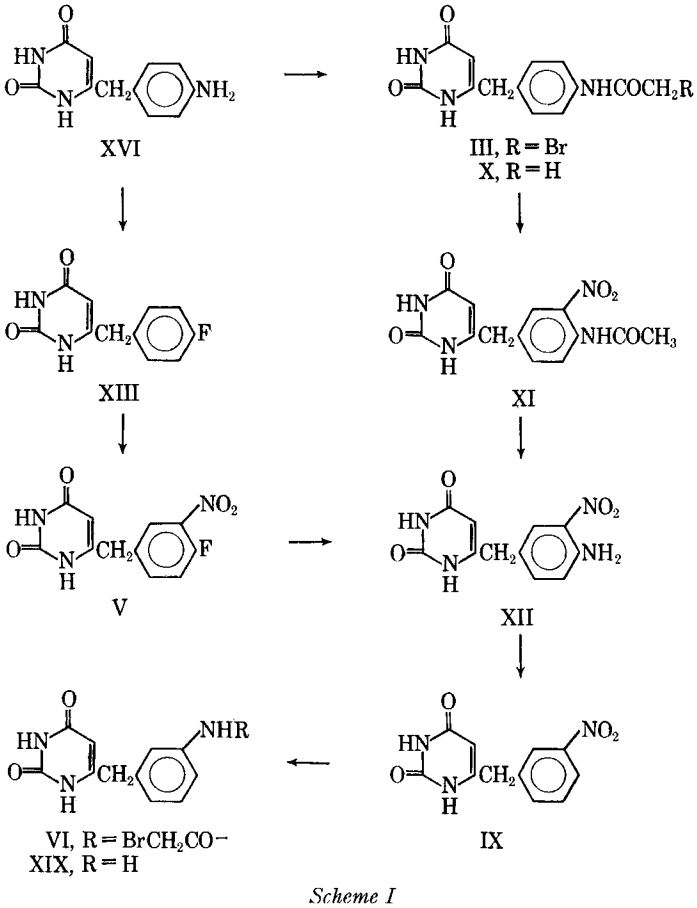
Conversion of 6-benzyluracil (I) to its *p*-amino derivative (XVI) by nitration and hydrogenation has been previously described (5). Reaction of XVI in *N*,*N*-dimethylformamide with bromoacetic anhydride at 0° proceeded smoothly to the bromoacetamide (III) which was uniform on TLC and showed the proper color reactions (14). Similarly, bromoacetylation of 6-(*p*-aminobenzyl)-5-bromouracil (XVII) (6) afforded the 5-bromo bromoacetamide (IV) (Table I).

Attempts to prepare 6-(*m*-aminobenzyl)uracil (XIX) by a primary synthesis starting with *m*-nitro- or *m*-acetamidophenylacetyl chloride were unsuccessful; the condensation of the acid chlorides with ethyl sodioacetoacetate gave black, resinous mixtures. Therefore, attention was turned to the conversion of 6-(*p*-aminobenzyl)uracil (XVI) to XIX by classical benzene chemistry. (Scheme I.)

Acetylation of XVI with acetic anhydride in *N*,*N*-dimethylformamide at 20° gave the *p*-acetamido derivative (X). Nitration of X with a mixture of 70% nitric acid and 96% sulfuric acid at 10–25° proceeded smoothly to the mononitro derivative (XI). That the nitro group had not entered the 5-position of the uracil was shown by nearly identical ultraviolet absorption of X and XI. The *N*-acetyl group of XI was removed with boiling 3 *N* aqueous hydrochloric acid to give a good yield of the nitroaniline derivative (XII). Diazotization of the aryl amino group of XII proceeded with difficulty due to a combination of poor reactivity and poor solubility; diazotization was eventually achieved, although poorly, by using 75% aqueous *N*,*N*-dimethylformamide. The resultant diazonium salt was then reduced *in situ* with hypophosphorus acid. The resultant 6-(*m*-nitrobenzyl)uracil (IX) was contaminated with two other minor products, but could be isolated in 21% yield by preparative TLC. Hydrogenation of IX to the *m*-aminobenzyl uracil (XIX) with a platinum oxide catalyst, followed by reaction with bromoacetic anhydride in *N*,*N*-dimethylformamide afforded the desired 6-(*m*-bromoacetamidobenzyl)uracil (VI).

Attempts to synthesize the 3-nitro-4-fluorobenzyl derivative (V) by diazotization of XII in 50% fluoboric acid with or without *N*,*N*-dimethylformamide as a solvent were unsuccessful due to the difficulty of diazotization. The desired V was then synthesized by an alternate method. Diazotization of 6-(*p*-aminobenzyl)uracil (XVI) in dilute aqueous fluoboric acid gave an insoluble diazonium fluoborate that was converted to XIII in 23% yield by heating at 150°. Nitration of XIII at 40° in a mixture of nitric and sulfuric acids gave V; that the nitro group had entered *ortho* to the fluorine atom was shown by conversion of V to XII with ammonia in *N*,*N*-dimethylformamide at 100°.

The 5-bromouracil derivatives, XIV and XV (Table I), were prepared by bromination of the corresponding uracil derivatives, XI and X, respectively. Hydrolysis of XIV with hot 3 *N* aqueous



Scheme I

hydrochloric acid afforded 6-(4′-amino-3′-nitrobenzyl)-5-bromouracil (XVIII).

EXPERIMENTAL

Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Ultraviolet spectra were determined in 10% ethanol (unless otherwise indicated) with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. All analytical samples gave a single spot on thin-layer chromatography (TLC) on Brinkmann Silica Gel GF when spots were detected by visual examination under ultraviolet light.

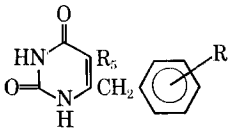
6-(p-Bromoacetamidobenzyl)uracil (III)—*Method A*—To a stirred solution of 540 mg. (2.64 mmoles) of XVI (5) in 5 ml. of *N,N*-dimethylformamide cooled in an ice bath was added 650 mg. (2.7 mmoles) of bromoacetic anhydride. The mixture was stirred at ambient temperature for 4 hr., then diluted with 5 ml. of water. The product was collected on a filter and recrystallized from aqueous *N,N*-dimethylformamide; yield, 420 mg. (50%) of white needles, m.p. 251–252° dec.; $\nu_{max.}$ 3400, 3140, 3000 (NH); 1700, 1680, 1600, 1530 (C=O, NH, C=C); 840 cm.$^{-1}$ (*p*-C_6H_4). See Table III for analytical data. The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen and a negative Bratton-Marshall test for aromatic amine (14).

6-(p-Fluorobenzyl)uracil (XIII)—A solution of 1.08 Gm. (5.26 mmoles) of XVI (5) in 100 ml. of water and 3 ml. of 50% fluoboric acid was cooled in an ice bath with magnetic stirring, then treated dropwise with a solution of 0.40 Gm. (5.8 mmoles) of $NaNO_2$ in 10 ml. of water at such a rate that the temperature was 3–5°. After an additional hour in the ice bath, the mixture was filtered and the diazonium fluoborate was washed with ice water, then dried *in vacuo;* 1.2 Gm. of white solid showing an N≡N band at 2280 cm.$^{-1}$ was obtained.

The diazonium salt was heated in a bath at 150° until gas evolution was essentially complete. The residue was recrystallized from ethanol; yield, 0.25 Gm. (23%) of white crystals, m.p. 274–276° dec.; $\nu_{max.}$ 3350, 3150, 3050 (NH); 1710, 1650, 1610 (NH, C=O, C=C); 1210 (C—F); 830 cm.$^{-1}$ (*p*-C_6H_4). See Table III for additional data.

6-(p-Chlorobenzyl)uracil (XX)—A solution of 1.27 Gm. (6.2 mmoles) of XVI (5) in 150 ml. water and 5 ml. of 12 *N* hydrochloric acid was cooled in an ice bath with stirring, then treated dropwise with a solution of 0.38 Gm. (5.5 mmoles) of $NaNO_2$ in 10 ml. of water at such a rate that the temperature was 3–5°. After being stirred an additional 10 min., the solution was treated with 0.5 Gm. of Cu_2Cl_2. The mixture was stirred an additional 2 hr. in the ice bath, then the product was collected on a filter and washed with water. Recrystallization from ethanol gave 0.38 Gm. (34%), of white plates, m.p. 257–259° dec.; $\nu_{max.}$ 3400, 3100, 3000 (NH);

TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	R_5	R	Method	Yield,[b] %	M.p. °C., dec.	Anal. Calcd.	Anal. Found	$\lambda_{max.}$ (10% EtOH) pH 6	$\lambda_{max.}$ (10% EtOH) pH 13
III	H	4-$BrCH_2CONH$-	*A*	50[c,d]	251–252	C, 46.2 H, 3.58 N, 12.4	C, 46.3 H, 3.72 N, 12.4	265	270
IV	Br	4-$BrCH_2CONH$-	*A*	43[c,d]	252–253	C, 37.4 H, 2.68 N, 10.1	C, 37.6 H, 2.75 N, 10.2	275	256 (sh) 290 (sh)
V	H	3-NO_2-4-F	*B*[e]	22[d]	280–281	C, 49.8 H, 3.04 N, 15.8	C, 50.0 H, 3.12 N, 15.7	263	283
VI	H	3-$BrCH_2CONH$-	*A*[f]	22[c,d]	269–271	C, 43.8[g] H, 3.96	C, 43.6 H, 3.60	270	285
IX	H	3-NO_2	Exptl.	21	288–291	C, 53.4 H, 3.67 N, 17.0	C, 53.3[h] H, 3.93 N, 16.9	267	283
X	H	4-CH_3CONH-	*A*[i]	49[d]	297–300	C, 60.2 H, 5.02 N, 16.2	C, 60.5 H, 5.21 N, 16.0	264	288
XI	H	3-NO_2-4-CH_3CONH-	*B*	71[j]	311–313	C, 51.3 H, 3.98 N, 18.4	C, 51.2 H, 4.07 N, 18.1	262	285
XII	H	3-NO_2-4-NH_2	*C*	79[k]	298–300	C, 50.4 H, 3.85 N, 21.5	C, 50.3 H, 3.90 N, 21.4	260	286
XIII	H	4-F	Exptl.	23[l]	274–276	C, 60.0 H, 4.13 N, 12.7	C, 60.1 H, 4.28 N, 12.6	265	288
XIV	Br	3-NO_2-4-CH_3CONH-	*D*[m]	68	257–258	C, 40.8 H, 2.89 N, 14.6	C, 40.8 H, 3.07 N, 14.5	280	301
XV	Br	4-CH_3CONH-	*D*	64	>360	C, 46.1 H, 3.58 N, 12.4	C, 46.1 H, 3.56 N, 12.2	250 (sh) 275	245 (sh) 295
XVI	Br	3-NO_2-4-NH_2	*C*	88[k]	>330	C, 38.7 H, 2.66 N, 16.4	C, 38.7 H, 2.84 N, 16.2	260	292
XX	H	4-Cl	Exptl.	34[l]	257–259	C, 55.8 H, 3.80 N, 11.8	C, 55.6 H, 3.94 N, 11.7	263	288

[a] All compounds were uniform on TLC and had infrared spectra compatible with their assigned structures. [b] Yield of analytically pure material. [c] The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen and a negative Bratton-Marshall test for aromatic amine (14). [d] Recrystallized from aqueous *N,N*-dimethylformamide. [e] Reaction performed at 40°. [f] See *Experimental* also. [g] Monohydrate. [h] Corrected for 2% ash from silica gel. [i] 30% excess of acetic anhydride employed at 20°. [j] Recrystallized from dimethylsulfoxide–ethanol. [k] Recrystallized from *N,N*-dimethylformamide–ethanol. [l] Recrystallized from ethanol. [m] Reaction performed at 80° in suspension. Solution occurred in 20 min. and the product was isolated by cooling.

1720, 1650 (uracil); 845 cm.$^{-1}$ (*p*-C_6H_4). See Table III for additional data.

6-(4′-Acetamido-3′-nitrobenzyl)uracil (XI)—*Method B*—To a stirred mixture of 10 ml. of 70% nitric acid and 10 ml. of 96% sulfuric acid cooled in an ice bath was added portionwise 3.0 Gm. (14.5 mmoles) of X at such a rate that temperature was 7–10°. After being stirred at ambient temperature for 1 hr., the mixture was poured on ice. The product was collected on a filter and washed with water, then ethanol. Recrystallization from hot dimethylsulfoxide by addition of ethanol gave 2.5 Gm. (71%) of yellow prisms, m.p. 311–313° dec.; $\nu_{max.}$ 3350, 3080, 3000 (NH); 1730, 1700, 1640, 1500 (NH, C=O, C=C, NO_2); 1330 cm.$^{-1}$ (NO_2). See Table III for additional data.

6-(4′-Amino-3′-nitrobenzyl)uracil (XII)—*Method C*—A mixture of 2.50 Gm. (10 mmoles) of XI and 30 ml. of 3 *N* aqueous hydrochloric acid was refluxed for about 18 hr., then spin evaporated *in vacuo*. The residue was recrystallized by solution in hot *N,N*-dimethylformamide, then addition of ethanol; yield, 1.5 Gm. (79%) of yellow crystals, m.p. 298–300° dec.; $\nu_{max.}$ 3450, 3300, 3150, 3000 (NH); 1700, 1650, 1570, 1500 (NH, C=C, C=O, NO_2); 1340 cm.$^{-1}$ (NO_2). See Table III for additional data.

This compound was also prepared by treatment of V with *N,N*-dimethylformamide saturated with ammonia at 100° in a Parr bomb for 9 hr. Recrystallization from water gave yellow crystals, m.p. 297–299° dec., that were identical with the preceding preparation.

6-(3′-Nitrobenzyl)uracil (IX)—A warm solution

of 1.7 Gm. (6.8 mmoles) of XII in 75 ml. of *N,N*-dimethylformamide and 1.5 ml. of 12 *N* aqueous hydrochloric acid was cooled in an ice bath with magnetic stirring, then treated with a solution of 0.55 Gm. (8 mmoles) of sodium nitrite in 20 ml. of water at such a rate that the temperature was 3–5°. After being stirred an additional 10 min. in the ice bath, the mixture was treated dropwise with 10 ml. of 10% hypophosphorus acid at such a rate that the temperature was 3–5°. After being stirred for 5 hr. in the ice bath, the mixture was stirred at ambient temperature for about 18 hr. The mixture was filtered, and the filtrate was diluted with 150 ml. of water. The product was collected on a filter and washed with water, then ethanol. The crude product was recrystallized from aqueous *N,N*-dimethylformamide; the resultant solid showed 3 spots on TLC. This product was further purified by preparative TLC on eight 20 × 20-cm. plates by the technique previously described (4). The major product, appearing in the second zone, was eluted with acetone; yield, 0.31 Gm. (21%) of yellow crystals, m.p. 288–291° dec. See Table III for additional data.

6-(3′-Bromoacetamidobenzyl)uracil (VI)—A suspension of 120 mg. (0.51 mmole) of IX in 50 ml. of glacial acetic acid was shaken with hydrogen at 2–3 Atm. in the presence of 50 mg. of platinum oxide catalyst; reduction was complete in 30 min. The filtered solution was spin evaporated *in vacuo*. The residue was dissolved in 5 ml. of 3 *N* aqueous hydrochloric acid. Spin evaporation *in vacuo* gave a residue of XIX hydrochloride that was recrystallized from 90% ethanol; yield, 70 mg. (54%) of XIX hydrochloride as white prisms, m.p. 279–281° dec.; $\nu_{max.}$ 3400 (NH); 2570, 2350, 2200 (NH^+); 1700, 1660, 1645, 1600–1500 (NH, C=O, C=C); 718 $cm.^{-1}$ (*m*-C_6H_4); $\lambda_{max.}^{H_2O}$ (pH 6): 279 mμ; (pH 13): 293 mμ.

Treatment of IX with bromoacetic anhydride by method *A* gave VI as off-white prisms, m.p. 269–271° dec., that was uniform on TLC, gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen, and a negative Bratton-Marshall test for aromatic amine (14). See Table III for additional data.

6-(p-Acetamidobenzyl)-5-bromouracil (XV)—*Method D*—To a solution of 300 mg. (1.2 mmoles) of X in 30 ml. of glacial acetic acid at 60° was added 200 mg. (1.25 mmoles) of bromine. Orange crystals rapidly separated. The mixture was cooled, then the product was collected on a filter and washed with ethanol. Recrystallization from aqueous *N,N*-dimethylformamide gave 250 mg. (54%) of white needles, m.p. above 360°; $\nu_{max.}$ 3400, 3250, 3000 (NH); 1700, 1650, 1620, 1560 (NH, C=C, C=O); 830 $cm.^{-1}$ (*p*-C_6H_4). See Table III for additional data.

ENZYME ASSAYS

Thymidine Phosphorylase—A 45–90% ammonium sulfate fraction from *E. coli* B was prepared as previously described (15). The final volume from 40 Gm. of frozen cells was 47 ml. in 0.05 *M* Tris buffer, pH 7.4.

Reversible Inhibition—Reversible inhibition of thymidine phosphorylase was performed as previously described with 0.4 m*M* 5-fluoro-2′-deoxyuridine (FUDR) as substrate in succinate–arsenate buffer (pH 5.9) containing 10% dimethylsulfoxide.

Irreversible Inhibition—The rate o cleavage of FUDR was previously observed to be proportional to the enzyme concentration when 0.4 m*M* FUDR was employed in the usual manner (15) as long as the total reaction did not exceed 0.5 absorbance units. The enzyme in pH 7.4 Tris buffer in the presence of 10% dimethylsulfoxide was stable over the 2-hr. incubation period at 37°. The inactivation experiments were performed as follows.

Incubation—In two tubes were placed 125 μl. of enzyme solution in 0.05 *M* Tris buffer (pH 7.4) and 0.55 ml. of deionized water in a 37° bath. After 5 min., 75 μl. of dimethylsulfoxide was added to tube 1 and 75 μl. of dimethylsulfoxide containing the inhibitor was added to tube 2. The contents were mixed, the time noted, then a 0.38-ml. aliquot was withdrawn from each tube as rapidly as possible and stored in an ice bath until ready for assay. The aliquot from the enzyme control was labeled C_1; the aliquot from the inhibitor tube was labeled I_1. The remainder in the two tubes (labeled C_2 and I_2) were then kept at 37° for the specified incubation time, then cooled in an ice bath until ready for assay.

Assay—An assay mix was prepared from 1.50 ml. of 4 m*M* FUDR, 7.50 ml. of 0.2 *M* succinate–arsenate buffer (pH 5.9), and 4.50 ml. of water; a larger amount can be made up if many assays are to be done, since the solution is stable at room temperature.

Three pairs of tubes are necessary to assay each time point for each solution; the C_1 and I_1 sets can be made up for assay during the 1–2-hr. incubation period. The back tube of each set serves as a zero time tube. In each back tube were placed 0.45 ml. of assay mix and 300 μl. of 3 *M* aqueous potassium hydroxide; the contents were mixed, then 50 μl. of C_1 incubation was added and the contents mixed again. In the three front tubes were placed 0.45 ml. of assay mix and 50 μl. of C_1 incubation mixture; the contents were mixed and the time noted. After 10–20 min., the time depending upon the activity of the enzyme (15), 300 μl. of 3 *M* aqueous potassium hydroxide was added and the contents mixed. The absorbance of each pair of tubes was read using the same 1-ml. cell for a pair of tubes. The C_2, I_1, and I_2 aliquots were assayed similarly. The average absorbance change between a front and back pair of tubes, which is proportional to the enzyme concentration, was plotted on a log scale against the incubation time on a linear scale (13). This procedure is satisfactory for a routine screen for a plus or minus answer on inactivation.

When a compound showed inactivation, a larger amount of inhibitor–enzyme mixture could be set up and a number of aliquots removed at varying times in order to obtain the half-life of inactivation.

The results of the inactivation experiments are shown in Table II.

REFERENCES

(1) Baker, B. R., and Kawazu, M., *J. Med. Chem.*, **10**, 313(1967).
(2) Baker, B. R., Kawazu, M., and McClure, J. D., *J. Pharm. Sci.*, **56**, 1081(1967).
(3) Baker, B. R., and Kawazu, M., *J. Med. Chem.*, **10**, 302(1967).
(4) Baker, B. R., Kawazu, M., Santi, D. V., and Schwan, T. J., *ibid.*, **10**, 304(1967).
(5) Baker, B. R., and Kawazu, M., *ibid.*, **10**, 311(1967).
(6) *Ibid.*, **10**, 316(1967).

(7) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. IV.
(8) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(9) *Reference 7*, chap. I, VIII–XII.
(10) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(11) Baker, B. R., and Rzeszotarski, W., *J. Med. Chem.*, **10**, November(1967).
(12) *Reference 7*, chap. VIII.
(13) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(14) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(15) Baker, B. R., *J. Med. Chem.*, **10**, 297(1967).

Method to Estimate Small Amounts of L-Triiodothyronine in D-Triiodothyronine

By IRVING B. EISDORFER and ALEX POST

One of the requirements for the use of D-triiodothyronine as a serum cholesterol-lowering agent is that it be essentially free of its optical isomer, L-triiodothyronine. A method for estimating small amounts of L-triiodothyronine in D-triiodothyronine is described. After removing any free triiodothyroacetic acid that may be present, the sample is reacted with stereospecific L-amino oxidase (from snake venom) to oxidize the L-triiodothyronine to triiodothyropyruvic acid. The triiodothyropyruvic acid is degraded to triiodothyroacetic acid, extracted, and chromatographed on paper to separate it from the unoxidized D-triiodothyronine. The final quantitative estimation is made by comparing the size and intensity of the colored spot, as visualized by an appropriate spray reagent, with those obtained from known mixtures of L- and D-triiodothyronine carried through the entire procedure. As little as 0.05 per cent L-triiodothyronine could be estimated in the various samples evaluated.

L-TRIIODOTHYRONINE (L-T3), the most potent of the naturally occurring thyroid hormones, is both a powerful calorigenic agent and an active serum cholesterol-lowering agent. Its use for the latter purpose is rather limited, however, since it cannot be used safely in patients suffering from angina pectoris and related diseases. In contrast, its optical antipode, D-triiodothyronine (D-T3), is potentially a very valuable agent for this purpose since it retains much of the serum cholesterol-lowering properties of the natural isomer without the liability of the latter's hypermetabolic activity.

Since L-T3 is such a potent calorigenic agent, its presence in any appreciable amount in lots of the D-T3 destined for clinical trials as a serum cholesterol-lowering agent would badly confound the results. For this reason, heroic efforts were made to limit the amount of L-T3 present in test lots of D-T3 to very small values (of the order of less than 0.2%). In this connection a procedure was required that would be capable of estimating the L-T3 content of samples of D-T3 in the 0–1% range. Since the analytical procedure was designed primarily for acceptance or rejection purposes, sensitivity was deemed more important than great accuracy, and values accurate to one or at most two significant figures were considered satisfactory.

Direct measurement of optical rotation as a means of determining the amount of L-T3 in samples of D-T3 proved to be rather too insensitive for this purpose. With a specific rotation of $-24.5°$ for D-T3, it was not found possible to reliably distinguish samples that contained 0.5% L-T3 from those that contained less than 0.1%. In any case, it was not possible by this procedure to detect 0.2% of L-T3 in samples of D-T3.

The most widely used procedure for determining small amounts of an L-amino acid in the presence of a large amount of its enantiomorph is by stereospecific enzymatic degradation with micromanometric measurement of gas taken up or released (1–5). While this procedure is reported to be capable of estimating as little as 0.1% of an L-amino acid in the presence of its D-isomer, the authors were not able to reliably detect less than 2% of L-T3 in samples of D-T3 manometrically. This may be due to the poor aqueous solubility of the triiodothyronines which prevented the use of an adequate size sample.

Although micromanometric procedures for determining L-amino acids in the presence of the D-isomers were not sensitive enough for the purposes of this work, stereospecific oxidation with an enzyme such as L-amino acid oxidase appeared to be a promising approach to the solution of this problem, provided a sensitive procedure could be

Received April 14, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101
Accepted for publication May 26, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

(7) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. IV.
(8) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(9) *Reference 7*, chap. I, VIII–XII.
(10) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(11) Baker, B. R., and Rzeszotarski, W., *J. Med. Chem.*, **10**, November(1967).
(12) *Reference 7*, chap. VIII.
(13) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(14) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(15) Baker, B. R., *J. Med. Chem.*, **10**, 297(1967).

Method to Estimate Small Amounts of L-Triiodothyronine in D-Triiodothyronine

By IRVING B. EISDORFER and ALEX POST

One of the requirements for the use of D-triiodothyronine as a serum cholesterol-lowering agent is that it be essentially free of its optical isomer, L-triiodothyronine. A method for estimating small amounts of L-triiodothyronine in D-triiodothyronine is described. After removing any free triiodothyroacetic acid that may be present, the sample is reacted with stereospecific L-amino oxidase (from snake venom) to oxidize the L-triiodothyronine to triiodothyropyruvic acid. The triiodothyropyruvic acid is degraded to triiodothyroacetic acid, extracted, and chromatographed on paper to separate it from the unoxidized D-triiodothyronine. The final quantitative estimation is made by comparing the size and intensity of the colored spot, as visualized by an appropriate spray reagent, with those obtained from known mixtures of L- and D-triiodothyronine carried through the entire procedure. As little as 0.05 per cent L-triiodothyronine could be estimated in the various samples evaluated.

L-TRIIODOTHYRONINE (L-T3), the most potent of the naturally occurring thyroid hormones, is both a powerful calorigenic agent and an active serum cholesterol-lowering agent. Its use for the latter purpose is rather limited, however, since it cannot be used safely in patients suffering from angina pectoris and related diseases. In contrast, its optical antipode, D-triiodothyronine (D-T3), is potentially a very valuable agent for this purpose since it retains much of the serum cholesterol-lowering properties of the natural isomer without the liability of the latter's hypermetabolic activity.

Since L-T3 is such a potent calorigenic agent, its presence in any appreciable amount in lots of the D-T3 destined for clinical trials as a serum cholesterol-lowering agent would badly confound the results. For this reason, heroic efforts were made to limit the amount of L-T3 present in test lots of D-T3 to very small values (of the order of less than 0.2%). In this connection a procedure was required that would be capable of estimating the L-T3 content of samples of D-T3 in the 0–1% range. Since the analytical procedure was designed primarily for acceptance or rejection purposes, sensitivity was deemed more important than great accuracy, and values accurate to one or at most two significant figures were considered satisfactory.

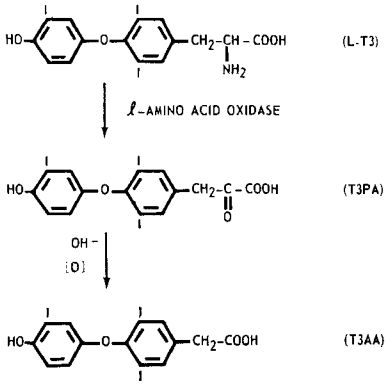
Direct measurement of optical rotation as a means of determining the amount of L-T3 in samples of D-T3 proved to be rather too insensitive for this purpose. With a specific rotation of $-24.5°$ for D-T3, it was not found possible to reliably distinguish samples that contained 0.5% L-T3 from those that contained less than 0.1%. In any case, it was not possible by this procedure to detect 0.2% of L-T3 in samples of D-T3.

The most widely used procedure for determining small amounts of an L-amino acid in the presence of a large amount of its enantiomorph is by stereospecific enzymatic degradation with micromanometric measurement of gas taken up or released (1–5). While this procedure is reported to be capable of estimating as little as 0.1% of an L-amino acid in the presence of its D-isomer, the authors were not able to reliably detect less than 2% of L-T3 in samples of D-T3 manometrically. This may be due to the poor aqueous solubility of the triiodothyronines which prevented the use of an adequate size sample.

Although micromanometric procedures for determining L-amino acids in the presence of the D-isomers were not sensitive enough for the purposes of this work, stereospecific oxidation with an enzyme such as L-amino acid oxidase appeared to be a promising approach to the solution of this problem, provided a sensitive procedure could be

Received April 14, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101
Accepted for publication May 26, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Conversion of L-T3 to T3AA



Scheme I

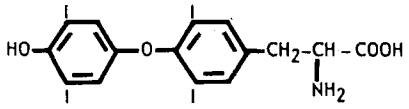
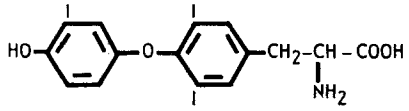
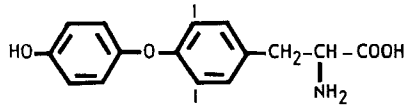
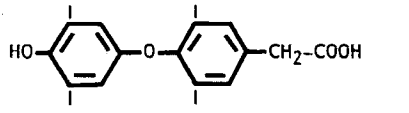
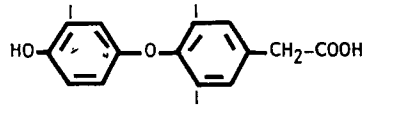
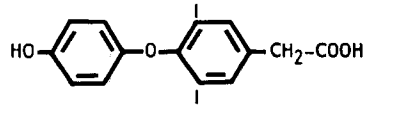
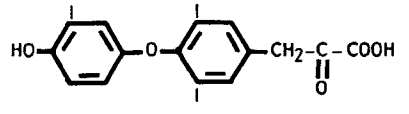
devised for estimating the very small amount of reactants that disappear or products that form. A number of workers (6–10) have reported that L-amino acid oxidase from snake venom oxidizes the L-iodothyronines to a mixture of the corresponding iodothyropyruvic and iodothyroacetic acids. The latter appears to be a degradation product of the former. Since procedures were at hand for separating and estimating small amounts of the various iodothyronines as well as their desamino derivatives by paper chromatography, this was explored as possible means for estimating the product of the oxidation reaction.

The method that was evolved and is reported here utilizes L-amino acid oxidase from snake venom to stereospecifically oxidize L-T3 in the sample of D-T3 to T3PA and T3AA. These are separated from D-T3 by solvent extraction and heated with alkali to convert the T3PA to T3AA (Scheme I). The L-T3 content of the sample is estimated from the amount of T3AA found after chromatographic separation of the digest extract. (The iodothyronines and derivatives are listed in Table I with their respective structures and abbreviations.)

Synthetic samples of D-T3 often contain small amounts of T3AA so it is essential that this material be removed before analysis for L-T3 lest the L-T3 content appear erroneously high. To this end a clean-up step has been incorporated in the procedure. The procedure as written also includes a chromatographic method for estimating the amount of T3AA present in the D-T3 sample. Since it is often valuable to know the D-T2 and D-T4 content of D-T3 samples and the same chromatographic solvent system is used as is used for the estimation of T3AA, a general procedure for this purpose is included also.

While the procedure described here was designed for estimating the L-T3 content of D-T3, it also worked equally well for the determination of L-T2 in D-T2, that is, for estimating the purity of the intermediate. There is good reason also to believe that it works satisfactorily for the determination of L-T4 in D-T4, as well as for the determination of L-T4 in D-T3, *etc.*

TABLE I—IODOTHYRONINES AND IODOTHYRONINE DERIVATIVES

Tetraiodothyronine	
	T-4
Triiodothyronine	
	T-3
Diiodothyronine	
	T-2
Tetraiodothyroacetic Acid	
	T4AA
Triiodothyroacetic Acid	
	T3AA
Diiodothyroacetic Acid	
	T2AA
Triiodothyropyruvic Acid	
	T3PA

PROCEDURE

Chromatography—Prepare a 12 × 18-in. sheet of Whatman 3 MM paper for spotting by drawing light pencil lines connecting the diagonal opposite corners. Also draw a light line through the intersection point of the lines parallel with the long sides of the paper. Finally draw a circle with a $^3/_4$-in. radius around the intersection point of the lines. The junction points of the circle and the lines are the spotting points.

After spotting, thread a 4-in. cotton cord wick (about 2 mm. thick) through a close fitting hole in

the center of the sheet so that about 1/4 in. protrudes above and the rest hangs below. Place the sheet between the sections of a chromatographic chamber made from two 12 × 18 × 3 in. rectangular glass trays with ground rims fitted face-to-face. Allow the wick to rest in a 1 × 3-in. Petri dish placed at the center of the bottom tray.

Equilibrate a mixture of 50 ml. of isoamyl alcohol, 50 ml. of tertiary amyl alcohol, and 100 ml. of 6 *N* aqueous ammonia solution. Transfer all of the lower phase and about 20 ml. of the upper phase to the bottom of the chamber without wetting the paper or the inside of the Petri dish. Allow the paper to come to equilibrium with the solvent vapors for 2 hr., and then start development of the chromatogram by adding about 75 ml. of the upper phase of the solvent mixture to the Petri dish through a small hole in the side of the lower tray with the aid of a long pipet.

Allow the chromatogram to develop until the solvent front reaches the nearest wall of the chamber, then remove and dry in air.

Emerson Reagent (Modified)—*Solution A*—Dissolve 2 Gm. of 4-aminoantipyrine hydrochloride in 2% aqueous sodium carbonate solution. Clarify by shaking with 1% of activated charcoal, filter, and store in a dark bottle in a refrigerator until used. Discard when the solution becomes noticeably yellow.

Solution B—Two per cent aqueous potassium persulfate solution.

Spray the front and back of the chromatogram lightly with solution *A*. Dry in a stream of air at room temperature and then respray with solution *B*. Examine after drying in air at room temperature. Colors are stable.

Venom (L-Amino Acid Oxidase)—Dehydrated *Crotalus adamanteus* venom, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Combine the contents of several bottles of venom, mix thoroughly, and standardize as described below. Transfer 10-mg. portions to individual vials, stopper tightly, and store in freezer until needed.

Venom Standardization—Pipet 5-ml. portions of a 0.10 mg./ml. solution of L-T_3 in 2% KOH into a series of beakers each of which contains 300 ml. of 0.1 *M* phosphate pH 7.2 buffer, 0.2 Gm. of KCl, and 7.5 ml. of 95% alcohol. Adjust the pH of each to 7.2 with dilute HCl or KOH. Add 10 mg. of snake venom to the first beaker, 20 mg. to the second, and 30 mg. to the third, *etc.*, then proceed as directed under *L-T3 Content*.

After extracting the product into 0.05 *N* NaOH, transfer to a 50-ml. volumetric flask, and dilute to volume. Determine the ultraviolet absorption spectrum of this solution in a 5-cm. cell over the range 300–360 mμ. Calculate the difference between the absorbance of the solution at 327.5 mμ and that of the point at the same wavelength on a straight line drawn between the points on the curve at 302.5 mμ and 350 mμ.

The smallest amount of venom that gives a maximum absorbance difference at the point chosen is the optimum weight to be used in the analysis.

Free T3AA Content—Dissolve 50 mg. of the D-T3 sample in 25 ml. of 2% KOH solution. Pipet 5 ml. of this solution into a 250-ml. separator containing 100 ml. of 0.2 *N* HCl. Extract three times with 30-ml. portions of dichloromethane (redistilled from anhydrous Na_2CO_3). Backwash the combined dichloromethane extract once with 30 ml. of 0.1 *N* HCl and once with distilled water. Retain the combined aqueous layers for determination of L-T3 content.

Evaporate the dichloromethane to small volume, transfer to a 50-ml. pear-shaped flask, and evaporate to dryness under vacuum. Dissolve the residue in 0.5 ml. of methanol and transfer by repeated spotting and drying under a stream of N_2 gas to a spotting point on a sheet of Whatman 3 MM paper prepared for chromatography. Rinse the flask twice with 0.25 ml. of methanol and transfer each in turn to the same spotting point.

Transfer 10, 20, and 30 mcg. of T3AA reference compound to other spotting points and chromatograph. Spray the dried chromatogram with Emerson reagent. Estimate the amount of T3AA in the sample from the relative size and intensity of the sample spot as compared to those of the corresponding standards.

L-T3 Content—*Enzymatic Oxidation*—Transfer the aqueous layer obtained under *Free T3AA Content* determination to a 600-ml. beaker. Heat the solution to about 37° and bubble air through it until the odor of the dichloromethane is no longer evident. Cool to room temperature and add 1 *N* KOH dropwise with stirring until the solution is approximately neutral to litmus paper. Add 7.5 ml. of 95% ethanol and 200 ml. of 0.1 *M* pH 7.2 phosphate buffer. Adjust to pH 7.2 ± 0.1 by dropwise addition of dilute HCl or KOH if necessary. Mix thoroughly with a mechanical stirrer for 30 min., and filter through Whatman No. 41 paper. Adjust the temperature of the filtrate to 37 ± 1°, add the required amount of L-amino acid oxidase, and bubble a very slow stream of filtered air through the gently stirred solution for 3 hr.

Transfer the solution to a separator, add 10 ml. of concentrated HCl, mix thoroughly, cool to room temperature, and extract 4 times with 40-ml. portions of redistilled dichloromethane. Collect the extracts in another separator, remove the aqueous phase, and break the slight residual emulsion by the addition of anhydrous sodium sulfate. Filter the clear dichloromethane layer into another separator and wash the residual salt mixture with two 15-ml. portions of dichloromethane. Wash the combined dichloromethane extract with 25 ml. of water and discard the aqueous phase. Finally, extract the dichloromethane solution four times with 10-ml. portions of 0.05 *N* NaOH.

Conversion of T3PA to T3AA—Heat the combined alkaline extract in a loosely stoppered vessel for 2 hr. on a steam bath. Cool to room temperature, add 1 ml. of concentrated HCl, and extract 3 times with 15-ml. portions of redistilled dichloromethane. Filter each portion of extract through a small bed of anhydrous Na_2SO_4 supported in a funnel by a small pledget of well-extracted adsorbent cotton. Evaporate the combined filtrates to dryness under vacuum in a 50-ml. pear-shaped evaporation flask.

Estimation of L-T3 Content—Dissolve the residue in 0.5 ml. of methanol and transfer, by repeated spotting and drying under a stream of N_2 gas to a spotting point on a sheet of Whatman 3 MM paper prepared for chromatography. Rinse the flask twice with 0.25-ml. portions of methanol and trans-

fer each in turn to the same spotting point. In a similar fashion transfer the residues from equivalent weight samples of pure D-T3,[1] pure D-T3 that has had 0.2%, and pure D-T3 that has had 0.4% of L-T3 added and carried through the entire procedure starting from *Free T3AA Content* to other spotting points on chromatographic sheet. Transfer to other spotting points a mixture of 10 mcg. each of L-T3 and T3AA as reference points.

Develop the chromatogram, dry thoroughly in air, and spray with Emerson reagent. Estimate the amount of L-T3 in the sample from the size and intensity of the T3AA spot as compared to that of the T3AA spot present in each of the known mixtures carried through the entire procedure.

T2 and T4 Content of D-T3 Samples—Transfer 10 μl. of a 10 mg./ml. solution of the sample dissolved in 2% ammonia in ethanol to one of the spotting points and 20 μl. to another. Transfer 2 μl. of a solution containing 1 mg./ml. each of T-2 and T-4 dissolved in 2% ammonia in ethanol to one of the other spotting points and 4 μl. to another. The sample spots should contain 100 and 200 mcg., respectively, and the standards should contain 2 and 4 mcg. each of T-2 and T-4. Develop the chromatogram. Spray the dried chromatogram with Emerson reagent, or alternatively immerse it in a 0.25% solution of ninhydrin in 1:100 acetic acid–acetone and allow to dry in air at room temperature. Estimate the amount of T-2 and T-4 present in the sample from the sizes and intensities of the spots as compared to those of the appropriate standards (Fig. 1).

RESULTS AND DISCUSSION

Paper Chromatography—Samples of D-T3 could contain D-T2, D-T4, T2AA, T3AA, and possibly T4AA, all of which are intermediates and side reaction products of the synthesis of D-T3. A chromatographic procedure was needed that would separate these compounds from each other. The chromatographic procedure used in this work does indeed accomplish this purpose. (Figs. 2–4.) Detection of all of these compounds at very low levels was accomplished by use of a modification of the Emerson reagent (10) for phenols. As originally described, potassium ferricyanide was used as the oxidant to bring out the color. This caused the colors to appear (red, orange, purple, *etc.*) on a yellow background. By substituting potassium persulfate for the potassium ferricyanide, it was possible to achieve equivalent color production on a white background with resultant increase in sensitivity (Table II).

Under certain circumstances, it was found useful to be able to distinguish the iodothyronines from the iodothyroacetic acids. Ninhydrin immersion proved to be a valuable means for making the thyronines visible without bringing out the desamino acids. Air drying at room temperature produced colors of equal sensitivity as those produced with the Emerson reagent. The latter, however, produced different shades of color with different degrees of iodine substitution while the former, of course, gave purple colors with all of the amino acids used. All of the compounds used in this work were visible even at very low levels under short wavelength U.V. light (256 mμ) which proved useful under certain circumstances. Table II lists the R_f values, colors, and limits of detection for each of the compounds used in this work.

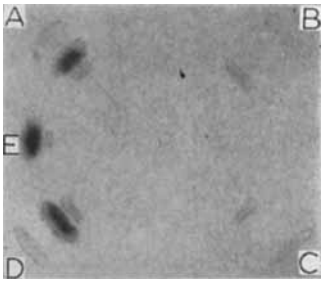


Fig. 1—Chromatogram of small amounts of T2, T4 in T3 sample. Key: A, *100 mcg. T-3 + 2 mcg. T-2 and T-4;* B, *4 mcg. T-2 and T-4;* C, *2 mcg. T-2 and T-4;* D, *100 mcg. T-3 + 4 mcg. T-2 and T-4;* E, *100 mcg. T-3.*

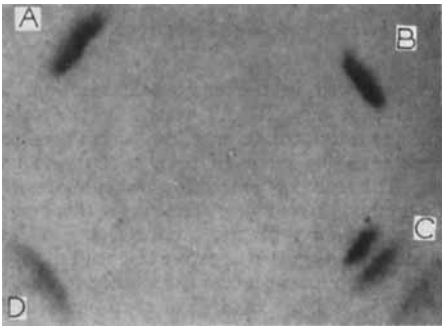


Fig. 2—Chromatogram of T2, T3, T4. Key: A, *10 mcg. T-3;* B, *10 mcg. T-4;* C, *10 mcg. T-2, T-3, T-4;* D, *10 mcg. T-2.*

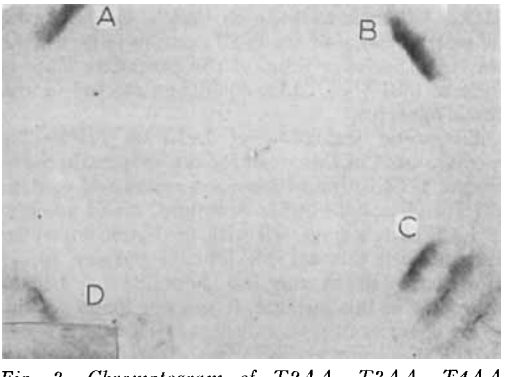


Fig. 3—Chromatogram of T2AA, T3AA, T4AA. Key: A, *10 mcg. T-3AA;* B, *10 mcg. T-4AA;* C, *10 mcg. T-2AA, T-3AA, T-4AA;* D, *10 mcg. T-2AA.*

Estimation of Free T3AA Content—Since final estimation of the amount of L-T3 present in the sample of D-T3 is dependent upon the amount of

[1] D-T3 and T3AA samples used as reference materials in this study were prepared at Smith Kline & French by standard procedures. D-T3 free of L-T3 was prepared by recrystallization of D-T3 samples until no T3AA spot was shown on the chromatogram after being carried through the above procedure. This indicated the presence of less than 0.05% of L-T3 in the D-T3 sample.

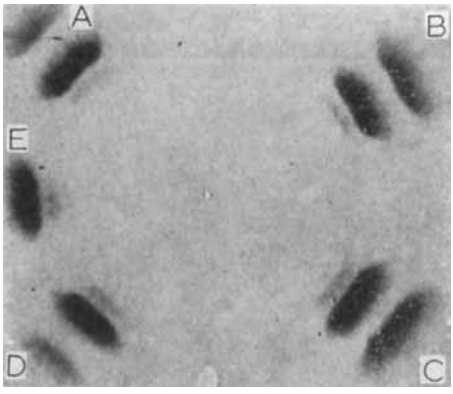


Fig. 4—Chromatogram of T3AA and T3. Key: A, *50 mcg. T-3 + 10 mcg. T-3AA;* B, *50 mcg. T-3 + 20 mcg. T-3AA;* C, *50 mcg. T-3 + 30 mcg. T-3AA;* D, *50 mcg. T-3 + 5 mcg. T-3AA;* E, *50 mcg. T-3.*

T3AA produced in the enzymatic reaction, it is essential that any T3AA present in the original sample (as intermediate or side reaction product of the synthesis) be removed prior to enzyme treatment. The solvent extraction scheme described in the method proved very efficient for this purpose. When tested on synthetic mixtures, the dichloromethane extracts gave a negative test with ninhydrin showing no significant loss of the iodothyronines into the extract, and the final dichloromethane extract gave a negative test with Emerson reagent indicating complete extraction of T3AA in the first two extractions. This was further confirmed by spectrophotometric examination of the two phases.

All of the samples of D-T3 evaluated in this study contained detectable amounts of T3AA (Table III). Although as little as 0.05% of T3AA was detected in some of the D-T3 samples (Fig. 5), this was not the lower limit of the procedure since as little as 0.01% of T3AA could be detected in synthetic mixtures.

Enzymatic Oxidation of L-T3 to T3PA—The medium used in this work for the enzymatic oxidation of L-T3 by snake venom L-amino acid oxidase, pH 7.2 phosphate buffer containing small amounts of KCl, corresponds well with media recommended for use with this enzyme for this purpose (6, 7). However, due to the low solubility of triiodothyronine in this medium, it was not found possible to get enough of the sample dissolved in the medium to achieve anywhere near the desired sensitivity. Reasoning, however, that it was only necessary to retain the L-T3 in solution and not the D-T3, a slow recrystallization step was incorporated in the procedure. This proved to be quite efficient as shown by the good sensitivity of the final procedure and the proportionate recovery of different amounts of added L-T3. The addition of a small amount of alcohol appeared to aid the enzymatic action as well as increase the solubility of the iodothyronines in the medium. The addition of too much alcohol inactivates the enzyme.

Conversion of T3PA to T3AA—Since it was reported that L-T2 was oxidized by snake venom oxidase to T2PA, and the latter partially oxidized to T2AA by peroxides formed by accompanying enzymes, it was presumed that L-T3 reacted the same way. This indeed proved to be the case, so it was not possible to accurately estimate the amount of L-T3 present in a sample until a means could be devised for converting it to one instead of two products.

TABLE III—PER CENT COMPOSITION OF D-T3 SAMPLES

No.[a]	Sample	L-T3	T3AA	D-T2	D-T4
1	GJ-3-254	1	0.05	1	1.5
2	GJ-3-176B	0	0.05	1	5
3	GJ-3-250B	0.75	0.05	1.5	2
4	GJ-3-186	0	0.05	1	<0.5
5	GJ-3-153A	0.1	0.05	1	1
6	PD-262	0	0.05	0.5	1.5
7	PD-102	0.1	0.1	1.5	3
8	AQ-3-5273	1.5	0.3	...	...
9	051-10A	1.5	0.2	...	...
10	RFS-2318-144A	1.5	0.3	...	...
11	O-23W	2	0.9	...	...
12	O-1N	1.5	1.25	...	...
13	O-167	1.5	0.75	...	...

[a] Samples *1–10:* prepared by the Organic Chemistry Laboratories, Smith Kline & French Laboratories, Philadelphia, Pa. Samples *11–13:* obtained from an outside supplier.

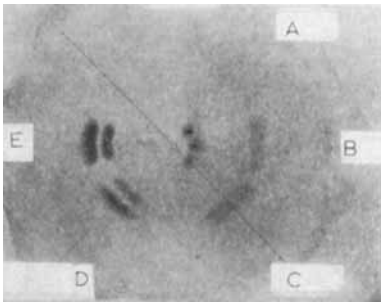


Fig. 5—Chromatogram of free T3AA from D-T3 samples. Key: A, *PD-102;* B, *PD-262;* C, *PD-263;* D, *T3 and T3AA, 10 mcg.;* E, *T3 and T3AA, 20 mcg.*

TABLE II—CHROMATOGRAPHIC CHARACTERISTICS OF IODOTHYRONINES AND DERIVATIVES

		Detection Techniques					
		Emerson Reagent		Ninhydrin Reagent		U.V. Light	
Compd.	R_f	Color	Sensitivity, mcg.	Color	Sensitivity, mcg.	Color	Sensitivity, mcg.
T2	0.47	Orange	1	Blue	1	Purple	1–2
T3	0.36	Red	1	Blue	1	Purple	1
T4	0.28	Purple	1	Blue	1	Purple	1
T2AA	0.60	Red	1	X	X	Purple	1–2
T3AA	0.50	Red	1	X	X	Purple	1
T4AA	0.41	Red	1	X	X	Purple	1

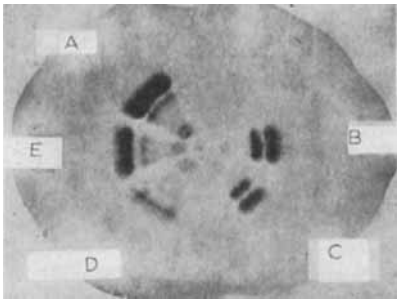


Fig. 6—Chromatogram of T3AA from L-*T3 in* D-*T3 samples, comparison with external T3AA standards. Key:* A, *O-IN;* B, *T3 + T3AA, 20 mcg.;* C, *T3 + T3AA, 10 mcg.;* D, *PD-102;* E, *0–167.*

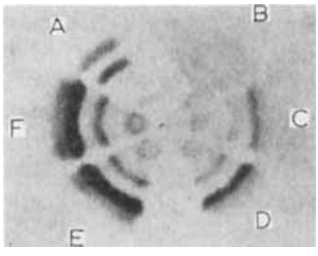


Fig. 7—Chromatogram of T3AA from L-*T3 in* D-*T3 samples, comparison with internal standards. Key:* A, *T3 + T3AA;* B, *PD-262;* C, *PD-262 + 0.25%* L-*T3;* D, *PD-262 + 0.50%* L-*T3;* E, *O-IN;* F, *O-23W(2).*

Since both T3PA and T3AA are strong absorbers in the U.V. and have maxima at slightly different wavelengths, attempts at conversion of one to the other were followed by this means. An initial attempt to prevent oxidation of the T3PA to T3AA by addition of catalase appeared promising but it failed to work reliably so it was discarded. Conversion of T3PA to T3AA by heating with dilute alkali proved to be a more reliable procedure. The appropriate conditions for this conversion were determined by following the reaction spectrophotometrically using known mixtures as well as enzyme digests.

L-T3 in D-T3—When samples of D-T3 containing known added amounts of L-T3 in the range of interest were taken through the procedure, the T3AA spots found on the chromatogram were quite adequate in sensitivity; however, comparison of their size and intensity with those of known amounts of T3AA spotted on the same sheet, showed rather less than complete recovery (Fig. 6). Whether these loses were due to incomplete recovery of L-T3 in the recrystallization step, incomplete oxidation by the enzyme, or to glass surface adsorption, *etc.*, is not known. Attempts to increase recovery by increasing the enzyme ratio proved of no avail. It was found, however, that there was a remarkable proportionality between the size and intensity of the T3AA spots found on the chromatograms and the L-T3 content of D-T3 samples that contained known added amounts of L-T3. Since it was not possible to use absolute reference standards, relative standards were used. That is, D-T3 samples containing known amounts (added) of L-T3 were run through the procedure with the unknown samples using the same enzyme, reaction times, *etc.*, and also spotting the samples and knowns on the same chromatogram, *etc.* Results obtained by this relative standardization procedure proved highly satisfactory if a bit tedious (Fig. 7).

TABLE IV—L-T2 CONTENT OF D-T2 SAMPLES

Sample	% L-T2
051-67B	Trace
051-73A	0.5
051-73B	1.5
051-8A	0.6
PD-39-281	0
051-25A	2
051-25B	0.25

L-T2 in D-T2—The procedure described for estimation of L-T3 in D-T3 also proved satisfactory for the estimation of L-T2 in D-T2, the immediate precursor of D-T3 in the synthetic process (Table IV).

Table III is a list of the amounts of T2, T4, T3AA, and L-T3 found in representative lots of D-T3 prepared at Smith Kline & French and elsewhere. It can be seen that it is possible to produce and analyze D-T3 of very low L-T3 content.

SUMMARY

A procedure has been developed for estimating as little as 0.05% of L-triiodothyronine in samples of D-triiodothyronine. It is also applicable to the determination of equally small amounts of L-diiodothyronine in samples of D-diiodothyronine and very probably for the determination of small amounts of L-tetraiodothyronine (thyroxine) in D-tetraiodothyronine.

A chromatographic procedure has also been described for separating and estimating diiodo-, triiodo-, and tetraiodothyronines in the presence of each other as well as in the presence of diiodo-, triiodo-, and tetraiodothyroacetic acids.

These procedures have been used to estimate the L-triiodothyronine and the D-diiodo and tetraiodothyronine contents of a number of lots of D-triiodothyronine derived from different sources.

REFERENCES

(1) Meister, A., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **192,** 535(1951).
(2) Parikha, J. R., Greenstein, J. P., Winitz, M., and Birnbaum, S. M., *J. Am. Chem. Soc.*, **80,** 953(1958).
(3) Greenstein, J. P., Birnbaum, S. M., and Otey, M. C., *J. Biol. Chem.*, **204,** 307(1953).
(4) Gilbert, J. B., Price, V. E., and Greenstein, J. P., *ibid.*, **180,** 473(1949).
(5) Zeller, E. A., "Advances in Enzymology," vol. 8, Nord, F. F., ed., Interscience Publishers, Inc., New York, N. Y., 1948, p. 459.
(6) Nakano, M., Danowski, T. S., and Utsumi, A., *Endocrinology*, **65,** 242(1959).
(7) Nakano, M., *ibid.*, **67,** 880(1960).
(8) Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **207,** 59(1954).
(9) Albright, E. C., Larson, F. C., Tomita, K., and Lardy, H. A., *Endocrinology*, **59,** 252(1956).
(10) Emerson, E., *J. Org. Chem.*, **8,** 417(1943).

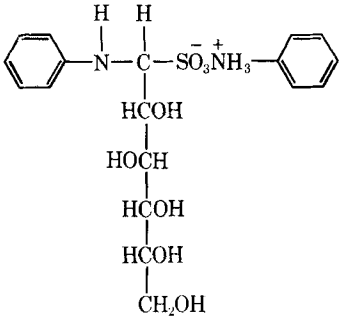
Reactions of Amine Drugs with Sugars I

Reaction of Amphetamine with Aldoses, Glycosylamines, and Bisulfite

By J. C. GRIFFIN* and G. S. BANKER

Amphetamine bisulfite was reacted with D-glucose, D-mannose, *N*-phenylisopropyl-D-mannosylamine, and *N*-phenyl-D-mannosylamine. The effect of an aryl or alkyl amine as the amino substituent at C-1 of the aldose on the formation of amino sulfonates was investigated. Alkyl substitution was found to yield bisulfite addition products, while aryl substitution yielded amino sulfonates. Mannose amphetamine sulfonate and glucose amphetamine sulfonate were synthesized.

THE REACTION of arylamine bisulfites with aldoses has been shown by Ingles (1) to produce amino sulfonates. The structure of an amino sulfonate of this type (D-glycero-D-ido-1-anilino-2,3,4,5,6-pentahydroxyhexylanilinium sulfonate) is shown in I.



I

The hydrolysis characteristics of these compounds (2) were found to be similar to those reported for glycosylamines derived from arylamines (3, 4). Decomposition of the sulfonate structure was observed when the amino sulfonate was dissolved in 10% acetic acid or 0.05 *N* sodium hydroxide solution (2).

The formation of bisulfite addition products, or hydroxysulfonates, by the reaction between inorganic bisulfites and aldehydes is well known (5, 6). The formation of aldose-bisulfite addition products is less well recognized with recent standard texts of organic chemistry (5, 6) indicating that aldoses will not form bisulfite addition products. The chemical literature does not uniformly concur in this view. As early as 1904 Kerp (7) reported the isolation of an impure glucose-sodium bisulfite addition product, while in more recent work Braverman (8) reported the preparation of a pure glucose sodium bisulfite addition product. The preparation of bisulfite addition products of D-glucose, D-galactose, D-mannose, and L-arabinose has also recently been reported by Ingles (9).

The stabilization of pharmaceuticals by the addition of inorganic bisulfites has been common practice for many years (10). The addition of bisulfite to dosage forms containing an amine drug and an aldose could provide the necessary reactants for the formation of aldose-bisulfite addition products as well as amino sulfonates. The implications of this reaction, to modification of amine drug absorption and activity as well as to the browning reaction of sugars, are manifold.

The synthesis of glucose bisulfite compounds of alkoxyamino pyridine by Friedman (11) represents a pharmaceutical application of amino sulfonate formation. The preparation of dialdehyde polysaccharide addition products from various amine bisulfites and dialdehyde starch has been claimed by Borchert (12). These products were of the hydroxy ammonium sulfonate type rather than amino sulfonates. Adams and Garber (13) prepared a number of hydroxy ammonium sulfonates but were unable to obtain products from D-glucose.

The objective of this study was to investigate the reaction of a primary aralkylamine drug with an aldose-bisulfite addition product. The amine drug selected for this phase of the study was dextroamphetamine. The aldose-bisulfite addition product selected was mannose sodium sulfonate.

A further purpose of this work was to examine the reaction of amphetamine bisulfite with the epimeric aldoses, D-glucose and D-mannose, and to examine the effect of an aryl- or alkylamine as

Received November 21, 1966, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication June 5, 1967.

Abstracted from a thesis submitted by John C. Griffin to the Graduate School, Purdue University, Lafayette, Ind., in partial fulfillment of Doctor of Philosophy degree requirements.

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the *N*-substituted constituent at C-1 of the aldose on the formation of amino sulfonates.

EXPERIMENTAL AND RESULTS

Reactions of Amines with Mannose Sodium Sulfonate

Synthesis of Mannose Sodium Sulfonate—D-Mannose,[1] 18 Gm. (0.1 mole), and sodium bisulfite,[2] 12 Gm. (0.115 mole), were separately dissolved in 18 ml. of distilled water with the aid of heat. The two solutions were mixed immediately after preparation. In 20 min. crystallization began and a solid cake formed in approximately 4 hr. The crystalline mass was separated from the mother liquor by filtration, washed with methanol–water (9:1), and finally with methanol. The product was recrystallized twice from a methanol–water (7:3) solvent system.

The recrystallized material had the following characteristics: m.p. 184°; $[\alpha]_D^{25}$ + 7.2 (c 3, 10% acetic acid); sulfite content [method of Adams and Garber (13)] 27.3% found, 28.1% theory.

Reaction of Amphetamine with Mannose Sodium Sulfonate—Mannose sodium sulfonate, 2.8 Gm. (0.01 mole) was added directly to 1.95 Gm. (0.0144 mole) of amphetamine base[3] and the mixture thoroughly mixed. A paste was obtained initially which gradually solidified as a white mass.

The crude product was extracted with 100 ml. of ether for 24 hr. to remove unreacted amphetamine. The ether extract contained 158 mg. of unreacted amphetamine.

The amphetamine content of the crude product was 38.7% as determined by hydrolyzing the adduct with base and extracting the liberated amine with chloroform. Sulfite content of the product was 18.9%. Theoretical values for the condensation product, amphetamine mannose sodium sulfonate, are: amphetamine, 39.0%; sulfite, 19.9%.

The infrared absorption spectrum of the compound between 9.75 and 12.25 μ, as determined by KBr pellet, is shown in Fig. 1 (spectrum A). Also shown in Fig. 1 are the absorption spectra in this region for amphetamine sodium sulfite (B), physical mixture of amphetamine sodium sulfite and mannose sodium sulfonate (C), and mannose sodium sulfonate (D). Amphetamine sodium sulfite was prepared by the addition of amphetamine base to an equimolar amount of sodium bisulfite.

Reaction of Amine Bisulfites with Aldoses

Preparation of Mannose Amphetamine Sulfonate—Amphetamine base, 13.6 Gm. (0.1 mole), was added to 14 ml. of water, and sulfur dioxide[4] from a compressed gas cylinder was bubbled through the suspension. Considerable evolution of heat and the formation of a white solid resulted almost immediately upon introduction of the sulfur dioxide. The white solid redissolved and formed a clear, slightly yellow solution on continued addition of sulfur dioxide. A solution of D-mannose, 9 Gm. (0.05 mole), in 9 ml. of water was prepared with the aid of heat and the mannose solution added to the amphetamine bisulfite solution. Crystallization began in approximately 2 min., and a solid cake was present in the beaker within 20 min. The crude product was suspended in 250 ml. of 95% ethanol and stirred for 1 hr. The product was separated by filtration and dried.

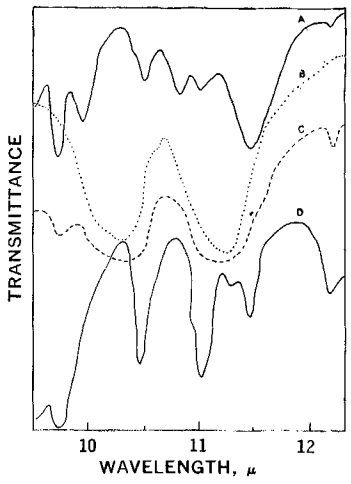


Fig. 1—Infrared absorption spectra. Key: A, *isolated compound;* B, *amphetamine sodium sulfite;* C, *physical mixture of amphetamine sodium sulfite and mannose sodium sulfonate;* D, *mannose sodium sulfonate.*

Free sulfite could not be detected in the product (14) after recrystallizing twice from methanol–water (8:2). Total combined sulfite, determined iodometrically, was 19.8%; theoretical 20.14% for mannose amphetamine sulfonate. Aqueous solutions of the compound gave a positive test for sugar with reagent tablets.[5] The melting point of the recrystallized material was 137°.

The ultraviolet absorption spectrum of the recrystallized product exhibited the characteristic absorption peaks of amphetamine. Amphetamine content, as determined by a chloroform extraction technique from alkaline solution, was 34.6%; theoretical value 34.0% for mannose amphetamine sulfonate.

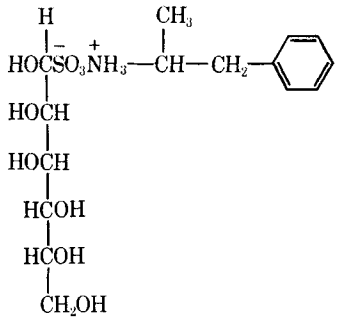
The infrared absorption of the compound in KBr disk showed the following absorption peaks: 3 μ, —OH, —NH; 5.1 μ, amine salt (15); 8.7 μ, R—SO_3^-; 13.5 and 14.5 μ, monosubstituted benzene.

The infrared absorption spectrum of the compound in potassium bromide disk appeared to be consistent with structure II.

Preparation of Glucose Amphetamine Sulfonate—A solution of amphetamine bisulfite, prepared from 13.6 Gm. (0.1 mole) amphetamine base, 14 ml. of water, and sulfur dioxide gas was added to a solution of D-glucose,[6] hydrous, 9.9 Gm. (0.05 mole), in 9 ml. of water. The solution was allowed to stand in an open beaker at room temperature. Crystallization began in about 4 hr. and was allowed to continue for 18 hr. The product was separated and washed in 250 ml. of ethanol (95%).

The characteristics of the isolated reaction product

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Mallinckrodt Chemical Co., St. Louis, Mo.
[3] Prepared from dextroamphetamine sulfate, Sigma Chemical Co., St. Louis, Mo.
[4] The Matheson Co., Inc., Joliet, Ill.
[5] Marketed as Clinitest tablets by the Ames Co., Inc., Elkhart, Ind.
[6] Mallinckrodt Chemical Co., St. Louis, Mo.



II

corresponded to those of mannose amphetamine sulfonate described above. Crystallization was, however, observed to be considerably slower than with the mannose reaction.

Anal.—Calcd. for $C_{15}H_{27}NO_9S$: C, 45.32; H, 6.85. Found: C, 46.5; H, 6.89.

Reactions of Amine Bisulfite with Glycosylamines

Reaction of N - Phenylisopropyl - D - mannosylamine with Amphetamine Bisulfite—A solution of *N*-phenylisopropyl-D-mannosylamine was prepared by refluxing a mixture of D-mannose (5.4 Gm., 0.03 mole), amphetamine (4.0 Gm., 0.03 mole), and methanol (25 ml.) for 20 min. after solution of the sugar was effected. A solution of amphetamine bisulfite, prepared from amphetamine base (4.0 Gm., 0.03 mole), water (0.54 ml., 0.03 mole), methanol (2 ml.), and sulfur dioxide was added to the *N*-phenylisopropyl-D-mannosylamine solution. The resulting solution was placed on a rotary vacuum evaporator and concentrated to small volume. The concentrated solution was placed in an open beaker and allowed to stand at room temperature. Crystallization started within a few minutes and was allowed to continue for 24 hr. The resulting crystalline product was separated, washed with ether, and recrystallized from 95% ethanol.

Physical and chemical characteristics of the recrystallized product were identical to those of mannose amphetamine sulfonate described previously.

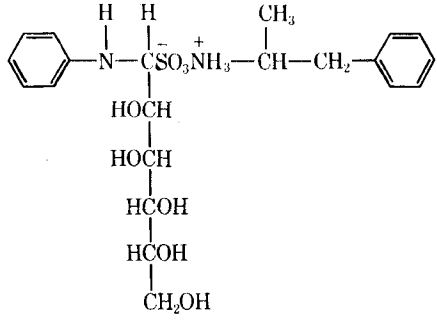
Reaction of N-Phenyl-D-mannosylamine with Amphetamine Bisulfite—A solution of *N*-phenyl-D-mannosylamine was prepared by refluxing a mixture of aniline,[7] 0.93 Gm. (0.01 mole), D-mannose, 1.8 Gm. (0.01 mole), and 10 ml. of methanol for 30 min. The mixture formed a clear solution in approximately 10 min. A white precipitate formed from the clear solution after refluxing for 25 min. Distilled water, 5 ml., was added to the reaction mixture and refluxing was continued. A clear solution resulted in 5 min. and refluxing was terminated at this point.

Sulfur dioxide was bubbled through a mixture of amphetamine, 1.36 Gm. (0.01 mole), distilled water, 0.18 ml. (0.01 mole), and methanol, 3 ml. A clear solution of amphetamine bisulfite resulted and was added to the preformed *N*-phenyl-D-mannosylamine. The solution was refluxed for 4 hr., transferred to a 50-ml. beaker, and allowed to stand at room temperature. Crystallization started within a few minutes and was allowed to continue for 24 hr.

The crystalline material was removed by filtration and washed first with methanol, then with absolute ethanol, and finally with ethyl ether. The product was recrystallized from methanol–water (8:2) and dried in a vacuum desiccator over calcium sulfate.

Sulfite content found, 16.3%; theoretical 16.9% for the amino sulfonate.

The ultraviolet absorption spectrum of the crystalline product dissolved in pH 10 buffer indicated the presence of both aniline and amphetamine chromophores and strongly suggests the structural formula III.



III

DISCUSSION

The reaction of an arylamine bisulfite with an aldose produces, as the normal reaction product, an amino sulfonate. The aralkylamine bisulfite, amphetamine bisulfite, when reacted with the aldoses, D-mannose and D-glucose, was observed to form a bisulfite addition product rather than an amino sulfonate.

A possible explanation of this difference in reaction products may be developed by an examination of the probable reaction mechanisms. Amino sulfonate formation may take place as a result of nucleophilic substitution of the amine at C-1 of the aldose, followed by addition of bisulfite. The strongly basic alkylamine bisulfites exist primarily as the protonated amine, in the system studied, and are thus no longer capable of nucleophilic substitution. The reaction products would thus appear to be limited to bisulfite addition products.

The reactions of *N*-phenyl-D-mannosylamine and *N* - phenylisopropyl - D - mannosylamine with amphetamine bisulfite provide information relative to the effect of the *N*-substituted constituent at C-1 of the aldose on the stability of amino sulfonates. The successful formation of the amino sulfonate, *N*-phenyl-D-mannose amphetamine sulfonate, and the failure to form the corresponding *N*-phenylisopropyl-D-mannose amphetamine sulfonate indicates that amino sulfonate synthesis requires, under the conditions used in this portion of the study, an arylamine as the *N*-substituted constituent at C-1 of the aldose.

Differences in the infrared spectra and absence of free amphetamine base in the product isolated from direct addition of amphetamine base to mannose sodium sulfonate would indicate that a new

[7] General Chemical Division, Allied Chemical and Dye Corp., New York, N. Y.

compound had been formed, possibly of the amino sulfonate type.

Failure to isolate an amino sulfonate from *N*-phenylisopropyl - D - mannosylamine and amphetamine bisulfite would suggest that the mannosylamine had reacted with bisulfite to form an unstable amino sulfonate. The amino sulfonate would then undergo expulsion of the amine, as in the hydrolytic reactions of glycosylamines (16), to form the more stable aldose bisulfite addition product. The reaction was undoubtedly driven in this direction as a result of the insolubility of the aldose bisulfite addition product which was observed to crystallize out of the reaction solution.

REFERENCES

(1) Ingles, D. L., *Australian J. Chem.*, **12**, 275(1959).
(2) *Ibid.*, **12**, 288(1959).
(3) Mitts, E., and Hixon, R. M., *J. Am. Chem. Soc.*, **66**, 483(1944).
(4) Pigman, W., Clevland, E. A., Couch, D. H., and Clevland, J. H., *ibid.*, **73**, 1976(1951).
(5) Roberts, J., and Caserio, M., "Basic Principles of Organic Chemistry," W. A. Benjamin, Inc., New York, N. Y., 1965, pp. 441, 618.
(6) Morrison, R. T., and Boyd, R. N., "Organic Chemistry," Allyn and Bacon, Inc., Boston, Mass., 1959, pp. 632, 766.
(7) Kerp, W., and Wöhler, P., *Arb. kais. Gesuncheitsamt.*, **32**, 88(1904); through *Chem. Abstr.*, **4**, 442(1910).
(8) Braverman, J. B. S., *J. Sci. Agr.*, **11**, 540(1953); through *Chem. Abstr.*, **48**, 1718*d*(1954).
(9) Ingles, D. L., *Australian J. Chem.*, **12**, 97(1959).
(10) Schroeter, L. C., *J. Pharm. Sci.*, **50**, 891(1961).
(11) Friedman, H. L., U. S. pat. 2,426,011(August 19, 1947).
(12) Borchert, P. J., U. S. pat. 3,098,869 (July 23, 1963).
(13) Adams, R., and Garber, J. D., *J. Am. Chem. Soc.*, **71**, 522(1949).
(14) Ingles, D. L., *Australian J. Chem.*, **14**, 302(1961).
(15) Thompson, W. E., Warren, R. J., Eisdorfer, I. B., and Zarembo, J. E., *J. Pharm. Sci.*, **54**, 1819(1965).
(16) Isbell, H. S., and Frush, H. L., *J. Org. Chem.*, **23**, 1309(1958).

Reactions of Amine Drugs with Sugars II

Synthesis and *In Vivo* Evaluation of Mannose Amphetamine Sulfonate and *N*-Phenylisopropyl-D-mannamine

By J. C. GRIFFIN* and G. S. BANKER

Mannose amphetamine sulfonate was synthesized from the reaction of amphetamine bisulfite with D-mannose. *N*-Phenylisopropyl-D-mannamine was synthesized by catalytic hydrogenation of *N*-phenylisopropyl-D-mannosylamine. The products were evaluated *in vivo* by the Williamson activity cage method. Mannose amphetamine sulfonate was found to have a significantly longer duration of activity and produced a higher level of mean activity, from 3.5 hr. to 4.5 hr. after administration, than dextroamphetamine sulfate. *N*-Phenylisopropyl-D-mannamine was found to be orally inactive at dosage levels of 10 and 50 mg./Kg. of body weight.

ADAMS *et al.* (1, 2) have extensively investigated the reaction of various amine bisulfites with aldehydes and ketones. The products isolated, using aldehydes such as benzaldehyde, were of the α-hydroxy sulfonate type. They were, however, unsuccessful in obtaining products with D-glucose. The formation of alkylamine bisulfite addition products with aldoses has been reported by Ingles (3).

The preparation of glycamines by catalytic hydrogenation of *N*-glucosides of alkylamines has been reported by a number of workers (4, 5). Mitts and Hixon (6) have reported on the properties of a number of alkylglucamines obtained by catalytic hydrogenation of glucosylamines.

The purpose of this work was the synthesis and *in vivo* evaluation of an aldose bisulfite addition product and a glycamine derived from an alkylamine drug.

EXPERIMENTAL

Synthesis of Mannose Amphetamine Sulfonate—The synthesis of this product was carried out by reacting dextroamphetamine bisulfite with D-mannose as described in the first paper of this series (7).

Synthesis of N-Phenylisopropyl-D-mannamine—*N*-Phenylisopropyl-D-mannosylamine was prepared by reacting dextroamphetamine,[1] 6.80 Gm. (0.05 mole), with D-mannose,[2] 9.00 Gm. (0.05 mole), in methanol, 5 ml., at 60–65° for 30 min. The resulting light amber colored solution of the mannosylamine was used without further purification for the catalytic hydrogenation.

Hydrogenation of the mannosylamine was carried

Received November 17, 1966, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication April 25, 1967.

Abstracted from a thesis submitted by John C. Griffin to the Graduate School, Purdue University, Lafayette, Ind., in partial fulfillment of Doctor of Philosophy degree requirements.

The authors express their appreciation to Dr. James Swarbrick and Dr. Jack N. Wells for their helpful comments on this paper.

Previous paper: Griffin, J. C., and Banker, G. S., *J. Pharm. Sci.*, **56**, 1098(1967).

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The purpose of this work was the synthesis and *in vivo* evaluation of an aldose bisulfite addition product and a glycamine derived from an alkylamine drug.

EXPERIMENTAL

Synthesis of Mannose Amphetamine Sulfonate—The synthesis of this product was carried out by reacting dextroamphetamine bisulfite with D-mannose as described in the first paper of this series (7).

Synthesis of N-Phenylisopropyl-D-mannamine—*N*-Phenylisopropyl-D-mannosylamine was prepared by reacting dextroamphetamine,[1] 6.80 Gm. (0.05 mole), with D-mannose,[2] 9.00 Gm. (0.05 mole), in methanol, 5 ml., at 60–65° for 30 min. The resulting light amber colored solution of the mannosylamine was used without further purification for the catalytic hydrogenation.

Hydrogenation of the mannosylamine was carried

Received November 17, 1966, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication April 25, 1967.

Abstracted from a thesis submitted by John C. Griffin to the Graduate School, Purdue University, Lafayette, Ind., in partial fulfillment of Doctor of Philosophy degree requirements.

The authors express their appreciation to Dr. James Swarbrick and Dr. Jack N. Wells for their helpful comments on this paper.

Previous paper: Griffin, J. C., and Banker, G. S., *J. Pharm. Sci.*, **56**, 1098(1967).

* Present address: The Upjohn Co., Kalamazoo, MI 49002

[1] Prepared from dextroamphetamine sulfate, Sigma Chemical Co., St. Louis, Mo.

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

out in a Parr type bomb using activated Raney nickel as the catalyst. The mannosylamine was mixed with 250 ml. of ethanol and 10 Gm. of the catalyst, and hydrogenation was carried out below 100° at 1,000 p.s.i. hydrogen pressure. The reaction was terminated after 24 hr. and the solvent reduced to a small volume under vacuum. The resulting crystalline product was filtered off and dried in a vacuum desiccator over calcium sulfate.

Amphetamine Assay—A qualitative test for amphetamine was considered necessary because of the possibility that the unsaturated benzene ring of the amphetamine moiety had been reduced to cyclohexane during the catalytic hydrogenation of the mannosylamine. Amphetamine exhibits a characteristic ultraviolet absorption spectrum which would be destroyed by hydrogenation of the benzene ring. A qualitative test, based on the ultraviolet absorption spectrum of amphetamine was, therefore, selected. Solutions of the mannamine and of dextroamphetamine sulfate in pH 10 buffer were prepared and the ultraviolet absorption spectra of the solutions were compared and found to be identical. The amphetamine content of the mannamine as determined spectrophotometrically was 46.3%; the theoretical amphetamine content is 45.3%.

In Vitro Acid-Base Stability—A quantitative test for amphetamine content, based on the extraction of amphetamine base from alkaline solutions by an organic solvent, was used to determine the acid-base stability of the mannamine. An aqueous solution containing 10.13 mg. of the mannamine per ml. was prepared and 1 ml. of this solution was added to each of three extraction tubes. One milliliter of 1 *N* sodium hydroxide solution was added to each tube and the time recorded. Ten milliliters of chloroform was added to one tube immediately after the addition of the sodium hydroxide solution. Extraction was carried out and the amphetamine content of the chloroform determined spectrophotometrically. The second sample was extracted after 1 hr. and the third sample after 9 hr. Results indicated that less than 50 mcg. of amphetamine was being extracted at each extraction, and that the value did not change during the course of the experiment.

Since the mannamine was apparently stable in the presence of 1 *N* sodium hydroxide, the procedure for the determination of its acid stability characteristics was considerably simplified. If the mannamine were unstable in acid solution, it would liberate the free base which would immediately convert to the salt form; subsequent addition of base would convert the salt form to the free base which could be extracted by chloroform.

The mannamine was found to be stable over a 9-hr. period in artificial gastric fluid.

In an experiment designed to study the partitioning of the mannamine between a pH 8 aqueous solution and chloroform, it was found that when 1 ml. of the aqueous solution containing the equivalent of 3.24 mg. of amphetamine per milliliter was shaken with 10 ml. of chloroform for 24 hr., the concentration of amphetamine present in the chloroform was 10 mcg./ml., thus indicating partitioning strongly in favor of the buffer. The product was found to have a solubility in chloroform equivalent to 50 mcg. of amphetamine per milliliter.

In Vivo Evaluation—For the *in vivo* evaluation by the Williamson[3] activity cage method, male Holtzman rats were selected as the test animal. Their initial weight was between 150 and 175 Gm. In all test runs, the rats were fasted for 10 to 12 hr. in an isolation cage prior to dosing and were preconditioned 30 min. to acquaint the animal with the cage. In each experiment three animals were run simultaneously. Each cage was used an equal number of times for each drug system and six observations were conducted for each of the drugs evaluated. All doses were administered as a solution. The control animals received 3.75 to 5.0 ml. of distilled water by stomach tube, corresponding to the volume of solution required to dose an equivalent weight test animal. All animals were maintained on a diet of Wayne Lab Blox and water *ad libitum* and were housed in an air-conditioned animal room at 23–25°. Animal colony size was 30 rats, maintained in two cages of 15 rats each.

Dosage was calculated on the basis of 10 mg. and 50 mg. of amphetamine per kilogram of body weight. Solutions of the test drugs were prepared in a concentration representing 1 or 5 mg. of amphetamine per 2.5 ml. of solution. All tests were conducted so that the starting time of the test period was between 7:30 and 8:00 p.m.

This procedure was used to evaluate dextroamphetamine sulfate, mannose amphetamine sulfonate, and *N*-phenylisopropyl-D-mannamine at a dosage level of 10 mg./Kg. and to establish control activity levels. *N*-Phenylisopropyl-D-mannamine was also evaluated at a dosage level of 50 mg./Kg.

At the end of the test period, the average number of responses per 0.5-hr. interval was calculated. A statistical analysis of the data was accomplished by first applying an over-all *F* test to determine if a significant difference existed in any of the possible combinations. If a significant F ($\alpha = 0.05$) was found, the Student Newman-Kuels test was used to evaluate each of the possible combinations (8). Table I presents the average activity, at each 0.5-hr.

TABLE I—MEAN ACTIVITY COUNTS PER 0.5-hr. PERIOD FOR DEXTROAMPHETAMINE COMPOUNDS[a] AND FOR THE CONTROL

Time, hr.	1 Control $\bar{X}$	2 H2 $\bar{X}$	3 5H2 $\bar{X}$	4 AMS $\bar{X}$	5 Amp. SO_4 $\bar{X}$
0.5	5.34	5.66	5.88	16.22	14.11
1.0	2.78	2.11	4.00	15.44	15.22
1.5	3.00	4.00	4.56	15.67	14.00
2.0	4.56	2.78	4.22	14.77	13.00
2.5	4.00	3.89	3.78	14.78	13.34
3.0	4.34	4.78	5.34	13.89	12.45
3.5	5.45	3.67	4.56	15.67	11.22
4.0	4.89	4.34	4.11	14.77	10.00
4.5	3.45	4.45	5.34	11.55	7.89
5.0	4.78	4.00	4.11	8.67	6.45
5.5	4.45	2.78	3.78	4.34	6.22
6.0	3.22	2.89	4.22	7.22	5.22

[a] H2, *N*-phenylisopropyl-D-mannamine; dosage level equivalent to 10 mg. dextroamphetamine per Kg. of body weight; $5XH_2$, *N*-phenylisopropyl-D-mannamine, dosage level equivalent to 50 mg. dextroamphetamine per Kg. of body weight; AMS, mannose amphetamine sulfonate, dosage level equivalent to 10 mg. of dextroamphetamine per Kg. of body weight; Amp. SO_4, dextroamphetamine sulfate, dosage equivalent to 10 mg. dextroamphetamine per Kg. of body weight.

[3] Williamson Development Co., Inc., West Concord Mass.

TABLE II—STATISTICAL ANALYSIS OF THE *In Vivo* DATA FOR SIGNIFICANCE OF DIFFERENCE BETWEEN THE VARIOUS DEXTROAMPHETAMINE COMPOUNDS AND BETWEEN THE COMPOUNDS AND THE CONTROL

	Significance at 0.05 Level[a]									
Time, hr.	1 × 2[b]	1 × 3	1 × 4	1 × 5	2 × 3	2 × 4	2 × 5	3 × 4	3 × 5	4 × 5
0.5	...	...	*	*	...	*	*	*	*	...
1.0	...	...	*	*	...	*	*	*	*	...
1.5	...	...	*	*	...	*	*	*	*	...
2.0	...	...	*	*	...	*	*	*	*	...
2.5	...	...	*	*	...	*	*	*	*	...
3.0	...	...	*	*	...	*	*	*	*	...
3.5	...	...	*	*	...	*	*	*	*	*
4.0	...	...	*	*	...	*	*	*	*	*
4.5	...	...	*	*	...	*	...	*	...	*
5.0	...	...	*	...	...	*	...	*	...	...
5.5	...	...	...	...	...	...	*	...	...	...
6.0	...	...	*	...	...	*	...	*	...	*

[a] An asterisk indicates a significant difference exists between the mean activity counts for the two drug systems being compared or between the drug system and the control at that 0.5-hr. period. [b] The numbers refer to the systems described in Table I and compare differences in mean activity between these systems at each 0.5-hr. period given.

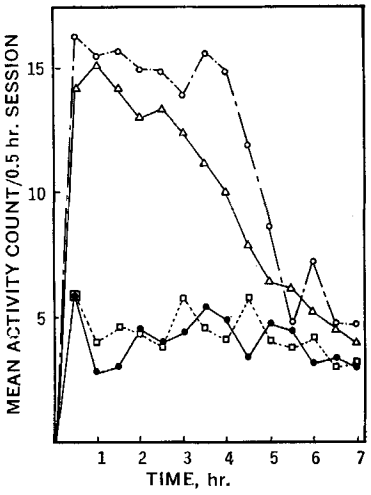

Fig. 1—In vivo *evaluation of amphetamine compounds. Key:* O, *AMS;* △, *amphetamine* SO_4; □, *5H2;* ●, *control. Equivalent dextroamphetamine dosage level and compound identification are the same as reported in Table I.*

interval, for all test groups. Results of the statistical analysis are given in Table II. Figure 1 is a plot of the mean activity exhibited by the animals used in the study at 0.5-hr. intervals.

DISCUSSION

Mannose amphetamine sulfonate and dextroamphetamine sulfate were found to have an immediate onset of pharmacologic action. The dextroamphetamine sulfate had a duration of activity of 4.5-hr. while the mannose amphetamine sulfonate had a 5-hr. duration of activity. Furthermore, the mannose amphetamine sulfonate produced a significantly higher CNS response than dextroamphetamine sulfate, from 3.5 hr. after administration, to 4.5 hr. after administration. No significant difference in the activity of dextroamphetamine sulfate and mannose amphetamine sulfonate was demonstrated during the first 3 hr. Further investigations of this and similar compounds would seem to be necessary for an elucidation of the mechanism responsible for the observed increased duration and level of activity produced by mannose amphetamine sulfonate.

N-Phenylisopropyl-D-mannamine was found to be inactive when administered orally to rats at a dosage level equivalent to 10 and 50 mg./Kg. of dextroamphetamine. This observation would, therefore, indicate that the compound was not degraded in the gastrointestinal tract to release the amphetamine moiety. It is highly improbable that the mannamine itself was absorbed, since the *in vitro* tests indicated a very low lipid solubility and almost no partitioning from an aqueous (pH 8) phase to a chloroform phase. From the results of this study, it could be assumed that the conversion of this class of amine drugs to glycamines would probably result in the formation of orally inactive compounds.

SUMMARY

Mannose amphetamine sulfonate was synthesized and evaluated *in vivo*. The compound was found to have a significantly longer duration of activity and produced a higher level of mean activity from 3.5 hr. to 4.5 hr. after administration than dextroamphetamine sulfate.

N-Phenylisopropyl-D-mannamine was synthesized and evaluated *in vitro* and *in vivo*. The compound was found *in vitro* to be stable over a 9-hr. period in both acidic and basic solutions. Solubility determinations and partitioning experiments indicated the product was relatively insoluble in chloroform and its partitioning between pH 8 buffer and chloroform was strongly in favor of the buffer. The mannamine was found to be inactive when administered orally to rats.

REFERENCES

(1) Adams, R., and Lipscomb, R. D., *J. Am. Chem. Soc.*, **71**, 519(1949).
(2) Adams, R., and Garber, J. D., *ibid.*, **71**, 522(1949).
(3) Ingles, D. L., *Australian J. Chem.*, **12**, 275(1959).
(4) Karrer, P., Saloman, H., Kunz, R., and Seebach, A., *Helv. Chim. Acta*, **18**, 1338(1935).
(5) Karrer, P., and Herkenrath, E., *ibid.*, **20**, 83(1937).
(6) Mitts, E., and Hixon, R. M., *J. Am. Chem. Soc.*, **66**, 483(1944).
(7) Griffin, J. C., and Banker, G. S., *J. Pharm. Sci.*, **56**, 1098(1967).
(8) Winer, J. B., "Statistical Principles in Experimental Design," McGraw-Hill Publishing Co., New York, N. Y., 1962, p. 82.

Comparative Cardio-Inhibitory Effects of Certain Cyclohexanol and Cyclohexylamine Derivatives

By D. C. KOSEGARTEN, J. J. DEFEO, and D. R. DEFANTI

Derivatives of cyclohexanol and cyclohexylamine were screened for cardioplegic effects on intact anesthetized rats and isolated guinea pig hearts. Evaluation of potency was based on the ability of the compounds to depress rat carotid blood pressure, and to produce reversible arrest of the isolated guinea pig heart. *trans*-2-*o*-Tolyl-*cis*-1,4-cyclohexanediol and *trans*-2-*o*-tolyl-*trans*-1,5-cyclohexanediol were equally effective and the most active compounds tested in producing a hypotensive response. *trans*-2-(*p*-Chlorophenyl)-*N*,*N*-dimethylcyclohexylamine was the least active and the only compound causing a pressor response. As a group, the cyclohexanol derivatives were more potent hypotensive agents than were the derivatives of cyclohexylamine. Conversely, the cyclohexylamines were more potent cardioplegic agents. The *cis* and *trans* isomers of 2-(*p*-chlorophenyl)-*N*,*N*-dimethylcyclohexylamine were equally the most active, with *trans*-2-*o*-tolyl-*cis*-1,4-cyclohexanediol the least active of the compounds studied. The minimum effective cardioplegic dose of 1-ethynyl-*trans*-2-*o*-tolylcyclohexanol was also the cardiotoxic dose as the heart failed to recover from arrest. Mechanism studies indicate an antagonistic effect between calcium and the negative inotropic action of these compounds.

TODAY "open-heart" surgery has changed the outlook for a great many cardiac patients, particularly those having congenital defects, who would have been labeled as hopeless cases slightly more than 10 years ago.

An important aid in the surgical treatment of intracardiac defects is the still heart. Under certain conditions, where a dry, motionless, and clearly visible operative field is desirable, an arrested and all but bloodless heart enables the surgeon to repair more accurately a particular defect.

Elective cardiac arrest has been experimentally and clinically produced by several methods; for a review see Stephenson (1). These consist chiefly of producing arrest by hypothermia, myocardial anoxia, pharmacological agent, or a combination of any or all three.

In 1959 Huitric *et al.* (2) reported that 2-(*o*-tolyl)-cyclohexanol was capable of producing reversible cardiac arrest, without affecting myocardial electrical properties. This finding stimulated the preparation of a number of cyclohexanol and cyclohexylamine derivatives by Huitric.

Previous pharmacologic studies by Smookler and DeFeo (3) and Smith (4) showed the degree of cardio-inhibitory activity to be related to the functional groups and stereochemistry of these compounds.

In continuation therefore, the present investigation was carried out on additional compounds prepared by Huitric, in an effort to determine their possible cardio-inhibitory activity and its relationship to molecular structure.

Received April 14, 1967, from the Department of Pharmacology, College of Pharmacy, University of Rhode Island, Kingston, RI 02881

Accepted for publication May 26, 1967.

Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Based on a thesis submitted by David C. Kosegarten to the Department of Pharmacology, College of Pharmacy, University of Rhode Island, in partial fulfillment of Master of Science degree requirements.

This work was generously supported by a grant from the Rhode Island Heart Association.

EXPERIMENTAL

Materials—The cyclohexylamine hydrochloride salts (Fig. 1) were freely soluble in water. The ethynyl derivatives of cyclohexanol (Fig. 2) were practically water insoluble, while the remaining cyclohexanol derivatives (Fig. 2) were slightly water soluble. Accordingly, the ethynyl derivatives were dissolved in 50% polyethylene glycol (PEG) 400, while aqueous solutions of the remaining cyclohexanol derivatives, as well as the cyclohexylamine hydrochloride salts were used throughout the investigation.

Rat Blood Pressure—Male albino rats (260 Gm., average weight) were anesthetized with an intraperitoneal injection of 1.2 Gm./Kg. of urethan. The effects were recorded on a slowly moving smoked kymograph by direct cannulation of the right carotid artery *via* a fluid bridge of normal saline connecting the cannula to a mercury manometer. Injections of the test compounds were made intravenously and not more than six injections were made during one experiment.

Dosage was adjusted according to the results obtained, in order to determine the dose of a particular compound that would produce a 50% drop in the blood pressure. Dose and response were subjected to linear regression computations and the dose producing a 50% drop in blood pressure (ED_{50}) calculated. The ethynyl derivatives of cyclohexanol could not be tested, as the solvent used for these compounds possessed an effect of its own on the blood pressure of the intact rat.

In an effort to account for the pressor response to TH-25 the experimental procedures were modified. The preparation was allowed to stabilize for 20 min. and then the following trials were carried out at 5-min. intervals: (*a*) occlusion of the left common carotid artery for 10 sec., (*b*) 0.06 ml. of epinephrine 1:100,000, (*c*) 4 mg./Kg. TH-25, (*d*) 20 mg./Kg. i.v. hexamethonium, (*e*) 10-min. pause to allow preparation to stabilize and then repeat tests (*a*),

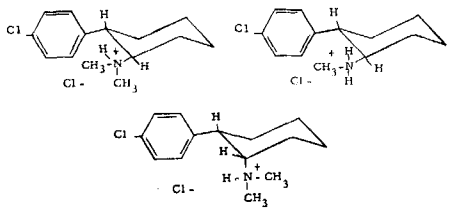

Fig. 1—Cyclohexylamine hydrochloride derivatives. Top left: trans-*2*-(p-*chlorophenyl*)-N,N-*dimethylcyclohexylamine hydrochloride* (*TH-25*). *Top right:* trans - *2* - (p - *chlorophenyl*) - N - *methylcyclohexylamine hydrochloride* (*TH-26*). *Bottom:* cis-*2*-(p-*chlorophenyl*)-N,N-*dimethylcyclohexylamine hydrochloride* (*TH-27*).

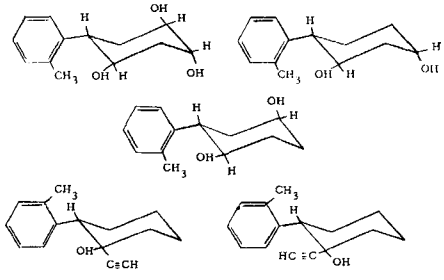

Fig. 2—Cyclohexanol derivatives. Top left: trans-*2*-o - *tolyl* - cis - *4* - *hydroxy* - trans - *5* - *hydroxycyclohexanol* (*CH-21*). *Top right:* trans-*2*-o-*tolyl*-trans-*1,5-cyclohexanediol* (*CH-20*). *Middle:* trans-2-o-*tolyl*-cis-*1,4-cyclohexanediol* (*CH-14*). *Bottom left: 1-ethynyl*-trans-*2*-o-*tolylcyclohexanol* (*AH-64*). *Bottom right: 1-ethynyl*-cis-*2*-o-*tolylcyclohexanol* (*AH-65*).

(*b*), and (*c*). The per cent change in blood pressure produced by TH-25 before and after the administration of hexamethonium was the index of activity studied.

Isolated Guinea Pig Heart—Guinea pigs of either sex were sacrificed by cervical dislocation. The heart was rapidly removed and was suspended after cannulation of the aorta in an Anderson-Craver perfusion apparatus. The perfusion solution used was that recommended by Chenoweth and Koelle (5), slightly modified by Smookler and DeFeo (3), and saturated with a continual flow of 95% oxygen and 5% carbon dioxide. The fluid level in the perfusion well of the apparatus was kept constant with the aid of a glass siphon tube placed in the upper end of the well. Excess fluid was thus collected with the aid of a pump and returned to the perfusion fluid reservoir. The preparation was maintained at 37.5° and the heart perfused under a head pressure of 54 cm. of water. Isotonic ventricular contractions were recorded on a slowly moving smoked kymograph. Only one compound was tested in each experiment of not more than five injections. A control dose of 50% PEG was injected at the beginning and end of each experiment involving the ethynyl derivatives of cyclohexanol.

A negative inotropic response of 95% or more was considered to be cardiac arrest and recovery was allowed to take place spontaneously. Dose and response were subjected to linear regression computations and the dose producing cardiac arrest (ED_{95}) calculated.

Isolated Rat Atria—Male albino rats were killed by cervical dislocation and hearts rapidly excised. The atria were freed of ventricular muscle and connective tissue, and suspended vertically in an 8-ml. tissue bath. The bathing solution (same as above) was continually aerated with a mixture of 95% oxygen and 5% carbon dioxide, and temperature maintained at 29° as suggested by Burn and Vane (6). Amplitude of isotonic contractions and the rate of spontaneous beat were recorded under a resting tension of approximately 0.75 Gm. on a smoked kymograph.

CH-20 and TH-27 were chosen to be tested in conjunction with a 4% calcium chloride solution, to study the mechanism involved in the cardioplegia produced by each series of compounds. At least 3 min. were allowed for the compounds to act on the suspended tissue. A minimum of three washes over a period of 5 min. were performed between trials, and the tissue allowed an additional 5-min. recovery period before proceeding to the next trial.

RESULTS AND DISCUSSION

Rat Blood Pressure—CH-21 in doses of 1.0 to 12.0 mg./Kg. produced a drop in mean blood pressure ranging from 22 to 80% of the normal. CH-14 in doses of 2.0 to 8.0 mg./Kg. produced a drop in mean blood pressure of 35 to 85%. CH-20 in doses of 2.0 to 8.0 mg./Kg. produced a drop in mean blood pressure of 42 to 63%. TH-26 in doses of 2.0 to 18.0 mg./Kg. produced a drop in mean blood pressure of 5 to 42%. TH-27 in doses of 2.0 to 14.0 mg./Kg. produced a drop in mean blood pressure of 20 to 57%.

Duration of response was not linear with the cyclohexanol derivatives, and varied from 50 to 400 sec. Conversely, the duration of the hypotensive response to the cyclohexylamine derivatives was linear from 25 to 74 sec. with 2.0 to 6.0 mg./Kg. of TH-26, and from 25 to 130 sec. with 2.0 to 8.0 mg./Kg. of TH-27.

The hypotensive ED_{50} of each of the above compounds is shown in Table I. Omitted from the table is TH-25, which in doses of 4.0 to 8.0 mg./Kg. produced a rise in mean blood pressure (Fig. 3) of 20 to 28%, with a linear duration in pressor response from 44 to 108 sec.

TABLE I—COMPARISON OF THE ED_{50} (HYPOTENSIVE) OF THE NONETHYNYL CYCLOHEXANOL DERIVATIVES AND THE CYCLOHEXYLAMINE DERIVATIVES ON THE BLOOD PRESSURE OF RATS

Compd.	Total Trials	Calcd. ED_{50}, mg./Kg.
trans-2-*o*-Tolyl-*cis*-4-hydroxy-*trans*-5-hydroxycyclohexanol (CH-21)	18	5.30
trans-2-*o*-Tolyl-*cis*-1,4-cyclohexanediol (CH-14)	11	3.95
trans-2-*o*-Tolyl-*trans*-1,5-cyclohexanediol (CH-20)	12	4.04
trans-2-(*p*-Chlorophenyl)-*N*-methylcyclohexylamine HCl (TH-26)	20	26.30
cis-2-(*p*-Chlorophenyl)-*N,N*-dimethylcyclohexylamine HCl (TH-27)	16	12.94

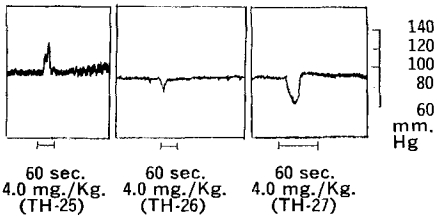

Fig. 3—Rat blood pressure response to each of the cyclohexylamine hydrochloride salts (4.0 mg./Kg.).

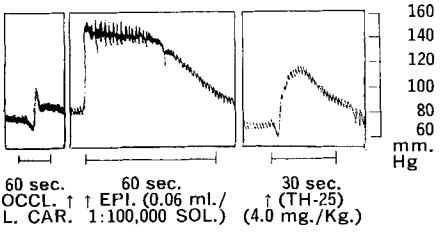

Fig. 4—The effect of trans-*2-*(p-*chlorophenyl*)-N,N-*dimethylcyclohexylamine hydrochloride (4.0 mg./Kg.) on rat blood pressure prior to hexamethonium (20 mg./Kg.).*

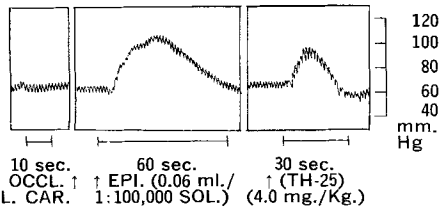

Fig. 5—The effect of trans-*2-*(p-*chlorophenyl*)-N,N-*dimethylcyclohexylamine hydrochloride (4.0 mg./Kg.) on rat blood pressure following hexamethonium (20 mg./Kg.).*

The effect of TH-25 on the rat blood pressure before and after i.v. hexamethonium is shown in Figs. 4 and 5. Prior to i.v. hexamethonium, occlusion of the left common carotid, epinephrine, and TH-25 each produced a transient pressor response of approximately 22%, 85%, and 60%, respectively.

Ten minutes after the slow i. v. injection of hexamethonium, occlusion of the left common carotid for 10 sec. failed to elicit the above-mentioned pressor response. However, second injections of epinephrine and TH-25 again produced transient pressor responses of approximately 60% and 42%, respectively.

The blood pressure results obtained with the nonethynyl cyclohexanol derivatives were typical of those previously obtained with other cyclohexanol derivatives (3). All produced an immediate transient drop in pressure. Although the magnitude of the effect was somewhat dose dependent, the duration of the effect was not. On the basis of the calculated ED_{50} (Table I), CH-20 and CH-14 were equally effective in lowering rat blood pressure. Substitution of a hydroxyl group in both the 4 and the 5 position of the cyclohexanol ring (CH-21) decreased hypotensive activity approximately 30%. It is well known that the activity and toxicity of parent compounds tend to decrease upon the introduction of hydroxyl groups into a chemical structure.

The difference in the hypotensive activity of the cyclohexylamines studied appears to be attributed to both the spatial configuration of the compounds and to the number of methyl groups substituted on the amino nitrogen. In the *trans* configuration, the secondary amine (TH-26) possessed hypotensive activity, while the tertiary amine in the *trans* configuration (TH-25), was the only compound studied that possessed pressor activity. Moreover, in the *cis* configuration, the tertiary amine (TH-27) was approximately twice as active as the secondary amine (TH-26).

Pressor activity of cyclohexylalkylamines has been reported to be due to a direct increase in peripheral vascoconstrictor activity (7, 8). In the preliminary investigation of the mechanism involved in the pressor response to TH-25, reflex vasoconstriction *via* baroreceptor activity was not demonstrated. Moreover, depletion of catecholamine stores by reserpine does not alter the pressor activity (9). Again, the compound appears to be acting directly on the peripheral vasculature.

Thus, with the exception of TH-25 and the ethynyl derivatives of cyclohexanol, the rapid and transient hypotensive response of the compounds suggested direct myocardial depression.

Isolated Guinea Pig Heart—The results obtained in the isolated heart studies are shown in Table II. Recovery to 50% of the original amplitude level was also recorded, and varied with the type of compound studied. At the minimum effective dose, CH-20 and CH-14 had the shortest mean recovery time of 21 and 87 sec., respectively; TH-25, TH-26, and TH-27 possessed intermediate mean recovery times of 202, 868, and 1205, respectively; while the longest mean recovery time of 1280 sec. occurred with AH-65. Moreover, the heart arrested by AH-64 failed to show any signs of recovery and the experiments were terminated after a minimum of 3400 sec. of systolic standstill.

TABLE II—COMPARISON OF THE ED_{95} (CARDIO-INHIBITORY) OF THE CYCLOHEXANOL AND CYCLOHEXYLAMINE DERIVATIVES ON THE ISOLATED GUINEA PIG HEART

Compd.	Total Trials	Calcd. ED_{95}, mg.
trans-2-*o*-Tolyl-*cis*-1,4-cyclohexanediol (CH-14)	15	6.80
trans-2-*o*-Tolyl-*trans*-1,5-cyclohexanediol (CH-20)	8	3.38
1-Ethynyl-*trans*-2-*o*-tolylcyclohexanol (AH-64)	11	2.52
1-Ethynyl-*cis*-2-*o*-tolylcyclohexanol (AH-65)	10	1.33
trans-2-(*p*-Chlorophenyl)-*N,N*-dimethylcyclohexylamine HCl (TH-25)	9	0.78
trans-2-(*p*-Chlorophenyl)-*N*-methylcyclohexylamine HCl (TH-26)	8	1.19
cis-2-(*p*-Chlorophenyl)-*N,N*-dimethylcyclohexylamine HCl (TH-27)	10	0.85

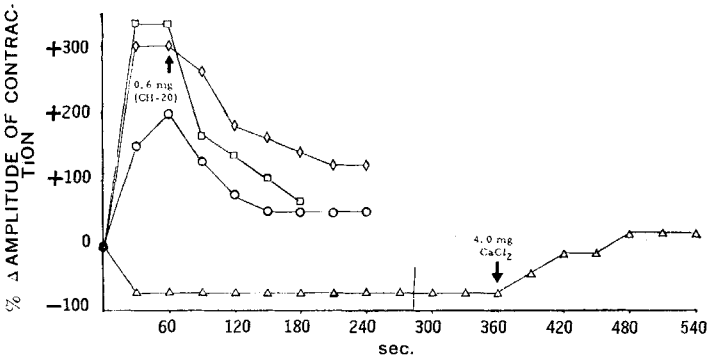

Fig. 6—The effects of trans-2-o-*tolyl* - trans - *1,5 - cyclohexanediol (0.6 mg.) and calcium chloride (4.0 mg.) on isolated rat atria. Key:* □, *4.0 mg.* $CaCl_2$; Δ, *0.6 mg. CH-20, followed by 4.0 mg.* $CaCl_2$; ◇, *4.0 mg.* $CaCl_2$, *followed by 0.6 mg. CH-20;* O, *4.0 mg.* $CaCl_2$ *and 0.6 mg. CH-20.*

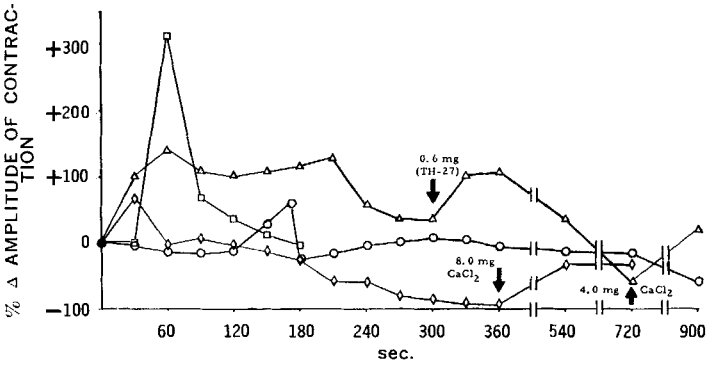

Fig. 7—The effects of cis-2-(p-*chlorophenyl*)-N,N - *dimethylcyclohexylamine hydrochloride (0.6 mg. and 1.2 mg.) and calcium chloride (4.0 mg. and 8.0 mg.) on isolated rat atria. Key:* □, *4.0 mg.* $CaCl_2$; Δ, *0.6 mg. TH-27, followed by 0.6 mg. TH-27 and 4.0 mg.* $CaCl_2$; ◇, *1.2 mg. TH-27, followed by 8.0 mg.* $CaCl_2$; O, *1.2 mg. TH-27 and 8.0 mg.* $CaCl_2$.

A 1.0-mg. quantity of CH-21 produced a mean negative inotropic effect of 10%. Due to insufficient compound, the higher dosage levels could not be investigated.

The antagonistic effect of 8 and 16 mg. of calcium chloride on the cardiac arrest produced by 1.0 mg. of AH-65 was also noted. In all cases, calcium produced a transient positive inotropic response, proportional in duration and magnitude to the concentration of calcium used, and followed by the resumption of the pre-existing cardioplegia.

The primary purpose of the investigation was to determine whether the compounds possessed cardioinhibitory activity. The results in Table II show that the cyclohexylamine derivatives were up to 8 times more active in producing cardioplegia than were the derivatives of cyclohexanol. In the latter series of compounds, it appears that hydroxyl substitution at the 5 instead of the 4 position of the cyclohexanol ring increases activity 2 times. Moreover, when this hydroxyl substituent is omitted and an ethynyl group substituted at the 1 position of the cyclohexanol ring, activity increases 3 and 5 times with the *trans* and *cis* isomer, respectively. The *trans* isomer (AH-64) possessed cardiotoxic properties as the arrested hearts failed to recover spontaneously and remained in systolic standstill until the experiments were terminated at the end of 90 min. The *cis* isomer (AH-65) did not show any signs of cardiotoxicity at the dose levels employed.

It will be recalled that the tertiary cyclohexylamine in the *trans* configuration (TH-25) possessed pressor activity. Nevertheless, both TH-25 and TH-27 were equally potent and the most active of all the compounds studied in arresting the isolated guinea pig heart. Moreover, these two isomers were approximately 25% more active than TH-26 in producing cardioplegia.

Isolated Rat Atria—The results are shown in Figs. 6 and 7. The rate of the spontaneous beat of the preparation increased 17% when 4.0 mg. of calcium chloride alone was added to the bath, and was unchanged when 0.6 mg. of CH-20 preceded 4.0 mg. of calcium chloride. The rate increased 13% if calcium preceded CH-20 and increased only 7% when both were administered simultaneously.

Conversely, 0.6 mg. of TH-27 caused a 42% reduction in the rate of atrial contraction. Moreover, a second dose of 0.6 mg. of TH-27 when added to the bath at this point led to a final reduction in rate of 67%, which was unaltered by 4.0 mg. of calcium chloride. Likewise, 1.2 mg. of TH-27 added to the bath initially caused a 65% reduction in rate which was also unaltered by a subsequent dose of 8.0 mg. of calcium chloride. A 55% reduction in rate occurred when 1.2 mg. of TH-27 and 8.0 mg. of calcium chloride were added to the bath simultaneously.

trans-2-*o*-Tolylcyclohexanol has been reported to reduce the contraction amplitude of isolated rabbit atria without affecting the rate, and calcium to restore the amplitude to a normal level (3). In the present study, CH-20 similarly affected spontaneously beating rat atria, and calcium also restored the amplitude of contraction to a control level. TH-27 produced both negative inotropic and chronotropic effects on the isolated rat atria. A positive inotropic response, the duration of which was inversely proportional to the dose employed, preceded in all instances the negative inotropic effect. Calcium was able to restore the amplitude to control level, but unable to alter the negative chronotropic

response which progressed throughout the entire tissue exposure time. If given simultaneously with either CH-20 or TH-27, the onset of the negative inotropic effect was delayed by the calcium.

SUMMARY AND CONCLUSIONS

As a group, the nonethynyl cyclohexanol derivatives were 2 to 7 times more potent than were the derivatives of cyclohexylamine in producing a hypotensive response in the intact rat. *trans*-2-*o*-Tolyl-*cis*-1,4-cyclohexanediol (CH-14) and *trans*-2-*o*-tolyl-*trans*-1,5-cyclohexanediol (CH-20) were equally effective and the most active, while the *trans* isomer of the tertiary cyclohexylamine (TH-25) was the least active and the only compound that produced a pressor response. The cyclohexylamine derivatives were up to 8 times more potent, in arresting the isolated guinea pig heart, than were the derivatives of cyclohexanol. The *trans* and *cis* isomers of the tertiary cyclohexylamine (TH-25 and TH-27) were equally effective and the most active; while *trans*-2-*o*-tolyl-*cis*-1,4-cyclohexanediol (CH-14) possessed the least amount of cardio-inhibitory activity among those compounds studied. An increase in the number of hydroxyl substituents on the cyclohexanol ring was accompanied by a decrease in both hypotensive and cardio-inhibitory activity. The minimum effective dose of 1-ethynyl-*trans*-2-*o*-tolylcyclohexanol (AH-64) in producing cardiac arrest was also the cardiotoxic dose; while the *cis* isomer (AH-65) showed the greatest cardio-inhibitory activity among the cyclohexanol derivatives studied. Spatial configuration was also significant in the blood pressure activity of the tertiary cyclohexylamines. The *cis* isomer (TH-27) produced a depressor response in contrast to the pressor response elicited by the *trans* isomer (TH-25). In the isolated atrial preparation, calcium antagonized the negative inotropic effects of *trans*-2-*o*-tolyl-*trans*-1,5-cyclohexanediol (CH-20) and *cis*-2-(*p*-chlorophenyl)-*N,N*-dimethylcyclohexylamine hydrochloride (TH-27). Moreover, calcium momentarily restored contraction of the heart arrested by 1-ethynyl-*cis*-2-*o*-tolylcyclohexanol (AH-65). In addition to its negative inotropic effect on the isolated atria, *cis*-2-(*p*-chlorophenyl)-*N,N*-dimethylcyclohexylamine hydrochloride (TH-27) also produced a negative chronotropic response which was unaffected by the administration of calcium. Decreased contraction of the myocardium appears to be responsible for the cardioplegia produced by the cyclohexanol compounds, since the pacemaker activity of the isolated atrial preparation is not affected. In addition to a decreased contraction of the myocardium, alteration of impulse formation or suppression of impulse conduction appear to be responsible for the cardioplegia produced by the cyclohexylamine derivatives. Preliminary studies of the pressor response to *trans*-2-(*p*-chlorophenyl)-*N,N*-dimethylcyclohexylamine hydrochloride (TH-25) indicate that the compound is capable of producing direct peripheral vasoconstriction.

REFERENCES

(1) Stephenson, H. E., Jr., "Cardiac Arrest and Resuscitation," E. V. Mosby, St. Louis, Mo., 1964, p. 427.
(2) Huitric, A. C., West, T. C., Durbin, R. A., and Bryan, G. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 132(1959).
(3) Smookler, H. H., and DeFeo, J. J., *J. Pharm. Sci.*, **51**, 736(1962).
(4) Smith, J. C., Jr., M. S. Thesis, University of Rhode Island, Kingston, R. I., 1963.
(5) Chenoweth, M. B., and Koelle, E. S., *J. Lab. Clin. Med.*, **31**, 600(1946).
(6) Burn, J. H., and Vane, J. R., *J. Physiol.*, **108**, 104 (1949).
(7) Marsh, D. F., *J. Pharmacol. Exptl. Therap.*, **93**, 338 (1948).
(8) Fellows, E. J., Macko, E., and McLean, R. A., *ibid.*, **100**, 267(1950).
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By B. KATCHEN and S. SYMCHOWICZ

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GRISEOFULVIN is given in large amounts and is insoluble in water. Its dose in man is 125–500 mg., and its water solubility is 15 mcg./ml. at 37°. If the digestive fluids are saturated with respect to griseofulvin at all times, and griseofulvin solubility in digestive fluids is the same as in distilled water, a minimum of 33 L. of digestive fluids must be cleared for complete absorption of 500 mg. of griseofulvin as shown by the following calculation: dose (500 mg.)/solubility (15 mg./L.) = 33 L. Clearly, oral griseofulvin dosage

Received December 16, 1966, from the Departments of Physiology and Biochemistry and Pharmaceutical Research, Research Division, Schering Corp., Bloomfield, NJ 07003
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REFERENCES

(1) Stephenson, H. E., Jr., "Cardiac Arrest and Resuscitation," E. V. Mosby, St. Louis, Mo., 1964, p. 427.
(2) Huitric, A. C., West, T. C., Durbin, R. A., and Bryan, G. H., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 132(1959).
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TABLE I—DISSOLUTION RATES OF GRISEOFULVIN PREPARATIONS

Dissolution Medium	Time, min.	% Dissolved Prepn. 1	Prepn. 2	Prepn. 3	Prepn. 4
Distilled water	5	4	4	0	0
	10	6	6	2	0
	15	9	7	5	34
	30	13	11	10	93
	45	18	13		100
	60	22	16	15	
Simulated intestinal fluid	5	6	7	3	0
	10	9	15	12	27
	15	11	24	26	69
	30	13	35	56	96
	45		45		100
	60		52	75	

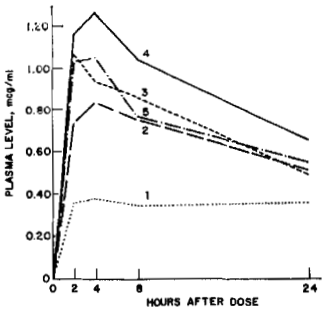

Fig. 1—Griseofulvin plasma levels. Plasma samples drawn at 0, 2, 4, 8, and 24 hr. postdrug. Each point is the mean of 10 values. Numeral near each curve is the preparation number.

TABLE II—GRISEOFULVIN PLASMA LEVELS (mcg./ml.) IN MAN FOLLOWING AN ORAL 500-mg. DOSE

Prepn.	Time, hr.	Subject[a] A	B	C	D	E	F	G	H	I	J	Mean	Area/24
1	2	(0.39)[b]	0.24	0.24	0.21	0.63	0.31	0.45	0.41	0.47	0.23	0.35	0.34
	4	(0.36)	0.25	0.31	0.10	0.76	0.27	0.64	0.46	0.44	0.17	0.38	
	8	(0.32)	0.24	0.34	0.20	0.55	0.21	0.68	0.33	0.33	0.21	0.34	
	24	(0.66)	0.32	0.33	0.55	0.38	0.27	0.37	0.47	0.22	0.32	0.36	
2	2	0.60	0.20	0.72	0.28	2.12	0.72	0.36	0.88	1.00	0.50	0.74	0.65
	4	0.60	0.24	0.59	0.66	1.80	0.78	1.67	0.70	0.86	0.47	0.84	
	8	0.71	0.80	0.59	0.21	1.44	0.74	1.45	0.50	0.69	0.36	0.75	
	24	0.83	0.61	0.59	0.19	0.57	0.48	0.48	0.45	0.50	0.43	0.51	
3	2	0.93	(0.62)	0.64	1.03	1.50	1.18	1.05	1.20	1.05	1.05	1.07	0.73
	4	1.07	(0.54)	0.47	1.00	1.22	0.96	0.95	0.78	0.94	1.11	0.94	
	8	0.88	(0.68)	0.36	0.90	1.47	0.76	0.81	0.55	1.13	0.86	0.86	
	24	0.76	(0.46)	0.28	0.46	0.55	0.58	0.20	0.35	0.62	0.59	0.49	
4	2	1.41	0.64	1.12	1.05	1.60	1.21	0.86	1.31	1.19	1.23	1.16	0.90
	4	1.01	1.00	0.90	1.61	1.40	1.26	1.90	0.93	0.48	1.10	1.26	
	8	1.00	0.71	0.79	1.20	1.60	0.80	1.90	0.64	0.91	0.83	1.04	
	24	0.99	0.45	0.64	0.79	0.80	0.61	0.62	0.44	0.54	0.61	0.65	
5	2	0.79	0.77	0.82	0.76	1.77	1.20	0.98	0.92	1.42	0.88	1.03	0.72
	4	0.79	0.34	0.82	0.78	0.94	1.10	1.17	1.03	1.40	1.01	1.05	
	8	0.44	0.44	0.62	0.63	1.57	0.52	0.98	0.71	1.11	0.64	0.77	
	24	0.69	0.69	0.42	0.40	0.64	0.39	0.45	0.45	0.60	0.81	0.56	

[a] Subjects A–E treated in hospital *A*; F–J, in hospital *B*. [b] Figures in parentheses used in place of missing results; values estimated *via* method of unweighted means.

forms must be carefully designed and evaluated to insure maximum drug availability.

Several factors affecting griseofulvin plasma levels were studied (1–6) but the effect of dissolution rate on griseofulvin absorption was not measured. This study will correlate dissolution rate of griseofulvin dosage forms and griseofulvin absorption in man.

EXPERIMENTAL

Materials—Five different 125-mg. griseofulvin preparations were studied.

Methods—At 1-week intervals, 5 different griseofulvin preparations were given to 10 humans in a three-factor repeat measure design (7). Each subject took 500 mg. griseofulvin before breakfast at approximately 8:00 a.m. and ate a standard meal at 10:00 a.m. The subjects were told to avoid, for the next 24 hr., the foods and drugs which interfere with the griseofulvin plasma assay (8).

Blood was drawn at 0, 2, 4, 8, and 24 hr. postdrug and the plasma samples were assayed fluorometrically for griseofulvin (9). All plasma levels were corrected for their zero-hour values.

Mean plasma level, which was the index of absorption, was calculated by dividing the area (determined by the trapezoidal rule) under the 24-hr. plasma level curve by 24.

Dissolution rates of single 125-mg. tablets or capsules were measured (10) in either 20 L. of distilled water or 20 L. of simulated intestinal fluid (dissolve 20 Gm. of pH 7.2 phosphate buffer powder[1] and an aqueous extract of 5 Gm. of enzyme powder[2] in 20 L. of distilled water).

RESULTS AND DISCUSSION

Dissolution Rates—Table I shows the dissolution rate of four preparations in distilled water and simulated intestinal fluid. Preparation *5* could not be analyzed because its contents clogged the 40-mesh screen of the sample holder; therefore, its

[1] Hartman-Leddon Co., Philadelphia, Pa.
[2] Marketed as VioKase by the VioBin Corp., Monticello, Ill.

TABLE III—ANALYSIS OF VARIANCE OF GRISEOFULVIN PLASMA LEVELS

Source	d.f.	*MS*	*F*	*p*
Between subjects	9			
Hospital	1	.0024	<1	>0.27
Subjects within hospital	8	.8722		
Within subjects	190			
Treatment	4	2.2643	21.77	<0.01
Treatment × hospital	4	0.0321	<1	>0.25
Treatment × subject within hospital	32	0.1040		
Hour	3	1.3310	8.20	<0.01
Hour × hospital	3	0.0621	<1	>0.25
Hour × subject within hospital	24	0.1624		
Treatment × hour	12	0.0137	4.22	<0.01
Treatment × hour × hospital	12	0.0082	<1	>0.25
Treatment × hour × subject	96	0.0324		

TABLE IV—ANALYSIS OF GRISEOFULVIN MEAN PLASMA LEVELS IN MAN BY DUNCAN'S MULTIPLE RANGE STATISTIC

Treatment	Area/24
1	0.34
2	0.65
5	0.72
3	0.73
4	0.90

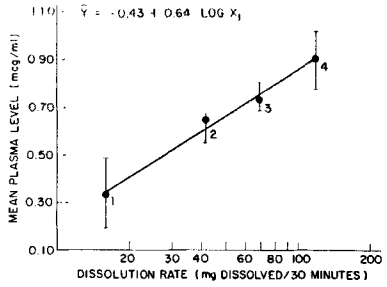

Fig. 2—Correlation of dissolution rate and mean griseofulvin plasma level. Correlation coefficient = 0.995 (p = 0.02). Brackets enclose 95% confidence intervals of regression line. Numeral beneath each point is the preparation number.

dissolution rate could not be correlated with absorption of griseofulvin.

In distilled water (Table I) the dissolution rates of preparations *1*, *2*, and *3* are very low and cannot be differentiated. However, in simulated intestinal fluid, except for preparation *1*, the dissolution rates are much higher and reveal differences between preparations related to their formulations. Thus, intestinal fluid is a more sensitive tool for evaluation of griseofulvin formulations.

Plasma Levels—The individual assay data are shown in Table II. Peak plasma levels, which vary from 0.35 to 1.26 mcg./ml., occur in 2 to 4 hr. (Fig. 1) and then decline slowly for the next 20–22 hr. at varying rates. Preparations *3* and *4*, which have the highest dissolution rates in simulated intestinal fluid, decline more rapidly than preparations *1* and *2*, which have the lowest dissolution rates in simulated intestinal fluid.

The sustained low plasma level of preparation *1* suggests slow and prolonged drug release *in vivo*. In contrast, the much higher peak plasma levels and disappearance rates of preparations *3* and *4* suggest early and rapid drug release *in vivo*. An analysis of variance procedure for repeat measure designs (Table III), which assumes a fixed effects model, was applied to the plasma levels.

The analysis shows treatments, time, and the treatment × time interaction are significant factors ($p < 0.01$). Duncan's multiple range test was applied to the area/24 values (Table IV) which were calculated for each set of plasma levels in Table II. The values not connected by a common line differ significantly ($p < 0.05$).

Correlation of Dissolution Rate and Griseofulvin Absorption—Because each preparation could not be characterized with a single dissolution rate constant, the authors arbitrarily selected amount dissolved in a given time as a measure of dissolution rate. A meaningful correlation between mean plasma level and dissolution rates in distilled water could not be found because most of the griseofulvin preparations have low dissolution rates in this solvent. However, a good correlation was found between mean griseofulvin plasma level and dissolution rate in simulated intestinal fluid. The best correlation is obtained with the logarithm of the 30-min. dissolution values (Fig. 2). The correlation coefficient is 0.995 ($p = 0.01$). The 15-min. dissolution values have a correlation coefficient of 0.96 ($p = 0.05$); the 10-min. values have a correlation coefficient of 0.82 ($p = 0.10$).

The high correlation coefficients in this study were obtained with preparations that differ widely in their composition and dissolution rates. This suggests that the ability of various griseofulvin formulations to increase plasma drug levels in man (1–3) might have been due to improved dissolution rates.

REFERENCES

(1) Duncan, W. A. M., MacDonald, G., and Thornton, M. J., *J. Pharm. Pharmacol.*, **14**, 217(1962).

(2) Atkinson, R. M., Bedford, C., Child, K. J., and Tomich, E. G., *Antibiot. Chemotherapy*, **12**, 225(1962).

(3) Crounse, R. G., *J. Invest. Dermatol.*, **37**, 529(1961).

(4) Kraml, M., Dubuc, J., and Beall, D., *Can. J. Biochem.*, **40**, 1449(1962).

(5) Marvel, J. R., Schlichting, D. A., Denton, C., Levy, E. J., and Cahn, M. M., *J. Invest. Dermatol.*, **42**, 197 (1964).

(6) Riegelman, S., Epstein, W. L., and Dayan, R. J., presented to the AMERICAN PHARMACEUTICAL ASSOCIATION, Las Vegas meeting, March 1962.

(7) Winer, B. J., "Statistical Principles in Experimental Design," McGraw-Hill Book Co., New York, N. Y., 1962.
(8) Child, K. J., Bedford, C., and Tomich, E. G., *J. Pharm. Pharmacol.*, **14**, 374(1962).
(9) Weinstein, G. D., and Blank, H., *Arch. Dermatol.*, **81**, 746(1960).
(10) Calesnick, B., Katchen, B., and Black, J., *J. Pharm. Sci.*, **54**, 1277(1965).

Conformational Analysis of 4-Hydroxycyclohexanone Oxime by NMR Spectroscopy

By WILLIAM F. TRAGER and ALAIN C. HUITRIC

The conformational free energy of the hydroxyl group of 4-hydroxycyclohexanone oxime has been found to be 0.33 and 0.61 Kcal./mole in pyridine and D_2O, respectively. These values are intermediate to those of the hydroxyl group of cyclohexanol and 4-hydroxycyclohexanone in the corresponding solvents. Comparison of the spectrum of 4-*tert*-butylcyclohexanone-3$_{(axial)}$,5,5-d_3 oxime with that of 4-hydroxycyclohexanone oxime and utilization of the long-range deshielding effect of the oximido group has permitted assignment of the individual resonances in the spectrum of the latter compound to specific hydrogens. Moreover, the comparison of spectra and use of the oximido group long-range deshielding effect has provided an independent check on the validity of the equilibrium positions calculated herein.

THE CONFORMATIONAL free energy of the hydroxyl group ($-\Delta G°_{OH}$) in cyclohexane systems has been the subject of extensive investigations over the past 10 years and is now well established. The results are summarized by Eliel and Schroeter (1). Values of 1.25 Kcal./mole in D_2O (2) and 0.83 in pyridine (3) have been obtained for cyclohexanol. It is only recently that comparative studies have been extended to six-membered saturated ring systems containing a hetero atom (4) or a trigonal carbon (5). Since the authors have found a significant difference in the values of $-\Delta G°_{OH}$ for cyclohexanol and 4-hydroxycyclohexanone at similar concentrations in a given solvent (5)[1] it was of interest to extend the investigation to the other six-membered ring compounds containing a trigonal carbon atom. The observation of an unusually large chemical shift difference between the geminal hydrogens adjacent to the oximido group in 4-*tert*-butylcyclohexanone oxime (6) pointed to 4-hydroxycyclohexanone oxime as an attractive system to study both phenomena.

DISCUSSION

Conformational Analysis—Conformational preference of the two chair conformers 1a and 1b, Fig. 1, was determined by the signal width method (5, 7–12) as described for 4-hydroxycyclohexanone (5). The mole fraction of conformer 1a (*Na*) was obtained from w, the width of the signal of H-4 (hydrogen Z) in the mobile system, and from w_e and w_a, the widths of the signals of H-1 in the two conformationally homogeneous models *trans*- and *cis*-4-*tert*-butylcyclohexanol-3$_{(axial)}$,5,5-d_3, II and III, respectively, by the following relationship (5):

$$Na = \frac{w - w_a}{w_e - w_a}$$

From the spectrum of II in pyridine, w_e was found to be 30.0 c.p.s., Jaa = 10.7, Jae = 4.4, and J_{gem} = 12.0 c.p.s.; and the spectrum of III in pyridine gave w_a = 11.0 c.p.s., Jea $\simeq$ Jee $\simeq$ 2.7 c.p.s. (5).

In pyridine the signal of Z has a width w, of 23 c.p.s. which leads to a value of 0.63 for the mole fraction *Na* of conformer 1a and a free energy difference, $-\Delta G°_{OH}$, of 0.33 Kcal./mole at 37°. In D_2O[2] the width of the signal measured from spectrum E is 24.8 c.p.s., leading to an *Na* value of 0.73 and $-\Delta G°_{OH}$ of 0.61 Kcal./mole at 37°. The addition of about two equivalents of diethylamine in pyridine did not cause any change in the width of the signal of Z. Addition of slightly over one equivalent of NaOH in D_2O caused an increase of about 0.5 c.p.s. in w. This suggests that there is very little, if any, ionization of the oxime in pyridine under the conditions of the measurements.

Assignment of Signals to Specific Hydrogens—The assignment of the signal of the Z hydrogen is obvious in all spectra. In the 60 Mc. spectra it occurs at τ 6.05 in D_2O and τ 5.91 in pyridine. In the 100 Mc. spectrum in pyridine it occurs at τ 5.88. There is also a slight difference in the chemical shift of the X hydrogen obtained from the 60 and 100 Mc. spectra in pyridine: τ 6.73 at 60 Mc. and

Received March 27, 1967, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication May 19, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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(7) Winer, B. J., "Statistical Principles in Experimental Design," McGraw-Hill Book Co., New York, N. Y., 1962.
(8) Child, K. J., Bedford, C., and Tomich, E. G., *J. Pharm. Pharmacol.*, **14**, 374(1962).
(9) Weinstein, G. D., and Blank, H., *Arch. Dermatol.*, **81**, 746(1960).
(10) Calesnick, B., Katchen, B., and Black, J., *J. Pharm. Sci.*, **54**, 1277(1965).

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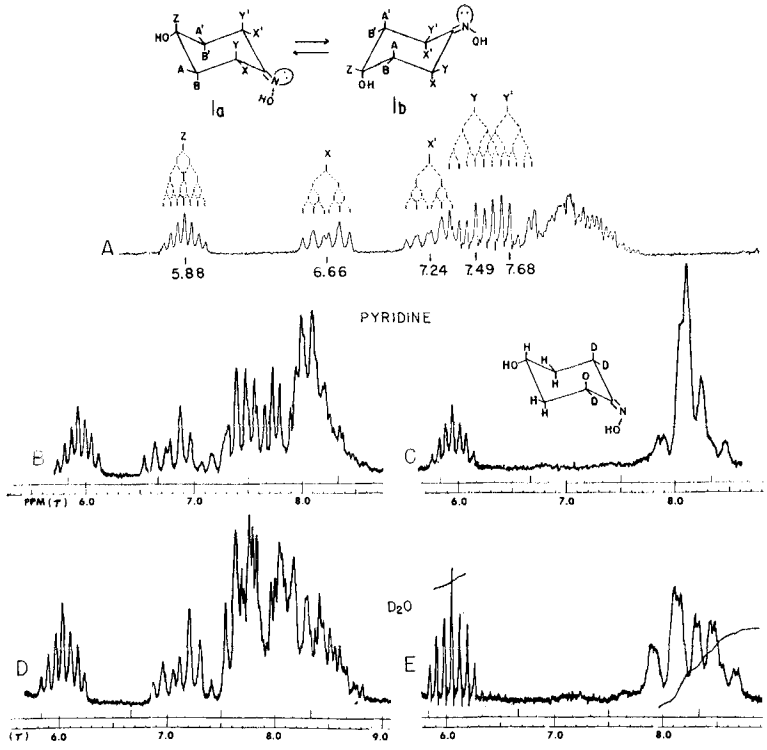
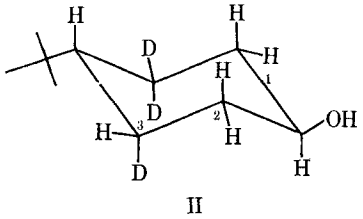
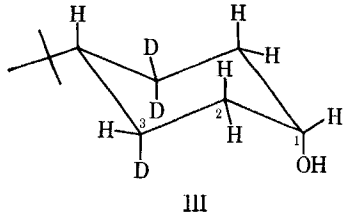


Fig. 1—NMR spectra of 4-hydroxycyclohexanone oxime (A, B, *and* D) *and the 2,2,6,6-*d_4 *analog* (C *and* E) *in pyridine and* D_2O *at 37°;* A *at 100 Mc. in pyridine,* B *and* C *at 60 Mc. in pyridine, and* D *and* E *at 60 Mc. in* D_2O.

τ 6.66 at 100 Mc. Small differences in observed chemical shifts obtained from 60 and 100 Mc. spectra are not uncommon and may arise from nonlinearity in frequency sweep with the 100 Mc. instrument, or also from nonfirst-order spectra. The 60 Mc. spectrum C of the deuterated analog shows that the difference in chemical shifts of A and B, or A′ and B′, is not sufficient to justify the first-order approximation in spectrum B (5). At 100 Mc. the complications from strong coupling and virtual coupling effects will be reduced. Comparison of spectra B and C establishes that the signals of the C-2 and C-6 hydrogens (X, X′, Y, and Y′) fall in the range between τ 6.50 and 7.85 and that the signals of the C-3 and C-5 hydrogens (A, A′, B, and B′) are above τ 7.75.



II



III

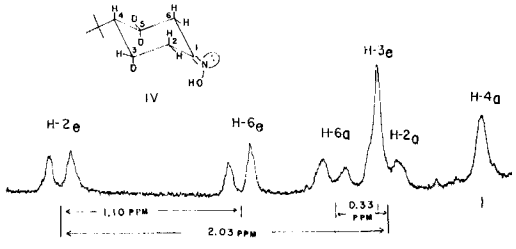


Fig. 2—NMR spectrum of 4-tert-*butylcyclohexanone-*$3_{(axial)}$,5,5-d_3 *oxime in pyridine at 100 Mc. at 37°.*

In a recent communication (6) dealing with the magnetic anisotropy of the oximido group in 4-*tert*-butylcyclohexanone-$3_{(axial)}$,5,5-d_3 oxime (IV) we have shown a large chemical shift difference between the equatorial hydrogens on C-2 and C-6 and have established the relationship between the signal of a given equatorial hydrogen at C-2 or C-6 and that of its geminal axial hydrogen. More recently experimental proof has been furnished (14) that the greatest deshielding effect of the oximido group results from the proximity of the hydroxyl group rather than from the unshared pair of electrons on the nitrogen as proposed by Saitô *et al.* (15–18). Other investigators have also presented evidence consistent with the largest deshielding effect of the hydroxyl group (19–24). On the basis of this information it is now possible to assign the signals of all hydrogens in IV as shown in the 100 Mc. spectrum measured in pyridine, Fig. 2.

The spectrum of IV, Fig. 2, provides an interesting check on the conformational equilibrium of I as calculated by the signal width method and also

permits the assignment of the signals of X, X′, Y, and Y′ in spectrum A, Fig. 1. Both spectra were measured at 100 Mc. in pyridine. In the spectrum of the conformationally homogeneous model IV there is a difference of 1.10 p.p.m. between chemical shifts of equatorial H-2 and H-6, and a difference of 0.33 p.p.m. between axial H-2 and axial H-6. The equatorial H-2 and H-6 in IV correspond to X and X′, respectively, in conformation Ia of 4-hydroxycyclohexanone oxime while in relationship to the oximido group X and X′ of conformer Ib correspond to axial H-2 and H-6, respectively, of IV. It follows, therefore, that the chemical shift difference between X and X′ should be 1.10 p.p.m. in pure conformer Ia and 0.33 p.p.m. in pure conformer Ib. In an equilibrium mixture of conformers Ia and Ib the observed spectrum at any given instant is that due to the weighted time average of the spectra arising from these two conformers. The observed difference between the chemical shifts of X and X′ in the equilibrium mixture will be a function of the populations of the two conformers. This difference for a population of 63% Ia and 37% Ib can be calculated by $[0.63(\nu_X - \nu_{X'})_{Ia} + 0.37(\nu_X - \nu_{X'})_{Ib}] = [0.63(1.10) + 0.37(-0.33)] = 0.57$ p.p.m.[3] The agreement with the observed experimental value of 0.58 p.p.m., spectrum A, is excellent.

A similar treatment for Y and Y′ yields valuable information about their relative chemical shifts and helps to unravel the complex pattern of peaks between about τ 7.35 and 7.85 resulting from the signals of Y and Y′. In conformer Ia hydrogens Y and Y′ are related to axial H-2 and H-6, respectively, of IV and their signals should be 33 c.p.s. apart, at 100 Mc., while in conformer Ib Y and Y′ are related to equatorial H-2 and H-6 of IV and their signals should be 110 c.p.s. apart. The experimental chemical shift difference between Y and Y′ in an equilibrium mixture of 63% Ia and 37% Ib should be $[0.63(\nu_Y - \nu_{Y'})_{Ia} + 0.37(Y - Y')_{Ib}] = [0.63(-0.33) + 0.37(1.10)] = 0.20$ p.p.m., or 20 c.p.s. at 100 Mc., with Y occurring at lower field. The experimental difference of 0.19 p.p.m. with the signal of Y at τ 7.49 and Y′ at τ 7.68 is in very good agreement and is consistent with the complexity of the partially overlapping signals and the relative intensities of the peaks.

Coupling Constants—Direct measurements of coupling constants from spectra A and E, Fig. 1, yield $J_{ZA} = 3.8$ and $J_{ZB} = 7.7$ c.p.s. for 4-hydroxycyclohexanone oxime in pyridine, and $J_{ZA} = 4.1$ and $J_{ZB} = 8.2$ for the deuterated analog in D_2O. The calculated values of J_{ZA} and J_{ZB}, based on the model compounds II and III, for an equilibrium mixture of 63% conformer A are:

$$J_{ZB} = 0.63\ Jaa + 0.37\ Jee = (0.63)(10.7) + (0.37)(27) = 7.7 \text{ c.p.s.}$$

$$J_{ZA} = 0.63\ Jae + 0.37\ Jea = (0.63)(4.4) + (0.37)(2.7) = 3.8 \text{ c.p.s.}$$

By similar calculations the theoretical values for an equilibrium of 73% of conformer Ia are: $J_{ZB} = 4.0$ and $J_{ZA} = 8.5$ c.p.s. The excellent agreement between observed and theoretical values suggests that in pyridine at 100 Mc. and in D_2O at 60 Mc. the difference in chemical shift between A and B is sufficient to be outside of the limits of serious complications from strong coupling effects (5) and that the first-order approximation is applicable to analysis of the Z signal. Strong coupling effects would cause a change in the relative values of observed coupling constants but would not change the sum of the coupling constants and therefore would not affect the width of the signal of Z.

Direct measurements of apparent coupling constants from the signals of X, X′, Y, and Y′ from spectrum A yield $J_{XY} = 14.2$, $J_{YB} = 9.4$, $J_{YA} = 4.0$, and $J_{XA} \simeq J_{XB} = 5.9$ c.p.s. This suggests unusual differences (26) in *Jaa*, *Jee*, *Jae*, and *Jea* between C-2 and C-3, or C-6 and C-5, vicinal hydrogens compared to the C-3 and C-4 system. The proximity of the sp^2 hydridized carbon appears to have some effect on the magnitudes of these vicinal coupling constants. The increase in geminal coupling constants by the proximity of an sp^2 carbon is reasonably well understood (27) and effects on vicinal coupling constants have been observed in α-halogenated cyclohexanone systems (9, 28–30), but in this system the chemical shift differences between the coupled hydrogens responsible for this part of the spectrum are not sufficient to truly justify the first-order approximation. This difficulty precludes speculation regarding the apparently anomalous vicinal coupling constants between hydrogens on C-2 and C-3 and on C-5 and C-6.

EXPERIMENTAL

The 60 Mc. spectra were determined on a Varian A-60 spectrometer at 37° on solutions of approximately 20% utilizing TMS as the internal standard for spectra determined in pyridine and DSS for the spectra determined in D_2O. Melting points were determined on a Fisher-Johns melting point apparatus.

4-Hydroxycyclohexanone Oxime—To a solution of 20% ammonium hydroxide (16.5 ml., 0.146 mole) was added hydroxylamine hydrochloride (4.6 Gm., 0.081 mole) with stirring. To the resulting solution was added, all at once, a solution of 4-hydroxycyclohexanone (8) (5.5 Gm., 0.048 mole) in 20 ml. of methanol. The reaction mixture was stirred for 20 min. then allowed to stand at room temperature for 22 hr. At the end of this period the reaction mixture was exhaustively extracted with ether. The ethereal extracts were combined, dried over anhydrous sodium sulfate, the mixture filtered, and the ether evaporated. The residue was taken up in ethyl acetate, treated with charcoal, then recrystallized from ethyl acetate yielding 3.4 Gm. (55%) of product, m.p 78–79°. Kahr and Berther (31) reported a boiling point of 161–164° at 11 mm. for 4-hydroxycyclohexanone oxime.

4-Hydroxycyclohexanone-2,2,6,6-d_4-oxime—This compound was obtained in a 30% yield by the slightly modified general method of Shriner, Fuson, and Curtin (32) for preparation of oximes. To a solution containing anhydrous pyridine (5 ml.), 99.8% D_2O (10 ml.), and hydroxylamine hydrochloride (1.0 Gm., 0.015 mole) was added 4-hydroxycyclohexanone-2,2,6,6-d_4 (8) (1.0 Gm., 0.0085 mole). The resulting solution was allowed to stand for 22 hr. then concentrated to approximately 2 ml. on a hot plate. The concentrated solution was extracted with 3 portions of ether, the ethereal

[3] The minus sign of $(\nu_X - \nu_{X'})_{Ib}$ results from axial H-6 being at lower field (larger ν) than axial H-2 in the model compound IV.

extracts combined, and dried over anhydrous calcium sulfate. The mixture was filtered and the ether evaporated. The residue was dissolved in a minimal amount of warm ethyl acetate, a crystal of 4-hydroxycyclohexanone oxime added, and crystallization allowed to proceed. The product was collected by filtration, m.p. 76–78°.

4 - tert - Butylcyclohexanone - $3_{(axial)}$,5,5 - d_3—This compound was obtained in 85% yield from the chromic acid oxidation of a mixture of *cis* and *trans*-4-*tert*-butylcyclohexanol-$3_{(axial)}$,5,5-d_3 (8) according to the method of Brown and Garg (33). The product was recrystallized from a water–methanol mixture, m.p. 49–50°. Ziegenbein and co-workers (34) reported a b.p. of 84–85° at 7.0 mm. and a m.p. of 49–50° for 4-*tert*-butylcyclohexanone.

4 - tert - Butylcyclohexanone - $3_{(axial)}$,5,5 - d_3-oxime—This compound was obtained in 92% yield by the slightly modified method of Ziegenbein and co-workers (34) for obtaining 4-*tert*-butylcyclohexanone oxime. To a solution of 20% ammonium hydroxide (19 ml., 0.11 mole), hydroxylamine hydrochloride (3.9 Gm., 0.056 mole), and methanol (75 ml.) was added 4-*tert*-butylcyclohexanone-$3_{(axial)}$,5,5-d_3 (5 Gm., 0.033 mole). The resulting solution was stirred for approximately 1 hr. at room temperature and then allowed to stand overnight. As the reaction progressed, the product precipitated, and after standing overnight it was collected by filtration. A second crop of product was obtained by adding water to the filtrate. The crystalline fractions were combined and recrystallized from a water–methanol mixture, m.p. 138–140°. Ziegenbein and co-workers (34) reported a m.p. of 133° for 4-*tert*-butylcyclohexanone oxime.

REFERENCES

(1) Eliel, E. L., and Schroeter, S. H., *J. Am. Chem. Soc.*, **87**, 5031(1965).
(2) Anet, F. A. L., *ibid.*, **84**, 1053(1962).
(3) Reisse, J., Celotti, J. C., Zimmerman, D., and Chiurdoglu, G., *Tetrahedron Letters*, **1964**, 2145.
(4) Chen, C. Y., and LeFèvre, R. J. W., *ibid.*, **1965**, 4057.
(5) Trager, W. F., Nist, B. J., and Huitric, A. C., *ibid.*, **1965**, 2931.
(6) Trager, W. F., and Huitric, A. C., *ibid.*, **1966**, 825.
(7) Trager, W. F., and Huitric, A. C., *J. Org. Chem.*, **30**, 3257(1965).
(8) Trager, W. F., Nist, B. J., and Huitric, A. C., *J. Pharm. Sci.*, **56**, 698(1967).
(9) Garbisch, E. W., Jr., *J. Am. Chem. Soc.*, **86**, 1780 (1964).
(10) Feltkamp, H., and Franklin, N. D., *Ann. Chem.*, **683**, 55(1965).
(11) Feltkamp, H., and Franklin, N. D., *J. Am. Chem. Soc.*, **87**, 1616(1965).
(12) Franklin, N. C., and Feltkamp, H., *Angew Chem. (Intern. Ed.)*, **4**, 774(1965).
(13) Trager, W. F., Nist, B. J., and Huitric, A. C., *Tetrahedron Letters*, **1965**, 267.
(14) Huitric, A. C., Roll, D. B., and DeBoer, J. R., *J. Org. Chem.*, **32**, 1661(1967).
(15) Saitô, H., Nukada, K., and Ohno, M., *Tetrahedron Letters*, **1964**, 2124.
(16) Saitô, H., and Nukada, K., *ibid.*, **1965**, 2117.
(17) Saitô, H., *J. Chem. Soc., Japan, Pure Chem. Sect.*, **85**, 724(1964).
(18) Saitô, H., and Nukada, K., *J. Mol. Spectry.*, **18**, 1 (1965).
(19) Karabatsos, G. J., Taller, R. A., and Vane, F. M., *J. Am. Chem. Soc.*, **85**, 2327(1963).
(20) Phillips, W. D., *Ann. N. Y. Acad. Sci.*, **70**, 817(1958).
(21) Lustig, E., *J. Phys. Chem.*, **65**, 491(1961).
(22) Slomp, G., and Wechter, W. J., *Chem. Ind.*, **1962**, 41.
(23) Mazur, R. H., *J. Org. Chem.*, **28**, 248(1963).
(24) Benson, W. G., and Pohland, A. E., *J. Org. Chem.*, **30**, 1129(1965).
(25) Huitric, A. C., Carr, J. B., and Trager, W. F., *J. Pharm. Sci.*, **55**, 211(1966).
(26) Huitric, A. C., Carr, J. B., Trager, W. F., and Nist, B. J., *Tetrahedron*, **19**, 2145(1963).
(27) Pople, J. A., and Bothner-By, A. A., *J. Chem. Phys.*, **42**, 1339(1965).
(28) Chen, C. Y., and LèFevre, R. J. W., *J. Chem. Soc.*, **1965**, 3700.
(29) Williamson, K. L., and Johnson, W. S., *J. Am. Chem. Soc.*, **83**, 4623(1961).
(30) Abraham, R. J., and Holker, J. S. E., *J. Chem. Soc.*, **1963**, 806.
(31) Kahr, K., and Berther, C., *Chem. Ber.*, **93**, 132(1960).
(32) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," 5th ed., John Wiley & Sons, Inc., New York, N. Y., 1964, p. 289, Method A.
(33) Brown, H. C., and Garg, C. P., *J. Am. Chem. Soc.*, **83**, 2952(1961).
(34) Ziegenbein, W., Schaffer, A., and Kaufhold, R., *Chem. Ber.*, **88**, 1906(1955).

Octamethyleneimine in the Mannich Reaction I

Substituted β-Amino Ketones and Substituted α-Amino Alcohols

By HEINO A. LUTS, J. F. GRATTAN, S. YANKELOWITZ*, and W. L. NOBLES†

A group of Mannich bases, utilizing octamethyleneimine as the amine component, has been prepared. Their preparation and biological activities are given.

OVER A period of years, a variety of Mannich bases has been prepared (1–26) for pharmacological testing. A typical Mannich condensation of the type discussed here may be illustrated as follows:

$$R{-}\overset{\overset{O}{\|}}{C}{-}CH_3 + HCHO + R_2NH{\cdot}HCl \rightarrow RCOCH_2CH_2NR_2{\cdot}HCl + H_2O$$

Certain substituted β-aminoketones and derivatives have been reported (2, 3, 5, 7) to show both local anesthetic and antispasmodic activity. The extensive literature dealing with

Received February 10, 1967, from Structure-Activity Research, Inc., Oxford, MS 38655, and Smith, Miller & Patch, New York, NY 10010
Accepted for publication May 23, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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extracts combined, and dried over anhydrous calcium sulfate. The mixture was filtered and the ether evaporated. The residue was dissolved in a minimal amount of warm ethyl acetate, a crystal of 4-hydroxycyclohexanone oxime added, and crystallization allowed to proceed. The product was collected by filtration, m.p. 76–78°.

4 - tert - Butylcyclohexanone - $3_{(axial)}$,5,5 - d_3—This compound was obtained in 85% yield from the chromic acid oxidation of a mixture of *cis* and *trans*-4-*tert*-butylcyclohexanol-$3_{(axial)}$,5,5-d_3 (8) according to the method of Brown and Garg (33). The product was recrystallized from a water–methanol mixture, m.p. 49–50°. Ziegenbein and co-workers (34) reported a b.p. of 84–85° at 7.0 mm. and a m.p. of 49–50° for 4-*tert*-butylcyclohexanone.

4 - tert - Butylcyclohexanone - $3_{(axial)}$,5,5 - d_3-oxime—This compound was obtained in 92% yield by the slightly modified method of Ziegenbein and co-workers (34) for obtaining 4-*tert*-butylcyclohexanone oxime. To a solution of 20% ammonium hydroxide (19 ml., 0.11 mole), hydroxylamine hydrochloride (3.9 Gm., 0.056 mole), and methanol (75 ml.) was added 4-*tert*-butylcyclohexanone-$3_{(axial)}$,5,5-d_3 (5 Gm., 0.033 mole). The resulting solution was stirred for approximately 1 hr. at room temperature and then allowed to stand overnight. As the reaction progressed, the product precipitated, and after standing overnight it was collected by filtration. A second crop of product was obtained by adding water to the filtrate. The crystalline fractions were combined and recrystallized from a water–methanol mixture, m.p. 138–140°. Ziegenbein and co-workers (34) reported a m.p. of 133° for 4-*tert*-butylcyclohexanone oxime.

REFERENCES

(1) Eliel, E. L., and Schroeter, S. H., *J. Am. Chem. Soc.*, **87**, 5031(1965).
(2) Anet, F. A. L., *ibid.*, **84**, 1053(1962).
(3) Reisse, J., Celotti, J. C., Zimmerman, D., and Chiurdoglu, G., *Tetrahedron Letters*, **1964**, 2145.
(4) Chen, C. Y., and LeFèvre, R. J. W., *ibid.*, **1965**, 4057.
(5) Trager, W. F., Nist, B. J., and Huitric, A. C., *ibid.*, **1965**, 2931.
(6) Trager, W. F., and Huitric, A. C., *ibid.*, **1966**, 825.
(7) Trager, W. F., and Huitric, A. C., *J. Org. Chem.*, **30**, 3257(1965).
(8) Trager, W. F., Nist, B. J., and Huitric, A. C., *J. Pharm. Sci.*, **56**, 698(1967).
(9) Garbisch, E. W., Jr., *J. Am. Chem. Soc.*, **86**, 1780 (1964).
(10) Feltkamp, H., and Franklin, N. D., *Ann. Chem.*, **683**, 55(1965).
(11) Feltkamp, H., and Franklin, N. D., *J. Am. Chem. Soc.*, **87**, 1616(1965).
(12) Franklin, N. C., and Feltkamp, H., *Angew Chem. (Intern. Ed.)*, **4**, 774(1965).
(13) Trager, W. F., Nist, B. J., and Huitric, A. C., *Tetrahedron Letters*, **1965**, 267.
(14) Huitric, A. C., Roll, D. B., and DeBoer, J. R., *J. Org. Chem.*, **32**, 1661(1967).
(15) Saitô, H., Nukada, K., and Ohno, M., *Tetrahedron Letters*, **1964**, 2124.
(16) Saitô, H., and Nukada, K., *ibid.*, **1965**, 2117.
(17) Saitô, H., *J. Chem. Soc., Japan, Pure Chem. Sect.*, **85**, 724(1964).
(18) Saitô, H., and Nukada, K., *J. Mol. Spectry.*, **18**, 1 (1965).
(19) Karabatsos, G. J., Taller, R. A., and Vane, F. M., *J. Am. Chem. Soc.*, **85**, 2327(1963).
(20) Phillips, W. D., *Ann. N. Y. Acad. Sci.*, **70**, 817(1958).
(21) Lustig, E., *J. Phys. Chem.*, **65**, 491(1961).
(22) Slomp, G., and Wechter, W. J., *Chem. Ind.*, **1962**, 41.
(23) Mazur, R. H., *J. Org. Chem.*, **28**, 248(1963).
(24) Benson, W. G., and Pohland, A. E., *J. Org. Chem.*, **30**, 1129(1965).
(25) Huitric, A. C., Carr, J. B., and Trager, W. F., *J. Pharm. Sci.*, **55**, 211(1966).
(26) Huitric, A. C., Carr, J. B., Trager, W. F., and Nist, B. J., *Tetrahedron*, **19**, 2145(1963).
(27) Pople, J. A., and Bothner-By, A. A., *J. Chem. Phys.*, **42**, 1339(1965).
(28) Chen, C. Y., and LèFevre, R. J. W., *J. Chem. Soc.*, **1965**, 3700.
(29) Williamson, K. L., and Johnson, W. S., *J. Am. Chem. Soc.*, **83**, 4623(1961).
(30) Abraham, R. J., and Holker, J. S. E., *J. Chem. Soc.*, **1963**, 806.
(31) Kahr, K., and Berther, C., *Chem. Ber.*, **93**, 132(1960).
(32) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," 5th ed., John Wiley & Sons, Inc., New York, N. Y., 1964, p. 289, Method A.
(33) Brown, H. C., and Garg, C. P., *J. Am. Chem. Soc.*, **83**, 2952(1961).
(34) Ziegenbein, W., Schaffer, A., and Kaufhold, R., *Chem. Ber.*, **88**, 1906(1955).

Octamethyleneimine in the Mannich Reaction I

Substituted β-Amino Ketones and Substituted α-Amino Alcohols

By HEINO A. LUTS, J. F. GRATTAN, S. YANKELOWITZ*, and W. L. NOBLES†

A group of Mannich bases, utilizing octamethyleneimine as the amine component, has been prepared. Their preparation and biological activities are given.

OVER A period of years, a variety of Mannich bases has been prepared (1–26) for pharmacological testing. A typical Mannich condensation of the type discussed here may be illustrated as follows:

$$R{-}\overset{\overset{O}{\|}}{C}{-}CH_3 + HCHO + R_2NH{\cdot}HCl \rightarrow RCOCH_2CH_2NR_2{\cdot}HCl + H_2O$$

Certain substituted β-aminoketones and derivatives have been reported (2, 3, 5, 7) to show both local anesthetic and antispasmodic activity. The extensive literature dealing with

Received February 10, 1967, from Structure-Activity Research, Inc., Oxford, MS 38655, and Smith, Miller & Patch, New York, NY 10010
Accepted for publication May 23, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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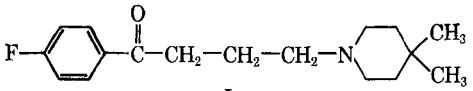
the Mannich reaction has been reviewed by Blicke (27) and later by Reichert (28).

Mannich and Lammering (2) reported that β-piperidinopropiophenone hydrochloride possesses local anesthetic activity. Denton and co-workers (7) in a series of papers described the antispasmodic activity of the Mannich bases. Under the conditions reported by these workers for determining antispasmodic activity, β-piperidinopropiophenone hydrochloride and 2-(β-piperidinopropionyl)-thiophene hydrochloride possess antispasmodic activity equal to that of β-diethylaminoethyl diphenyl acetate hydrochloride[1] and greater than that of papaverine. Burckhalter and Johnson (20) reported the antibacterial activity of certain Mannich bases derived from α,β-unsaturated ketones. Mercier and his associates indicated that the diethylamino Mannich base from acetophenone was adrenolytic, hypotensive, and ganglioplegic (10). β-Amino ketones derived from hexamethyleneimine were reported to possess CNS tranquilizing ability as well as local anesthetic activity (29, 30).

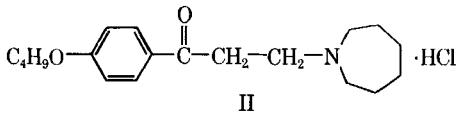
Most of the compounds prepared to the present have been alkylamino, dialkylamino, or 5- or 6-membered heterocyclic rings; only a few large ring amines have been prepared. In the last paper (26), heptamethyleneimine was used; now an attempt has been made to extend the Mannich reaction to a larger nitrogen-containing ring and to evaluate the compounds thus obtained. The authors have anticipated that the enlargement of the carbon content of the molecule would increase the lipid solubility and, because of the different shape of the amine ring (as compared to simple dialkylamines), it was felt also that these compounds might possibly exhibit different biological properties.

The demonstration that larger nitrogen-containing ring systems or highly branched amines in Mannich bases tend to elicit (26) a significant pharmacological activity which may or may not be particularly pronounced in Mannich bases containing smaller ring systems has influenced the continuation of this work. Recently, more evidence, in a degree, of this has come to our attention in the work of Simpson (31) that 4-fluoro-4-(4,4-dimethylpiperidino)butyrophenone acted as an antipsychotic agent and lowered blood cholesterol (I).



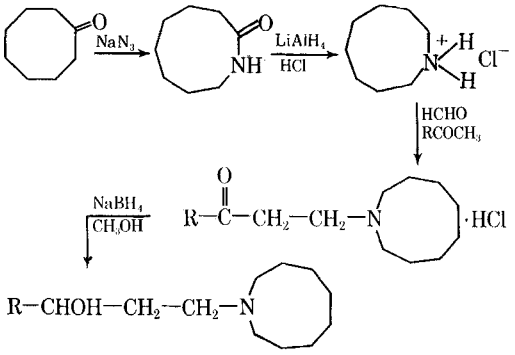
I

Zaidler and Kudrin (32) noted that 4′-butoxy-3-[hexahydro-(1*H*)azepine-1-yl]propiophenone hydrochloride has relatively high antiarrhythmic activity (II).



II

The method of preparing the octamethyleneimine was that of Blicke and associates (33). The Mannich reaction was carried out according to the original work of Tollens, in 1903 (34), and the Mannich base so obtained was reduced with sodium borohydride (35). (Scheme I.)



Scheme I

PHARMACOLOGY

The preliminary pharmacological results of these β-amino ketones and γ-amino alcohols, listed in Table I, are indicated below.

Phenylquinone Writhing Test in Mice—Good activity: 40% decrease of writhing movements as compared to untreated controls. Slight activity: 10–40% decrease of the writhing movements as compared to the untreated controls.

Hexobarbital[2] Induced Sleep in Mice—Good potentiation: 40% increase in sleeping time over the untreated controls. Slight potentiation: 10–40% increase in sleeping time over the untreated controls.

Tremorine Test in Mice—Induced tremors (T), lacrimation (L), and salivation (S). Good activity: one or more factors (T, L, S) decreased by 75–100% as compared with untreated controls. Slight activity: one or more factors (T, L, S) decreased by 50–75% as compared with untreated controls.

Compound *1* demonstrated a mild depressant effect, good activity in the phenylquinone test, possessed a transient hypotensive activity, and had an ocular anesthetic effect.

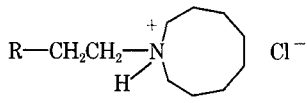
Compound *2* acted as a mild depressant and affected muscle tone. It had a good antipyretic and hypotensive activity. In the hexobarbital-induced sleep test in mice it demonstrated a good potentiation.

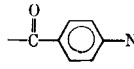
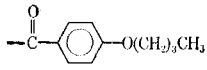
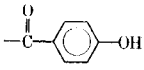
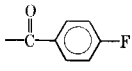
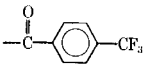
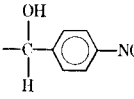
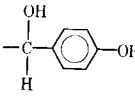
Compound *3* also exhibited a mild depressant

[1] Trademarked as Trasentin by Ciba Pharmaceutical Co., Summit, N. J.

[2] Trademarked as Evipal by Winthrop Laboratories, New York, N. Y.

TABLE I—MANNICH BASES



Compd.	R	Formula	M.p., °C.	Anal.—C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1		$C_{17}H_{25}ClN_2O_3$	190–191	59.94	59.82	7.39	7.31	8.22	8.18
2		$C_{21}H_{34}ClNO_2$	163	68.54	68.60	9.31	9.26	3.81	3.76
3		$C_{17}H_{26}ClNO_2$	160–161	65.47	65.34	8.40	8.31	4.49	4.43
4		$C_{17}H_{25}NOFCl$	138–139	65.09	65.17	8.03	7.92	4.45	4.51
5		$C_{18}H_{25}ClNOF_3$	153–154	59.41	59.56	6.95	6.82	3.85	3.97
6		$C_{15}H_{24}NSOCl$	142–143	59.68	59.55	8.01	7.89	4.67	4.56
7	 *d,l*	$C_{17}H_{27}ClN_2O_3$	182–186	59.58	59.71	8.23	8.32	8.17	8.22
8	 *d,l*	$C_{71}H_{28}ClNO_2$	144–146	65.06	65.27	8.99	8.81	4.45	4.57

effect and an effect on muscle tone. This compound demonstrated a slight sympathomimetic activity, and elicited a good phenylquinone writhing response. It also demonstrated good activity as an analgesic in the "hot plate" test method, and activity in a reversed hexobarbital test. It was also a good antipyretic.

Compound *4* acted as a mild depressant, with some effect on muscle tone, by increasing tonus. A slight phenylquinone writhing effect and a good antipyretic effect were demonstrated. There was only transient hypotensive activity.

Compound *5* acted as a mild depressant, possessing also a slight to good phenylquinone writhing effect. Good antipyretic effect was demonstrated.

Compound *6* possessed a transient hypotensive activity with a slight anti-parkinsonism effect.

Compound *7* decreased spontaneous motor activity and demonstrated some sympathomimetic activity. It had only a slight phenylquinone writhing effect. A slight analgesic effect was elicited with the "hot plate" test. Good potentiation of hexobarbital-induced sleep was noted; only slight ocular anesthetic activity was demonstrated.

Compound *8* acted as a mild depressant but showed a slight phenylquinone writhing effect. However, good activity in hexobarbital-induced sleep potentiation was noted.

EXPERIMENTAL[3]

β-Amino Ketones—The preparation of the β-amino ketones was accomplished by the following general procedure. A solution of 0.05 mole of the appropriate acetophenone, 0.05 mole of octamethyleneimine hydrochloride, 1.5 Gm. of paraformaldehyde, and 140 ml. of absolute ethanol was refluxed for 1 hr. Three-hundred milligrams of paraformaldehyde was added, and the solution was refluxed for another hour. The ethanol was then boiled off until only 25 ml. of solution remained. After cooling to room temperature, 100 ml. of acetone and 100 ml. of anhydrous ether were added. The mixture was cooled and filtered. The crystals were twice washed with 30 ml. of anhydrous ether and recrystallized from ethanol–ether.

γ-Amino Secondary Alcohols—The preparation of the γ-amino secondary alcohols was patterned after the method of Chaikin and Brown (35). The γ-amino ketone (0.05 mole) was suspended in 100 ml. of methanol and to this was added 0.1 mole of sodium borohydride. The addition was conducted at a rate such as to maintain the temperature between 20–40°. After the evolution of hydrogen had subsided, the methanol was removed first by

[3] Microanalysis was performed by Mr. G. Roberts, Jr., Florham Park, N. J. All melting points are uncorrected.

distilling, later under vacuum. The residue was suspended in 100 ml. of distilled water and extracted with three 100-ml. portions of ether. The ether was then removed under diminished pressure and the alcohol recrystallized to analytical purity from an ethanol solution.

SUMMARY

The compounds evaluated indicate that the larger ring amine components in Mannich bases show pharmacological potentialities and should be included as amine components in series. More extensive work is in progress.

REFERENCES

(1) Mannich, C., and Kresche, W., *Arch. Pharm.*, **250**, 647(1912); through *Chem. Abstr.*, **7**, 2746(1913).
(2) Mannich, C., and Lammering, D., *Ber.*, **55**, 3510 (1922).
(3) Blicke, F. F., and Blake, E. S., *J. Am. Chem. Soc.*, **52**, 235(1930).
(4) Nisbet, H. B., and Gray, C. G., *J. Chem. Soc.*, **1933**, 839.
(5) Levvy, G. A., and Nisbet, H. B., *ibid.*, **1938**, 1053.
(6) Wilson, W., and Kyi, Z. Y., *ibid.*, **1952**, 1321.
(7) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, **71**, 2048(1949).
(8) Denton, J. J., Schedl, H. P., Neier, W. B., and Brookfield, M., *ibid.*, **72**, 3792(1950).
(9) Weijlard, J., Orahovats, P. D., Sullivan, A. P., Jr., Purdue, G., Heath, F. K., and Pfister, K., *ibid.*, **78**, 2342 (1956).
(10) Mercier, F., Mercier, J., and Sectier, M. R., *J. Physiol. Paris*, **45**, 186(1954); through *Chem. Abstr.*, **47**, 11527(1953).
(11) Issekutz, B., Pórszász, J., Issekutz, L., and Nádor, K., *Acta Physiol. Acad. Sci. Hung.*, **6**, 95(1954); through *Chem. Abstr.*, **49**, 3394(1955).
(12) DaRe, R., Verlicchi, L., and Sertnikar, I., *J. Org. Chem.*, **25**, 1097(1960).
(13) Bockstahler, E. R., and Wright, D. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 542(1957).
(14) Florestano, H. J., and Bahler, M. E., *ibid.*, **45**, 320 (1956).
(15) Profit, E., *Chem. Tech. Berlin*, **3**, 210(1951); through *Chem. Abstr.*, **46**, 688(1952).
(16) Profit, E., *Chem. Tech. Berlin*, **4**, 241(1952); through *Chem. Abstr.*, **47**, 10532(1953).
(17) Profit, E., *Chem. Tech. Berlin*, **5**, 13(1953); through *Chem. Abstr.*, **48**, 7608(1954).
(18) Fuson, R. C., *Chem. Rev.*, **16**, 1(1935).
(19) Schranfstatter, E., and Deutsch, S., *Chem. Ber.*, **81**, 489(1948).
(20) Burckhalter, J. H., and Johnson, S. R., *J. Am. Chem. Soc.*, **73**, 4835(1951).
(21) Nobles, W. L., Britton, S. B., and Caldwell, H. C., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 641, 644(1954); Nobles, W. L., and Caldwell, H. C., *ibid.*, **44**, 273(1955); Nobles, W. L., and Britton, S. B., *ibid.*, **44**, 717(1955); Nobles, W. L., and Burckhalter, J. H., *ibid.*, **47**, 77(1958); Nobles, W. L., and Blanton, C. D., *J. Pharm. Sci.*, **51**, 878(1962); Nobles, W. L., and Luts, H. A., *ibid.*, **51**, 273(1962).
(22) Levvy, G. A., and Nisbet, H. B., *J. Pharmacol. Exptl. Therap.*, **65**, 129(1939); through *Chem. Abstr.*, **33**, 2985(1939).
(23) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Holcomb, W. F., and Rawlins, A. L., *J. Am. Chem. Soc.*, **68**, 1894(1946).
(24) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Jones, P. A., Holcomb, W. F., and Rawlins, A. L., *ibid.*, **70**, 1363(1948).
(25) Luts, H. A., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 273(1962).
(26) *Ibid.*, **54**, 67(1965).
(27) Blicke, F. F., "Organic Reactions," vol. I, John Wiley & Sons, New York, N. Y., 1942, p. 303.
(28) Reichert, B., "Die Mannich-Reaction," Deutscher Apotheker Verlag, Stuttgart, Germany, 1959.
(29) Kost, A. N., Kudrin, A. N., Terent'ev, P. B., and Erskov, V. V., *Vestn. Mosk.*, Ser II, **15**, (No. 3), 66(1960); through *Chem. Abstr.*, **54**, 24791(1960).
(30) Kudrin, A. N., Ershov, V. V., Troshina, A. E., Polyakova, N. B., Uspenskii, V. A., Terent'ev, P. B., and Yakovleva, I. A., *Farmakol. i Toksikol.*, **25**, 437(1962).
(31) Simpson, G. N., Kunz, E., and Watts, P. S., *Psychopharmacologia*, **8**, 223(1965).
(32) Zaidler, Ya. I., and Kudrin, A. N., *Farmakol. i Toksikol.*, **28**, 662(1965).
(33) Blicke, F. F., and Doorenbos, N. J., *J. Am. Chem. Soc.*, **76**, 2317(1954).
(34) van Marle, C. M., and Tollens, B., *Ber.*, **36**, 1351 (1903).
(35) Chaikin, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, **71**, 122(1949).

Acetylation of Acetaminophen in Tablet Formulations Containing Aspirin

By K. T. KOSHY*, A. E. TROUP, R. N. DUVALL, R. C. CONWELL, and L. L. SHANKLE

The degradation of aspirin in commercial products containing acetaminophen was found to be accompanied by the formation of diacetyl-*p*-aminophenol (DAPAP) or *p*-acetoxyacetanilid. This reaction product was not detectable using conventional acetaminophen analytical procedures, but was detected qualitatively by means of thin-layer chromatography. A procedure utilizing partition–column chromatography and gas–liquid chromatography was developed for the determination of DAPAP in products containing aspirin, acetaminophen, and caffeine. Commercial products were found to contain various levels of DAPAP up to 4 mg./tablet. Magnesium stearate was found to accelerate the reaction markedly.

THE STABILITY of aspirin *per se* in pharmaceutical dosage forms has received considerable attention and the problem is widely recognized. However, reports of the interaction of aspirin with other drugs, such as amines and phenols, have only recently begun to appear in the literature. Troup and Mitchner (1) reported that phenylephrine underwent acetylation in tablets containing aspirin. Under accelerated conditions, the mono-, di-, and triacetylated products were formed. Even at 24° the secondary amine group

Received April 11, 1967, from the Pharmaceutical Research and Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication June 2, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
* Present address: The Upjohn Co., Kalamazoo, MI 49001

distilling, later under vacuum. The residue was suspended in 100 ml. of distilled water and extracted with three 100-ml. portions of ether. The ether was then removed under diminished pressure and the alcohol recrystallized to analytical purity from an ethanol solution.

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(3) Blicke, F. F., and Blake, E. S., *J. Am. Chem. Soc.*, **52**, 235(1930).
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(5) Levvy, G. A., and Nisbet, H. B., *ibid.*, **1938**, 1053.
(6) Wilson, W., and Kyi, Z. Y., *ibid.*, **1952**, 1321.
(7) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, **71**, 2048(1949).
(8) Denton, J. J., Schedl, H. P., Neier, W. B., and Brookfield, M., *ibid.*, **72**, 3792(1950).
(9) Weijlard, J., Orahovats, P. D., Sullivan, A. P., Jr., Purdue, G., Heath, F. K., and Pfister, K., *ibid.*, **78**, 2342 (1956).
(10) Mercier, F., Mercier, J., and Sectier, M. R., *J. Physiol. Paris*, **45**, 186(1954); through *Chem. Abstr.*, **47**, 11527(1953).
(11) Issekutz, B., Pórszász, J., Issekutz, L., and Nádor, K., *Acta Physiol. Acad. Sci. Hung.*, **6**, 95(1954); through *Chem. Abstr.*, **49**, 3394(1955).
(12) DaRe, R., Verlicchi, L., and Sertnikar, I., *J. Org. Chem.*, **25**, 1097(1960).
(13) Bockstahler, E. R., and Wright, D. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 542(1957).
(14) Florestano, H. J., and Bahler, M. E., *ibid.*, **45**, 320 (1956).
(15) Profit, E., *Chem. Tech. Berlin*, **3**, 210(1951); through *Chem. Abstr.*, **46**, 688(1952).
(16) Profit, E., *Chem. Tech. Berlin*, **4**, 241(1952); through *Chem. Abstr.*, **47**, 10532(1953).
(17) Profit, E., *Chem. Tech. Berlin*, **5**, 13(1953); through *Chem. Abstr.*, **48**, 7608(1954).
(18) Fuson, R. C., *Chem. Rev.*, **16**, 1(1935).
(19) Schranfstatter, E., and Deutsch, S., *Chem. Ber.*, **81**, 489(1948).
(20) Burckhalter, J. H., and Johnson, S. R., *J. Am. Chem. Soc.*, **73**, 4835(1951).
(21) Nobles, W. L., Britton, S. B., and Caldwell, H. C., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 641, 644(1954); Nobles, W. L., and Caldwell, H. C., *ibid.*, **44**, 273(1955); Nobles, W. L., and Britton, S. B., *ibid.*, **44**, 717(1955); Nobles, W. L., and Burckhalter, J. H., *ibid.*, **47**, 77(1958); Nobles, W. L., and Blanton, C. D., *J. Pharm. Sci.*, **51**, 878(1962); Nobles, W. L., and Luts, H. A., *ibid.*, **51**, 273(1962).
(22) Levvy, G. A., and Nisbet, H. B., *J. Pharmacol. Exptl. Therap.*, **65**, 129(1939); through *Chem. Abstr.*, **33**, 2985(1939).
(23) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Holcomb, W. F., and Rawlins, A. L., *J. Am. Chem. Soc.*, **68**, 1894(1946).
(24) Burckhalter, J. H., Tendick, F. H., Jones, E. M., Jones, P. A., Holcomb, W. F., and Rawlins, A. L., *ibid.*, **70**, 1363(1948).
(25) Luts, H. A., and Nobles, W. L., *J. Pharm. Sci.*, **51**, 273(1962).
(26) *Ibid.*, **54**, 67(1965).
(27) Blicke, F. F., "Organic Reactions," vol. I, John Wiley & Sons, New York, N. Y., 1942, p. 303.
(28) Reichert, B., "Die Mannich-Reaction," Deutscher Apotheker Verlag, Stuttgart, Germany, 1959.
(29) Kost, A. N., Kudrin, A. N., Terent'ev, P. B., and Erskov, V. V., *Vestn. Mosk.*, Ser II, **15**, (No. 3), 66(1960); through *Chem. Abstr.*, **54**, 24791(1960).
(30) Kudrin, A. N., Ershov, V. V., Troshina, A. E., Polyakova, N. B., Uspenskii, V. A., Terent'ev, P. B., and Yakovleva, I. A., *Farmakol. i Toksikol.*, **25**, 437(1962).
(31) Simpson, G. N., Kunz, E., and Watts, P. S., *Psychopharmacologia*, **8**, 223(1965).
(32) Zaidler, Ya. I., and Kudrin, A. N., *Farmakol. i Toksikol.*, **28**, 662(1965).
(33) Blicke, F. F., and Doorenbos, N. J., *J. Am. Chem. Soc.*, **76**, 2317(1954).
(34) van Marle, C. M., and Tollens, B., *Ber.*, **36**, 1351 (1903).
(35) Chaikin, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, **71**, 122(1949).

Acetylation of Acetaminophen in Tablet Formulations Containing Aspirin

By K. T. KOSHY*, A. E. TROUP, R. N. DUVALL, R. C. CONWELL, and L. L. SHANKLE

The degradation of aspirin in commercial products containing acetaminophen was found to be accompanied by the formation of diacetyl-*p*-aminophenol (DAPAP) or *p*-acetoxyacetanilid. This reaction product was not detectable using conventional acetaminophen analytical procedures, but was detected qualitatively by means of thin-layer chromatography. A procedure utilizing partition–column chromatography and gas–liquid chromatography was developed for the determination of DAPAP in products containing aspirin, acetaminophen, and caffeine. Commercial products were found to contain various levels of DAPAP up to 4 mg./tablet. Magnesium stearate was found to accelerate the reaction markedly.

THE STABILITY of aspirin *per se* in pharmaceutical dosage forms has received considerable attention and the problem is widely recognized. However, reports of the interaction of aspirin with other drugs, such as amines and phenols, have only recently begun to appear in the literature. Troup and Mitchner (1) reported that phenylephrine underwent acetylation in tablets containing aspirin. Under accelerated conditions, the mono-, di-, and triacetylated products were formed. Even at 24° the secondary amine group

Received April 11, 1967, from the Pharmaceutical Research and Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication June 2, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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was found to be acetylated to the extent of 14% after 34 months of storage.

Jacobs *et al.* (2), in a study of a capsule formulation containing aspirin and codeine, observed that acetylcodeine was formed but apparently only at elevated temperatures in the presence of moisture. No acetylcodeine was detected in commercial samples stored for 32 months at room temperature.

More recently, Schwartz and Amidon (3) recognized the possibility that aspirin might react with amino groups of proteins.

Preliminary work in these laboratories had indicated the possibility of an interaction between aspirin and acetaminophen (APAP) in pharmaceutical preparations. This paper summarizes the investigation of this interaction and shows, in particular, the extent to which it is taking place in commercial products.

Initial qualitative identification of the reaction product diacetyl-*p*-aminophenol (DAPAP) or *p*-acetoxyacetanilid was accomplished by means of thin-layer chromatography. Present assay methods for APAP, such as the column-partition method of Koshy (4), or a similar method by Levine and Hohmann (5), or the N.F. XII method (6) would not distinguish between APAP and DAPAP. Colorimetric methods which depend upon hydrolysis of APAP also lack specificity since DAPAP is readily hydrolyzed to *p*-aminophenol.

A quantitative method applicable to aspirin, APAP, and caffeine mixtures containing 0.01 to 20 mg. of DAPAP per tablet was developed. The procedure consists of the removal of caffeine, aspirin, and salicylic acid by a modification of the partition-column method developed by Levine (7) and removal of APAP and *p*-aminophenol (PAP) by a silicic acid column with water as the stationary phase. The residue obtained after passing the sample through the three columns was analyzed for DAPAP content by gas-liquid chromatography.

EXPERIMENTAL

Preparation of DAPAP—Twenty-seven grams of APAP was added to a mixture of 40 ml. acetic anhydride and 40 ml. reagent grade pyridine. This was refluxed 4 hr. on a steam bath. A precipitate formed when the mixture was allowed to cool to room temperature. The precipitate was collected by filtration and washed with 500 ml. of cold water to remove the pyridine and acetic anhydride. The residue was suspended in 150 ml. of hot water and heated almost to boiling. Small amounts of methanol were added until complete dissolution occurred. The solution was allowed to cool slowly to obtain the product, m.p. 152–156°. [Lit. m.p. 151–154° (8).]

Anal.—Calcd. for $C_{10}H_{11}NO_3$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.85; H, 6.00; N, 7.54.

Qualitative Detection of DAPAP—One ground tablet was shaken thoroughly on a mechanical shaker with 10 ml. of chloroform and filtered, and the filtrate was evaporated to dryness under a stream of air. The residue was shaken with 1 ml. 3A alcohol and filtered. Twenty microliters of the filtrate was spotted on a 0.25-mm. Silica Gel G (Brinkmann Instruments, Inc.) plate previously activated by heating for 1 hr. at 105°. Twenty microliters of a reference solution of pure DAPAP (300 mcg./ml.) was also spotted for comparison. The plate was developed in a solvent mixture of chloroform–acetone–acetic acid (80:18:2) and allowed to dry. The DAPAP appeared as a yellow spot under long wavelength (350 mμ) U.V. light after about 10-min. irradiation with short wavelength (254 mμ) U.V. light.

For samples containing salicylamide the same chromatography system was used except aluminum oxide (neutral alumina AG7, Bio·Rad Laboratories) was used instead of silica gel.

Quantitative Determination of DAPAP—*Column Preparation*—All solvents used on the columns were water saturated. Three glass chromatographic columns, each 19 × 450 mm. (Fischer and Porter Co.), were used. The first column contained 10 Gm. of 100 mesh, analytical reagent grade silicic acid (Mallinckrodt Chemical Works) thoroughly mixed with 7 ml. of 4 *N* sulfuric acid. The column was washed with 100 ml. ethyl ether which had been freshly distilled over zinc and potassium hydroxide. The second column contained 10 Gm. silicic acid thoroughly mixed with 7 ml. water and was washed with 100 ml. chloroform N.F. (Allied Chemical, General Chemical Division). The third column contained 20 Gm. of diatomaceous earth[1] thoroughly mixed with 20 ml. 1 *N* sodium bicarbonate solution. The column was washed with 200 ml. chloroform N.F. It was necessary to acid wash the diatomaceous earth before it could be used. This was accomplished by adding a sufficient quantity of concentrated hydrochloric acid to the diatomaceous earth to form a thin slurry. The mixture was heated on a steam bath for about 1 hr. The diatomaceous earth was then washed free of acid with distilled water and dried for 16 hr. at 105°.

Sample Preparation—Five noneffervescent or two effervescent tablets were ground and shaken with 20 ml. of chloroform for about 5 min. The mixture was filtered through Whatman No. 541 filter paper into a 25-ml. volumetric flask and brought to volume by washing the residue with a small amount of chloroform.

Column Operation—Five milliliters of the sample solution together with 10 ml. of ether were added to the first column. When the liquid level reached the top of the silicic acid column, 10 ml. of ether was added. When the liquid level again reached the top of the silicic acid, 50 additional ml. of ether was added. The entire eluate was collected in a 150-ml. beaker. The eluate was evaporated to dryness using a current of air. (*Caution:* no heat.) The residue was dissolved in about 10 ml. of chloroform and transferred quantitatively to the second column using an additional 10 ml. of chloroform to rinse

[1] Marketed as Celite 545 by the Johns-Manville Corp., New York, N. Y.

the beaker. The rinsings were added to the column after the liquid just reached the top of the silicic acid. Another 50 ml. of chloroform was added to the column and the entire eluate was collected in a 150-ml. beaker and diluted to 100 ml. to reduce the aspirin concentration for more effective separation. The eluate was transferred quantitatively to the third column. When the solution level reached the top of the diatomaceous earth column, 100 ml. of chloroform was added in small portions. The eluate was collected in a 250-ml. beaker and evaporated down to a few milliliters on a steam bath. The concentrate was quantitatively transferred to a small vial and the evaporation was continued to dryness. The amount of DAPAP in the residue was determined by gas–liquid chromatography.

Gas–Liquid Chromatography—An F & M model 810 gas chromatograph with a flame ionization detector was used with an aluminum column $1^1/_2$-ft. by $^1/_4$-in. o.d. containing 5% Carbowax 20 M (Union Carbide Corp.) on Chromosorb G 70/80 mesh (Johns-Manville). The column temperature was 225° and both detector and injection port temperatures were 275°. Gas flow rates were: helium, 60 ml./min.; hydrogen, 65 ml./min.; and air, 300 ml./min. The sample residue was dissolved in 1–6 ml. of acetone N.F. depending upon the DAPAP content. Then, depending upon DAPAP concentration, 10 to 30 μl. of the sample was injected using a Beckman liquid sampling syringe. The amount of DAPAP was determined by comparison of sample peak height with a standard injected concurrently.

Salicylic Acid Determination—Salicylic acid was determined according to the method reported by Gold and Campbell (9).

RESULTS AND DISCUSSION

Initial qualitative evidence for the occurrence of a reaction between aspirin and APAP was obtained by thin-layer chromatography. A mixture of aspirin, APAP, and magnesium stearate with 1% water added was stored for 2 weeks at 50°. A portion of the sample was dissolved in ethyl acetate and passed through a diatomaceous earth column containing sodium bicarbonate as stationary phase to remove aspirin and salicylic acid. The effluent was evaporated to dryness using a current of air, the residue redissolved in acetone, and 20 μl. spotted on a silica gel plate. Development was with chloroform–ethyl alcohol–acetic acid (88:10:2). A spot was revealed by iodine vapor which did not correspond to salicylic acid, aspirin, or APAP. It was postulated that DAPAP was formed. Another solvent system, as described under *Experimental*, was developed which would separate DAPAP from other materials in commercial samples containing aspirin, APAP, and caffeine and two possible degradation products found in such mixtures, salicylic acid and *p*-aminophenol (PAP). Results are shown in Fig. 1. Samples 2 and 3 established that DAPAP could be separated from the other ingredients. Although the spot in sample 4 at R_f 0.52 is slightly lower than the DAPAP known, it was readily recognizable as DAPAP because of the yellow color exhibited by DAPAP under U.V. irradiation. Subsequent analysis of this same sample indicated 1.8 mg. of DAPAP per tablet.

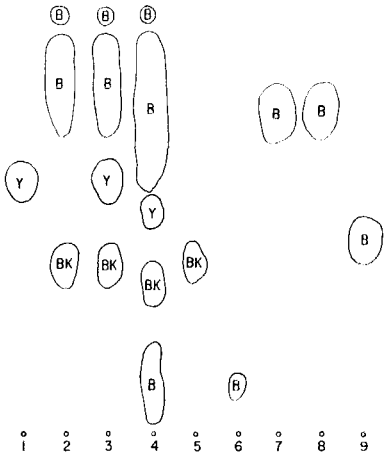

Fig. 1—Thin-layer chromatogram of aspirin, APAP, caffeine mixtures, and possible degradation products. Key: 1, DAPAP; 2, aspirin, APAP, caffeine mixture; 3, 2 plus DAPAP; 4, product A; 5, APAP; 6, PAP; 7, aspirin; 8, SA; 9, caffeine. Color under U.V. irradiation; B, *blue;* BK, *black;* Y, *yellow.*

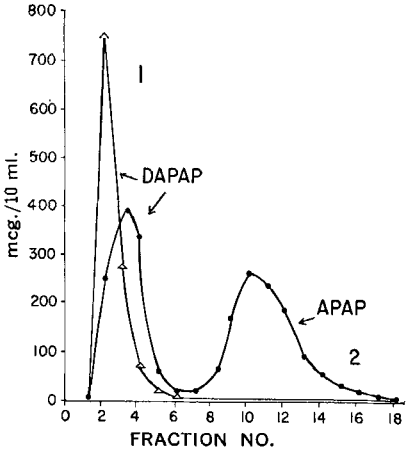

Fig. 2—Elution curve of APAP and DAPAP from silicic acid–water column. Key: 1, chloroform elution; 2, ether elution.

A procedure utilizing both partition column chromatography and gas chromatography was developed for the determination of DAPAP in products containing aspirin, APAP, and caffeine. The procedure involved removal of caffeine using a silicic acid–sulfuric acid column, APAP and PAP using a silicic acid–water column, and aspirin and salicylic acid by means of a diatomaceous earth–sodium bicarbonate column. DAPAP content in the final eluate was determined by gas chromatography. It was attempted, initially, to determine DAPAP in the eluate spectrophotometrically using its absorbance at 246 mμ. However, traces of aspirin coming through in the eluate caused erratic recoveries; whereas no interference was found using gas chromatography.

Figure 2 shows the elution of APAP and DAPAP from the silicic acid–water column. When the

Table I—Recovery of DAPAP from Tablet Mixtures[a] Containing Aspirin, APAP, and Caffeine

DAPAP Added, mcg.	Found Analyst A	Found Analyst B	Av. % Recovery
20830	20700	20430	98.8
6096	6020	5980	98.5
2083	1950	1950	93.6
1002	977	961	96.7
601	591	604	99.4
401	407	394	99.9
200	196	...	98.0
100	105	104	104.5
75.2	80.5	81.8	107.9
52.1	53.7	...	103.1
25.0	27.5	23.3	101.6

[a] Each mixture contained 324 mg. aspirin, 162 mg. APAP, and 33 mg. caffeine.

column is eluted with ether the DAPAP comes off first, followed closely by the APAP. However, if chloroform is used as the eluant, the DAPAP elution is sharper and the APAP is completely retained. This column could be used to determine DAPAP as a contaminant in APAP.

One discovery noted during the course of developing the assay is worthy of note. After a few inconsistent high recoveries, each step in the procedure was critically examined and it was found that DAPAP was formed during the evaporation step of the eluate from the first column since at that time a steam bath was being used. This phenomenon emphasizes that heat should be avoided prior to aspirin–APAP separation.

Recovery studies were performed on a typical formulation containing aspirin, APAP, caffeine, and various common lubricants and excipients. Known amounts of DAPAP in chloroform were added to the powder mixture prior to analysis. Results are shown in Table I. It is apparent that this analytical method is applicable to a wide concentration range of DAPAP limited only by its solubility in chloroform (about 4% w/v). The high recoveries for samples containing less than 100 mcg. may be attributed to the presence of trace impurities, since it was necessary to operate the gas chromatograph at high sensitivities for low DAPAP levels. The over-all accuracy and precision were probably as good as could be expected, especially in view of the complexity of these mixtures.

Commercial products, of unknown age, containing aspirin and APAP, were purchased from retail outlets and analyzed for DAPAP and salicylic acid (SA) content. Results are shown in Table II. As can be seen, aspirin–APAP interaction is taking place to varying extents in the samples analyzed. Product *A*, an effervescent tablet, was found to contain considerable quantities of DAPAP ranging from 1.24–4.13 mg./tablet. Product *B*, a press-coated tablet, contained lower levels of DAPAP ranging from 36–140 mcg. per tablet. Product *C*, a compressed tablet, contained only trace quantities of DAPAP. Product *D*, a compressed tablet, contained salicylamide which interfered in the analysis of salicylic acid and DAPAP. However, using the eluate from the partition–column chromatography procedure, DAPAP could be detected qualitatively using thin-layer chromatography in two samples of this product. By running various levels of a standard DAPAP solution simultaneously with the samples, the DAPAP content of the samples was estimated to be 15 mcg./tablet.

Inspection of the data in Table II indicates a relationship between the rate of formation of SA and the rate of formation of DAPAP. To study this further, tablets of product *A* from the same batch were heated for 6 weeks at 50° and analyzed weekly for SA and DAPAP content. Results, shown in Fig. 3, indicate a linear relationship between the two degradation products. The same trend exists in various samples stored at ambient conditions, although at lower SA and DAPAP levels the correlation is not so good.

The effect of various lubricants on aspirin stability has been the subject of numerous investigations. Alkaline earth stearates are reportedly responsible for poor aspirin stability in plain aspirin tablets (10) and aspirin, phenacetin, and caffeine combina-

Table II—DAPAP and SA Content of Commercial Products Containing Aspirin and APAP

Product	Comp.	Sample	SA, mg./Tab.	DAPAP, mg./Tab.
A	324 mg. aspirin 130 mg. APAP	1	32.4	1.24
		2	45.0	1.40
		3	31.4	1.64
		4	25.5	1.77
		5	49.3	1.90
		6	37.7	2.84
		7	50.2	3.57
		8	44.5	3.70
		9	62.7	4.13
B	227 mg. aspirin 194 mg. APAP 33 mg. caffeine	1	0.72	36
		2	1.33	52
		3	1.57	96
		4	1.48	103
		5	1.01	140
C	230 mg. aspirin 125 mg. APAP 30 mg. caffeine	1	0.69	0
		2	0.37	5
		3	0.31	10
D	Aspirin APAP Caffeine Salicylamide	1	...	Detected qualitatively
		2	...	Detected qualitatively

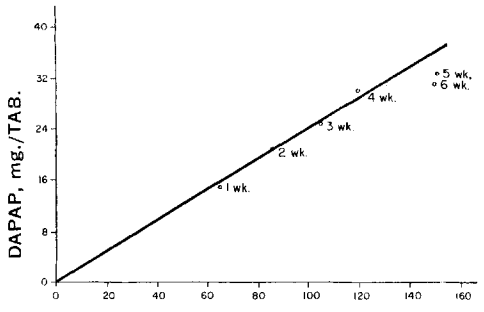

Fig. 3—Product A stored at 50°.

tions (11, 12). Magnesium stearate also has been shown to accelerate the formation of acetylphenylephrine in aspirin–phenylephrine combinations (1). Consequently, the relative effects of water, stearic acid, and magnesium stearate on the rate of formation of SA and DAPAP in aspirin–APAP mixtures were studied. Powder mixtures containing (*a*) 3.25 Gm. aspirin, 1.75 Gm. APAP, and 0.05 Gm. water; (*b*) mixture (*a*) plus 0.05 Gm. stearic acid; and (*c*) mixture (*a*) plus 0.05 Gm. magnesium stearate were sealed in small bottles and stored for 4 weeks at 50°. Subsequent DAPAP analysis of these mixtures indicated 67 mcg./Gm. in (*a*), 50 mcg./Gm. in (*b*), and 48.4 mg./Gm. in (*c*). Thus, magnesium stearate had a marked accelerating effect on the rate of DAPAP formation. Consequently, this lubricant is contraindicated in such formulations.

The pharmacological significance of the presence of DAPAP in these products is not known since information in the literature concerning this compound is scanty. Stockelbach (13) disclosed in a U. S. patent that DAPAP has valuable medicinal properties particularly as an antipyretic, antineuralgic, and sedative remedy. Charlier *et al.* (14) list DAPAP as one of the compounds studied for influence on bile secretion and toxicity, but did not give any data for DAPAP.

REFERENCES

(1) Troup, A. E., and Mitchner, H., *J. Pharm. Sci.*, **53**, 375(1964).
(2) Jacobs, A. L., Dilatush, A. E., Weinstein, S., and Windheuser, J. J., *ibid.*, **55**, 893(1966).
(3) Schwartz, M. A., and Amidon, G. L., *ibid.*, **55**, 1464 (1966).
(4) Koshy, K. T., *ibid.*, **53**, 1280(1964).
(5) Levine, J., and Hohmann, J. R., *J. Assoc. Offic. Anal. Chem.*, **49**, 533(1966).
(6) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 11.
(7) Levine, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 687 (1957).
(8) Broyles, M. H., and Easley, W. K., *J. Org. Chem.*, **25**, 2233(1960).
(9) Gold, G., and Campbell, J. A., *J. Pharm. Sci.*, **53**, 52(1964).
(10) Trakman, Yu. G., *Sb. Nauchn. Tr. Tsentr. Aptechn. Nauchn.-Issled. Inst.*, **2**, 85(1961); through *Chem. Abstr.*, **58**, 1306*h*(1963).
(11) Ribeiro, D., Stevenson, D., Samyn, J., Milosovich, G., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 226(1955).
(12) Nazareth, M. R., and Huyck, L. C., *ibid.*, **50**, 620 (1961).
(13) Stockelbach, F. E., U. S. pat. 1,034,528(1912).
(14) Charlier, R., Prost, M., and Bruckner, P., *Arzneimittel-Forsch.*, **9**, 210(1959).

Micellar Solubilization of Testosterone I

In Aqueous Solutions of Polysorbates

By ARVIND L. THAKKAR and NATHAN A. HALL

The solubility of testosterone in aqueous solutions of partially purified polysorbate 20, 40, and 60 was determined by measuring the area under the ultraviolet spectral peaks. There was solubilization below the critical micelle concentration. A linear relationship was observed between the amount of testosterone solubilized and the per cent polysorbate concentration. Polysorbate 60 exhibited the greatest solubilizing capacity and polysorbate 20 the least. The *Z*-value method for determining solvent polarity was employed to investigate the polarity of the environment in which testosterone was solubilized. At low polysorbate concentrations the *Z*-value was similar to that in water. As the polysorbate concentration increased, the *Z*-value decreased. This decrease in *Z*-value paralleled the increase in solubility of testosterone.

RECENT REVIEWS (1, 2) in pharmaceutical literature indicate that the phenomenon of solubilization continues to be of interest. In 1949 Ekwall and Sjöblom (3) published the first study on the solubilization of steroid hormones. They reported the solubility of testosterone in a 10% sodium oleate, 20% sodium myristyl sulfate, and 20% sodium cholate solutions. Nakagawa (4) has measured the solubility of testosterone in 20% aqueous solution of polyoxyethylene sorbitan monolaurate containing about 12 ethyleneoxide groups. Sjöblom and co-workers (5–7), in their

Received March 7, 1967, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication June 5, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Abstracted in part from a thesis to be presented by Arvind L. Thakkar to the Graduate School, University of Washington, Seattle, in partial fulfillment of Doctor of Philosophy degree requirements.

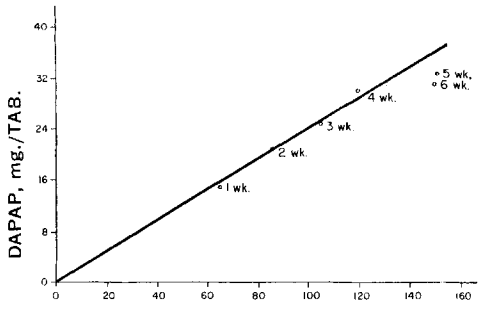

Fig. 3—Product A stored at 50°.

tions (11, 12). Magnesium stearate also has been shown to accelerate the formation of acetylphenylephrine in aspirin–phenylephrine combinations (1). Consequently, the relative effects of water, stearic acid, and magnesium stearate on the rate of formation of SA and DAPAP in aspirin–APAP mixtures were studied. Powder mixtures containing (*a*) 3.25 Gm. aspirin, 1.75 Gm. APAP, and 0.05 Gm. water; (*b*) mixture (*a*) plus 0.05 Gm. stearic acid; and (*c*) mixture (*a*) plus 0.05 Gm. magnesium stearate were sealed in small bottles and stored for 4 weeks at 50°. Subsequent DAPAP analysis of these mixtures indicated 67 mcg./Gm. in (*a*), 50 mcg./Gm. in (*b*), and 48.4 mg./Gm. in (*c*). Thus, magnesium stearate had a marked accelerating effect on the rate of DAPAP formation. Consequently, this lubricant is contraindicated in such formulations.

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REFERENCES

(1) Troup, A. E., and Mitchner, H., *J. Pharm. Sci.*, **53**, 375(1964).
(2) Jacobs, A. L., Dilatush, A. E., Weinstein, S., and Windheuser, J. J., *ibid.*, **55**, 893(1966).
(3) Schwartz, M. A., and Amidon, G. L., *ibid.*, **55**, 1464 (1966).
(4) Koshy, K. T., *ibid.*, **53**, 1280(1964).
(5) Levine, J., and Hohmann, J. R., *J. Assoc. Offic. Anal. Chem.*, **49**, 533(1966).
(6) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 11.
(7) Levine, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 687 (1957).
(8) Broyles, M. H., and Easley, W. K., *J. Org. Chem.*, **25**, 2233(1960).
(9) Gold, G., and Campbell, J. A., *J. Pharm. Sci.*, **53**, 52(1964).
(10) Trakman, Yu. G., *Sb. Nauchn. Tr. Tsentr. Aptechn. Nauchn.-Issled. Inst.*, **2**, 85(1961); through *Chem. Abstr.*, **58**, 1306*h*(1963).
(11) Ribeiro, D., Stevenson, D., Samyn, J., Milosovich, G., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 226(1955).
(12) Nazareth, M. R., and Huyck, L. C., *ibid.*, **50**, 620 (1961).
(13) Stockelbach, F. E., U. S. pat. 1,034,528(1912).
(14) Charlier, R., Prost, M., and Bruckner, P., *Arzneimittel-Forsch.*, **9**, 210(1959).

Micellar Solubilization of Testosterone I

In Aqueous Solutions of Polysorbates

By ARVIND L. THAKKAR and NATHAN A. HALL

The solubility of testosterone in aqueous solutions of partially purified polysorbate 20, 40, and 60 was determined by measuring the area under the ultraviolet spectral peaks. There was solubilization below the critical micelle concentration. A linear relationship was observed between the amount of testosterone solubilized and the per cent polysorbate concentration. Polysorbate 60 exhibited the greatest solubilizing capacity and polysorbate 20 the least. The *Z*-value method for determining solvent polarity was employed to investigate the polarity of the environment in which testosterone was solubilized. At low polysorbate concentrations the *Z*-value was similar to that in water. As the polysorbate concentration increased, the *Z*-value decreased. This decrease in *Z*-value paralleled the increase in solubility of testosterone.

RECENT REVIEWS (1, 2) in pharmaceutical literature indicate that the phenomenon of solubilization continues to be of interest. In 1949 Ekwall and Sjöblom (3) published the first study on the solubilization of steroid hormones. They reported the solubility of testosterone in a 10% sodium oleate, 20% sodium myristyl sulfate, and 20% sodium cholate solutions. Nakagawa (4) has measured the solubility of testosterone in 20% aqueous solution of polyoxyethylene sorbitan monolaurate containing about 12 ethyleneoxide groups. Sjöblom and co-workers (5–7), in their

Received March 7, 1967, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication June 5, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Abstracted in part from a thesis to be presented by Arvind L. Thakkar to the Graduate School, University of Washington, Seattle, in partial fulfillment of Doctor of Philosophy degree requirements.

work with estrogens and corticosteroids, have made extensive investigations of the effect upon solubilization of modifications in the structure of the steroid solubilizate.

Although previous reports on micellar solubilization of steroids have presented information about how the nature of the solubilizate may affect solubilization, little detailed data are available on the effect of the nature of the solubilizer. As part of a study of the effect of different surfactants upon the solubilization of a single steroid, this paper deals with the study of testosterone solubilized in some nonionic surfactants of the polyoxyethylene sorbitan ester series.

It is well known that the electronic absorption spectrum of a substance is appreciably altered when the substance is in the micellarly solubilized form. Riegelman *et al.* (8) have drawn certain qualitative conclusions about the location of molecules solubilized in micellar solutions based upon the alterations in their absorption spectra. Bjaastad and Hall (9) have recently demonstrated the use of the *Z*-value method, an empirical means of solvent polarity developed by Kosower (10), for investigating the polarity of the environment of some solubilized ketones. This method is based upon the assumption that the polarity of the layers of solvent immediately surrounding a molecule or ion will differ appreciably from that of the bulk solution. Kosower has defined *Z*-value as the energy of electronic transition of 1-ethyl-4-carbomethoxy pyridinium iodide, a substance with an ultraviolet spectrum remarkably sensitive to solvent polarity. Application of this method involves determining the spectral characteristics of the solubilizate in a number of solvents of known *Z*-values and establishing a relationship between *Z*-value and the energy of transition which is readily obtained from the wavelength of maximum absorbance. From this relationship the *Z*-value of a solvent or a medium such as a surfactant solution may be derived once the wavelength of maximum absorbance of the solubilizate in the particular solvent or medium is known. Bjaastad and Hall have pointed out the assumptions which must be made in order to apply the *Z*-value method to solubilized systems.

Testosterone is a steroid of relatively simple structure. The ultraviolet absorption spectrum of this α,β-unsaturated ketone is solvent sensitive. The polarity of the environment of testosterone solubilized in aqueous solutions of polysorbates was investigated by the *Z*-value method and is reported in this paper.

EXPERIMENTAL

Materials—Three nonionic surfactants, having the same hydrophilic portion in their molecules but differing in the length of the carbon atom chain of their lipophilic portion, were selected. These were polysorbate 20, polyoxyethylene-20-sorbitan monolaurate; polysorbate 40, polyoxyethylene-20-sorbitan monopalmitate; and polysorbate 60, polyoxyethylene-20-sorbitan monostearate.[1] The commercial samples of the three nonionic agents were partially purified by partitioning between 5 *N* sodium chloride solution and ethyl acetate by the method of Weibull (11). Samples of partially purified polysorbates were dried over anhydrous sodium sulfate, placed in a 60° oven for 24 hr. to remove traces of any organic solvent, and stored in a desiccator until used. Removal of polyethylene and polyoxyethylene glycols was followed by a thin-layer chromatographic procedure (12). Solutions of polysorbates were prepared using water redistilled in the presence of alkaline permanganate from an all-glass distilling apparatus. The critical micelle concentrations of these partially purified surfactants, determined in this laboratory and elsewhere, with benzopurpurin 4B (13), have been reported as 1.0×10^{-2}, 5.5×10^{-3}, and 4.6×10^{-3} Gm./100 ml., respectively (14, 15).

Testosterone N.F.[2] was used as received. Solvents used where of reagent grade.

Solubility Determinations—To a series of screw cap vials containing 10 ml. of polysorbate solutions of known concentration, an excess of testosterone was added. The caps (lined with aluminum foil) were placed on the vials which were then rotated in a water bath[3] at 25.0° ± 0.1° for 96 hr. Although the solutions showed an initial supersaturation, all were found to attain solution equilibrium within the 96-hr. period. The contents of the vials were filtered through a Swinney filter adapter containing a 0.45 μ Millipore filter disk.[4] The ultraviolet absorption spectra were recorded with a Cary model 14 automatic recording spectrophotometer. Matched cylindrical silica absorption cells, 1 and 10 mm. light path, were used. For the determination of the concentration, ultraviolet spectra were recorded at 1 mμ/sec., from 350 mμ to 210 mμ, and the area under the spectral peak determined by multiplying the height of the peak by its width at one-half the height. The area under the peak was found to give a direct relationship to the concentration of testosterone. The observed solubility in each solution (S) was divided by the solubility in water (S_0) to give the relative solubility in the surfactant concentration.

Z-Value Determinations—In order to relate the ultraviolet absorbance of testosterone to the polarity of the solvent, the wavelength of maximum absorbance ($\lambda_{max.}$) of testosterone was determined in a number of solvents of known *Z*-values. The $\lambda_{max.}$ was first estimated from spectra scanned at 1 mμ/sec., then accurate determinations of $\lambda_{max.}$ were made by recording the spectra at 0.25 mμ/sec. for 20 mμ in the vicinity of the maxima. The reported

[1] Marketed as Tween 20, Tween 40, and Tween 60, respectively, by Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Generously supplied by Schering Corp., Bloomfield, N. J.
[3] Gyrotory water bath shaker, model G76, New Brunswick Scientific Co., Inc., New Brunswick, N. J.
[4] While this work was in progress, Cahn (16) reported the presence of surfactants in Millipore filters. The experimental procedures used in this work were checked to assess the effect of the surfactant contributed by the filter. Neither the quantity of testosterone solubilized nor its ultraviolet absorption spectrum was affected by the quantity of surfactant leached from the filter disk.

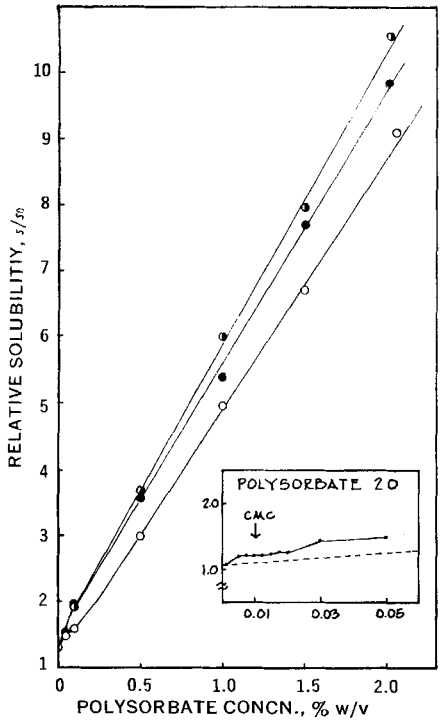

Fig. 1—Relative solubility of testosterone versus *concentration of polysorbate 20* (○), *polysorbate 40* (●), *and polysorbate 60* (◑). *Inset: polysorbate 20 solutions at low surfactant concentrations. Dashed line indicates an extrapolation of the linear portion of the larger graph.*

values of $\lambda_{max.}$ were obtained from an average of three scans and were precise to ± 0.5 mμ.

Spectral measurements for determination of $\lambda_{max.}$ of solubilized testosterone were made using undiluted solutions. In some cases, however, the saturated solutions were too concentrated to permit spectral measurements even with the cells of the shorter light path length of 1 mm. It was necessary in these cases to dilute the saturated solutions with the appropriate surfactant solution. Dilution was kept to a minimum, however. The effect of testosterone concentration upon $\lambda_{max.}$ and Z-value was investigated in some detail for solutions containing polysorbate 20.

RESULTS AND DISCUSSION

Solubility Determinations—The relationship between relative solubility of testosterone and polysorbate concentrations is shown in Fig. 1. After the initial stages of solubilization, the plots are linear, indicating that the amount of testosterone solubilized is directly proportional to polysorbate concentration. Similar observations for other steroids have been made by Sjöblom (5–7) and by Guttman *et al.* (17). The values of the slopes of the linear portions of the curves multiplied by the aqueous solubility of testosterone, in mg./100 ml., gave the solubilizing capacities of polysorbates in terms of mg. of testosterone per Gm. of polysorbate. Table I lists the solubilizing capacities on both a weight and molar basis. Since polysorbates are heterogeneous, the limitation of the significance of the molar values should be borne in mind. The results (Table I and Fig. 1) show that polysorbate 60 has the greatest capacity and polysorbate 20 the least.

The order of solubilizing capacities of the three polysorbates can be directly related to the length of their alkyl portions. In an idealized picture of spherical micelles these alkyl portions may be visualized as being directed inward. An increase in their length would result in micelles of larger size; thus polysorbate 60 micelles would have the largest relative size. It is interesting to note that the solubilizing capacities of the three polysorbates are of similar order of magnitude.

TABLE I—SOLUBILIZING CAPACITY OF POLYSORBATES FOR TESTOSTERONE AT 25°

Surfactant	Solubility: mg. Testosterone/ Gm. Surfactant	Solubility: mole Testosterone/ mole Surfactant[a]
Polysorbate 20	8.29	3.529×10^{-2}
Polysorbate 40	8.95	3.984×10^{-2}
Polysorbate 60	9.64	4.385×10^{-2}

[a] Calculated from the theoretical molecular weights of 1228, 1284, and 1312 for polysorbate 20, 40, and 60, respectively.

TABLE II—SPECTRAL PROPERTIES OF TESTOSTERONE IN SOLVENTS OF VARYING POLARITY

Solvent	Z (9)	$\lambda_{max.}$, mμ ± 0.5 mμ
Water, distilled	94.6	2490
80% Methanol (v/v)	87.1	2448
70% Ethanol	84.5	2445
90% Methanol	85.5	2435
80% Ethanol	84.8	2435
95% Methanol	84.5	2428
90% Ethanol	82.5	2425
95% Ethanol	81.2	2420
Methanol	83.6	2415
Ethanol	79.6	2410
Isopropanol	76.6	2408
Acetonitrile	71.3	2383
Diethylether, water saturated	66.1	2338
n-Hexane	60.4	2313

TABLE III—EFFECT OF TESTOSTERONE CONCENTRATION UPON $\lambda_{max.}$ AND Z-VALUES IN SOLUTIONS OF POLYSORBATE 20

Polysorbate 20 Concn., %	Testosterone % Saturation	Testosterone Concn., mg./100 ml.	$\lambda_{max.}$, mμ	Z
0.01	40	1.15	2490	95.17
	20	0.57	2490	95.17
0.05	40	1.27	2489	94.97
	20	0.64	2486	94.45
0.10	100	3.41	2476	92.57
	20	0.68	2482	93.65
	10	0.34	2483	93.89
0.50	100	6.45	2442	86.13
	50	3.23	2437	85.17
	20	1.29	2437	85.17
	10	0.65	2437	85.17
1.00	100	10.75	2429	83.65
	50	5.38	2426	83.05
	20	2.15	2422	82.29
	10	1.98	2427	83.25

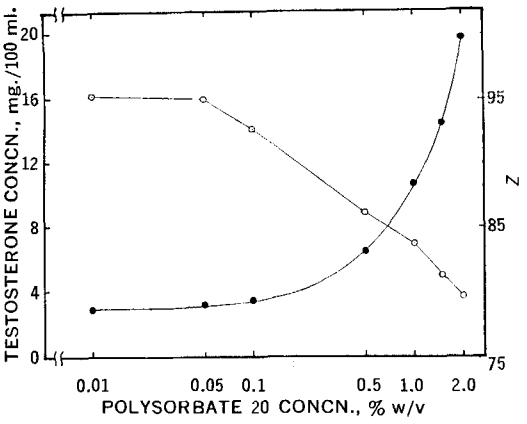

Fig. 2—The relationship between concentration of polysorbate 20 (logarithm), solubility of testosterone (●), and Z-value (○).

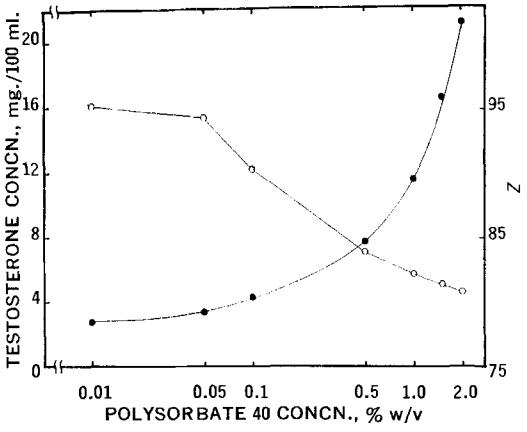

Fig. 3—The relationship between concentration of polysorbate 40 (logarithm), solubility of testosterone (●), and Z-value (○).

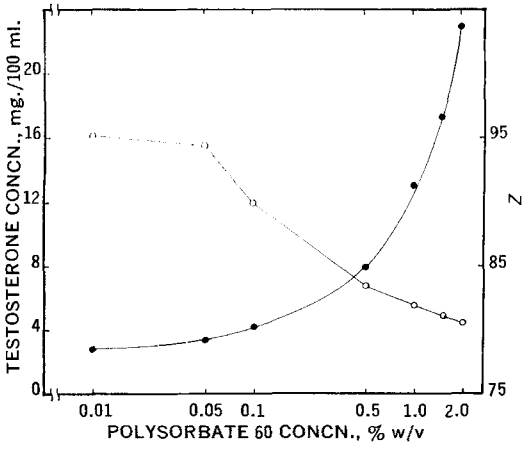

Fig. 4—The relationship between concentration of polysorbate 60 (logarithm), solubility of testosterone (●), and Z-value (○).

In general, solubilization is associated with the presence of micelles, there being little or no solubilization below the CMC. In the present study it was found that testosterone was solubilized below the CMC. This aspect was investigated in some detail with solutions of polysorbate 20 and is illustrated in the inset of Fig. 1. Moilliet *et al.* (18) have suggested that solubilization below the CMC may be explained by the formation of mixed micelles below the usual CMC due to an affinity between the surfactant and the solubilizate. In case of polysorbate 20 and testosterone such an explanation seems plausible.

Z-Value Determinations—The wavelengths of maximum absorbance of testosterone in a wide range of solvents are given in Table II.

The energy of electronic transition for testosterone was calculated from the relationship (19):

$$E_t = 2.859 \times 10^4/\lambda_{max.} \text{ in m}\mu \text{ (Kcal./mole)}$$

When a plot of Z *versus* E_t was made, a straight line with a negative slope was obtained. A negative slope indicates a $\pi \rightarrow \pi^*$ electronic transition (20). By the method of least squares, the slope and intercept were calculated and the following equation obtained for the straight line:

$$Z = -4.00\, E_t + 554.45$$

The Z-values of surfactant solutions were calculated from this equation. Since it was necessary to dilute some of the surfactant solutions saturated with testosterone, the effect of testosterone concentration upon $\lambda_{max.}$ and Z-values was investigated for solutions of polysorbate 20. This is shown in Table III.

It may be seen that any changes in $\lambda_{max.}$ due to dilution are within ± 0.5 mμ, the precision of the method. The corresponding changes in Z should not be significant. This is in agreement with previous observations made with solubilized ketones (9, 14).

Figures 2, 3, and 4 show the relationships among the concentration of polysorbate, solubility of testosterone, and Z-value. At 0.01% polysorbate concentration the Z-value is the same as that of water, and the solubility of testosterone is not appreciably greater than that in water. This concentration is approximately the CMC for polysorbate 20, and for polysorbate 40 and 60 it is higher than the CMC. With increasing polysorbate concentrations and consequent increase in testosterone solubility, the Z-value decreases, indicating an environment of decreasing polarity. The behavior is similar for the three polysorbates.

As Bjaastad and Hall (9) have pointed out, decreasing polarity might be explained by an increase in concentration and organization of micelles as the concentration of surfactant is increased. It may also be due to the solubilized testosterone encountering less polar regions of micellar interior. Guttman *et al.* (17) have suggested that the steroids, prednisolone, methylprednisolone, and fluoromethylone, are associated, by hydrogen bonding, with the polyethylene chain of the nonionic surfactant oxyethylated tertiary octylphenol formaldehyde polymer.[5] Testosterone with its hydroxyl group at C-17 should be capable of association with the ethylene oxide portion on the surface of polysorbate micelles. This would account for the high

[5] Triton WR-1339, Rohm and Haas, Philadelphia, Pa.

Z-value of low concentration polysorbate solutions in which one may expect testosterone molecules to be oriented in the hydrated polyoxyethylene surface of the micelles. The fact that the ethylene oxide chain lengths of the three polysorbates are identical and the observation that the values of testosterone solubility are nearly the same in low concentrations of all the three polysorbates support this view. The decrease in Z-value possibly results from multilayer adsorption. This proposal is substantiated by similar order of testosterone solubilization in higher polysorbate concentrations. If solubilization involved incorporation of testosterone within micellar interior, one would expect a more pronounced difference in the solubilities with a change in lipophilic moiety.

The magnitude of Z-values in 2% polysorbate solutions are comparable to those of ethanol and isopropanol, which also indicates that the environment of solubilized testosterone is quite polar. Although the hydrocarbon interior of micelles is believed to contain some water (21), its polarity might be expected to be lower than those of the alcohols.

Further studies utilizing ionic surfactants with comparable alkyl chain lengths are being conducted and will be reported in a future communication.

REFERENCES

(1) Swarbrick, J., *J. Pharm. Sci.*, **54**, 1229(1965).
(2) Mulley, B. M., in "Advances in Pharmaceutical Sciences," Bean, M. S., Beckett, A. M., and Carless, J. E., eds., Academic Press Inc., New York, N. Y., 1964.
(3) Ekwall, P., and Sjöblom, L., *Acta Chem. Scand.*, **3**, 1179(1949).
(4) Nakagawa, T., *J. Pharm. Soc. Japan*, **73**, 469(1953); through *Chem. Abstr.*, **47**, 9563g(1953).
(5) Sjöblom, L., *Acta Acad. Abo. Math. Phys.*, **20**, 164 (1956).
(6) Blomquist, C., and Sjöblom, L., *Acta Chem. Scand.*, **18**, 2405(1964).
(7) Sjöblom, L., and Sundblom, N., *ibid.*, **18**, 1996(1964).
(8) Riegelman, S., Allawala, N. A., Hrenoff, M. K., and Strait, L. A., *J. Colloid Sci.*, **13**, 208(1958).
(9) Bjaastad, S. G., and Hall, N. A., *J. Pharm. Sci.*, **56**, 504(1967).
(10) Kosower, E. M., *J. Am. Chem. Soc.*, **80**, 3253(1958).
(11) Weibull, B. W., *Proc. Intern. Congr. Surface Activity, 3rd, Cologne, C*, **1960**, 121.
(12) Thakkar, A. L., Kuehn, P. B., and Hall, N. A., *Am. J. Pharm.*, **139**, 122(1967).
(13) Becher, P., *J. Phys. Chem.*, **66**, 374(1962).
(14) Bjaastad, S. G., and Brown, K. F., *Australasian J. Pharm.*, **45**, S116(1964).
(15) Bjaastad, S. G., Hall, N. A., and Thakkar, A. L., *J. Pharm. Sci.*, **54**, 1529(1965).
(16) Cahn, R. D., *Science*, **155**, 195(1967).
(17) Guttman, D. E., Hamlin, W. E., Shell, J. W., and Wagner, J. G., *ibid.*, **50**, 305(1961).
(18) Moilliet, J. L., Collie, B., and Black, W., "Surface Activity," E. and F. N. Spon Ltd., London, England, 1961, p. 51.
(19) Bauman, R. P., "Absorption Spectroscopy," John Wiley & Sons, Inc., New York, N.Y., 1962, pp. 7–8.
(20) Kosower, E. M., *J. Am. Chem. Soc.*, **80**, 3261(1958).
(21) Sasaki, H., Okuyama, H., and Saito, S., *Bull. Chem. Soc. Japan*, **29**, 752(1956); **30**, 186(1957).

Steric Aspects of Adrenergic Drugs VII

Certain Pharmacological Actions of D(−)-Pseudoephedrine

By J. B. LAPIDUS, ARTHUR TYE, and P. N. PATIL

The ability of D(−)-pseudoephedrine to influence the actions of selected adrenergic drugs was examined in both *in vivo* and *in vitro* experiments. In anesthetized dogs, pretreatment with D(−)-pseudoephedrine can reduce or block the pressor effects of the other ephedrine isomers and (+)-amphetamine. Delayed potentiation was observed with norepinephrine and epinephrine. D(−)-Pseudoephedrine did not affect the pressor effects resulting from bilateral carotid occlusion, and exhibited no unique properties on the rabbit aortic strip. D(−)-Pseudoephedrine had no intrinsic activity on the isolated rat vas deferens but could block the contractions caused by tyramine or D(−)-ephedrine, and potentiate the effects of norepinephrine. D(−)-Pseudoephedrine and D(−)-ephedrine compete for α-adrenergic sites in the reserpine pretreated rat vas deferens and can protect these receptors from dibenamine blockade. It appears that D(−)-pseudoephedrine acts at both catecholamine uptake sites and at α-receptors, but apparently lacks intrinsic effects at the latter site.

In previous papers (1, 2), the authors have presented evidence that D(−)-pseudoephedrine has a unique vascular activity when compared with other isomers of ephedrine. It has virtually no pressor activity (2), yet is a diastereoisomer of D-(−)-ephedrine, the most active pressor member of the four ephedrine isomers. Certain stereochemical consequences of this relationship led to the belief that D(−)-pseudoephedrine should, in fact, be able to antagonize the pressor response to D(−)-ephedrine (1). D(−)-Ephedrine and D(−)-pseudoephedrine have the same absolute configuration at the β-carbon atom (that bearing the hydroxyl group) (Fig. 1). If the assumption is made that the amino group, the hydroxyl group, and the phenyl ring are all involved in the drug–receptor interaction (3), then these two diastereoisomers represent a

Received February 1, 1967, from the Division of Chemical Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210
Accepted for publication May 17, 1967.
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(20) Kosower, E. M., *J. Am. Chem. Soc.*, **80**, 3261(1958).
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Experiments on isolated rat vas deferens were carried out with the technical assistance of Mr. D. Enright.
Previous paper: Tye, A., Baldesburger, R., LaPidus, J. B., and Patil, P. N., *J. Pharmacol. Exptl. Therap.*, to be published.

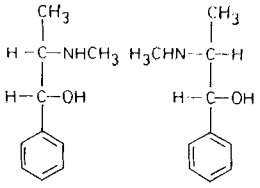


Fig. 1—Planar structures of D(-)-ephedrine (left) and D(-)-pseudoephedrine (right).

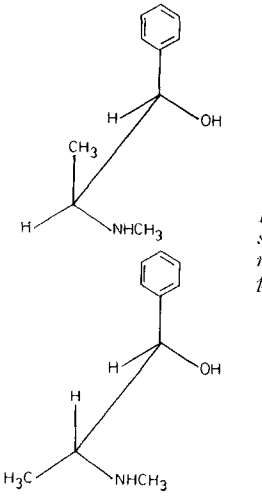


Fig. 2—Three-dimensional structures of D(-)-ephedrine (top) and D(−)-pseudoephedrine (bottom).

unique case in which the active groups in both molecules can occupy the same three points in space. This is not apparent from the Fischer projection formulas shown in Fig. 1, but becomes clear when the compounds are represented as in Fig. 2.

This structural relationship, coupled with the observed differences in pressor activity of these two compounds, led to the conjecture that D(−)-pseudoephedrine is capable of interacting with the same receptor(s) as D(−)-ephedrine, but apparently does so in a biologically unproductive manner. In other words, D(−)-pseudoephedrine may have affinity but very little or no intrinsic activity.

This paper is primarily concerned with the interactions of D(−)-pseudoephedrine with other isomers of ephedrine and related agents.

METHODS

In Vivo Experiments—Mongrel dogs of either sex, weighing 7–11 Kg., were anesthetized with sodium barbital, 330 mg./Kg. i.p. All experimental animals were pretreated with atropine sulfate, 1 mg./Kg. Blood pressure was recorded through the right carotid artery *via* a mercury manometer. In experiments involving bilateral carotid occlusion the femoral blood pressure was recorded and sodium pentobarbital,[1] 35 mg./Kg., was used as an anesthetic. The trachea was always cannulated. Respiration was recorded, when desired, on a kymograph through a tambour. Electrocardiograms were recorded on a Sanborn Twin Viso recorder.

[1] Nembutal, Abbott Laboratories, North Chicago, Ill.

In Vitro Experiments—Spirally cut aortic strips of the rabbit were prepared according to the method described by Furchgott and Bhadrakom (4). After the animal was rendered unconscious by a sharp blow on the head, a long incision was made on the skin and thoracic and abdominal contents were resected to expose the whole length of aorta. An incision was then made at the descending level of the aortic arch, and a long glass rod (about 3 mm. in diameter) passed gradually in it. The whole length of the aorta was thus removed from the rabbit. The fat and other connective tissue was carefully separated. During this procedure the aorta was kept moist throughout by means of Kreb's bicarbonate solution (5). The whole length of the aorta was cut spirally to give a strip 3–4 mm. wide. Strips 4 cm. in length were mounted in jacketed 10-ml. muscle chambers containing Kreb's bicarbonate at 37.5°. The contractions of the aortic strip were magnified by a lever in a ratio of 1:20, and counterbalanced to exert 4 Gm. of tension. The preparations were oxygenated by 95% O_2 and 5% CO_2 and allowed to stabilize for at least 2 hr. before any drug induced contractions were recorded *via* a frontal point lever on a slowly moving smoked kymograph. The cumulative dose-response curves were obtained as described by Van Rossum (6). No attempt was made to make two dose-response curves from the same strip. It required 10 to 15 min. to reach a plateau after addition of each dose of drug. In antagonism studies, the preparation was exposed to the antagonist for 10 min. and the dose-response curve of the agonist then obtained in presence of the antagonist.

Rats weighing 100–150 Gm. were killed by a sharp blow on the head. A vas deferens was isolated from all connective tissues and suspended in a jacketed 10-ml. muscle chamber which contained Tyrode's solution at 37–37.5°. The composition of the Tyrode's solution was as follows: NaCl, 137 m*M*; KCl, 2.68 m*M*; $CaCl_2(2H_2O)$, 1.76 m*M*; $MgCl_2(6H_2O)$, 0.88 m*M*; NaH_2PO_4, 0.36 m*M*; $NaHCO_3$, 12 m*M*; glucose, 5.5 m*M*; demineralized water was used to make the solution. A mixture of oxygen (95%) and CO_2 (5%) was bubbled through the solution. Drug-induced contractions were recorded on a smoked drum *via* a light lever (magnification: 20, tension approximately 350 mg.) which was always under the influence of low vibrations produced by a small electric motor.

Receptor protection experiments as introduced by Furchgott (7) were carried out on isolated rat vas deferens. Dibenamine (10^{-6} *M*) was selected as an irreversible α-adrenergic blocker. These experiments were carried out in the reserpine-pretreated (5 mg./Kg. i.p. 8 to 16 hr. previously) rats, because D(−)-ephedrine is classified as a mixed acting amine on isolated rat vas deferens (8). Therefore, norepinephrine liberated from the nerve endings would complicate the protection of α-adrenergic receptors by D(−)-ephedrine.

DRUGS

The optical isomers of ephedrine were prepared by the method described by LaPidus *et al.* (1). For each experiment fresh solutions of the isomers were made in physiological saline by the addition of a small amount of dilute hydrochloric acid. The following drugs were obtained from commercial

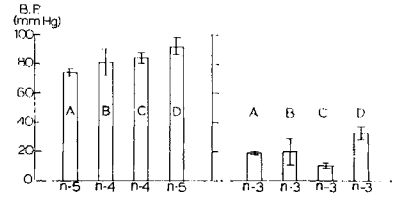


Fig. 3—Reduction of pressor effects of D(-)-ephedrine L(+)-ephedrine, D(+)-amphetamine, and L(+)-pseudoephedrine by 30-min. pretreatment with D(-)-pseudoephedrine in anesthetized dog. Key: A, *D(-)-ephedrine, 0.33 mg./Kg.;* B, *L(+)-ephedrine, 0.99 mg./Kg.;* C, *L(+)-pseudoephedrine, 1.65 mg./Kg.;* D, *D(+)-amphetamine, 0.36 mg./Kg.; vertical lines indicate standard errors of mean [control data from Patil* et al. *(2)];* n, *number of dogs;* B.P., *blood pressure rise in mm. of Hg over preinjection level; left, control; right, D(-)-pseudoephedrine.*

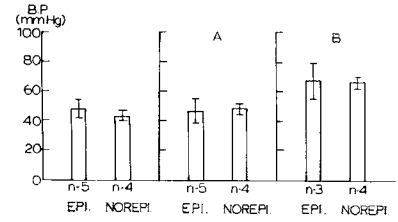


Fig. 4—Effect of D(-)-pseudoephedrine (3.3 mg./Kg.) on the pressor responses to epinephrine (EPI), *1.5 mg./Kg. and norepinephrine* (NOREPI) *0.4 mcg./Kg. in anesthetized dogs. Key:* B.P., *blood pressure rise in mm. of Hg over preinjection level;* n, *number of observations;* A, *epinephrine and norepinephrine given 10 to 20 min. after D(-)-pseudoephedrine;* B, *epinephrine and norepinephrine given 45 to 60 min. after D(-)-pseudoephedrine; vertical lines indicate standard errors of the mean; right, control; middle and left, D(-)-pseudoephedrine.*

sources: D(+)-amphetamine sulfate, norepinephrine bitartrate,[2] reserpine phosphate,[3] epinephrine bitartrate,[4] tyramine HCl, and dibenamine HCl. All the weights given refer to free base except for amphetamine which refers to its sulfate. The terminology used for ephedrine isomers has been described previously (2).

Whenever possible the standard errors of the mean were calculated; significance between two means was tested by *t* test. Otherwise, for qualitative purposes, at least 3 observations were made in different animals. Since most of these drugs may affect the responses of one another, each observation was made in an individual animal.

RESULTS

The Effects of Pretreatment with D(−)-Pseudoephedrine—In preliminary experiments eight dogs were pretreated with D(−)-pseudoephedrine (0.33, 3.3, 9.9, 16.5 mg./Kg.) 30 min. before a challenging dose of D-(−)-ephedrine was given. [The blocking ability of D(−)-pseudoephedrine was found to be maximal when the pretreatment interval was

[2] Levophed, Winthrop Laboratories, New York, N. Y.
[3] Serpasil, Ciba Pharmaceutical Co., Summit, N. J.
[4] Suprarenin, Winthrop Laboratories, New York, N. Y.

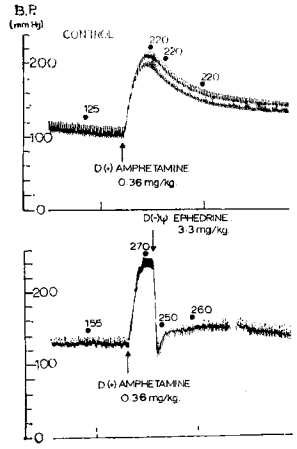


Fig. 5—Antagonistic effect of D(-)-pseudoephedrine on the pressor effects of D(+)-amphetamine in anesthetized dogs. Key: B.P., *blood pressure in mm. of Hg; heat rate (beats/min.) indicated by numbers above the blood pressure tracing; time mark, 10 min.*

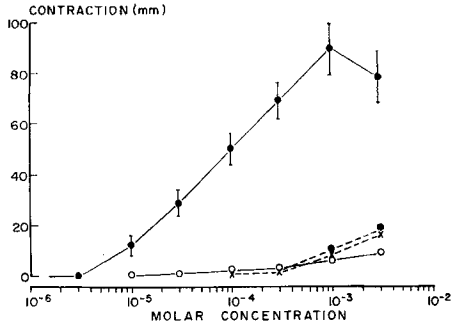


Fig. 6—Effect of ephedrine isomers on the contraction of the rabbit aortic strip. D(-)-ephedrine (●–●), L(+)-ephedrine (○—○), L(+)-pseudoephedrine (×----×), D(-)-pseudoephedrine (●···●). Nine to 12 strips used for each isomer. Vertical lines indicate standard errors of the mean. In the case of L(+)-ephedrine, L(+)-pseudoephedrine, and D(-)-pseudoephedrine, standard errors markedly overlapped each other.

reasonably short.] Doses of 9.9 or 16.5 mg./Kg. completely blocked the blood pressure, heart rate, and respiratory effect of D(−)-ephedrine (0.33 mg./Kg.). D(−)-Pseudoephedrine itself shows temporary depressor effects in anesthetized animals (2). Animals pretreated with 3.3 mg./Kg. of D-(−)-pseudoephedrine 30 min. before challenging with one of the following amines: D(−)-ephedrine, 0.33 mg./Kg.; L(+)-ephedrine, 0.99 mg./Kg.; L(+)-pseudoephedrine, 1.65 mg./Kg.; or D(+)-amphetamine sulfate, 0.36 mg./Kg., exhibited marked reduction of the pressor effects of these amines (Fig. 3).

At the 0.33-mg./Kg. dose level D(−)-pseudoephedrine did not significantly modify the pressor effects of epinephrine or norepinephrine. However, when the dose of D(−)-pseudoephedrine was increased to 3.3 mg./Kg., a potentiation of pressor effects was noted. This potentiation was more

TABLE I—CONTRACTION OF RABBIT AORTIC STRIPS TO 10^{-3} *M* D(−)-EPHEDRINE AFTER DOSE RESPONSE CURVES OF OTHER ISOMERS OF EPHEDRINE ARE COMPLETED

	Mean Contraction, mm. (± S.E.M.)			
	Control	After L(+)-Ephedrine	After L(+)-Pseudoephedrine	After D(−)-Pseudoephedrine
D(−)-Ephedrine (10^{-3} *M*)	89, ± 11	14, ± 5	48, ± 15	28, ± 9
No. of observations	10	9	6	9
P value compared with control		<0.001	<0.05	<0.01

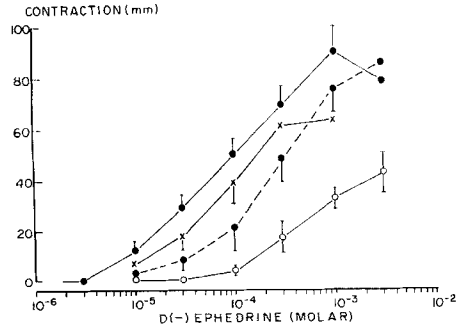


Fig. 7—Dose-response curves of D(-)-ephedrine in the presence of L(+)-ephedrine (10^{-3} M) (O—O), L(+)-pseudoephedrine (×—×) (10^{-3} M), and D(-) pseudoephedrine (10^{-3} M) (●---●). Vertical lines indicate standard errors of the mean. Average of 6, strips for each isomer.

marked 45 to 60 min. after administration (Fig. 4). The pressor effects of epinephrine (in terms of both duration and magnitude) tended to be reduced if it was given 10 to 20 min. after a dose of 9.9 mg./Kg. of D(−)-pseudoephedrine. In four experiments the average control rise in blood pressure to 0.75 mcg./Kg. of epinephrine was 29 mm. of Hg (S.E. ± 4) and that after D(−)-pseudoephedrine was 18 mm. of Hg (S.E. ± 7). Under similar conditions, the pressor effects of norepinephrine, 0.4 mcg./Kg., were not affected. In four experiments the average control rise in blood pressure was 44 mm. of Hg (S.E. ± 7), and that after D(−)-pseudoephedrine was 44 mm. of Hg (S.E. ± 18). While the intensity of the response to norepinephrine remained the same, the duration was increased.

D(−)-Pseudoephedrine was tested for its ability to modify bilateral carotid occlusion pressor effects. In doses of 3.3 or 9.9 mg./Kg. (two animals for each dose), it did not affect the carotid occlusion pressor effects up to 30 to 60 min. after injection.

When D(−)-pseudoephedrine, 3.3 mg./Kg., was given at the height of pressor responses to D(−)-ephedrine, 0.33 mg./Kg. (3 experiments), an immediate reduction in pressor response occurred in all experiments (Fig. 5). In control experiments the pressor effects of D(−)-ephedrine and D(+)-amphetamine sulfate persisted for 15 to 20 min. The increase in heart rate was not effectively antagonized.

Rabbit Aortic Strip—D(−)-Ephedrine is the only isomer which showed a marked contraction of the aortic strips. Other isomers—namely, L(+)-ephedrine, L(+)-pseudoephedrine, and D(−)-pseudoephedrine did not show any appreciable effects. The maximal effects produced by less active isomers were less than 20% of the effect produced by D(−)-ephedrine. The negative log ED_{50} of D(−)-ephedrine is 4.19 (S.E. ± 0.08) which is obtained by plotting every single dose-response curve and from log transformed values. The results are illustrated in Fig. 6.

If $10^{-3}M$ concentration of D(−)-ephedrine was added in the tissue bath after complete dose-response curves of lesser active isomers were constructed, there was a marked reduction of the maximal effects of ephedrine (Table I).

In the other series of experiments, the dose-response curves of D(−)-ephedrine were constructed in the presence of 10^{-4} *M* and 10^{-3} *M* concentrations of less active isomers. In the lower concentrations there was not any significant shift of the dose-response curve of D(−)-ephedrine. In higher concentrations, however, there was a significant shift to the right. L(+)-Ephedrine appeared to be the most effective in antagonizing the action of D(−)-ephedrine. The height of the dose-response curve of D(−)-ephedrine in the presence of 10^{-3} *M* L(+)-ephedrine was depressed (Fig. 7 and Table II).

Rat Vas Deferens—Tyramine, 10^{-4} *M*, and D(−)-ephedrine, 10^{-4} *M*, produce a maximum or near maximum contraction of rat vas deferens (9). D(−)-Pseudoephedrine, in concentrations of 10^{-4} *M* and 10^{-3} *M*, markedly antagonized the effects of tyramine and D(−)-ephedrine. D(−)-Pseudoephedrine did not show any intrinsic effects (Tables III and IV). The same concentrations of D(−)-pseudoephedrine produced a clear cut potentiation of norepinephrine (Fig. 8).

In receptor protection experiments, both D(−)-

TABLE II—NEGATIVE LOG ED_{50} VALUES OF D(−)-EPHEDRINE IN THE PRESENCE OF 10^{-3} *M* CONCENTRATION OF L(+)-EPHEDRINE, L(+)-PSEUDOEPHEDRINE, AND D(−)-PSEUDOEPHEDRINE ON THE RABBIT AORTIC STRIP

	Concn. of Antagonist, 10^{-3} *M*			
	Control	L(+)-Ephedrine	L(+)-Pseudoephedrine	D(−)-Pseudoephedrine
D(−)-Ephedrine ED_{50}	4.19 (S.E. ± 0.08)	3.36 (S.E. ± 0.11)	4.01 (S.E. ± 0.07)	3.65 (S.E. ± 0.11)
No. of observations	10	6	6	6
P value compared with control		<0.001	<0.05	<0.01

TABLE III—REDUCTION OF TYRAMINE INDUCED CONTRACTIONS OF RAT VAS DEFERENS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF D(−)-PSEUDOEPHEDRINE

	Tyramine (10^{-4} *M*) Mean Contraction, mm. (± S.E.M.)					
	Control[a]	After 10^{-5} *M* D(−)-Pseudoephedrine[b]	Control[a]	After 10^{-4} *M* D(−)-Pseudoephedrine[b]	Control[a]	After 10^{-3} *M* D(−)-Pseudoephedrine[b]
	152, ± 10	121, ± 5	133, ± 8	85, ± 4	137, ± 12	25, ± 10
n[c]	9	9	8	8	6	6
P value	<0.01		<0.001		<0.001	

[a] Controls were obtained on the contralateral side of the same rat. [b] Added 3 min. prior to tyramine. [c] *n*, number of observations.

TABLE IV—REDUCTION OF D(−)-EPHEDRINE INDUCED CONTRACTIONS OF RAT VAS DEFERENS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF D(−)-PSEUDOEPHEDRINE

	D(−)-Ephedrine (10^{-4} *M*) Mean Contraction, mm. (±S.E.M.)					
	Control[a]	After 10^{-5} *M* D(−)-Pseudoephedrine[b]	Control[a]	After 10^{-4} *M* D(−)-Pseudoephedrine[b]	Control[a]	After 10^{-3} *M* D(−)-Pseudoephedrine[b]
	56, ± 7	47, ± 5	36, ± 6	8, ± 3	50, ± 8	2, ± 2
n[c]	6	6	8	8	8	8
P value	>0.05		<0.001		<0.001	

[a] Controls were obtained on the contralateral sides of the same rat. [b] Added 3 min. prior to D(−)-ephedrine. [c] *n*, number of observations.

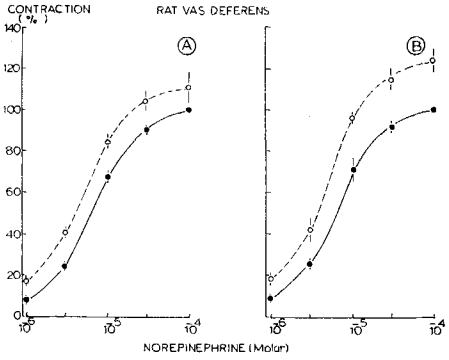


Fig. 8—Key: A, *potentiation of norepinephrine in the presence of* 10^{-4} M *D(-)-pseudoephedrine;* B, *potentiation of norepinephrine in the presence of* 10^{-3} M *D(-)-pseudoephedrine. Each point of the curve represents an average of eight observations. Vertical lines indicate standard errors of the mean. Vas deferens was exposed to D(-)-pseudoephedrine for 3 min. before a dose-response curve of norepinephrine was repeated.*

pseudoephedrine (10^{-3} *M*) and D(−)-ephedrine (10^{-3} *M*) completely protected the α-adrenergic receptors from dibenamine blockade in the reserpine pretreated tissues. Results are summarized in Table V.

D(−)-Ephedrine produced an average 41 mm. (S.E. ± 7.8) contraction of the reserpine pretreated vas deferens. Under similar conditions, lower concentrations, 3×10^{-4} *M*, of D(−)-pseudoephedrine and D(−)-ephedrine also protected the α-adrenergic receptors to the extent of 26% (S.E. ± 11) and 44% (S.E. ± 6.9), respectively.

DISCUSSION

D(−)-Ephedrine and D(−)-pseudoephedrine are each capable of attachment to three identical points on a receptor (Fig. 2). Since the pressor effects of D(−)-pseudoephedrine are markedly inferior to those of D(−)-ephedrine (2), it was anticipated that it might antagonize the actions of the latter compound. The authors found that both *in vivo* and *in vitro* D(−)-pseudoephedrine does indeed markedly reduce the effects of D(−)-ephedrine and other "indirectly" acting sympathomimetic amines such as amphetamine and tyramine. It is interesting to note that D(−)-pseudoephedrine does not have any "intrinsic" effects on isolated rat vas deferens and at concentrations in which it antagonizes tyramine and ephedrine, potentiates the effects of norepinephrine. This led to the conclusion that D(−)-pseudoephedrine possibly acts at the catecholamine uptake sites. In other words, it has "cocaine-like" effects at the sympathetic nerve endings.

TABLE V—PROTECTION OF α-ADRENERGIC RECEPTORS BY D(−)-PSEUDOEPHEDRINE AND D(−)-EPHEDRINE IN RESERPINE PRETREATED (5 mg./Kg., 8–16 hr.) RAT VAS DEFERENS

Norepinephrine (10^{-4} *M*) Mean Contraction, mm. (± S.E.M.)					
Control[a]	Tissue Incubated with Dibenamine,[b] 10^{-6} *M*	Control[a]	Tissue Incubated with D(−)-Pseudoephedrine[c] (10^{-3}*M*) and Dibenamine,[b] 10^{-6} *M*	Control[a]	Tissue Incubated with D(−)-Ephedrine[c] (10^{-3}*M*) and Dibenamine,[b] 10^{-6} *M*
141, ± 5	0	148, ± 3	140, ± 6	150, ± 1	151, ± 4
n^d = 8		*n* = 5		*n* = 5	

[a] Controls were obtained from the contralateral side on the same rat. [b] Incubation time was 10 min., then tissue washed 4 times and 10 min. after the last wash norepinephrine was added. [c] Five minutes prior to dibenamine, D(−)-pseudoephedrine and D(−)-ephedrine were added in the tissue bath. [d] *n*, number of observations.

By using receptor protection experiments, it was possible to demonstrate that D(−)-pseudoephedrine also competes for the α-adrenergic sites. However, this effect must be very weak because the effects of exogenous norepinephrine are potentiated (*i.e.*, the competition of D(−)-pseudoephedrine at α-receptors is overcome by norepinephrine, the uptake of which is being prevented in the nerve endings). Thus, D(−)-pseudoephedrine is capable of acting at the catecholamine uptake sites and the α-adrenergic sites; however, it lacks intrinsic activity at the latter site.

The pharmacological effects of L(+)-pseudoephedrine and L(+)-ephedrine are similar on the aortic strips which support the previous report that there is a small difference between the pressor effects of these L-diastereoisomers in the dog (2).

D(−)-Ephedrine is the only isomer of ephedrine which produces a marked contraction of rabbit aortic strips. In the presence of 10^{-3} *M* concentrations of L(+)-ephedrine, L(+)-pseudoephedrine, or D(−)-pseudoephedrine, the dose-response curves of D(−)-ephedrine are shifted to the right.

On the isolated rat vas deferens D(−)-pseudoephedrine did not show any intrinsic effects. However, the contractions due to tyramine and D(−)-ephedrine were markedly antagonized by 10^{-4} *M* and 10^{-3} *M* D(−)-pseudoephedrine, while the effects of norepinephrine were potentiated. In addition, D(−)-pseudoephedrine and D(−)-ephedrine also compete for the α-adrenergic sites in the reserpine pretreated animals. D(−)-Pseudoephedrine appears to act at both the catecholamine uptake site and the α-adrenergic site, but apparently lacks intrinsic effects at the latter site.

SUMMARY

In anesthetized dogs, pretreatment (30 min.) with D(−)-pseudoephedrine, 3.3 mg./Kg., can reduce or block the pressor effects of D(−)-ephedrine, L(+)-ephedrine, L(+)-pseudoephedrine, and D(+)-amphetamine. D(−)-Pseudoephedrine also promptly reduces the pressor effects, if given during the height of response. The pressor effects of norepinephrine and epinephrine appeared to be potentiated by D(−)-pseudoephedrine. Such a potentiation was best seen 45 to 60 min. after the administration of the isomer. The pressor effects due to bilateral carotid occlusion were unaffected by D(−)-pseudoephedrine.

REFERENCES

(1) LaPidus, J. B., Tye, A., Patil, P., and Modi, B., *Med. Chem.*, **6**, 76(1963).
(2) Patil, P. N., Tye, A., and LaPidus, J. B., *J. Pharmacol. Exptl. Therap.*, **148**, 158(1965).
(3) Ariens, E. J., "Adrenergic Mechanisms," Vane, J. R., ed., Little, Brown and Co., Boston, Mass., 1960, p. 253.
(4) Furchgott, R. F., and Bhadrakom, S., *J. Pharmacol. Exptl. Therap.*, **108**, 129(1953).
(5) Umbriet, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques and Tissue Metabolism," 4th ed., Burgess Publishing Co., Minneapolis, Minn., 1964, p. 132.
(6) Van Rossum, J. M., *Arch. Intern. Pharmacodyn.*, **143**, 299(1963).
(7) Furchgott, R. F., *J. Pharmacol. Exptl. Therap.*, **111**, 265(1954).
(8) Patil, P. N., LaPidus, J. B., Campbell, D., and Tye, A., *ibid.*, **155**, 13(1967).
(9) Patil, P. N., LaPidus, J. B., and Tye, A., *ibid.*, **155** 1(1967).

Differentiating Nonaqueous Titration of Salicylic Acid and Acetylsalicylic Acid Combination

By SONG-LING LIN

Synthetic mixtures of salicylic acid and acetylsalicylic acid are differentiated by potentiometric nonaqueous titration. With sodium methoxide or tetrabutylammonium hydroxide titrant and glass-calomel electrode system, dimethylformamide is found to be the best differentiating solvent. The effect of the solvent in titration and differentiation of salicylic acid and acetylsalicylic acid in water and dimethylformamide is illustrated and interpreted. Various solvent–titrant–electrode combinations are employed to explore their effects on the sensitivity of the differentiating titration. The presence of water in the titration solvent is demonstrated to be highly detrimental and undesirable on differentiation. The proposed procedure is simple, accurate, and applicable even when there is a disproportionate concentration of the components. The work suggests that by a proper combination of solvent, titrant, and electrode system, it should be possible to differentiate potentiometrically both components of acidic or basic mixtures whose dissociation constants are well below the theoretical limit of 16.

A VARIETY OF procedures (1–8) have been proposed for the analysis of acetylsalicylic acid and one of its degradation products, salicylic acid. These methods involve colorimetry, chromatography, and spectrophotometry. However, a search of the literature for titrimetric techniques applicable to differentiating salicylic acid–acetylsalicylic acid combination was not successful. It was the purpose of this study to develop such a procedure.

Received March 17, 1967, from the Research Department, Ciba Pharmaceutical Co., Summit, NJ 07901
Accepted for publication June 23, 1967.
The author expresses his appreciation to Dr. L. Lachman and Dr. T. Bates for their interest and discussions during the course of this investigation. The assistance of Mrs. Caroline Wade is also gratefully acknowledged.

By using receptor protection experiments, it was possible to demonstrate that D(−)-pseudoephedrine also competes for the α-adrenergic sites. However, this effect must be very weak because the effects of exogenous norepinephrine are potentiated (*i.e.*, the competition of D(−)-pseudoephedrine at α-receptors is overcome by norepinephrine, the uptake of which is being prevented in the nerve endings). Thus, D(−)-pseudoephedrine is capable of acting at the catecholamine uptake sites and the α-adrenergic sites; however, it lacks intrinsic activity at the latter site.

The pharmacological effects of L(+)-pseudoephedrine and L(+)-ephedrine are similar on the aortic strips which support the previous report that there is a small difference between the pressor effects of these L-diastereoisomers in the dog (2).

D(−)-Ephedrine is the only isomer of ephedrine which produces a marked contraction of rabbit aortic strips. In the presence of 10^{-3} *M* concentrations of L(+)-ephedrine, L(+)-pseudoephedrine, or D(−)-pseudoephedrine, the dose-response curves of D(−)-ephedrine are shifted to the right.

On the isolated rat vas deferens D(−)-pseudoephedrine did not show any intrinsic effects. However, the contractions due to tyramine and D(−)-ephedrine were markedly antagonized by 10^{-4} *M* and 10^{-3} *M* D(−)-pseudoephedrine, while the effects of norepinephrine were potentiated. In addition, D(−)-pseudoephedrine and D(−)-ephedrine also compete for the α-adrenergic sites in the reserpine pretreated animals. D(−)-Pseudoephedrine appears to act at both the catecholamine uptake site and the α-adrenergic site, but apparently lacks intrinsic effects at the latter site.

SUMMARY

In anesthetized dogs, pretreatment (30 min.) with D(−)-pseudoephedrine, 3.3 mg./Kg., can reduce or block the pressor effects of D(−)-ephedrine, L(+)-ephedrine, L(+)-pseudoephedrine, and D(+)-amphetamine. D(−)-Pseudoephedrine also promptly reduces the pressor effects, if given during the height of response. The pressor effects of norepinephrine and epinephrine appeared to be potentiated by D(−)-pseudoephedrine. Such a potentiation was best seen 45 to 60 min. after the administration of the isomer. The pressor effects due to bilateral carotid occlusion were unaffected by D(−)-pseudoephedrine.

REFERENCES

(1) LaPidus, J. B., Tye, A., Patil, P., and Modi, B., *Med. Chem.*, **6**, 76(1963).
(2) Patil, P. N., Tye, A., and LaPidus, J. B., *J. Pharmacol. Exptl. Therap.*, **148**, 158(1965).
(3) Ariens, E. J., "Adrenergic Mechanisms," Vane, J. R., ed., Little, Brown and Co., Boston, Mass., 1960, p. 253.
(4) Furchgott, R. F., and Bhadrakom, S., *J. Pharmacol. Exptl. Therap.*, **108**, 129(1953).
(5) Umbriet, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques and Tissue Metabolism," 4th ed., Burgess Publishing Co., Minneapolis, Minn., 1964, p. 132.
(6) Van Rossum, J. M., *Arch. Intern. Pharmacodyn.*, **143**, 299(1963).
(7) Furchgott, R. F., *J. Pharmacol. Exptl. Therap.*, **111**, 265(1954).
(8) Patil, P. N., LaPidus, J. B., Campbell, D., and Tye, A., *ibid.*, **155**, 13(1967).
(9) Patil, P. N., LaPidus, J. B., and Tye, A., *ibid.*, **155** 1(1967).

Differentiating Nonaqueous Titration of Salicylic Acid and Acetylsalicylic Acid Combination

By SONG-LING LIN

Synthetic mixtures of salicylic acid and acetylsalicylic acid are differentiated by potentiometric nonaqueous titration. With sodium methoxide or tetrabutylammonium hydroxide titrant and glass-calomel electrode system, dimethylformamide is found to be the best differentiating solvent. The effect of the solvent in titration and differentiation of salicylic acid and acetylsalicylic acid in water and dimethylformamide is illustrated and interpreted. Various solvent–titrant–electrode combinations are employed to explore their effects on the sensitivity of the differentiating titration. The presence of water in the titration solvent is demonstrated to be highly detrimental and undesirable on differentiation. The proposed procedure is simple, accurate, and applicable even when there is a disproportionate concentration of the components. The work suggests that by a proper combination of solvent, titrant, and electrode system, it should be possible to differentiate potentiometrically both components of acidic or basic mixtures whose dissociation constants are well below the theoretical limit of 16.

A VARIETY OF procedures (1–8) have been proposed for the analysis of acetylsalicylic acid and one of its degradation products, salicylic acid. These methods involve colorimetry, chromatography, and spectrophotometry. However, a search of the literature for titrimetric techniques applicable to differentiating salicylic acid–acetylsalicylic acid combination was not successful. It was the purpose of this study to develop such a procedure.

Received March 17, 1967, from the Research Department, Ciba Pharmaceutical Co., Summit, NJ 07901
Accepted for publication June 23, 1967.
The author expresses his appreciation to Dr. L. Lachman and Dr. T. Bates for their interest and discussions during the course of this investigation. The assistance of Mrs. Caroline Wade is also gratefully acknowledged.

Although salicylic acid and acetylsalicylic acid as individual components can be readily determined by titration in nonaqueous media, the titrimetric analysis of mixtures of these compounds has not been reported. This may be due to the fact that the difference between the pKa's, in water, of salicylic acid (2.97) and acetylsalicylic acid (3.49) (9) has been considered to be too small to accomplish the differentiation, even in nonaqueous solvents.

Theoretical expressions for the titration of a mixture of two monoprotic acids can be treated as in the titration involving a single diprotic acid. Auerbach and Smolczyk (10) have shown theoretically that no inflection occurs at the first equivalence point in the potentiometric titration curve of polyfunctional acids in aqueous solvents unless the ratio of the first to the second dissociation constant is greater than 16. It is difficult, in fact, to locate the inflection accurately even at a ratio of 100. Kolthoff and Stenger (11) state that, in water, an accurate and satisfactory differentiation of acids or bases can be obtained only with a pKa (H_2O) difference of approximately 4.0. When titration is performed in nonaqueous solvents, differentiation is possible even with a pKa (H_2O) difference of about or below 2.0. Many dibasic acids which give only a single inflection in water give two sharp inflections in nonaqueous solvents. For example, the two acidic groups of succinic acid (pK_1 of 4.2 and pK_2 of 5.6) were differentiated in acetone (12). In this investigation, the resolution of acid mixtures with a pKa (H_2O) difference of only 0.52 [or with Ka (H_2O) difference of only 3] is successfully accomplished by selecting the proper combinations of titrant, solvent, and electrode system.

In this investigation, differentiating nonaqueous titration was performed with a Fisher automatic titrimeter. Various solvent–titrant–electrode combinations were employed to explore their effects on the sensitivity of differentiation. The feasibility of resolution and characteristics of titration curves are markedly different from one solvent–titrant–electrode system to another. The presence of water in the titration solvent is shown to be highly detrimental and undesirable on differentiation. The degree of water tolerance with sodium methoxide titrant is threefold as resistant as with tetrabutylammonium hydroxide titrant.

With sodium methoxide or tetrabutylammonium hydroxide in benzene–methanol as titrant and a glass–calomel electrode pair as the electrode system, dimethylformamide was found to be the best differentiating solvent. The suppression of the sharpness of the inflections for both compounds in differentiating titration as compared with the titration of individual components is realized. Acetylsalicylic acid offers sharper inflection than salicylic acid in differentiating titration, although the latter component demonstrates better inflection than its acetylated derivative when titrated individually. The proposed procedure is simple, accurate, and applicable even when there is a disproportionate concentration of the components. Preliminary treatment of the sample is unnecessary, and tedious separation and extraction techniques are obviated.

The work suggests that, by a proper combination of solvent, titrant, and electrode system, it would be possible to titrate and differentiate potentiometrically both components of an acidic and basic mixture whose dissociation constants are well below the theoretical limit of 16 proposed by Auerbach and Smolczyk (10). The findings obtained will serve as guidelines for future work on the differentiating nonaqueous titration of acetylsalicylic acid–salicylic acid–acetic acid mixtures.

EXPERIMENTAL

Apparatus—Titrations were performed potentiometrically with a Fisher automatic titrimeter, model 36, equipped with a glass electrode (Beckman No. 40498) and a sleeve-type calomel electrode (Beckman No. 40463). For this study, the calomel electrode was modified by replacing the aqueous saturated potassium chloride solution in the bridge with a saturated solution of potassium chloride in methanol. Other electrodes evaluated were platinum electrode (Fisher 13-639-102) and antimony electrode (Fisher 9-313-212). A 25-ml. automatic buret, graduated to 0.05 ml., with a titrant storage container, was used to prevent any contamination of the titrant.

Reagents and Solutions—Acetylsalicylic acid U.S.P. (Mallinckrodt) was dried at 60° for 4 hr. and was used without further purification. Analysis by the official U.S.P. assay procedure indicated a purity of better than 99.9%. This meets the U.S.P. requirements for the allowable salicylic acid content (<0.1%). The salicylic acid used was U.S.P. grade. Analysis by U.S.P. method showed a purity of 99.8%. A 0.1 *N* solution of sodium methoxide in benzene–methanol (10:1) was prepared and standardized potentiometrically against a reference standard benzoic acid dissolved in dimethylformamide. A 0.1 *N* solution of tetrabutylammonium hydroxide in benzene–methanol (10:1) was prepared by the silver oxide method and standardized potentiometrically against a reference standard benzoic acid dissolved in dimethylformamide. Both titrants were protected from the atmosphere by an Ascarite guard tube. Dimethylformamide was purified by distillation (b.p. 153°). Other chemicals and solvents used in this study were reagent grade and used as received without further purification.

Differentiating Titration of Salicylic Acid and Acetylsalicylic Acid—Approximately 0.50 meq. of salicylic acid and 0.50 meq. of acetylsalicylic acid, accurately weighed, were dissolved in 50 ml. of solvent or solvent mixture in a 150-ml. beaker covered with a rubber plate having holes for the passage of the electrodes and the buret tip. The solution, under constant stirring, was titrated potentiometrically with 0.1 *N* sodium methoxide solution or 0.1 *N* tetrabutylammonium hydroxide solution, using a modified calomel and glass electrode system. A blank titration was also performed. Titration curves were obtained by plotting the potential reading, *E*, in millivolts (mv.) *versus* volume (ml.) of titrant. The exact end point was determined by plotting $(\Delta E/\Delta \bar{V})$ *versus* ml. The titration solvents evaluated in this study included dimethylformamide, pyridine, nitromethane, nitrobenzene, acetone, methyl ethyl ketone, methyl isobutyl ketone, methanol, ethanol, isopropanol, and *tert*-butylalcohol. Prior to carrying out the differentiating titration of salicylic acid and acetylsalicylic acid in each solvent, the individual compounds were titrated separately.

Electrode Systems—The six electrode combinations employed were glass–calomel, platinum–calomel, antimony–calomel, antimony–platinum, glass–antimony, and glass–platinum. The titration solvents used in the evaluation of these electrode systems included dimethylformamide, nitromethane, acetonitrile, and methyl isobutyl ketone. The concentration of the acids, volume of solvent, and titrants were the same as above.

Variation of Salicylic Acid-to-Acetylsalicylic Acid Ratio—A series of differentiating titrations was performed in which the milliequivalent ratio of salicylic acid to acetylsalicylic acid was varied from 1 to 1 to greater than 10 to 1. Amounts of salicylic acid and acetylsalicylic acid calculated to give the desired milliequivalent ratio of components were accurately weighed and dissolved in 50 ml. of dimethylformamide. The solution was titrated potentiometrically with 0.1 *N* sodium methoxide solution or 0.1 *N* tetrabutylammonium hydroxide solution using the glass–calomel electrode system.

Effect of Varying Water Concentration in Titration Solvent on Differentiating Titration—Accurately weighed quantities of salicylic acid and acetylsalicylic acid were dissolved in 50 ml. of water–dimethylformamide solvent mixture composed of varying amounts of water. The solution was titrated potentiometrically with 0.1 *N* sodium methoxide solution or 0.1 *N* tetrabutylammonium hydroxide solution, using the glass–calomel electrode pair.

RESULTS AND DISCUSSION

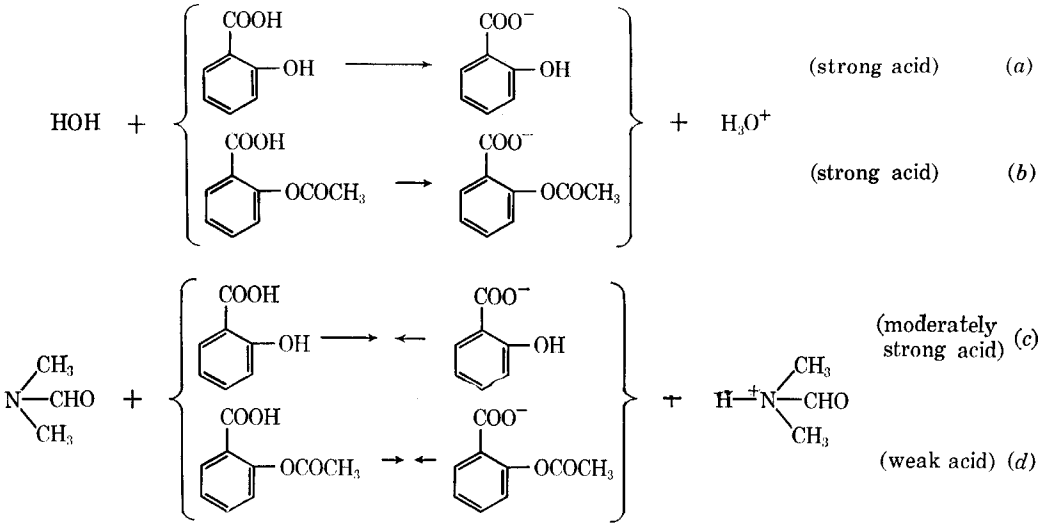
An ideal solvent for the satisfactory differentiation of an acid mixture should be sufficiently weak in basicity to permit the titration of the stronger acidic component and sufficiently weak in acidity to permit the titration of the weaker acidic component. From a practical standpoint, the differentiating quality of the solvent can be realized from the characteristics of the potentiometric titration curves obtained. A well-defined inflection will be observed for the potentiometric titration of the stronger acid if the solvent is a good differentiating solvent. The better the differentiating ability of the solvent, the greater the inflection will be. In preliminary experiments a series of solvents and combinations of solvents were employed as titration solvents to test their differentiating ability. Titrations were effected potentiometrically with 0.1 *N* sodium methoxide or 0.1 *N* tetrabutylammonium hydroxide using a glass–modified calomel electrode system. Of the solvent systems tested, dimethylformamide was found to yield the most satisfactory end points in the differentiating titration of the salicylic acid–acetylsalicylic acid mixture, despite the fact that dimethylformamide has been reported to be of only limited use for the titration of acids and their mixtures because of strong acid–solvent interaction (13). The autoprotolysis constant for this solvent, as determined from potentiometric measurements, was found to be 10^{-18} (14).

When an electrically neutral acid (HA) is dissolved in the solvent (SH), species with various charges are formed upon the ionization of the solvent:

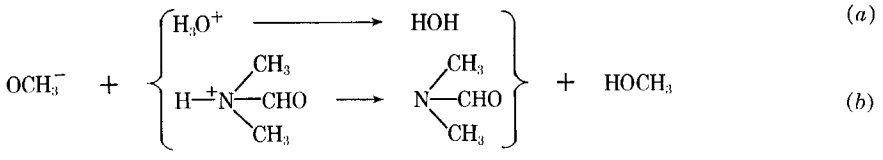
$$SH + HA \rightleftarrows A^- + SH_2^+ \qquad \text{(Eq. 1)}$$

The reaction is not solvent-independent since reactants and products differ significantly in their charge densities, and therefore, in their ability to accept hydrogen bonds or form ion pairs in the solvent. The effect of the solvent in titration and differentiation of salicylic acid and acetylsalicylic acid in water and dimethylformamide is illustrated in Schemes I–III.

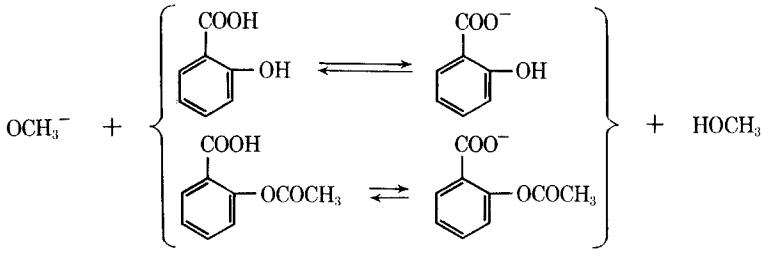
It was observed that salicylic acid and acetylsalicylic acid, alone or in combination, are readily dissolved in dimethylformamide but slowly dissolved in water. This may be explained by the important role of the nature of the solvent molecule on the strength of the solute–solvent interactions. The nature of the solution also varies with the solvent. Ions and molecules have a tendency to achieve the lowest possible energy state, and the formation of certain structure in the solution process is one way of attaining this. The greater the solvation, the lower the resulting energy of the solute, and its stability in solution is increased. This is achieved when a charged species is surrounded by suitably oriented dipoles of a solvent of high dielectric constant. In water, both anions and cations are well solvated and thus shielded from each other because of van der Waal forces and other intermolecular attractions. Anions, because of their great solvation energies, are well stabilized in solution, and the forward reactions of Scheme I (*a* and *b*), are greatly favored. In other words, since the anion is much more densely charged than the electrically neutral solvent molecule, the strong anion–solvent interactions due to hydrogen bonding lower the energy of anion relative to the electrically neutral solvent molecule. As a result, the equilibrium in water is shifted further to the right as compared to a solvent incapable of forming hydrogen bonding with the anions. Upon the addition of alkoxide ion as titrant during the titration, the solvated proton reacts quantitatively with alkoxide ion as shown in Scheme II(*a*). Consequently, salicylic acid and acetylsalicylic acid can be titrated individually in water as shown in Fig. 1 (curves 1 and 2). Parker (15) and Buckingham (16) have shown that anions are solvated to a greater extent



Solvolysis (Reaction with Solvent)
Scheme I



Titration of the Solvated Proton (Reaction with the Titrant)
Scheme II



Net Reaction
Scheme III

than cations in water and small anions are much more solvated than large anions. However, in water, the difference in energy between anions of salicylic acid and acetylsalicylic acid might not be great enough to permit the successful differentiation of a salicylic acid–acetylsalicylic acid mixture as evidenced by Fig. 1, curve 3.

When dimethylformamide replaced water as the solvent, the situation was changed. This is due to the fact that dimethylformamide and water possess different solvent characteristics. (*a*) Water is amphiprotic in nature, while dimethylformamide donates electrons but does not accept them. (*b*) The strongest base that can exist in water is the solvated hydroxide ion, while on the other hand, a much stronger base system is possible in dimethylformamide. (*c*) The dielectric constants of water and dimethylformamide are 80 and 36, respectively, and the higher the dielectric constant of a solvent, the lower the electrostatic interaction and less will be the chance for the dissolved species bearing an opposite charge to interact. (*d*) The positive charge on the small hydrogen atoms of water can come much closer to an anion than can a positive charge on an atom other than hydrogen. Hence, the possible interaction in dimethylformamide is of the weaker ion–dipole interaction, neither hydrogen bonding nor ion-pairing being operative. Consequently, the anions in dimethylformamide are much less solvated and more reactive than those in water. In other words, solute molecules dissolved in dimethylformamide yield anions which have much greater free energies than the anions produced from solutes dissolved in water. As illustrated in Scheme I (*c* and *d*), the forward reaction becomes less favorable relative to the backward

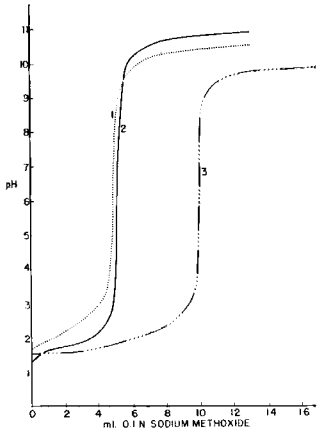


Fig. 1—Typical curves for the titration of salicylic acid and acetylsalicylic acid dissolved in water, individually and in combination, with 0.1 N sodium methoxide in benzene–methanol (10:1), using glass-modified calomel electrode system. Key: 1, acetylsalicylic acid; 2, salicylic acid; 3, salicylic acid and acetylsalicylic acid.

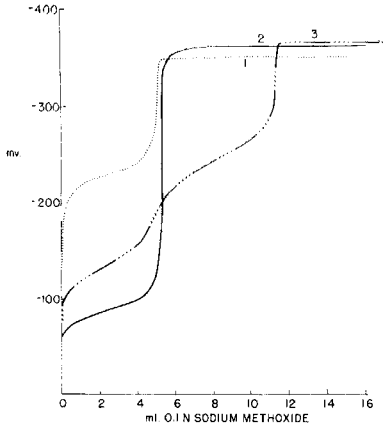


Fig. 2—Typical curves for the titration of salicylic acid and acetylsalicylic acid dissolved in dimethylformamide, individually and in combination, with 0.1 N sodium methoxide in benzene–methanol (10:1), using glass-modified calomel electrode system. Key: 1, acetylsalicylic acid; 2, salicylic acid; 3, salicylic acid and acetylsalicylic acid.

reaction when water is replaced with dimethylformamide. The equilibrium in dimethylformamide, therefore, does not lie as far to the right as it does in water. As the result, a greater sensitivity in end point detection was noted for the individual components with water (curves 1 and 2, Fig. 1) than with dimethylformamide (curves 1 and 2, Fig. 2) as titration solvent. Zaugg (17), Prue and Sherrington (18), and Parker (15) have pointed out that in dimethylformamide anions are much less solvated than cations, and large polarizable anions may be slightly more solvated than small anions. Even more important is the fact that the difference in energy between anions of different sizes and charge densities is much greater in dimethylformamide than in water. Consequently, differentiating titration of a salicylic acid–acetylsalicylic acid mixture can be readily achieved in dimethylformamide (curve 3, Fig. 2), but not in water (curve 3, Fig. 1). When the combination of drugs was titrated in dimethylformamide, two distinct inflections were obtained, the first corresponding to the salicylic acid content and the second representing the acetylsalicylic acid end point. In this connection, Kolthoff *et al.* (19) have emphasized that, in the interpretation of difference in acid strength in different solvents, one must consider the degree of anion solvation and the formation and dissociation of ion pairs in addition to the basic characteristics and dielectric constant of the solvent. Additional studies are presently underway concerning the basic behavior of salicylic acid and acetylsalicylic acid in water and dimethylformamide; the results will be presented in a future publication (20).

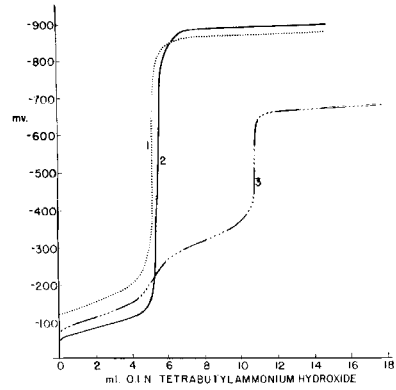


Fig. 3—Typical curves for the titration of salicylic acid and acetylsalicylic acid dissolved in dimethylformamide, individually and in combination, with 0.1 N tetrabutylammonium hydroxide in benzene–methanol (10:1), using glass-modified calomel electrode system. Key: 1, acetylsalicylic acid; 2, salicylic acid; 3, salicylic acid and acetylsalicylic acid.

With dimethylformamide as the titration solvent, the titration curves in Fig. 2 were obtained with sodium methoxide, whereas those in Fig. 3 were obtained with tetrabutylammonium hydroxide as the titrant. The sharp breaks in the titration curves (curves 1 and 2, Figs. 2 and 3) indicated excellent quantitative recoveries of acetylsalicylic acid and salicylic acid when titrated individually. As would be expected, a greater sensitivity in end point detection was realized for the individual components with the more basic titrant, tetrabutylammonium hydroxide, than with sodium methoxide titrant. A comparison of the sharpness of the inflection, as shown in Table I by the magnitude of $(\Delta E/\Delta \bar{V})_{max.}$, expressed in mv./ml., indicates that for acetylsalicylic acid the sharpness of the inflection was doubled when the quaternary ammonium hydroxide titrant replaced the alkoxide titrant. The steeper inflection given by salicylic acid over acetylsalicylic acid in dimethylformamide is predictable from their respective dissociation constants in water. When the combination of drugs was titrated (curve 3, Figs. 2 and 3), satisfactory differentiation was obtained with either titrant. When sodium methoxide was substituted by tetrabutylammonium hydroxide as titrant, as indicated in

TABLE I—DETERMINATION OF SALICYLIC ACID AND ACETYLSALICYLIC ACID, INDIVIDUALLY AND IN COMBINATION, IN DIMETHYLFORMAMIDE USING GLASS–CALOMEL ELECTRODE SYSTEM

Curve Ref.[a]	Compd.	Sodium Methoxide		Tetrabutylammonium Hydroxide	
		$(\Delta E/\Delta \bar{V})_{max.}$	Recovery, %	$(\Delta E/\Delta \bar{V})_{max.}$	Recovery, %
1	Acetylsalicylic acid	1000[b]	100.04 ± 0.32[c]	2500	99.50 ± 0.49
2	Salicylic acid	1450	99.94 ± 0.56	2800	99.98 ± 0.40
3	Salicylic acid	70	99.14 ± 0.36	80	100.54 ± 0.67
	Acetylsalicylic acid	550	99.87 ± 0.81	1850	98.98 ± 0.71

[a] Numbers correspond to curves in Figs. 2 and 3. [b] Expressed in mv./ml. of titrant added. [c] Standard deviation is based on at least 5 determinations.

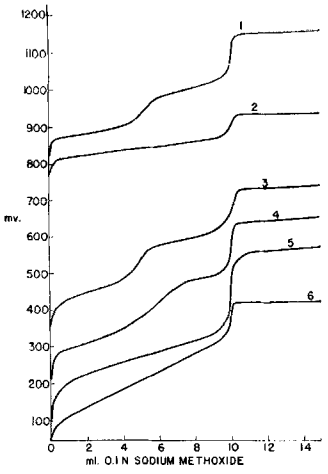


Fig. 4—Typical curves for differentiating titration of salicylic acid and acetylsalicylic acid in various solvents. Key: 1, dimethylformamide; 2, pyridine; 3, nitromethane; 4, nitrobenzene; 5, alcoholic solvent; 6, ketonic solvent. The titrant was 0.1 N sodium methoxide in benzene–methanol (10:1).

Table I, the sharpness of the inflection remained fairly unchanged for salicylic acid, while the sharpness of the inflection for acetylsalicylic acid was more than tripled. This may be explained, at least in part, by the fact that when tetrabutylammonium hydroxide is used as titrant, the potential span available is two to three times larger than when sodium methoxide is employed. The suppression of the sharpness of the inflections during differentiating titration of mixtures, as compared with the titration of individual components, is further shown from the data in Table I. With sodium methoxide as the titrant, the magnitude of $(\Delta E/\Delta \bar{V})_{max.}$ obtained during differentiation is suppressed about twentyfold for salicylic acid and approximately twofold for acetylsalicylic acid. When the mixture was titrated with tetrabutylammonium hydroxide, a more drastic suppression of the magnitude of $(\Delta E/\Delta \bar{V})_{max.}$ was observed with salicylic acid, giving a 35-fold reduction of the sharpness of potential break during differentiation as compared with the value obtained from the titration of salicylic acid alone. It is interesting to note that acetylsalicylic acid offers a sharper inflection than salicylic acid during the differentiating titration, in spite of the fact that salicylic acid demonstrates better inflection than its acetylated derivative when titrated individually.

Using a glass–methanol modified calomel electrode system, the effect of various solvents on the sensitivity of differentiation was investigated with either sodium methoxide (Fig. 4) or tetrabutylammonium hydroxide (Fig. 5) as the titrant. Basic solvents other than dimethylformamide, such as pyridine, while excellent for the individual components, do not permit differentiation in the case of acetylsalicylic acid–salicylic acid mixture. Figure 4, curve 2, indicates a single end point corresponding to the total acid present when titration is performed in pyridine. An explanation of this is that both acids react nearly completely with the pyridine which tends to level all acids so that their strengths become indistinguishable. This phenomenon has been termed the "leveling effect" (21). When sodium methoxide was used as the titrant, no resolution was observed in acetonitrile, the ketones, and alcohols. Using tetrabutylammonium hydroxide as titrant, similar results were obtained in these solvents with the exception of *tert*-butyl alcohol (curve 5, Fig. 5). When the titration is performed in nitromethane, differentiation is readily accomplished with the sodium methoxide titrant (curve 3, Fig. 4) but not with tetrabutylammonium hydroxide titrant (curve 4, Fig. 5). On the contrary, when the titration is carried out in *tert*-butyl alcohol, resolution is realized with tetrabutylammonium hydroxide titrant (curve

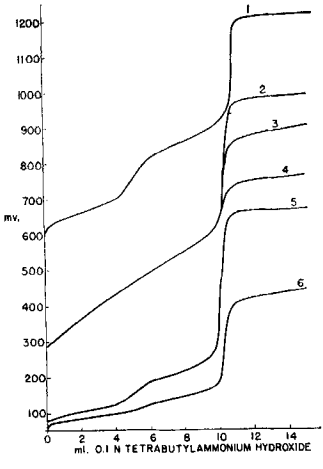


Fig. 5—Typical curves for differentiating titration of salicylic acid and acetylsalicylic acid in various solvents. Key: 1, dimethylformamide; 2, ketonic solvents; 3, acetonitrile; 4, nitromethane; 5, tert-butyl alcohol; 6, other alcoholic solvent. The titrant was 0.1 N tetrabutylammonium hydroxide in benzene–methanol (10:1).

TABLE II—DIFFERENTIATING TITRATION OF SALICYLIC ACID AND ACETYLSALICYLIC ACID IN VARIOUS SOLVENTS USING GLASS–CALOMEL ELECTRODE SYSTEM AND SODIUM METHOXIDE TITRANT

Solvent	No. of Determinations	Recovery, % Salicylic Acid	Recovery, % Acetylsalicylic Acid
Dimethylformamide	5	99.14 ± 0.36[a]	99.87 ± 0.81
Nitromethane	5	98.87 ± 0.49	100.06 ± 0.48
Nitrobenzene	5	100.81 ± 1.52	98.72 ± 0.98
Nitromethane–benzene (1:10)	3	99.12 ± 0.62	98.72 ± 0.65
Nitromethane–chloroform (1:10)	3	98.92 ± 0.75	100.81 ± 0.62
Nitromethane–dimethylformamide (1:10)	4	100.18 ± 0.40	99.42 ± 0.60
Dimethylformamide–nitrobenzene (1:10)	4	98.62 ± 0.64	100.70 ± 0.55
Dimethylformamide–benzene (1:8)	3	One end point[b]	
Dimethylformamide–chloroform (1:8)	3	One end point	

[a] Standard deviation is based on at least 4 determinations. [b] Corresponds to salicylic acid plus acetylsalicylic acid.

TABLE III—EFFECT OF ELECTRODE COMBINATION ON DIFFERENTIATING TITRATION OF SALICYLIC ACID AND ACETYLSALICYLIC ACID

Curve Ref.[a]	Electrode Combination	End Points Observed,[b] No. Dimethylformamide $NaOCH_3$	Dimethylformamide $(Bu)_4NOH$	Nitromethane $NaOCH_3$	Nitromethane $(Bu)_4NOH$
1	Glass–calomel	2	2	2	1
2	Platinum–calomel	0	2	2	1
3	Antimony–calomel	1	2	2	1
4	Antimony–platinum	2	1[d]	1	1
5	Glass–antimony	2[c]	2	2	1
6	Glass–platinum	2[c]	2	2[c]	1[c]

[a] Numbers correspond to curves in Figs. 6 and 7. [b] Observation is based on at least 4 determinations. [c] Abnormal titration curve was observed. [d] One end point corresponding to salicylic acid plus acetylsalicylic acid.

5, Fig. 5) and not with the sodium methoxide titrant (curve 5, Fig. 4). Although it is possible to differentiate the acids in nitrobenzene (curve 4, Fig. 4), a smaller and less well-defined inflection is obtained for the stronger acid in the mixture. Consequently, the importance of the proper selection of the solvent–titrant combination for a successful differentiating titration is clearly exemplified by the plots in Figs. 4 and 5.

Pifer *et al.* (22) point out that the inclusion in the titration solvent system of an excess of a solvent having a low dielectric constant can increase markedly the sensitivity in the detection of the end point. This useful principle has been applied satisfactorily to the titration of acetophenetidin (23) and the differentiation of acetophenetidin–caffeine mixture (24). However, an attempt to sharpen the end points by the incorporation of an excess of benzene or chloroform in dimethylformamide, nitrobenzene, or nitromethane was not successful with sodium methoxide as the titrant. Although quantitative recoveries, as reported in Table II, were obtained for both components dissolved in nitromethane and mixed solvents containing a large excess of chloroform or benzene in nitromethane, the sharpness of the inflection remains fairly unaltered. The incorporation of an excess of a solvent having a low dielectric constant in dimethylformamide or nitrobenzene not only prevented the differentiation, but a single end point corresponding to the total acid present in the system was obtained.

The effect of six electrode combinations on the resolution of salicylic acid and acetylsalicylic acid was explored, using either sodium methoxide or tetrabutylammonium hydroxide as titrant. Dimethylformamide and nitromethane were employed as the titration solvents. The electrode systems and the number of end points subsequently observed for the differentiating titration are listed in Table III. It was observed that the feasibility of the differentiation and the characteristics of titration curves were markedly different from one titrant–solvent–electrode system to another. For example, with dimethylformamide as the titration solvent and sodium methoxide as the titrant, various degrees of differentiation were observed with six electrode couples investigated. As depicted in the family of curves on the left side of Fig. 6, two distinct inflections were obtained with the glass–calomel (curve 1) and the antimony–platinum (curve 4) electrode pairs, one potential break representing total acid was realized with the antimony–calomel (curve 3) electrode system, no discernible inflection was attainable with the platinum–calomel (curve 2) electrode couple, and abnormal titration curves near the second equivalent points were encountered with the antimony–glass (curve 5) and the platinum–glass (curve 6) electrode combinations. When tetrabutylammonium hydroxide replaced sodium methoxide as titrant, as shown in the family of curves on the right side of Fig. 6, all electrode systems, with the exception of the antimony–platinum (curve 4) electrode pair, gave satisfactory but different degrees of resolution for the salicylic acid–acetylsalicylic acid mixture dissolved in dimethylformamide. When titration was performed

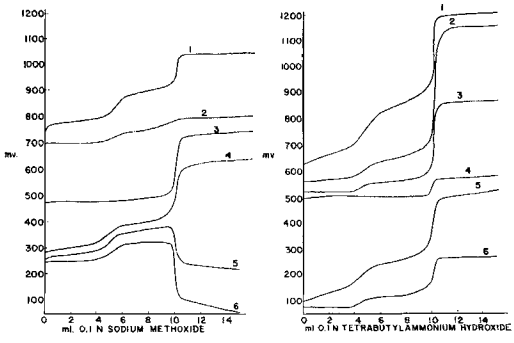


Fig. 6—Effect of electrode combination on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid dissolved in dimethylformamide. Right series of curves were obtained with sodium methoxide as titrant, whereas the left series of curves were obtained with tetrabutylammonium hydroxide. The numbers above the curves correspond to those in Table III.

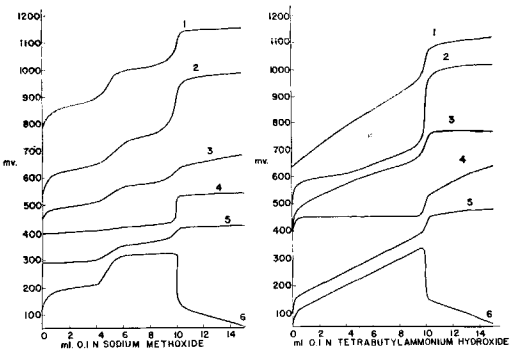


Fig. 7—Effect of electrode combination on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid dissolved in nitromethane. Left series of curves were obtained with sodium methoxide as titrant, and the right series of curves were obtained with tetrabutylammonium hydroxide. The numbers above the curves correspond to those in Table III.

in nitromethane, tetrabutylammonium hydroxide proved unsuitable as a differentiating titrant. As shown in the family of curves on the right side of Fig. 7, tetrabutylammonium hydroxide titrant did not permit the differentiation in any of six electrode combinations studied, whereas the sodium methoxide titrant allowed the differentiation to be accomplished in four out of the six electrode systems investigated, the exceptions being the antimony–platinum (one end point representing the total acid) and the glass–platinum (abnormal titration curve) electrode couples as demonstrated in the family of curves on the left side of Fig. 7. Lin and Blake (25, 26) reported the same phenomenon in the differentiating titration of acetylsalicylic acid and barbiturate combinations dissolved in methyl isobutyl ketone, using glass–calomel or antimony–calomel electrode pairs. They found that resolution was achieved with the sodium methoxide titrant but not with tetrabutylammonium hydroxide as the titrant. Further studies were performed using sodium methoxide as the titrant and acetonitrile as the titration solvent or using tetrabutylammonium hydroxide as the titrant and methyl isobutyl ketone as the titration solvent. However, no differentiation of salicylic acid–acetylsalicylic acid mixtures was observed with any of the six electrode systems investigated.

Quantitative recoveries, together with the corresponding maximum potential change per unit volume of titrant added, for both salicylic acid and acetylsalicylic acid obtained in various titrant–solvent–electrode combinations are tabulated in Table IV. Excellent results were obtained for all combinations. For each titrimetric condition, acetylsalicylic acid offers a sharper potential break than salicylic acid in the differentiating titration, although salicylic acid shows better inflection than acetylsalicylic acid when it is titrated separately. In general, the tetrabutylammonium hydroxide titrant gave sharper inflections than the sodium methoxide titrant for acetylsalicylic acid, the weaker acid presented in the acid mixture to be differentiated, while only slight differences in the sharpness of the inflections for salicylic acid was noted between the two titrants. Another example to illustrate the importance of the suitable solvent–titrant combination on the successfulness of the resolution can be easily deduced from Table III. With the antimony–calomel electrode system, for example, differentiation is readily achieved with a combination of dimethylformamide–tetrabutylammonium hydroxide or nitromethane–sodium methoxide but is unsuccessful with the dimethylformamide–sodium methoxide or nitromethane–tetrabutylammonium hydroxide system.

Since salicylic acid is one of the hydrolytic degradation products of acetylsalicylic acid, the effect of varying the ratio of concentrations of the components on the sensitivity of the differentiating titration was studied. The data for a series of titrations in which the milliequivalent ratio of salicylic acid to acetylsalicylic acid was varied from 1 to 1 to about 12 to 1 are reported in Table V. Typical titration curves obtained with the sodium methoxide titrant are shown in Fig. 8 and those obtained with tetrabutylammonium hydroxide titrant are depicted in Fig. 9. With sodium methoxide as the titrant, two discernible inflections in the titration curve were obtained when the milliequivalent ratio of salicylic acid to acetylsalicylic acid was not greater than about 5 to 1. When the ratio was greater

TABLE IV—DIFFERENTIATING TITRATION OF SALICYLIC ACID AND ACETYLSALICYLIC ACID IN VARIOUS TITRANT–SOLVENT–ELECTRODE COMBINATIONS

Titrant[a]	Solvent	Electrode Pair	Salicylic Acid $(\Delta E/\Delta V)_{max.}$	Salicylic Acid Recovery, %	Acetylsalicylic Acid $(\Delta E/\Delta V)_{max.}$	Acetylsalicylic Acid Recovery, %
Sodium methoxide	Dimethyl-formamide	Glass-calomel[b]	70[c]	99.14 ± 0.36[d]	550	99.87 ± 0.81
		Antimony–platinum	50	100.72 ± 0.69	800	100.02 ± 0.50
	Nitromethane	Glass–calomel	100	98.87 ± 0.49	300	100.06 ± 0.48
		Platinum–calomel	80	99.50 ± 0.81	400	99.62 ± 0.60
		Antimony–calomel	45	100.71 ± 0.45	60	100.40 ± 0.38
		Antimony–glass	55	98.62 ± 0.48	80	99.89 ± 0.72
Tetrabutylammonium hydroxide	Dimethyl-formamide	Glass–calomel	80	100.54 ± 0.67	1850	98.98 ± 0.71
		Platinum–calomel	60	101.17 ± 0.50	3150	100.06 ± 0.45
		Antimony–calomel	45	99.42 ± 0.48	2100	99.62 ± 0.82
		Antimony–glass	55	98.62 ± 0.46	900	100.80 ± 0.54
		Platinum–glass	50	97.86 ± 0.72	1000	100.42 ± 0.65
	tert-Butyl alcohol	Glass–calomel	50	100.32 ± 0.52	2500	98.71 ± 0.66

[a] Titrant was prepared in 10:1 benzene–methanol solvent mixture. [b] Electrolyte bridge in calomel electrode was saturated potassium chloride in methanol. [c] Expressed in mv./ml. of titrant added. [d] Standard deviation is based on at least 4 determinations.

TABLE V—EFFECT OF SALICYLIC ACID-TO-ACETYLSALICYLIC ACID RATIO ON SENSITIVITY OF DIFFERENTIATING TITRATION

Curve Ref.[a]	Amt. Weighed, meq. Salicylic Acid	Amt. Weighed, meq. Acetylsalicylic Acid	Recovery, %: Sodium Methoxide Titrant — Salicylic Acid	Recovery, %: Sodium Methoxide Titrant — Acetylsalicylic Acid	Recovery, %: Tetrabutylammonium Hydroxide Titrant — Salicylic Acid	Recovery, %: Tetrabutylammonium Hydroxide Titrant — Acetylsalicylic Acid
1	0.50	0.50	100.71 ± 0.54[b]	98.62 ± 0.68	101.04 ± 0.42	99.92 ± 0.60
2	0.50	0.40	99.46 ± 0.64	99.60 ± 0.50	100.75 ± 0.77	99.77 ± 0.54
3	0.50	0.33	100.40 ± 0.38	99.62 ± 0.71	100.80 ± 0.65	98.42 ± 0.68
4	0.50	0.20	98.79 ± 0.71	101.37 ± 0.82	99.98 ± 0.54	98.32 ± 0.57
5	0.50	0.17	99.98 ± 0.78	100.04 ± 0.62	98.78 ± 0.80	100.57 ± 0.60
6	0.50	0.13	100.84 ± 0.78	99.00 ± 0.92	99.37 ± 0.64	100.10 ± 0.71
7	0.50	0.10	100.32 ± 0.66	100.94 ± 0.90	100.60 ± 0.50	100.94 ± 0.65
8	0.50	0.07	One end point[c]		99.72 ± 0.84	98.70 ± 0.72
9	0.50	0.40	One end point		One end point	

[a] Numbers correspond to curves in Figs. 8 and 9. [b] Standard deviation is based on at least 4 determinations. [c] Corresponds to salicylic acid plus acetylsalicylic acid.

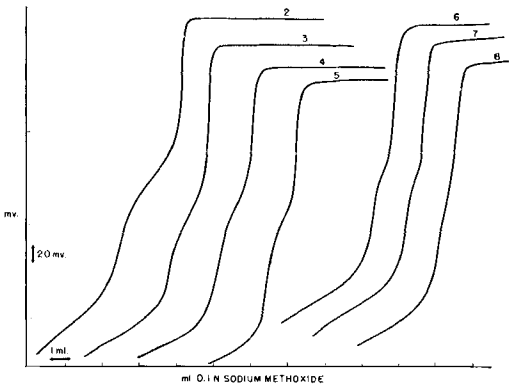


Fig. 8—Effect of salicylic acid-to-acetylsalicylic acid ratio on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid dissolved in dimethylformamide. The numbers above the curves correspond to those in Table V. The titrant was 0.1 N sodium methoxide in benzene–methanol (10:1).

than this, only one end point representing total acid was realized. The curves in Fig. 8 demonstrate clearly the effect of the ratio on the resolution of the two end points. With tetrabutylammonium hydroxide as the titrant, satisfactory resolution was realized when the salicylic acid-to-acetylsalicylic acid ratio was as high as 7 to 1. However, the salicylic acid inflection began to blend with the acetylsalicylic acid inflection at an 11 to 1 ratio. In Fig. 9, curve 8, two end points are clearly defined and recoveries of both components are quantitative. However, in curve 9, only one end point corresponding to the total acid is discernible. Conceivably these ratios could be extended in dimethylformamide by using either more diluted titrants or larger sample sizes.

Water is present as a common impurity in nearly all nonaqueous solvents. The presence of water in the titration solvent can interfere in the titration of acids in protophilic (basic) solvents, of bases in protogenic (acidic) solvents, and of acids and bases in inert solvents. This is due to the amphiprotic nature of water. However, the presence of water in the quaternary ammonium titrant has shown to exhibit a profound stabilizing action on the titrants at a sacrifice of its basic strength (27). The weak acid properties of water are evident in basic solvents, such as dimethylformamide (28, 29), pyridine (30), and ethylenediamine (28). In this investigation, the effect of adding varying quantities of water into the dimethylformamide titration solvent on the sensitivity of differentiation was examined. Equal milliequivalent concentrations of salicylic acid and acetylsalicylic acid were used for all titrations. This requirement was necessary in order to prevent any concentration effect (31). A suitable quantity of water or dimethylformamide was delivered from microburets into the beaker containing accurately weighed samples of the acids. Since a well-defined inflection will be realized for the potentiometric titration of the stronger acid if the solvent employed has good differentiating power, a new and simple method was employed to define the resolution power of the solvent system. This is depicted in Fig. 10 for sodium methoxide titrant and in Fig. 11 for tetrabutylammonium hydroxide titrant. Only a representative set of curves obtained are shown for clarity. The magnitude of the inflection for the stronger acid which is the determining factor for satisfactory differentiation was determined by extrapolating the two straight portions of the

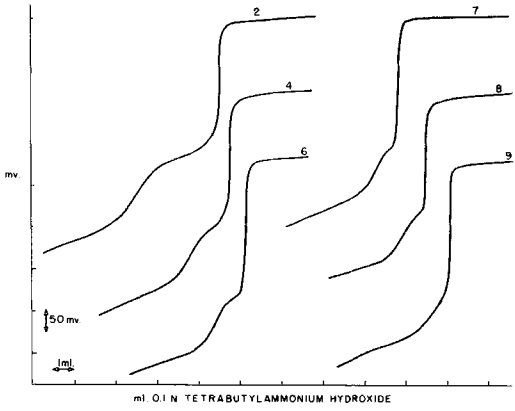


Fig. 9—Effect of salicylic acid-to-acetylsalicylic acid ratio on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid dissolved in dimethylformamide. The numbers above the curves correspond to those in Table V. The titrant was 0.1 N tetrabutylammonium hydroxide in benzene–methanol (10:1).

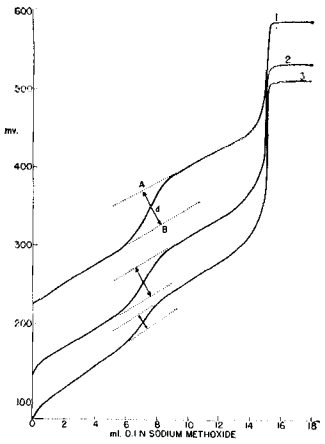


Fig. 10—Effect of water in water–dimethylformamide solvent mixture on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid. Key: 1, 0% v/v water; 2, 6% v/v water; 3, 15% v/v water.

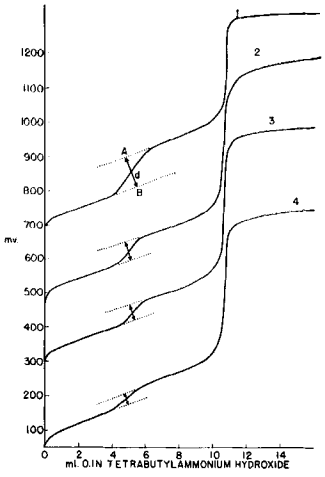


Fig. 11—Effect of water in water–dimethylformamide solvent mixture on sensitivity of differentiating titration of salicylic acid and acetylsalicylic acid. Key: 1, 0% v/v water; 2, 6% v/v water; 3, 10% v/v water; 4, 20% v/v water.

TABLE VI—EFFECT OF WATER CONCENTRATION IN TITRATION SOLVENT ON SENSITIVITY OF DIFFERENTIATING TITRATION

% Water in Water–Dimethylformamide Solvent Mixture	d, cm. Sodium Methoxide Titrant	d, cm. Tetrabutylammonium Hydroxide Titrant
0	2.55[a]	5.08
1	2.40	4.63
2	2.33	4.30
4	2.12	3.75
6	1.97	2.94
8	1.80	2.50
10	1.65	2.12
12	1.41	1.82
15	1.08	1.51
18	0.90	1.33
20	0.75	1.07
25	0.56	0.95

[a] Average of 3 determinations.

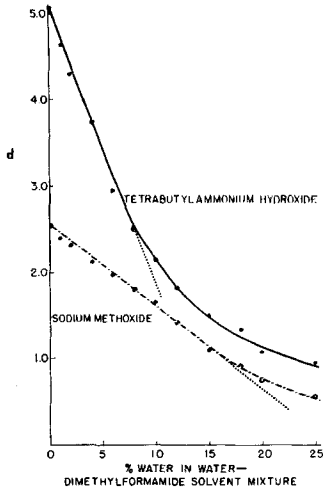


Fig. 12—Relationship between d and concentration of water in water–dimethylformamide solvent mixture during differentiating titration of salicylic acid and acetylsalicylic acid. Glass-modified calomel electrode system was employed.

titration curve and then drawing a straight line through the end point and verticle to the extrapolated line to obtain interception points A and B. The distance of the line AB = d is measured in cm. The magnitude of d obtained in various water–dimethylformamide solvent mixtures is reported in Table VI. The higher the concentration of water in the solvent mixture, the smaller the d value, and the weaker the differentiating ability of the solvent mixture. As expected, water has a detrimental effect on the differentiation. The presence of only a small percentage in the solvent is sufficient to reduce markedly the size and sharpness of the inflections obtained. The potential jump for salicylic acid was reduced gradually upon the progressive addition of water. This is well illustrated in Figs. 10 and 11. Although the recovery is quantitative for both components in the mixture, higher recoveries were obtained when the solvent mixture contained higher water concentrations. However, this is coincided with the increasing blank obtained as the water concentration is increased. When the magnitude of inflection of the stronger acid was plotted *versus* per cent v/v of water in the water–dimethylformamide mixed solvent, Fig. 12 was obtained. At the same concentration of water, larger d values were obtained with the tetrabutylammonium titrant than with the sodium methoxide titrant. This indicates that relatively larger inflections were obtained with the former titrant than with the latter titrant at any particular level of water concentration in the mixed solvent. A linear relationship exists up to 15% of water when the sodium methoxide titrant was employed, as compared to 8% of water when the quaternary ammonium hydroxide titrant was used. The slope of the linear portion of the curve for the quaternary

ammonium titrant is approximately three times steeper than that of the sodium methoxide titrant. This correlation demonstrates that the rate of water tolerance with the sodium methoxide titrant is threefold as resistant as with the quaternary ammonium hydroxide titrant. Since this investigation was designed to provide potentiometric differentiating data for analytical purposes rather than accurate fundamental data, the volume shrinkage during mixing of the solvents and the dielectric constants of such solvent mixtures were not precisely controlled. It is possible that the dielectric constants of such mixtures as measured in the ordinary manner do not represent the effective dielectric constant in the immediate surroundings of the ions. The greater polarity of the water as compared to the dimethylformamide may cause the titration in the immediate vicinity of the ions to be richer in water than the bulk of the solution (32–34). The qualitative pictures obtained in Table VI and Fig. 12 indicate that a progressive increase of dielectric constant with increasing water concentration has a harmful effect on the sensitivity of differentiation.

The proposed nonaqueous differentiating procedures make possible the simple and accurate determination of the combination of acetylsalicylic acid and salicylic acid. Preliminary treatment of the sample is unnecessary, and tedious separation and extraction techniques are obviated. The differentiating titration is achieved in various solvent–titrant–electrode combinations. The technique is applicable even when there is a disproportionate concentration of the components. This work suggests that, by a proper combination of solvent, titrant, and electrode system, it should be possible to titrate potentiometrically both components of acidic or basic mixture whose dissociation constants not only approach but are well below the theoretical limit of 16 proposed by Auerbach and Smolczyk (10).

REFERENCES

(1) Leeson, L. J., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 329(1958).
(2) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 20.
(3) "The National Formulary," 11th ed., J. B. Lippincott Co., Philadelphia, Pa., 1960 p. 13.
(4) Heuermann, R. F., and Levine, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 276(1958).
(5) Levine, J., *J. Pharm. Sci.*, **50**, 506(1961).
(6) Tinker, R. B., and McBay, A. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 315(1954).
(7) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723(1950).
(8) Reed, R. C., and Davis, W. W., *J. Pharm. Sci.*, **54**, 1533(1965).
(9) "The Merck Index," 7th ed., Merck and Co., Rahway, N. J., 1960, p. 13.
(10) Auerbach, F., and Smolczyk, E., *Z. Physik. Chem.*, **110A**, 65(1924).
(11) Kolthoff, I. M., and Stenger, V. A., "Volumetric Analysis," 2nd ed., vol. I, Interscience Publishers, Inc., New York, N. Y., 1942.
(12) Fritz, J. S., and Yamamura, S. S., *Anal. Chem.*, **29**, 1079(1957).
(13) Cundiff, R. H., and Markunas, P. C., *ibid.*, **30**, 1447 (1958).
(14) Teze, M., and Schaal, R., *Bull. Soc. Chim. France*, **1962**, 1372.
(15) Parker, A. J., *Quart. Rev. (London)*, **16**, 163(1962).
(16) Buckingham, A. D., *Discussions Faraday Soc.*, **24**, 151(1957).
(17) Zaugg, H. E., *J. Am. Chem. Soc.*, **82**, 2903(1960).
(18) Prue, J. E., and Sherrington, P. J., *Trans. Faraday Soc.*, **57**, 1796(1961).
(19) Kolthoff, I. M., Bruckenstein, S., and Chantooni, M. K., *J. Am. Chem. Soc.*, **83**, 3927(1961).
(20) Lin, S., Lachman, L., and Higuchi, T., to be published.
(21) Luder, W. F., and Zuffanti, S., "Electronic Theory of Acids and Bases," John Wiley & Sons, New York, N. Y., 1946, p. 104.
(22) Pifer, C. W., Wollish, E. G., and Schmall, M., *Anal. Chem.*, **25**, 310(1953).
(23) Lin, S. L., and Blake, M. I., *J. Pharm. Sci.*, **54**, 1512 (1965).
(24) Lin, S. L., and Blake, M. I., *Anal. Chem.*, **38**, 549 (1966).
(25) Lin, S. L., and Blake, M. I., *J. Pharm. Sci.*, **55**, 781 (1966).
(26) *Ibid.*, **56**, 43(1967).
(27) Harlow, G. A., *Anal. Chem.*, **34**, 1487(1962).
(28) Fritz, J. S., *ibid.*, **24**, 306(1952).
(29) Deal, V. Z., and Wyld, G. E. A., *ibid.*, **27**, 47(1955).
(30) Harlow, G. A., and Wyld, G. E. A., *ibid.*, **30**, 73 (1958).
(31) van der Heijde, H. B., and Dahmen, E. A. M. F., *Anal. Chim. Acta*, **13**, 378(1957).
(32) Scatchard, G., *J. Chem. Phys.*, **9**, 34(1941).
(33) Kilpatrick, M., *Chem. Rev.*, **30**, 159(1942).
(34) Amis, E. S., *J. Am. Chem. Soc.*, **60**, 428(1956).

Emulsifying Properties of a New Polysaccharide Gum

By OSCAR E. ARAUJO

A recently developed polysaccharide gum produced by bacterial fermentation, now commercially available, was investigated as a potential emulsifying agent for pharmaceutical systems. Mineral oil emulsions having a fixed oil to water ratio were prepared using concentrations of the new gum ranging from 0.1 to 0.9 per cent. Emulsions containing 5 to 40 per cent acacia were also prepared and served as a basis for comparison. Rheological measurements, particle size studies, and organoleptic observations were performed on all the emulsions. A Stormer viscosimeter was used to obtain the flow properties, while a standard microscopic technique was employed for the particle size analysis. The results showed the plastic nature of the emulsions prepared with the new gum and the existence of a definite yield value. These emulsions showed good over-all stability over a period of 6 months, with little change in viscosity or particle size. The general appearance of the emulsions containing the new gum was creamier than that of those prepared with acacia, and the emulsions containing more than 0.2 per cent of the polysaccharide exhibited no phase separation after 6 months.

THE EVALUATION of potential suspending and emulsifying agents which would increase the stability and cosmetic elegance of heterogeneous pharmaceutical systems has been the object of much investigation.

In recent years, research by the U. S. Department of Agriculture resulted in the production of a heteropolysaccharide from the bacterial fermentation of glucose by *Xanthomonas campestris* (1). Jeanes *et al.* (2) reported several of the properties of what was at the time referred to as polysaccharide B-1459: this xantham gum has since been produced commercially.[1] These authors had shown upon analysis that the major constituents of the new polysaccharide were D-mannose, D-glucose, and D-glucuronic acid present as the potassium salt.

A study of the acute toxicity of and the skin sensitivity to the gum was carried out and no evidence of either was found (3). Government clearance for food and drug use is still pending final FDA approval.

Carnie (4) discussed some of the properties of solutions of the gum and the effects of heating, pH, concentration, and homogenization on these preparations. Kabre *et al.* (5, 6) studied the rheological characteristics of this xantham gum and evaluated it as a suspending agent for zinc oxide and sulfamethazine.

The above studies along with preliminary experimentation revealed certain unusual properties of this polysaccharide which indicated its potential as an emulsifying agent and led to the present investigation. Emulsions using acacia, a well-known naturally occurring polysaccharide, served as the basis for comparison.

Received April 14, 1967, from the Department of Pharmacy, College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication June 21, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

[1] Marketed as Kelzan by the Kelco Co., Los Angeles, Calif.

EXPERIMENTAL

Preparation of Emulsions—All the emulsions were prepared using a 4:6 mineral oil to water ratio. The xantham gum was used in concentrations of 0.1 to 0.9%, in increments of 0.1%, and acacia in concentrations of 5 to 40%, in increments of 5%. The carefully measured liquids were placed in a Waring blender, the emulsifying agent added to the surface of the liquids, and the entire mixture blended for 4 min. The resulting emulsions were passed twice through a hand homogenizer to further standardize the products. The distilled water used to prepare all emulsions contained 0.2% benzoic acid as a preservative. All emulsions were allowed to equilibrate for 2 hr. before any measurements were made.

pH Measurements—A Photovolt electronic pH meter model 110 was used to measure the pH of the emulsions.

Rheological Measurements—A Stormer viscosimeter equipped with the modified cup and bob as described by Fischer (7) was employed. A weight hanger with various slotted weights provided the shearing stress. Calibration data obtained with a standard oil were used to calculate the instrumental constant, K_v, from the expression:

$$K_v = \eta \frac{\text{r.p.m.}}{W} \qquad \text{(Eq. 1)}$$

where W represents the shearing stress in Gm., r.p.m., the rate of shear, and η, the viscosity of the standard oil in cps.

The plastic viscosity was calculated according to the expression:

$$U = K_v \frac{W - W_f}{\text{r.p.m.}} \qquad \text{(Eq. 2)}$$

where W_f is the extrapolated yield value intercept in Gm., and U is the plastic viscosity in cps.

The yield value was obtained from the expression:

$$f = \frac{2\pi}{60} \times K_v \times W_f \times \frac{1}{2.303 \log R_c/R_b} \qquad \text{(Eq. 3)}$$

All rheological measurements were performed at 20 ± 0.1°.

Organoleptic Observations—The emulsions were examined as to appearance, odor, and texture; any

change in these characteristics over a period of 6 months was noted.

Particle Size Analysis—The diluting medium used to prepare the slides for the microscopic analysis consisted of a 75% aqueous glycerin solution. Several other vehicles were tried, including propylene glycol as suggested by Knoechel (8); the medium selected provided the best contrast and least particle mobility.

One milliliter was withdrawn from well below the surface of the emulsion by means of a syringe equipped with an 18 gauge, 3.5 in. length needle. The sample was then introduced into a 100-ml. volumetric flask containing approximately 50 ml. of the diluting fluid, mixed thoroughly and brought to a volume of 100 ml. One drop of the diluted emulsion was then placed on a glass slide and a cover slip carefully placed over the liquid. Approximately 15 min. was permitted to elapse to allow the particles to rise and assume a fixed position on the slide.

The particle count was performed with the aid of a standard microscope equipped with a 43× objective, a 10× ocular, a movable micrometer stage, and a micrometer eyepiece. The latter contained a movable cross hair, the traverse of which was measured by a revolving drum marked into 100 divisions. By proper calibration each division was then equivalent, under the above conditions, to a fraction of a micron.

The number of particles counted for each sample varied from 400 to 1600 depending on the range of particle distribution. A preliminary scan of the entire slide provided an over-all picture of the spread of particle size, helping to establish the number to be counted. The degree of aggregation or clustering was also noted in this examination.

Using the movable micrometer stage and a table of random numbers, "coordinates" were chosen to insure a random selection of fields and to prevent duplicate measurements. The size frequency tally obtained from these counts was used to calculate the arithmetic mean diameter, d_m, the geometric mean diameter, d_g, and the volume-surface mean diameter, d_{vs}, according to the following expressions:

$$d_m = \frac{\Sigma n_i d_i}{\Sigma n_i} \qquad \text{(Eq. 4)}$$

$$d_g = \frac{\Sigma n_i \ln d_i}{\Sigma n_i} \qquad \text{(Eq. 5)}$$

$$d_{vs} = \frac{\Sigma n_i d_i^3}{\Sigma n_i d_i^2} \qquad \text{(Eq. 6)}$$

where n_i is the number of globules having a class diameter of d_i in microns (7, 9).

RESULTS AND DISCUSSION

Preliminary Studies—In order to evaluate effectively the emulsifying ability of the xantham gum, it was necessary not only to prepare the emulsions in exactly the same manner each time, but also to maintain a constant oil–water ratio. These precautions would eliminate all the variables affecting the quality of the emulsions except the nature and concentration of the emulsifier.

Preliminary experiments were performed to establish the range of concentrations of the new gum to be used in the study. The results showed that a 1.0% aqueous mucilage of xantham gum was a firm gel, while those mucilages containing less than 0.1% were too dilute. Therefore, the range of concentrations selected was 0.1 to 0.9%.

Emulsions of castor, cod liver, and mineral oils were prepared using equal parts of oil and water; 0.25 and 0.5% of the xantham gum served as the emulsifier. Mineral oil was selected for the investigation on the basis of the over-all appearance of the emulsions.

The oil–water ratio selected for the detailed experimentation was the result of an appraisal of the general appearance of mineral oil emulsions prepared with ratios of 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2. Again the xantham gum was used in concentrations of 0.25 and 0.5%. Emulsions having a 4:6 oil–water ratio were chosen by the above criterion for use throughout the study.

Since emulsions containing acacia were to be used for the purpose of comparison, it was necessary to determine if the 4:6 ratio also provided satisfactory emulsions with acacia. Accordingly, a trial mineral oil emulsion with acacia was prepared and judged satisfactory.

Having established the range of concentrations of xantham gum to be used in the investigation, similar preliminary experiments were conducted with acacia, resulting in the selection of concentrations ranging from 5 to 40%.

Organoleptic Qualities—The emulsions prepared with the new gum exhibited a creamy white appearance. The lone exception involved the product containing 0.1% which appeared to be incompletely emulsified; this observation was confirmed by examining the emulsion under a microscope, which clearly showed large oil lakes throughout the sample, making a globule count impossible. No unpleasant odor developed in any of the emulsions during a 6-month shelf-life. The general texture when applied to the skin was smooth, leaving no gummy residue. This quality would seem to be important from a lotion formulation standpoint. The preparations having concentrations above 0.5% had a gel-like consistency easily broken up by shaking. Creaming was observed to a slight degree only with the 0.2% emulsion after storage of 6 months.

The emulsions prepared with acacia did not have a creamy appearance and possessed an off-white coloration. A gummy film characteristic of acacia preparations remained on the skin upon drying. The acacia emulsions having concentrations of 25% and less showed creaming after a period of 6 months, the extent of which increased with decreasing concentration.

pH Considerations—The pH of each emulsion was taken initially and after 6 months. The pH of the emulsions with the xantham gum ranged from 3.5 to 4.0 while that of those prepared with acacia varied from 4.2 to 4.4. The measurements indicate essentially no change in the pH of the emulsions prepared with either emulsifier.

Rheological Observations—The emulsions prepared with the new gum showed certain unusual rheological characteristics. Figure 1 clearly indicates the plastic nature of the emulsions and the existence of a definite yield value. As the shearing stress is increased, a value is reached for each of the emulsions above which a constant plastic viscosity is given by the linear portion of

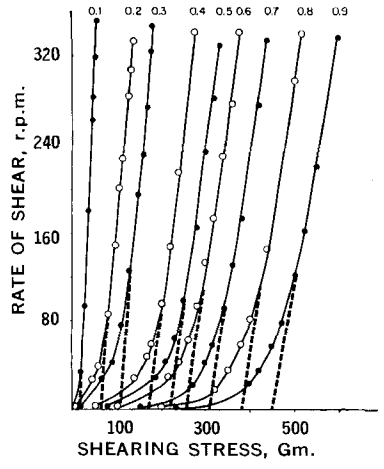


Fig. 1—Rheograms of emulsions containing various per cent concentrations of xantham gum.

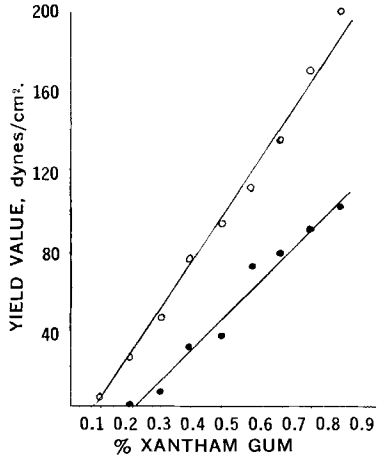


Fig. 2—Yield values of emulsions containing the xantham gum. Key: ●, minimum yield value; ○, Bingham yield value.

the rheogram. Extrapolation of this linear segment to the shearing stress axis gives the Bingham yield value, in Gm.

A minimum yield value was established for each of the xantham gum emulsions by determining the minimum number of Gm. which produced rotation of the bob on the Stormer viscosimeter. Each emulsion, except the 0.1% semiemulsified product, displayed a minimum yield value, thereby precluding any pseudoplasticity.

The minimum and extrapolated yield values for the various concentrations of the xantham gum are shown in Fig. 2. There appears to be a linear relationship between concentration and both yield values over the range studied. In addition, the Bingham yield value for the most concentrated emulsion is less than 200 dynes/cm.², well within the range of the stress of a hand-shaken bottle.

Figure 3 indicates that the plastic viscosities increase with increasing concentrations of the gum, as did the yield values. The increase, however, appears to decrease exponentially so that the overall range of plastic viscosity was only 25 to 86 cps. Consequently, the 0.9% xantham gum emulsion which has the highest plastic viscosity still has sufficient mobility upon agitation to be easily poured from a bottle.

Rheologically, the ideal emulsifying agent should possess the same qualities as those of the ideal suspending agent: high viscosity at the negligible shear of shelf storage and low viscosity upon agitation, causing the preparation to be free-flowing and easily spread on the skin (10).

The new polysaccharide appears to lack one desirable feature of an emulsifying agent, that of thixotropy. Since the upcurve and downcurve of the rheograms were found to be coincidental, there was no time-dependence in the rheological reconstitution of the emulsions upon removal of the shearing stress. This property held true for all concentrations of gum but only affects pourability in those emulsions of concentrations above 0.6%, where agitation must be maintained to remove the contents from the bottle.

The rheograms for the acacia emulsions are shown

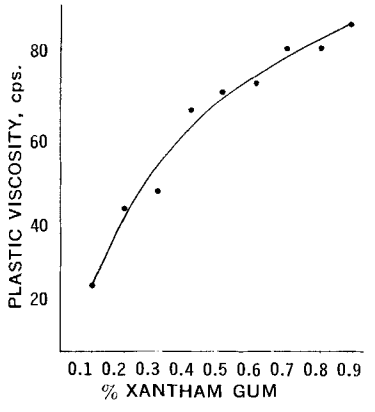


Fig. 3—Plastic viscosities of emulsions containing xantham gum.

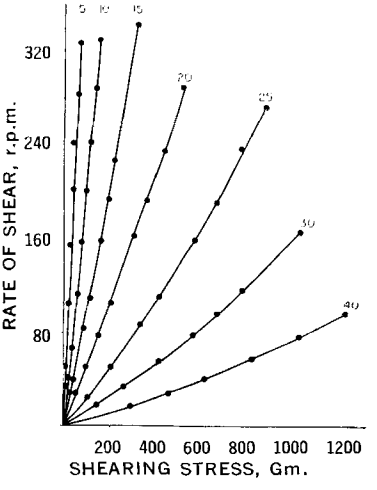


Fig. 4—Rheograms of emulsions containing various per cent concentrations of acacia.

TABLE I—PARTICLE SIZE OF EMULSION GLOBULES

% Xantham Gum	Diam., μ			% Acacia	Diam., μ		
	d_m	d_g	d_{vs}		d_m	d_g	d_{vs}
0.2	1.38	1.13	11.1	5	7.53	5.33	19.7
0.3	1.46	1.18	12.1	10	6.47	5.37	11.6
0.4	1.81	1.21	25.9	15	5.81	4.40	13.7
0.5	2.00	1.27	28.9	20	2.39	2.10	3.60
0.6	2.08	1.24	33.3	25	2.05	1.84	2.84
0.7	2.26	1.30	31.5	30	1.83	1.72	2.26
0.8	2.52	1.38	27.2	40	<1.00	...	...
0.9	3.19	1.55	26.4				

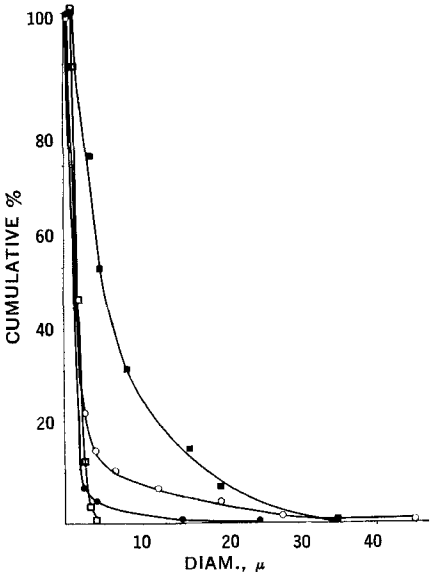


Fig. 5—Particle size distribution of emulsions containing extremes of concentration of acacia and xantham gum. Key: ■, 5% acacia; □, 30% acacia; ●, 0.2% xantham gum; O, 0.9% xantham gum.

in Fig. 4. The quasi-Newtonian characteristics of the dilute emulsions and the pseudoplastic nature of the more concentrated ones are evident. The rheological pattern is quite similar to that of the aqueous mucilages of the gum (11).

Regardless of the emulsifying agent used or its concentration, the products showed no significant change in rheological properties after a 6-month shelf-life, indicating good consistency stability.

Particle Size Studies—Table I gives the mean globule diameters for all the emulsions prepared in this study. The ranges of the arithmetic and geometric mean diameters of the emulsions prepared with the xantham gum from low to high concentration are narrower than those for the acacia emulsions. However, the emulsion containing 40% acacia contained only particles of 1 μ and smaller; no larger particles were observed even when the entire slide was scanned.

The volume-surface mean diameters are much larger for the emulsions prepared with the new polysaccharide, indicating the presence of a number of particles in the 25 to 45 μ range. However, in Fig. 5, which shows the cumulative frequency distribution curves for the extremes of concentration of both emulsifiers, it can be seen that the xantham gum emulsions have a considerably larger percentage of 1-μ diameter globules than do the acacia emulsions.

Figure 6 shows the log normal plots for the emulsions prepared with acacia. Whereas these emulsions appeared to follow a log normal distribution, those prepared with the new gum did not. This may be explained by the fact that in most cases more than 90% of the globules in the xantham gum emulsions had a diameter of less than 1 μ.

Interestingly, the emulsions with xantham gum increase in particle diameter and in the number of very large globules as the concentration is increased; the acacia emulsions, on the other hand, show a decrease in globule size with an increase in concentration. The explanation is probably involved in the rheological nature of the two emulsifying agents. The xantham gum may produce a strong film around the globules and as the concentration is increased, the well protected particles resist breakdown by the blender and homogenizer. At lower concentrations of the new gum, where the film may be more vulnerable, the larger particles may be broken down, producing a lower mean diameter. It was further noted that samples of the xantham gum emulsions, when viewed microscopically, show clusters of globules which remained in this aggregated state without coalescing. Some globule coalescence was observed with the acacia emulsions, as two globules in close proximity would eventually merge, sometimes within 24 hr. There did not appear to be as many clusters of particles in the

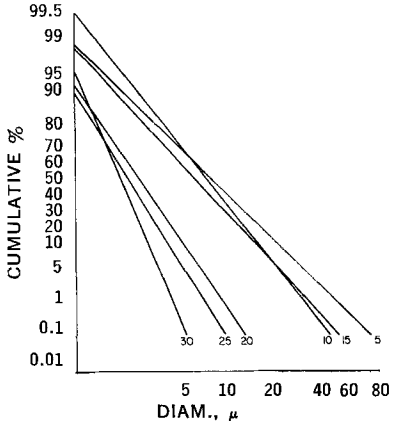


Fig. 6—Log probability plot of particle size distribution of emulsions containing various per cent concentrations of acacia.

acacia emulsions. Regardless of the emulsifier used, re-examinations of all emulsions showed no significant change in globule pattern or over-all mean diameters after 6 months on the shelf. These results were corroborated by the previously mentioned rheological stability of the emulsions after 6 months. If a general increase in particle size due to coalescence had occurred, the over-all viscosity of the emulsions would have decreased (12).

The above findings further contribute to the belief that thickness and strength of the emulsifier film adsorbed at the globule surface play a more important role in the stability of emulsions than does the initial particle size distribution.

SUMMARY

Mineral oil emulsions with a 4:6 oil to water ratio were prepared using various concentrations of a new polysaccharide.

The emulsions prepared with the new xantham gum demonstrated desirable rheological characteristics from a stability standpoint. The plastic nature of the xantham gum emulsions, as well as the corresponding yield values, were examined in detail. Their particle size distribution was broader in general than the emulsions prepared with acacia. Globule coalescence of the emulsions containing the xantham gum appeared to be minimal.

The emulsions containing more than 0.2% of the new gum did not exhibit creaming after 6 month's storage. The xantham gum did not leave a tacky film on the skin after drying. There appeared to be no evidence of bacterial decomposition in any of the emulsions, as evidenced by no significant change in pH or viscosity.

A much lower concentration of xantham gum than of acacia was required to produce stable emulsions. As a result of this investigation, it was felt that the new polysaccharide showed good potential as an emulsifier. For the mineral oil emulsions prepared in this study, the optimum concentration of the xantham gum appeared to be 0.4 to 0.5%.

REFERENCES

(1) U. S. Department of Agriculture, Ag. Res. Service Bulletin CA-N-9, 1959.
(2) Jeanes, A., Pittsley, J. E., and Senti, F. R., *J. Appl. Polymer Sci.*, **5**, 519(1961).
(3) Booth, A. N., Hendrickson, A. P., and DeEds, F., *Toxicol. Appl. Pharmacol.*, **5**, 478(1963).
(4) Carnie, G. A. R., *Australasian J. Pharm.*, **45**, S80 (1964).
(5) Kabre, S. P., DeKay, H. G., and Banker, G. S., *J. Pharm. Sci.*, **53**, 492(1964).
(6) *Ibid.*, **53**, 495(1964).
(7) Fischer, E. K., "Colloidal Dispersions," John Wiley & Sons, Inc., New York, N. Y., 1950, pp. 159–168.
(8) Knoechel, E. L., and Wurster, D. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 1(1959).
(9) Orr, C., and Dallavalle, J. M., "Fine Particle Measurement," The Macmillan Co., New York, N. Y., 1959, pp. 13–37.
(10) Martin, A. N., "Physical Pharmacy," Lea & Febiger Publishing Co., Philadelphia, Pa., 1960, p. 620.
(11) Araujo, O. E., *J. Pharm. Sci.*, **55**, 636(1966).
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Effect of Probenecid on Riboflavin Absorption and Excretion in Man

By WILLIAM J. JUSKO and GERHARD LEVY*

An oral dose of probenecid given 1 hr. prior to oral administration of riboflavin or riboflavin-5′-phosphate (FMN) to normal humans produced a marked decrease in the excretion rate of the vitamin during the early excretion phase but had no significant effect during the later (post-absorptive) phase. Oral administration of probenecid prior to intramuscular injection of FMN increased the apparent "half-life" of the initial rapid phase of riboflavin excretion. The urinary recovery of riboflavin following oral or intramuscular administration of the vitamin was usually somewhat decreased by probenecid. These results are consistent with our earlier suggestion that riboflavin is excreted in part by renal tubular secretion. It appears that probenecid also inhibits the specialized transport process responsible for the intestinal absorption of riboflavin in man. The complex kinetics of riboflavin distribution and elimination in man are discussed and additional evidence is presented in support of enterohepatic cycling of the vitamin.

THE AUTHORS have shown recently that riboflavin and riboflavin-5′-phosphate (FMN) are absorbed from the gastrointestinal tract of man by a site-specific and saturable specialized transport process rather than by passive diffusion (1, 2). An analysis of published data has led also to the conclusion that the renal excretion of riboflavin occurs not only by glomerular filtration but apparently also by renal tubular secretion (3).

The purpose of the study to be described here was to determine the effect of probenecid on the excretion of riboflavin. Probenecid inhibits a

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Accepted for publication June 13, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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REFERENCES

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number of specialized renal tubular transport processes (4, 5). An effect of this drug on the excretion of riboflavin would constitute additional support for the suggestion that riboflavin excretion involves active tubular secretion.

Some of the results of this study have bearing also on the absorption, distribution, and disposition of riboflavin and provide additional support for our earlier suggestion that riboflavin is subject to enterohepatic cycling (2).

EXPERIMENTAL

Seven healthy male volunteers, 24 to 38 years of age, served as test subjects. They received 1 Gm. of probenecid[1] (0.5 Gm. in one instance) in the morning after an overnight fast and then ate a standard breakfast. Exactly 1 hr. after probenecid administration, three subjects received 10 to 150 mg. riboflavin or FMN[2] dissolved in 200 ml. of water. Four of the subjects were given an intramuscular injection of approximately 30 mg. of riboflavin as FMN[3] 1 hr. after probenecid administration. The exact amount of FMN injected was determined by direct analysis of an equivalent volume of the parenteral solution measured in the same syringe used for the injection. The injected amounts averaged 28.4 mg. riboflavin equivalent. Similar experiments were carried out without preadministration of probenecid. The order of experiments was randomized with an interval of at least 2 weeks between them.

Urine was collected at frequent intervals for 24 to 48 hr. The standard breakfast, urine collections, and other details of the protocol were essentially the same as described in previous reports (1, 2).

Analyt cal Methods—Riboflavin and FMN in the urine were determined fluorimetrically by the method of Burch *et al.* (6) and by a modified U.S.P. assay procedure (7) using the Turner fluorometer, model 111, with primary filter 47-B and secondary filter 2A-12. The modifications of the two assays have been described in a previous publication (1).

At least two 24-hr. blank urine collections were carried out in each subject and all data were corrected for blank values. The data reported in the tables and figures are based on results obtained with the modified U.S.P. assay. Probenecid and probenecid metabolites (in urines collected after probenecid administration) were found to have no effect on the riboflavin assays.

RESULTS AND DISCUSSION

In the first part of this study, three subjects received various doses of riboflavin or FMN 1 hr. after oral administration of 1 Gm. probenecid. Typical examples of the effect of probenecid on the excretion of riboflavin after oral administration of FMN are shown in Figs. 1 and 2. Probenecid produced a pronounced decrease in the excretion rate of riboflavin during the early phase of riboflavin excretion, but had no detectable effect during the later (post-absorptive) phase of excretion. The effect of probenecid on the early excretion rates and on the total urinary recovery of riboflavin after oral administration of various doses of the vitamin is summarized in Table I for all experiments. In each instance, probenecid produced not only a marked decrease in the excretion rate of riboflavin during the first 2 to 3 hr. after administration of the vitamin, but caused also a statistically significant decrease in the total urinary recovery of the vitamin ($p < 0.05$, paired comparisons).

Bahal (5) found that 1 Gm. probenecid inhibited the elimination of sulfisomidine and procainamide for at least 8 hr. This is consistent with the reported plasma half-life of probenecid which, though

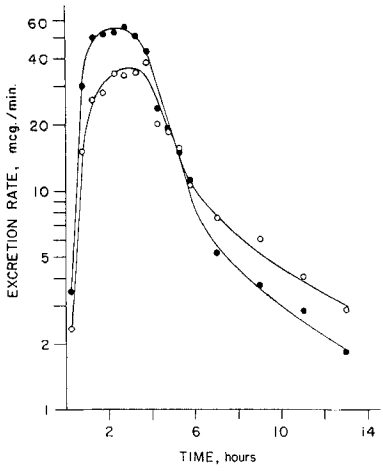


Fig. 1—Urinary excretion rate of riboflavin as a function of time after oral administration of FMN (equivalent to 30 mg. riboflavin) in aqueous solution with (○) and without (●) preadministration of 1 Gm. probenecid. Subject L.

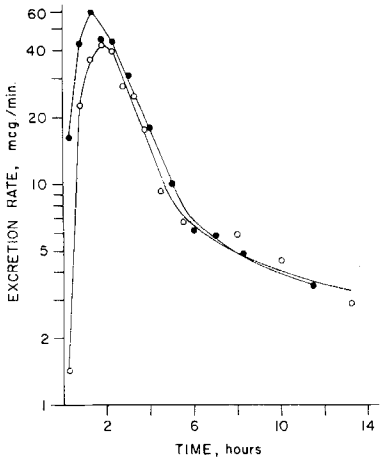


Fig. 2—Urinary excretion rate of riboflavin as a function of time after oral administration of FMN (equivalent to 30 mg. riboflavin) in aqueous solution with (○) and without (●) preadministration of 1 Gm. probenecid. Subject J.

[1] Benemid Tablets, Merck, Sharpe and Dohme, West Point, Pa.
[2] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.
[3] Hyrye Injection, Durst Co., Philadelphia, Pa.

TABLE I—EFFECT OF PROBENECID ON INITIAL EXCRETION RATES AND TOTAL URINARY RECOVERY OF RIBOFLAVIN AFTER ORAL ADMINISTRATION OF RIBOFLAVIN (FR) AND RIBOFLAVIN-5′-PHOSPHATE (FMN)

Subject	Drug; Dose, mg.[a]	Probenecid Dose, Gm.	Excretion Rate, mcg./min. Time, hr.: 0.5	1	1.5	2	2.5	% Urinary Recovery[b]
J	FR; 10	0	22	35	32	16	14	63[c]
		0.5	5	22	21	13	12	43
J	FR; 30	0	11	39	47	59	64	44
		1.0	5	35	34	32	33	36[c]
J	FMN; 30	0	16	42	59	45	43	44
		1.0	1	23	36	42	39	38
J	FMN; 150	0	18	58	71	75	73	15
		1.0	1	7	16	17	20	8
A	FMN; 30	0	5	28	44	37	42	37
		1.0	1	14	25	30	33	36
L	FMN; 30	0	3	30	50	51	53	49
		1.0	2	15	26	28	34	44

[a] FMN doses are expressed as riboflavin equivalents. [b] Thirty-six hour collection period, except where *Footnote c* designated. [c] Twenty-four hour collection period.

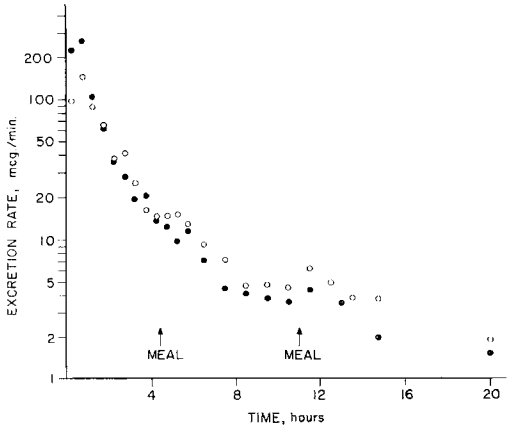


Fig. 3—Urinary excretion rate of riboflavin as a function of time after intramuscular injection of FMN (equivalent to 30 mg. riboflavin) with (○) and without (●) preadministration of 1 Gm. probenecid. Subject M. Note the secondary excretion rate maxima after each meal.

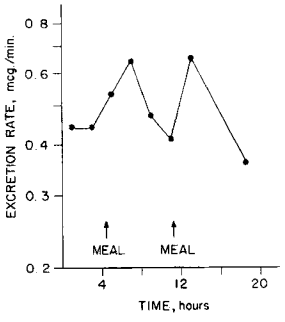


Fig. 4—Urinary excretion rates of riboflavin as a function of time in a subject (F) not receiving riboflavin except for the amount obtained from a normal diet.

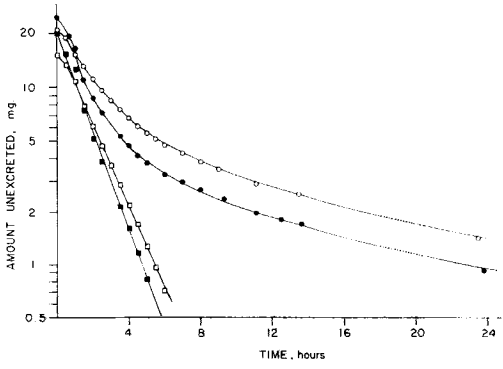


Fig. 5—Urinary excretion of riboflavin as a function of time after intramuscular injection of FMN (equivalent to 30 mg. riboflavin) with (open symbols) and without (solid symbols) preadministration of 1 Gm. probenecid. The lower curves (squares) are the residuals obtained by resolving the upper curves (circles) into two components by the "backward projection" technique.

apparently dose-dependent, is around 6 hr. for a 1-Gm. dose (8). The fact that riboflavin excretion was inhibited only during the first 2 to 3 hr. after administration of probenecid suggested to the authors that the latter inhibits the absorption rather than the excretion of riboflavin. While there appears to be no previous report of an intestinal absorption inhibiting effect of probenecid in man, it is of interest that Binder and co-workers have found recently that probenecid inhibits the active intestinal transport of certain amino acids in the hamster (9).

It was desirable, in view of the results obtained from oral administration of riboflavin, to determine the effect of probenecid on the elimination of parenterally administered riboflavin. A typical example of the effect of probenecid on the excretion rate of riboflavin as a function of time after intramuscular administration of FMN is shown in Fig. 3. The time course of riboflavin excretion observed in these experiments is similar to that found in previous studies (1, 2) where it was noted that the excretion of the vitamin exhibits an initial rapid and a subsequent slow phase. In addition, secondary excretion rate maxima are evident in Fig. 3. These maxima were found in about one-half of the experiments and have been noted also and consistently after oral administration of large doses of the vitamin (2). These secondary maxima are thought to

TABLE II—EFFECT OF PROBENECID ON URINARY RECOVERY AND INITIAL APPARENT HALF-LIFE OF RIBOFLAVIN AFTER INTRAMUSCULAR INJECTION OF FMN[a]

Subject	Injection Site[b]	% Urinary Recovery		Initial Apparent Half-Life[c]	
		Without Probenecid	With Probenecid	Without Probenecid	With Probenecid
J	L	68	66	1.1 (2.7)	1.8 (2.8)
N	A	88	75	1.2 (4.2)	1.4 (5.7)
M	A	102	79	0.8 (2.5)	1.0 (4.0)
F	L	59	83	0.9 (3.3)	1.7 (2.9)

[a] Average dose of 28.4 mg. riboflavin equivalent (range: 27.8 mg. to 29.0 mg.) based on an analysis of the parenteral solution at the time of administration. [b] L, vastus lateralis muscle (leg); and A, deltoid muscle (arm). [c] Figures in parentheses represent the number of "half-lives" during which the residuals yielded a straight line when plotted in semilogarithmic form.

be due to enterohepatic cycling of riboflavin. The possiblity that the secondary peaks observed in the previous study were due to delayed gastric emptying of a portion of the orally administered FMN is ruled out by the occurrence of secondary peaks in this study where the vitamin was administered parenterally. The secondary peaks are not due to absorption of additional riboflavin from dietary sources since the urinary excretion of riboflavin by subjects deriving the vitamin only from dietary sources is very much lower than that observed in the present study. It is of interest however that excretion rate maxima are observed after meals even in subjects receiving only dietary riboflavin (Fig. 4). The excretion rate maxima observed in the latter instance are at least one order of magnitude lower than those shown in Fig. 3. Similar pronounced secondary maxima have been observed recently in the serum levels of dipyridamole and have been ascribed also to enterohepatic cycling (10). The occurrence of such enterohepatic cycling precludes a rigorous pharmacokinetic analysis of riboflavin absorption, distribution, and elimination.

An effect of probenecid on riboflavin excretion after FMN injection becomes evident upon resolving a semilogarithmic plot of the amount of riboflavin unexcreted as a function of time after drug administration into two components by backward projection of the apparently linear terminal portion of the excretion curve. A good example of this is shown in Fig. 5. A semilogarithmic plot of the residuals was linear in all instances up to the time of occurrence of a secondary excretion rate maximum. As a matter of convenience, the rate of decline of the initial linear portion of the semilogarithmic plot of the residuals is described in terms of an "initial apparent half-life." It must be emphasized, however, that this designation is without rigorous pharmacokinetic basis since the actual kinetic model describing the distribution and elimination of riboflavin is considerably more complex than one involving only two parallel unidirectional exponential processes.

The initial apparent "half-lives" and urinary recoveries of riboflavin obtained with and without pre-administration of probenecid are listed in Table II. In all subjects, the initial apparent "half-life" of riboflavin was increased by probenecid but this difference was slightly short of statistical significance ($p > 0.05$; < 0.1). Also, in three of the four subjects, the urinary recovery of the vitamin was decreased by probenecid.

A comparison of the results obtained by the modified U.S.P. and Burch methods, which gives an indication of the relative concentrations of riboflavin and FMN in the urine (2), showed that probenecid had no noticeable effect on the metabolic fate of the vitamin. As noted in the previous study, the urine contained mainly free riboflavin regardless of the form or route of administration.

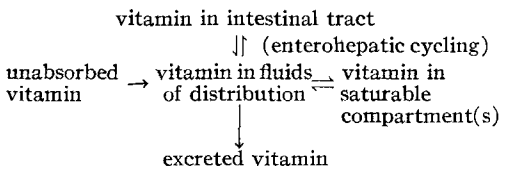
The results of the parenteral study suggest that probenecid does have an inhibitory effect on the renal excretion of riboflavin, presumably by inhibiting an active tubular secretory process (3). While the duration of this effect appears to be relatively short, it is believed unlikely to be due to a decreased rate of absorption of FMN from the site of injection since no such effect of probenecid is known to the authors. On the other hand, Rennick has found an active renal tubular secretory process for riboflavin in the chicken and noted that this process is inhibited by probenecid (11). She suggested that the vitamin shares the transport system for organic bases since the renal tubular secretion of riboflavin was inhibited also by tolazoline. In addition, Markkanen has found that probenecid decreases the basal riboflavin excretion in man and rabbits whose riboflavin intake was limited to that derived from the normal diet (12).

The incomplete recovery of riboflavin observed in most experiments in the present and in previous studies (1, 2), and the decreased urinary recovery of parenterally administered vitamin upon pre-administration of probenecid suggest that the elimination of riboflavin involves at least two parallel pathways.[4] One of these is the urinary excretion of riboflavin; the other process may involve biotransformation, extra-renal excretion, and/or a reversible association of the vitamin with proteins resulting in the formation of certain flavoproteins. The occasional complete urinary recovery of parenterally administered riboflavin (*Reference 13* and subject *M* in the present study) implies that biotransformation and extra-renal excretion may be either quantitatively unimportant or nonexistent. This is consistent with recent evidence that riboflavin is not degraded in rat tissues (14). The third suggested route, namely the reversible association[5] of riboflavin with certain proteins, must be treated kinetically as a bi-directional process involving a compartment of limited capacity. This concept is supported by the fact that nutritionally deficient subjects retain a large fraction of a small test dose of riboflavin (15) and that subjects in

[4] The various phosphorylation-dephosphorylation cycles to which riboflavin is subject in the body have been discussed in a previous publication (2) and are neglected in the present discussion.

[5] This refers to the formation of covalent bonds between riboflavin and proteins in extravascular tissues rather than complex formation with plasma proteins.

negative nitrogen balance (*i.e.*, during excessive catabolism of tissue proteins) show an increased basal excretion of the vitamin (16). These considerations lead to the kinetic model for riboflavin shown in Scheme I.



Scheme I

Even this model, though of considerable complexity, neglects certain additional processes such as phosphorylation-dephosphorylation cycles, formation of FAD, and synthesis of the vitamin by intestinal microorganisms. The time course of riboflavin elimination, as found in this and previous studies in this laboratory (1, 2), is qualitatively similar to that of bromosulfophthalein and rose bengal for which similar reversible multiple compartment models of limited capacity have been suggested (17, 18).

It is reasonable to assume that the saturable compartment is near capacity in subjects who are in exceptionally good nutritional status with respect to riboflavin. Subject *M* of the present study professed to have been taking 5 mg. of riboflavin a day in the form of multiple vitamin tablets four or five times weekly. He discontinued this regimen about 1 week prior to the first parenteral injection of riboflavin. At this time his basal excretion of riboflavin (1.4 mg./day) was indicative of an exceptionally good nutritional status with respect to the vitamin since the usual basal excretion rate is about 1.0 mg./day. (His basal excretion decreased to this level about 3 weeks after the first parenteral injection of riboflavin. During this time he did not use the dietary supplement.) This subject excreted 102% of the first intramuscular dose of FMN which was given without probenecid. It is conceded that this represents very limited evidence but it is noteworthy that none of the other subjects used vitamin preparations prior to the study and that the urinary recovery of riboflavin in these subjects was always incomplete.

The results of this study suggest that probenecid inhibits the urinary excretion of riboflavin. This is consistent with the previous conclusion that the renal excretion of riboflavin apparently involves active tubular secretion (3). It appears also that probenecid inhibits the active intestinal transport of riboflavin although the evidence is by no means unequivocal. This latter point must be established by appropriate animal experiments which await the development of suitable experimental methods since classical techniques used in active transport studies do not appear to be satisfactory for riboflavin (unpublished data). Efforts in this direction are presently being pursued in this laboratory.

ADDENDUM

Effect of Probenecid on Plasma Protein Binding of Riboflavin—Ultrafiltration studies, using human plasma containing 0.1 mg.% riboflavin or FMN alone and with 10 mg.% probenecid, respectively, showed that probenecid has no measurable effect on the plasma protein binding of the vitamin. Details of the binding of riboflavin and FMN by plasma proteins will be reported in a future communication. The concentrations of vitamin and probenecid used in this study reflect the maximum concentration in the plasma obtained upon administration of 30 mg. FMN intramuscularly and 1 Gm. probenecid orally (3, 8, 13). The lack of effect of probenecid on the plasma protein binding of riboflavin suggests that probenecid does not affect the distribution of the vitamin in the body.

REFERENCES

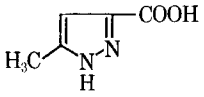
(1) Levy, G., and Jusko, W. J., *J. Pharm. Sci.*, **55**, 285(1966).
(2) Jusko, W. J., and Levy, G., *ibid.*, **56**, 58(1967).
(3) Levy, G., and Jusko, W. J., *ibid.*, **55**, 1322(1966).
(4) Beyer, K. H., *Pharmacol. Rev.*, **2**, 227(1950).
(5) Bahal, S. M., Ph.D. Thesis, Temple University, Philadelphia, Pa., 1965.
(6) Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457(1948).
(7) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 907.
(8) Dayton, P. G., Yu, T. F., Chen, W., Berger, L., West, L. A., and Gutman, A. B., *J. Pharmacol. Exptl. Therap.* **140**, 278(1963).
(9) Binder, H. J., Katz, L. A., Spencer, R. P., and Spiro, H. M., *J. Clin. Invest.*, **45**, 1854(1966).
(10) Mellinger, T. J., and Bohorfoush, J. G., *Arch. Intern. Pharmacodyn.*, **163**, 471(1966).
(11) Rennick, B. R., *Proc. Soc. Exptl. Biol. Med.*, **103**, 241(1960).
(12) Markkanen, T., Toivanen, P., Toivanen, A., and Sotaniemi, E., *Scand. J. Clin. Lab. Invest.*, **15**, 511(1963).
(13) Stripp, B., *Acta Pharmacol. Toxicol.*, **22**, 353(1965).
(14) Yang, C. A., and McCormick, D. B., *Federation Proc.*, **26**, 305(1967).
(15) Najjar, V. A., and Holt, L. E., Jr., *Bull. Johns Hopkins Hosp.*, **69**, 476(1941).
(16) Windmueller, H. G., Anderson, A. A., and Michelson, O., *Am. J. Clin. Nutr.*, **15**, 73(1964).
(17) Winkler, K., in "The Biliary System," Taylor, W., ed., F. Davis Co., Philadelphia, Pa., 1965, p. 551.
(18) Erjavec, M., and Vakselj, M., *Rad. Diagn.*, **7**, 123 (1966).

Absorption, Metabolism, and Excretion of 5-Methylpyrazole-3-carboxylic Acid in the Rat, Dog, and Human

By DAVID L. SMITH, JOHN G. WAGNER, and GEORGE C. GERRITSEN

5-Methylpyrazole-3-carboxylic acid is completely absorbed and rapidly excreted in the rat, dog, and human. After 1- or 9-mg./Kg. doses to the rat, the urinary excretion half-life was 1.0 hr., with 57 per cent of the dose excreted unchanged and the remainder as an acidic conjugate. Pretreatment of rats with high doses of the drug did not significantly alter the absorption, metabolism, or excretion of the drug. After oral and intravenous administration of about 7 mg./Kg. to the dog, all of the drug was excreted unchanged with a half-life of 1.8 hr. (average of blood and urine data). After oral and intravenous doses of 37.5 mg. to six human volunteers, the average half-life for the excretion of radioactivity was 1.9 hr. At this dose the human converts an average of 31 per cent of the drug to the same metabolite produced by the rat. In the human, the following averages were not statistically different after oral and intravenous administration: total urinary excretion, urinary excretion half-life, total fecal excretion, half-life of disappearance from plasma, apparent volume of distribution, area under the plasma curve, and percentage of drug metabolized. The human data have been summarized by general equations, which were derived for a simple kinetic model. The average first-order rate constants ($hr.^{-1}$) for the six humans were as follows (average of oral and intravenous data): over-all loss, 0.32; excretion of drug, 0.22; formation of metabolite, 0.10; excretion of metabolite, 1.5.

IN PREVIOUS studies (1) 5-methylpyrazole-3-carboxylic acid (I) was isolated as a metabolite



I

from the urine of rats treated with 3,5-dimethylpyrazole and was shown to be 116 times more potent than tolbutamide as a hypoglycemic agent in the intact, fasted, glucose-primed rat (2).

Studies in the rat with ^{14}C-labeled 3,5-dimethylpyrazole (3) showed the oral dose to be completely absorbed and rapidly excreted as four metabolites: 5 - methylpyrazole - 3 - carboxylic acid (13%), conjugated 5-methylpyrazole-3-carboxylic acid (14%), conjugated 4-hydroxy-3,5-dimethylpyrazole (71%), and an unidentified metabolite (1%).

All of the hypoglycemic activity of 3,5-dimethylpyrazole in the rat appeared to be due to I, its active metabolite. Unlike 3,5-dimethylpyrazole, I stimulates glucose utilization by adipose tissue *in vitro* and depresses blood sugar of eviscerated rats (2). It was not known whether the human would metabolize 3,5-dimethylpyrazole to the active carboxy metabolite and, if so, how much would be produced. It was therefore decided to undertake the development of the active metabolite as an antidiabetic agent. As part of this research effort, ^{14}C-labeled I (also referred to as the drug) was prepared, and its absorption, metabolism, and excretion compared in the rat, dog, and human.

EXPERIMENTAL

Materials—^{14}C-labeled I[1] was prepared from ethyl acetopyruvate-4-^{14}C and hydrazine (4). Its chemical and radiochemical purity was established by melting point, elemental analysis, infrared spectroscopy, and paper and thin-layer chromatography.

Rat Studies—Charles River CD male rats, weighing 140–200 Gm., were used. They were fasted overnight prior to the experiment (water was allowed *ad libitum*), and were housed individually in stainless steel metabolism cages. Rats *1–3* were dosed orally with 1.315 mg. (9 mg./Kg.) of labeled I (specific activity: 1.33 μc./mg.), dissolved in 0.5 ml. of 0.25% sterile methylcellulose (MC) vehicle. Sequential urine samples were collected from each rat at 2, 4, 6, 8, 15, 24, 48, and 72 hr. Since biological studies suggested that tachyphylaxis to the drug develops in rats (unpublished results) and since a change in the metabolism of the drug could explain this phenomenon, rats *10–15* were treated orally with 25 mg./Kg. of unlabeled I dissolved in 0.5 ml. of 0.25% MC vehicle twice daily for 3 days and 3 times on the fourth day. The rats were fasted on the fourth night and dosed orally with 0.184 mg. (1.0 mg./Kg.) of labeled drug (specific activity: 0.57 μc./mg.)

Received February 6, 1967, from the Control Analytical Research and Development Department, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication June 13, 1967.

The authors thank the following individuals: R. C. Thomas for the synthesis of the ^{14}C-labeled drug, R. L. Johnston for assistance with the dog studies, A. L. Pulliam for analytical assistance, J. I. Northam, R. H. Baker, Jr., and C. M. Metzler for statistical and interpretive analysis of the data, and A. K. Herd and L. H. MacDonald for preparation of the dosage forms for human use.

[1] This compound was prepared by Dr. R. C. Thomas, The Upjohn Co.

in 4.6 ml. of physiological saline on the morning of the fifth day. The controls (rats *4–9*) were treated exactly like rats *10–15* except that they received only vehicle on days 1 through 4. Rats *4–15* were hydrated by oral administration of 25 ml./Kg. of physiological saline on the afternoon of the fourth day. Sequential urine samples were collected from rats *4–15* at 0.5, 1, 2, 3, 4, 6, 7, 8, and 24 hr. after oral administration of the ^{14}C-labeled drug.

Dog Studies—The ^{14}C-labeled I was administered both orally and intravenously in crossover fashion to two fasted beagles (one male, one female). One week was allowed between the oral and intravenous doses. In each case the dose was approximately 7.5 mg. (10 μc.). The oral dose was given as a powder in a soft gelatin capsule and the intravenous dose was dissolved in 5 ml. of water. Several blood and sequential urine samples were collected for 72 hr. The urine was collected by catheter.

Human Studies—Oral and intravenous doses of ^{14}C-labeled I were administered to six adult male volunteers in crossover fashion. Three of the subjects received the drug intravenously and the other three received it orally on the first week and then, after a 2-week rest period, the drug was administered again with the routes of administration crossed over. The ages and weights of the volunteers are given in Table V. The intravenous dose was 10 ml. of a 3.75 mg./ml. sterile aqueous solution of the labeled drug (specific activity: 1.33 μc./mg.). The oral dose was 37.5 mg. of the drug (1.33 μc./mg.) mixed with lactose in a mortar (average particle size about 70 μ; all particles less than 107 μ), and packed in capsules. The volunteers were fasted overnight and for 6 hr. after drug administration. To facilitate urine collection, each subject received 8 fl. oz. of water 1 hr. prior to drug administration and at 0, 1.5, 4, 6.5, and 10 hr. Plasma and sequential urine samples were collected for 3 days as indicated in Tables II and III. Fecal samples were collected at 24, 48, and 72 hr.

General Counting Techniques—The samples were counted in duplicate using Packard Tri-Carb liquid scintillation spectrometers, models 314EX-2A and 314X; corrections for counting efficiency were made using ^{14}C-toluene as the internal standard. Urine was counted directly by dissolving 0.1 to 0.5 ml. in 15 ml. of diotol counting solution, which was prepared by the procedure of Herberg (5). Plasma and homogenized fecal samples (0.5 to 0.9 Gm.) were placed in sample sacs made from dialysis tubing, and dried overnight. The dried samples were burned by the Schöniger technique, and the evolved $^{14}CO_2$ trapped in a 1 *M* solution of 2-phenylethylamine in methanol. For counting, an aliquot of the latter was mixed with an equal volume of a solution consisting of 4.6 Gm. PPO and 8 mg. POPOP per liter of toluene.

Paper Chromatography—The following paper chromatographic systems were employed, using Whatman No. 2 paper: BAW system, *n*-butanol–acetic acid–water (2:1:1; R_f values: I, 0.76; metabolite, 0.72); BPW system, *n*-butanol–piperidine–water (81:2:17; R_f values: I, 0.37; metabolite, 0.32). The radioactive zones were measured with a 4π paper-strip scanner, Vanguard model 880. The percentage of the total radioactivity represented by each peak was determined by cutting out the peaks and weighing them.

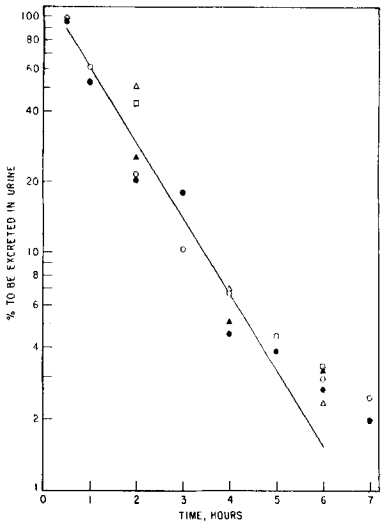


Fig. 1—Rate of urinary excretion of equivalents of 5-methylpyrazole-3-carboxylic acid-^{14}C in the rat. Key: △, rat 1; ▲, rat 2; □, rat 3; ○, rats 4–9; ●, rats 10–15. Average $t_{1/2}$ = 1.0 hr.

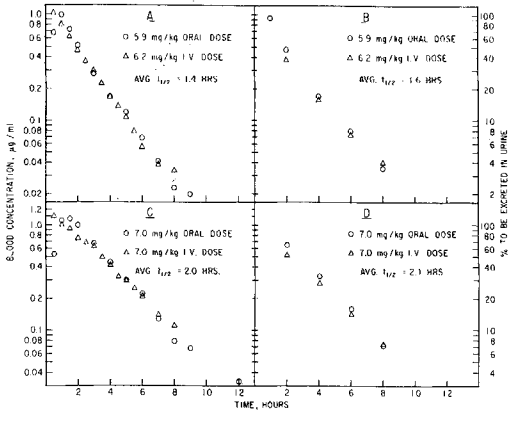


Fig. 2—Rate of excretion of 5-methylpyrazole-3-carboxylic acid-^{14}C in the dog. Key: A, blood levels in the female; B, urinary excretion by the female; C, blood levels in the male; D, urinary excretion by the male.

RESULTS

Rat Studies—After oral administration of the ^{14}C-labeled drug to three rats at a dose of 9 mg./Kg., 88.4% of the radioactivity was found in the urine and 0.2% in the feces. By paper chromatography, an average of 57% of the urinary radioactivity was unchanged drug while the remainder was a single metabolite. The excretion of radioactivity in the urine by the rat obeyed first-order kinetics with a half-life of about 1.0 hr. (Fig. 1).

The six drug-pretreated rats excreted an average of 98.8% of the dose in the urine with 54.0% unchanged. The six nonpretreated rats given the 1-mg./Kg. dose excreted an average of 100.3% of the dose in the urine with 59.7% as unchanged drug. For these two groups there was no significant difference in average percentage of radio-

TABLE I—BLOOD HALF-LIFE, URINARY EXCRETION HALF-LIFE, AND APPARENT VOLUME OF DISTRIBUTION OF 5-METHYLPYRAZOLE-3-CARBOXYLIC ACID-^{14}C IN THE DOG

Dog	Sex	Route	Half-Life, hr. Blood	Half-Life, hr. Urine	Apparent V_d, L.	Apparent V_d, % Body Wt.
1	M	i.v.	2.12 ± 0.09[a]	2.15	4.21	39.5
1	M	Oral	2.06	1.95	4.08	38.7
		Mean	2.09	2.03	4.15	39.1
2	F	i.v.	1.43 ± 0.08[a]	1.65	5.24	43.1
2	F	Oral	1.42	1.45	5.20	40.5
		Mean	1.43	1.55	5.22	41.8
		Over-all mean	1.76	1.79	4.69	40.5

[a] Straight line least squares fit (95% confidence limits given).

TABLE II—AVERAGE PLASMA CONCENTRATIONS OF DRUG EQUIVALENTS AFTER ADMINISTRATION OF A 37.5-mg. DOSE OF 5-METHYLPYRAZOLE-3-CARBOXYLIC ACID-^{14}C TO THE HUMAN

Time, hr.	mcg./ml. ± S.E.M. i.v. Route	Oral Route
0.00	0.00 ± 0.00	0.00 ± 0.00
0.05	2.78 ± 0.08	...
0.3	2.08 ± 0.03	...
0.5	1.83 ± 0.05	0.78 ± 0.24
0.8	1.59 ± 0.05	...
1.0	...	1.42 ± 0.29
2.0	1.23 ± 0.04	1.24 ± 0.04
3.0	...	0.89 ± 0.04
4.0	0.58 ± 0.04	0.55 ± 0.02
5.0	0.32 ± 0.03	0.38 ± 0.01
7.0	0.13 ± 0.02	0.16 ± 0.01
9.0	...	0.073 ± 0.005
10.0	0.041 ± 0.006	...
12.0	...	0.024 ± 0.003
24.0	0.00 ± 0.00	0.00 ± 0.00

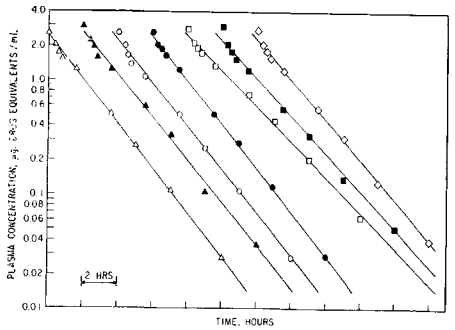


Fig. 3—Plasma levels of equivalents of 5-methylpyrazole-3-carboxylic acid-^{14}C in humans after a 37.5-mg. intravenous dose (log scale). Each curve is displaced 2 hr. from the one preceding it, and the last point on each curve is at 12.0 hr. Key: △, subject No. 1; ▲, No. 2; ○, No. 3; ●, No. 4; □, No. 5; ■, No. 6; ◇, average of No. 1–6.

activity represented by unchanged drug in each of the urine collection intervals. For example, in the first 30 min. 63.7 ± 3.0% (±S.D.) of the radioactivity excreted by the nonpretreated rats was unchanged drug, whereas 61.8 ± 1.5% of the total excreted during this interval by the pretreated rats was unchanged. Therefore, pretreatment with drug for 4 days did not alter the excretion pattern of labeled drug given on the fifth day. Both the rate of excretion (Fig. 1) and the total amount of labeled drug excreted were essentially identical in both the pretreated and nonpretreated groups.

Dog Studies—*Intravenous Dose*—After intravenous administration of approximately 7 mg./Kg. to two dogs, 104.6% of the dose was recovered in the urine and 0.3% in the feces. By paper chromatography, all of the urinary radioactivity was unchanged drug. The whole blood concentrations are plotted in Fig. 2. In the case of one dog, both the whole blood and plasma levels of radioactivity were measured. The plasma levels, which paralleled the blood levels, averaged 28% higher. The elimination of drug from the blood of both dogs obeyed first-order kinetics with half-lives of 1.40 and 2.15 hr. for the female and male dog, respectively (Fig. 2, A and C). No radioactivity was detected in the blood of either dog at 24 hr. The urinary excretion of radioactivity into the urine also obeyed first-order kinetics with half-lives of 1.65 and 2.15 hr. for the female and male dog, respectively (Fig. 2, B and D).

Oral Dose—After oral administration of approximately 7 mg./Kg. to two dogs, an average of 100.2% of the dose was recovered in the urine, and 0.1% in the feces. By paper chromatography all the urinary radioactivity was unchanged drug.

In both dogs the peak levels of radioactivity in the blood were attained within 1 hr. after the oral dose (Fig. 2). The elimination of radioactivity from the blood obeyed first-order kinetics with half-lives of 1.25 and 1.75 hr. for the female and male dog, respectively (Fig. 2, A and C). After the oral dose, urinary excretion of the radioactivity obeyed first-order kinetics with half-lives of 1.45 and 1.95 hr. in the female and male dog, respectively (Fig. 2, B and D).

The half-lives calculated from the blood and urine levels after oral and intravenous doses in the dog are summarized in Table I.

Human Studies—*Intravenous Dose*—The average concentrations of drug equivalents in plasma and their standard errors at several time intervals after the 37.5-mg. intravenous dose are summarized in Table II, and the data for the individual subjects are plotted in Fig. 3. The log plasma concentration time curves show two segments, the first representing rapid equilibration with body fluids (average half-life ~7 min.); the second segment is rate-determining, having an average half-

TABLE III—CUMULATIVE URINARY EXCRETION OF DRUG EQUIVALENTS AFTER ADMINISTRATION OF A 37.5-mg. DOSE OF 5-METHYLPYRAZOLE-3-CARBOXYLIC ACID-^{14}C TO HUMANS

Time, hr.	mg. ± S.E.M. i.v. Route	Oral Route
0	0.00 ± 0.00	0.00 ± 0.00
1	8.19 ± 1.66	1.92 ± 0.66
2	14.88 ± 1.41	...
3	...	12.20 ± 1.64
5	27.13 ± 1.06	27.31 ± 1.01
9	31.18 ± 0.50	31.54 ± 0.78
11	32.26 ± 0.56	...
12	...	34.07 ± 0.49
24	33.18 ± 0.84	34.76 ± 0.43

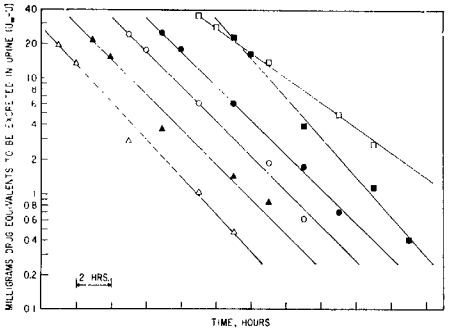


Fig. 4—Urinary excretion of equivalents of 5-methylpyrazole-3-carboxylic acid-^{14}C by humans after a 37.5-mg. intravenous dose (log scale). Each curve is displaced 2 hr. and the first point on each curve is at 1.0 hr. Subject identification is the same as that in Fig. 3.

TABLE IV—PERCENTAGE AS METABOLITE IN THE HUMAN URINE COLLECTIONS[a]

Urine Collection Interval, hr.	% as Metabolite[b] ± S.E.M. i.v. Route	Oral Route
0–1	16.7 ± 1.7	15.0 ± 1.7
1–2	30.0 ± 3.0	...
1–3	...	30.3 ± 3.0
2–5	32.6 ± 2.6	...
3–5	...	36.2 ± 4.9
5–9	35.3 ± 2.8	40.9 ± 1.9
9–11	38.7 ± 5.1	...
9–12	...	39.1 ± 4.7
11–24	33.5 ± 2.8	...
12–24	...	35.9 ± 3.7
0–24	38.6 ± 2.8	33.7 ± 3.2

[a] Average of six subjects. [b] The remainder was excreted as intact drug.

life (±S.E.M.) of 1.66 ± 0.05 hr. in the six subjects.

The urinary excretion of drug equivalents following the intravenous dose is presented in Table III. The urine data for the individual subjects are plotted in Fig. 4. The average half-life (±S.E.M.) for the excretion of drug equivalents in urine after the intravenous dose was 1.91 ± 0.15 hr.

An average (±S.E.M.) of 71.4 ± 2.8% of the urinary radioactivity was found to be unchanged drug (Table IV); the remainder (28.6 ± 2.8) was present as a metabolite, which corresponded by

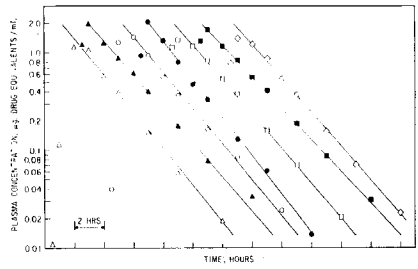


Fig. 5—Plasma levels of equivalents of 5-methylpyrazole-3-carboxylic acid-^{14}C in humans after a 37.5-mg. oral dose (log scale). The displacement of the curves from the origin and the subject identification are the same as in Fig. 3.

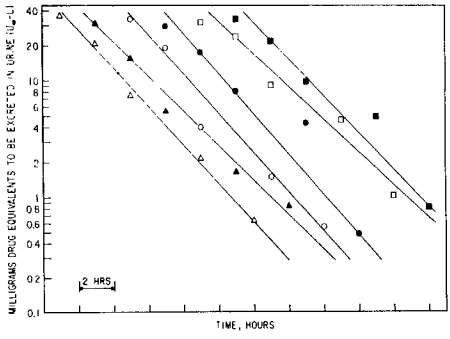


Fig. 6—Urinary excretion of equivalents of 5-methylpyrazole-3-carboxylic acid-^{14}C by humans after a 37.5-mg. oral dose. The displacement of the curves from the origin is the same as Fig. 4. Subject identification is the same as that in Fig. 3.

paper chromatography to the same metabolite excreted by the rat.

An average cumulative total (±S.E.M.) of 0.003 ± 0.002, 0.009 ± 0.002, and 0.009 ± 0.002 equivalents of the intravenous 37.5-mg. dose was found in the feces at 24, 48, and 72 hr., respectively.

Oral Dose—The average concentrations of drug equivalents and their standard errors in plasma at several time intervals after the 37.5-mg. oral dose are summarized in Table II and the data for the individual subjects are plotted in Fig. 5. The average plasma half-life (±S.E.M.) for the six subjects was 1.71 ± 0.05 hr. The average plasma levels beyond about 2 hr. were actually higher after the oral dose than after the intravenous dose.

The urinary excretion of drug equivalents following the oral dose is presented in Table III, and the data for the individual subjects are plotted in Fig. 6. The average (±S.E.M.) half-life for the excretion of drug equivalents into the urine after the oral dose was 2.22 ± 0.20 hr.

Following the oral dose, an average (±S.E.M.) of 66.3 ± 3.2% of the radioactivity recovered in the urine was unchanged drug, and the remainder was excreted as the metabolite (Table IV).

An average (±S.E.M.) cumulative total of 0.016 ± 0.014, 0.052 ± 0.038, and 0.058 ± 0.038 equivalents of the oral 37.5-mg. dose was found in the feces at 24, 48, and 72 hr., respectively.

TABLE V—CALCULATED CONSTANTS FROM HUMAN PLASMA DATA: AREAS UNDER PLASMA CURVES, PLASMA RATE CONSTANTS, AND APPARENT VOLUMES OF DISTRIBUTION

Subject[c]	i.v. Route: Plasma Area, mcg. hr./ml.	i.v. Route: Plasma K,[a] hr.$^{-1}$	i.v. Route: Vd,[b] L.	Oral Route: Plasma Area, mcg. hr./ml.	Oral Route: Plasma K,[a] hr.$^{-1}$	Oral Route: Vd,[b] L.
1 (29, 65)[d]	5.43	0.435	13.1	3.86	0.431	21.9
2 (30, 73)	5.92	0.424	12.6	6.02	0.379	14.7
3 (50, 72)	5.22	0.432	14.9	5.02	0.402	17.5
4 (27, 65)	5.62	0.434	13.1	5.55	0.448	14.1
5 (43, 85)	7.03	0.356	14.7	5.20	0.406	16.3
6 (26, 68)	6.17	0.389	12.6	5.92	0.366	16.5
Mean	5.90	0.412	13.5	5.26	0.405	16.8
S.E.M.	±0.31	±0.013	±0.4	±0.32	±0.013	±1.1

[a] $t_{1/2}$ (hr.) = 0.693/K. [b] $Vd = \frac{U_\infty}{(\text{plasma area})\ (\text{plasma } K)}$ (see Table VI for U_∞ values). [c] Subjects *1*, *2*, and *3* received the intravenous dose on week 1 and the oral dose on week 4. Subjects *4*, *5*, and *6* received the oral dose on week 1 and the intravenous dose on week 4. [d] Age and weight (Kg.), respectively.

TABLE VI—CALCULATED CONSTANTS[a] FROM HUMAN URINARY EXCRETION DATA: INFINITE URINARY EXCRETION (U_∞), URINARY RATE CONSTANTS, AND LAG TIMES (t_0)

Subject	i.v. Route: U_∞, mg.	i.v. Route: Urine K,[b] hr.$^{-1}$	i.v. Route: t_0, hr.	Oral Route: U_∞, mg.	Oral Route: Urine K,[b] hr.$^{-1}$	Oral Route: t_0, hr.
1	31.05	0.433	0.003	36.52	0.338	1.034
2	31.46	0.410	0.085	33.63	0.381	0.786
3	33.57	0.330	0.042	35.27	0.389	1.046
4	31.96	0.347	0.328	35.05	0.264	0.546
5	36.87	0.243	0.944	34.36	0.239	0.866
6	34.32	0.406	0.074	35.81	0.261	0.823
Mean	33.21	0.362	0.243	35.10	0.312	0.852
S.E.M.	±1.51	±0.029	±0.047	±0.49	±0.027	±0.075

[a] Using these fitting constants, the urinary excretion data for each subject may be fitted to the equation: $U = U_\infty[1 - e^{-K(t-t_0)}]$, where U = the milligrams of drug equivalents excreted at time, t, in hours. [b] $t_{1/2}$ (hr.) = 0.693/K.

DISCUSSION

Absorption—The drug is very efficiently absorbed in the rat, dog, and human as shown by the essentially quantitative recovery of the dose in the urine after oral administration. In the dog the drug was absorbed so rapidly that the blood levels after oral administration were essentially identical to those after intravenous administration (Fig. 2). The superimposition of the oral and intravenous blood level curves does not mean, however, that oral absorption was complete in the 20 sec. that was required for intravenous administration, but rather that the oral absorption rate and excretion rate counterbalance each other, *i.e.*, since the intravenous dose gets in faster, a larger fraction is being excreted sooner.

The drug was absorbed very rapidly in the human. The absorption rate could not be estimated even though the first blood sample was taken 0.5 hr. post-drug administration. Nearly quantitative absorption of the oral dose in the human is shown by the fact that the total urinary excretion (Table III) and the areas under the plasma curves (Table V) were not significantly different after oral and intravenous administration. Based on the drug equivalents excreted in the urine (Table VI), the average per cent absorbed was 92.7% of the oral dose. This is a minimum estimate since this method of calculation gives only 89.0% absorbed estimated from the intravenous data.[2]

[2] The fact that the urinary excretion is lower after the intravenous dose than after the oral dose may indicate nonquantitative intravenous administration.

The per cent absorbed is 88.1 and 105.7% calculated from oral/intravenous ratios of the areas under the plasma curves and urinary output, respectively. Since the 72-hr. fecal excretion averaged only about 0.2% of the oral dose, the actual absorption may be as high as 99.8% of the dose.

Distribution—The apparent volumes of distribution (V_d) of the drug in the male and female dogs, after both routes of administration, are summarized in Table I. The apparent volumes are based on the plasma concentrations. Expressed as per cent of body weight, the V_d was very similar in the two dogs and was essentially identical after oral and intravenous administration. The average V_d (40.5% of body weight) is about twice the volume of extracellular water, but is less than total body water.

In the human, the early portion of the plasma curves after the intravenous dose reveals a rapid disappearance phase, having an average half-life (estimated from residuals) of about 7 min. in the six subjects, which undoubtedly reflects distribution and equilibration of the drug in body fluids. The apparent volumes of distribution of the drug in the six subjects, after both routes of administration, are summarized in Table V. The apparent volumes of distribution are independent of route of administration. The average V_d in the six subjects was found to be 15.2 L. (21.5% of their average body weight). This volume corresponds (perhaps fortuitously) to extracellular fluid volume as determined by insulin and sucrose (6).

Metabolism—The dog excreted all of the drug

TABLE VII—CALCULATED CONSTANTS[a] FROM URINARY EXCRETION DATA: INFINITE URINARY EXCRETION (U_∞), RATE CONSTANTS (K), AND LAG TIMES (t_0) FOR THE DRUG AND ITS METABOLITES

		Drug			Metabolite		
		Urine			Urine		
Subject	Route	U_∞, mg.	K, hr.$^{-1}$	t_0, hr.	U_∞,[b] mg.	K, hr.$^{-1}$	t_0, hr.
1	i.v.	23.93	0.451	−0.156	7.12	0.390	0.546
2	i.v.	23.62	0.407	−0.076	7.84	0.417	0.503
3	i.v.	21.51	0.343	−0.188	12.08	0.313	0.455
4	i.v.	20.33	0.347	0.132	11.64	0.346	0.641
5	i.v.	24.63	0.272	0.925	11.56	0.226	1.049
6	i.v.	27.50	0.417	0.007	6.84	0.361	0.348
Mean		23.59	0.373	0.107	9.51	0.342	0.590
S.E.M.		1.04	0.027	0.170	1.02	0.027	0.100
1	Oral	24.57	0.356	1.020	12.15	0.294	1.064
2	Oral	20.67	0.393	0.705	12.90	0.367	0.924
3	Oral	20.74	0.416	1.033	14.79	0.349	1.075
4	Oral	20.70	0.314	0.425	12.72	0.244	0.817
5	Oral	22.88	0.257	0.801	11.52	0.208	1.037
6	Oral	28.84	0.275	0.799	7.02	0.214	0.971
Mean		23.07	0.335	0.797	11.85	0.279	0.981
S.E.M.		1.32	0.026	0.091	1.06	0.028	0.040

[a] Using these fitting constants, the urinary excretion of drug and metabolite for each subject may be fitted to the equation: $U = U_\infty[1 - e^{-K(t-t_0)}]$, where U = mg. of drug equivalents excreted at time t. [b] Expressed as drug equivalents.

unchanged; two paper chromatographic systems revealed only a single zone corresponding to intact drug. The 15 rats excreted an average of 57% of the drug unchanged and the remainder as an acidic metabolite. The percentage of drug metabolized was essentially identical at the 1-mg./Kg. and 9-mg./Kg. dose levels. This metabolite can be converted to the free drug with acid but not with sulfatase or glucuronidase and is therefore believed to be a glycine conjugate. The R_f values of this metabolite correspond to one of the metabolites of 3,5-dimethylpyrazole in two paper chromatographic systems (2).

In the human, an average (±S.E.M.) of 71.4 ± 2.8% of the intravenous dose and 66.3 ± 3.2% of the oral dose was excreted as unchanged drug. The remainder of the dose in the human was excreted as a metabolite, which corresponded by paper chromatography to the one excreted by the rat. The distribution of the drug and its metabolite in each sequential urine sample from the rat and human was also determined, in order to calculate the individual rates of excretion of the drug and its metabolite (*vide infra*).

Effect of Pretreatment with Drug on Its Absorption and Metabolism in the Rat—The data strongly suggest that tachyphylaxis to the drug in rats is not due to altered metabolism. Absorption from the gastrointestinal tract was also not altered with repeated administration. Paper chromatography indicated that the metabolite was identical in both the pretreated and nonpretreated groups and the amounts of metabolite excreted were essentially the same in the two groups. Since no alteration in metabolism occurs, tachyphylaxis cannot be explained on the basis of drug metabolism.

Excretion—The average half-life for the excretion of radioactivity by the rats was 1.0 hr. Excretion by the dog was slightly slower; the half-lives obtained from blood level and urine data were essentially identical, averaging about 1.8 hr. in the two dogs.

The over-all (plasma and urine) half-life for the excretion of drug equivalents in the six subjects was essentially identical to that found in the dog, averaging 1.9 ± 0.1 hr. The plasma levels and rates of elimination of drug equivalents from the plasma were remarkably uniform in the humans. The half-lives for the appearance of radioactivity in the urine of the humans were obtained by fitting the data by the method of least squares to the first-order equation (Eq. 1):

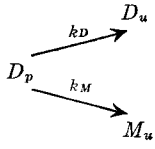
$$U = U_\infty[1 - e^{-K(t - t_0)}] \qquad \text{(Eq. 1)}$$

where U is the milligrams of drug equivalents excreted to time t, U_∞ is the amount excreted at infinite time, K is the observed rate constant for excretion ($t_{1/2} = 0.693/K$), and t_0 is a "lag time" which corrects for the delays caused by absorption, metabolism, and distribution.

Kinetics of Metabolism and Excretion in the Human—Since the over-all rate constant for the loss of radioactivity in the human was known to be a composite of the rates of excretion of the unchanged drug and the formation of its metabolite, it seemed desirable to assay for each of these compounds in all of the sequential urine collections. Hopefully, the individual rate constants could then be estimated. The percentages of radioactivity as metabolite in each of the sequential human urine samples, determined by quantitative paper chromatography, are presented in Table IV. After the first hour, the average ratio of drug to metabolite remained constant within the error of the assay. From the distribution of radioactivity in each sample, the cumulative excretion and the 24-hr. distribution[3] of the drug and its metabolite were calculated. The cumulative excretion data and Eq. 1 were used to estimate the over-all rate constant for the appearance in the urine of the drug and its metabolite. The constants of Eq. 1 which were obtained by this procedure are summarized in Table VII.

[3] The 24-hr. distribution obtained by this procedure was in excellent agreement with that found by paper chromatography of a pooled sample consisting of one-tenth of every sequential urine sample.

Model I—After both oral and intravenous administration, the average rate constant for the appearance of drug in the urine is not significantly different from the corresponding average constant for the appearance of metabolite (Fig. 7), *i.e.*, the ratio of drug to metabolite remains relatively constant with time. This suggests the following simple model:



where D_p is the amount of drug in the body and D_u and M_u are the amounts of drug and metabolite excreted in the urine. This model describes the data only in the mathematical sense; it ignores at least one of the rate constants involved in the transfer of drug in the plasma to metabolite in the urine. The over-all rate constant (K) for the appearance of drug or metabolite in the urine is equal to the sum of k_D and k_M. This model requires that the experimentally determined ratio of D_u to M_u be constant with time and equal to the ratio of k_D to k_M. Also, according to this model, the over-all rate constant (K) for the appearance of drug in the urine must be equal to the rate constant for the appearance of metabolite in the urine as well as to the rate constant for the disappearance of drug from the plasma. The model is described by the following equations (7):

$$D_p = D_0 e^{-Kt} \quad \text{(Eq. 2)}$$

$$D_u = (k_D D_0/K)(1 - e^{-Kt}) \quad \text{(Eq. 3)}$$

$$M_u = (k_M D_0/K)(1 - e^{-Kt}) \quad \text{(Eq. 4)}$$

$$K = k_D + k_M \quad \text{(Eq. 5)}$$

where D_0 is a constant equal to the amount of drug absorbed, and t is the time. The elimination of drug from the plasma data obeys Eq. 2 and the excretion of drug into the urine after the intravenous dose obeys Eq. 3, but the appearance of both drug and metabolite after the oral dose are described best by Eqs. 6 and 7, which include the correction term, t_0:

$$D_u = (k_D D_0/K)[1 - e^{-K(t - t_0)}] \quad \text{(Eq. 6)}$$

$$M_u = (k_M D_0/K)[1 - e^{-K(t - t_0)}] \quad \text{(Eq. 7)}$$

Fitting the average human urine data to Eqs.

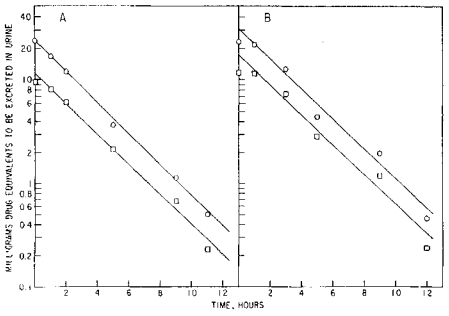


Fig. 7—Urinary excretion of 5-methylpyrazole-3-carboxylic acid-^{14}C and its metabolite (log scale) as determined by paper chromatography. Average of 6 subjects. Key: A, *37.5-mg. i.v. dose;* B, *37.5-mg. oral dose;* O, *drug;* □, *metabolite.*

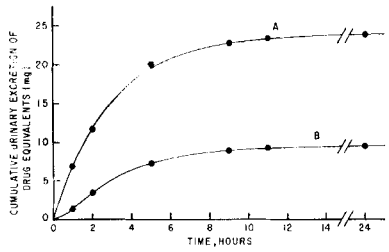


Fig. 8—Average cumulative excretion of 5-methylpyrazole-3-carboxylic acid-^{14}C and its metabolite in the 6 humans after the intravenous dose. Curves are calculated from Eqs. 3 and 13; K = *0.34,* k_D = *0.24,* k_m = *0.10,* k'_m = *1.5; points are experimental values. Key:* A, *drug;* B, *metabolite.*

TABLE VIII—COMPARISON OF OBSERVED AND CALCULATED[a] CUMULATIVE URINARY EXCRETION OF 5-METHYLPYRAZOLE-3-CARBOXYLIC ACID AND ITS METABOLITE FOLLOWING 37.5-mg. ORAL AND INTRAVENOUS DOSES

		Av. Excreted by the 6 Subjects, mg.					
		Drug			Metabolite		
Time, hr.	Route	Obs.	Calcd.	Deviation	Obs.	Calcd.	Deviation
1	i.v.	6.88	6.41	0.47	1.31	1.30	0.01
2	i.v.	11.57	11.33	0.24	3.31	3.65	0.34
5	i.v.	19.83	19.11	0.72	7.29	7.38	0.09
9	i.v.	22.42	22.38	0.04	8.76	8.94	0.18
11	i.v.	23.05	22.71	0.34	9.21	9.21	0.00
24	i.v.	23.55	23.51	0.04	9.45	9.49	0.04
				Mean 0.31			Mean 0.11
1	Oral	1.63	1.26	0.37	0.29	0.06	0.23
3	Oral	10.55	11.20	0.65	4.29	5.41	1.12
5	Oral	18.53	16.60	1.93	8.78	8.32	0.46
9	Oral	20.98	21.12	0.14	10.42	10.76	0.34
12	Oral	22.48	22.26	0.22	11.37	11.37	0.00
24	Oral	22.94	23.00	0.06	11.61	11.77	0.10
				Mean 0.56			Mean 0.38

[a] From Eqs. 8–11.

TABLE IX—ESTIMATES OF PARAMETERS OF MODEL *II* FOR METABOLISM AND EXCRETION OF 5-METHYLPYRAZOLE-3-CARBOXYLIC ACID AFTER INTRAVENOUS ADMINISTRATION

Parameter	Subject 1	2	3	4	5	6	Av.
k_m (hr.$^{-1}$)	0.111	0.108	0.139	0.130	0.051	0.081	0.103
k'_m (hr.$^{-1}$)	1.14	1.76	1.41	1.31	0.618	2.21	1.32
k_D (hr.$^{-1}$)	0.377	0.325	0.251	0.222	0.084	0.332	0.256
V_d (L.)	13.4	13.5	16.6	15.5	38.9	14.3	15.2

6 and 7 (the oral and intravenous doses were treated separately), gave the following constants for excretion of the drug and metabolite:

	Intravenous Dose	Oral Dose
D_0 (mg.)	32.95	34.76
K (hr.$^{-1}$)	0.339	0.305
k_D (hr.$^{-1}$)	0.242	0.202
k_M (hr.$^{-1}$)	0.097	0.103

The cumulative excretion of drug and metabolite after the 37.5-mg. intravenous and oral doses is described, therefore, by Eqs. 8–11.

i.v. dose

$$D_u = 23.52\,[1 - e^{-0.34(t\,-\,0.06)}] \quad \text{for } t \geq 0.06 \text{ hr.} \quad \text{(Eq. 8)}$$

$$M_u = 9.49\,[1 - e^{-0.34(t-0.56)}] \quad \text{for } t \geq 0.56 \text{ hr.} \quad \text{(Eq. 9)}$$

oral dose

$$D_u = 23.02\,[1 - e^{-0.31(t-0.83)}] \quad \text{for } t \geq 0.83 \text{ hr.} \quad \text{(Eq. 10)}$$

$$M_u = 11.78\,[1 - e^{-0.31(t-0.98)}] \quad \text{for } t \geq 0.98 \text{ hr.} \quad \text{(Eq. 11)}$$

The experimental data are compared with the values calculated from Eqs. 8–11 in Table VIII. The deviation of the average cumulative urinary excretion of the six humans from that calculated from the above equations averaged less than 1.3% of the dose.

Model II—Although model *I* yields equations which give a satisfactory mathematical description of the data, it has an obvious defect. The appearance of the metabolite in the urine must be effected by *at least* two rate processes, first the formation of the metabolite with a rate constant k_m, and second, its excretion with a rate constant k'_m, *viz.*:

$$\begin{array}{l} \overset{k_D}{\longrightarrow} D_u \\ D_p \xrightarrow{k_m} M_p \xrightarrow{k'_m} M_u \end{array}$$

Since first-order kinetics are nearly obeyed (Figs. 3–6), one of these processes must be much faster than the other.

Since rapid metabolism followed by slow excretion of the metabolite would not be consistent with the observed ratio of drug to metabolite, the rate-determining step is undoubtedly the formation of the metabolite (k_m). Therefore, the rate constant k_M in model *I* must be essentially equal to k_m. Since the rate constant for excretion of metabolite is larger than the rate constant for formation, the plasma levels of metabolite are undoubtedly quite low compared to plasma levels of drug, *i.e.*, the plasma half-life obtained by measurement of total radioactivity (Table III) reflects primarily intact drug.

The magnitude of k'_m, the rate constant for the excretion of the metabolite, was determined by both analog and digital computer techniques. Using the above constants for D_0, K, and k_D, and setting k_m equal to k_M, the analog computer[4] gave an excellent fit for both the intravenous and oral data when k'_m was set at approximately 1.5 hr.$^{-1}$, *i.e.*, when the half-life for the excretion of the metabolite is about 28 min. An analog computer plot of the intravenous data is presented in Fig. 8.

The digital computer was also used as a tool for the fitting of the data to model *II*. A digital computer program,[5] which simultaneously fits both the urine and plasma data, was used to estimate the rate constants. Equations 3, 12, and 13, which describe model *II* for the intravenous dose, were incorporated into the computer program.

$$C_D + C_M = D_0[(K - k'_m)V_d]^{-1} [(k_D - k'_m)e^{-Kt} + k_m e^{-k'_m t}] \quad \text{(Eq. 12)}$$

$$M_u = k_m D_0 K^{-1}[1 + k'_m(K - k'_m)^{-1} e^{-Kt} - K(K - k'_m)^{-1} e^{-k'_m t}] \quad \text{(Eq. 13)}$$

where C_D and C_M represent the concentrations of drug and metabolite in the plasma, and the other terms have already been defined. These equations satisfactorily describe not only the average data, but also the data of the individual subjects. The parameters obtained are presented in Table IX. The average values do not differ significantly from those obtained by the use of the analog computer. For the best fit of the oral data, at least one more rate constant (representing gastrointestinal absorption) would have to be introduced into model *II*.

REFERENCES

(1) Smith, D. L., Forist, A. A., and Dulin, W. E., *J. Med. Chem.*, **8**, 350(1965).
(2) Gerritsen, G. C., and Dulin, W. E., *J. Pharmacol. Exptl. Therap.*, **150**, 491(1965).
(3) Smith, D. L., Forist, A. A., and Gerritsen, G. C., *ibid.*, **150**, 491(1965).
(4) Knorr, L., and MacDonald, J., *Ann.*, **279**, 217(1894).
(5) Herberg, R. J., *Anal. Chem.*, **32**, 42(1960).
(6) Deane, N., Schreiner, G. E., and Robertson, J. S., *J. Clin. Invest.*, **30**, 1463(1951).
(7) Frost, A. A., and Pearson, R. G., "Kinetics and Mechanism," John Wiley & Sons, Inc., New York, N. Y., 1953, chap. 8.

[4] The assistance of Dr. R. H. Baker, Jr., in the fitting of the data by the analog computer is gratefully acknowledged.
[5] This program was written by Dr. Carl L. Metzler, The Upjohn Co.

Determination of Terpin Hydrate by Gas Chromatography

By LEON KURLANSIK, CAROLYN DAMON, and EDWARD F. SALIM

Two gas chromatographic procedures have been developed for the determination of terpin hydrate using dodecyl alcohol as an internal standard. Methods involving direct chromatographic comparison of the two materials as well as formation and measurement of the corresponding trimethylsilyl (TMS) ethers have been tested. Agreement between the methods is ±1 per cent.

TERPIN HYDRATE, *cis-p*-menthane-1,8-diol hydrate, has been the subject of several analytical investigations including acetylation (1–3) and aquametry (4). The latter procedure is an example of the application of Karl Fischer reagent to quantitative determination of organic functional groups.

Gas-liquid chromatography has been applied to the separation of terpin hydrate from oils and perfumes (5, 6). Volatility of the compound at relatively low temperatures is conducive to gas chromatographic analysis. Two quantitative methods for the determination of terpin hydrate are presented which incorporate the highly desired essentials of simplicity, specificity, and accuracy. Method *A* is a direct chromatographic comparison of terpin hydrate to dodecyl alcohol as an internal standard and method *B* is the formation and chromatographic measurement of terpin hydrate and dodecyl alcohol as their trimethylsilyl ether derivatives.

EXPERIMENTAL

Reagents—Hexamethyldisilazane (HMDS) (Matheson Coleman & Bell), trimethylchlorosilane (TMCS) (Eastman Kodak), and dodecyl alcohol (Analabs, Inc.) were used without purification.

Terpin Hydrate Reference Standard—Terpin hydrate N.F. was recrystallized twice from water–alcohol (5:1). The material was analyzed by differential scanning calorimetry (99.86%) and nuclear magnetic resonance (99.92%). The analytical evaluations are calculated on an anhydrous basis using the water content determined by Karl Fischer titrimetry.

Apparatus—An F & M model 810 dual column gas chromatograph with a dual flame ionization detector and a Minneapolis Honeywell recorder was used.

Received March 29, 1967, from the Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication June 6, 1967.

The authors thank Candace Plato and Dr. Arthur Jacobson for analytical evaluations of the terpin hydrate reference standard.

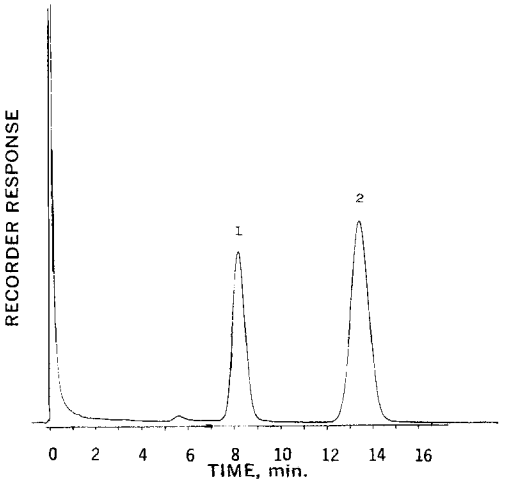


Fig. 1—Chromatogram of an alcohol solution of terpin hydrate and dodecyl alcohol on a Carbowax 20M column. Key: 1, dodecyl alcohol; 2, terpin hydrate.

Method *A*

Columns—Stainless steel columns (6 ft. × 1/8 in.) packed with 10% Carbowax 20 M on Diatoport S, 60–80 mesh (prepared by F & M Scientific Co.).

Chromatography Conditions—Column temperature, 170°; injection port temperature, 170°; detector temperature, 170°; amplifier gain 5 × 10^8 ohms at an attenuation of 2; helium flow, 40 ml./min.; hydrogen 44 ml./min.; air, 360 ml./min.

Internal Standard Solution—Dissolve about 1 Gm. of dodecyl alcohol, accurately weighed, in 100.0 ml. of absolute alcohol.

Procedure—Transfer about 200 mg. of terpin hydrate, accurately weighed, into a 50-ml. volumetric flask and dissolve in about 30 ml. of absolute alcohol. Add 10.0 ml. of internal standard solution, dilute to volume with absolute alcohol, and mix. A 0.5-μl. portion of this solution was injected into the chromatograph. A typical chromatogram is shown in Fig. 1.

Calculation—The per cent of anhydrous terpin hydrate is calculated by the formula:

$$H \times \frac{C \times V}{W} \times F \times 100$$

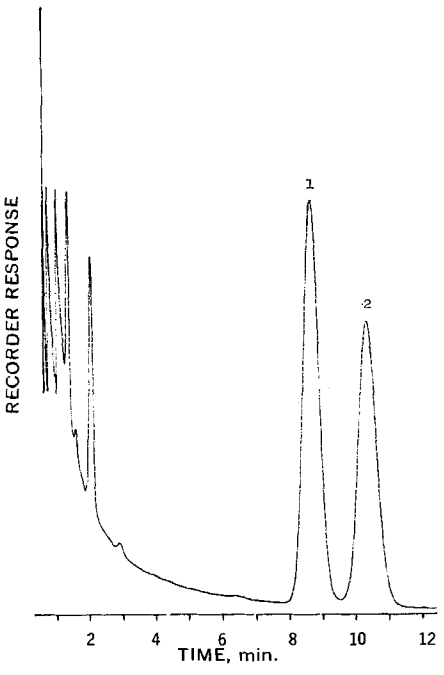


Fig. 2—Chromatogram of ether derivatives of terpin hydrate and dodecyl alcohol on a SE-30 column. Key: 1, terpin hydrate (TMS derivative); 2, dodecyl alcohol (TMS derivative).

in which H = peak ht. of terpin hydrate / peak ht. of internal std.

C = concentration, in mg./ml., of internal standard preparation,

V = volume, in ml., of internal standard added in procedure,

W = weight, in mg., of terpin hydrate taken calculated on an anhydrous basis using a water content determined by Karl Fischer titrimetry,

F = relative response of equal weights of dodecyl alcohol (DDA) and terpin hydrate (TH) standard as determined by peak height measurement (DDA/TH).

Method *B*

Columns—Stainless steel columns (6 ft. × 1/8 in.) packed with 10% SE-30 on Diatoport S, 60–80 mesh (prepared by F & M Scientific Co.)

Chromatography Conditions—Column temperature, 140°; injection port temperature, 145°; detector temperature, 150°; amplifier gain 5 × 10^8 ohms at an attenuation of 1; helium flow, 65 ml./min.; hydrogen, 58 ml./min.; air, 370 ml./min.

Internal Standard Solution—Dissolve about 2 Gm. of dodecyl alcohol, accurately weighed, in 100.0 ml. of pyridine.

Procedure—Transfer about 200 mg. of terpin hydrate, accurately weighed, into a 50-ml. volumetric flask and dissolve in about 25 ml. of pyridine. Add 10.0 ml. of internal standard solution, dilute to volume with pyridine, and mix. Transfer 1.0 ml. to a screw-cap vial, add 0.3 ml. of hexamethyldisilazane, 0.2 ml. of trimethylchlorosilane, stopper the vial, and shake vigorously for 1 min. Allow to stand for 1 hr. and inject 0.4 μl. of supernatant liquid into the chromatograph. A typical chromatogram is shown in Fig. 2.

Calculation—The formula given in method *A* may be used for calculation of terpin hydrate recoveries. However, for this procedure H is the ratio of the peak heights for the corresponding trimethylsilyl esters of terpin hydrate and dodecyl alcohol, and F is the relative response determined for the TMS ethers.

Table I—Recovery of Terpin Hydrate for Commercial Samples by Method *A*

Sample	Terpin Hydrate, mg. Added	Terpin Hydrate, mg. Found	Recovery, %[a] (Anhydrous)
A	201.5	180.2	98.8
	203.5	181.9	98.7
	...	...	98.9[b]
B	198.6	177.8	98.8
	200.4	178.2	98.2
	210.4	187.7	98.5
C	202.4	181.1	99.3
	210.5	187.5	99.1
	207.4	186.2	99.6
D	201.8	182.0	99.8
	204.0	183.8	99.6

[a] Based on water content determined by Karl Fischer titrimetry: sample A, 9.54%; sample B, 9.45%; sample C, 9.87%; sample D, 9.63%. [b] Average of 12 determinations by three analysts with S.D. ± 0.27.

Table II—Recovery of Terpin Hydrate for Commercial Samples by Method *B*

Sample	Terpin Hydrate, mg. Added	Terpin Hydrate, mg. Found	Recovery, %[a] (Anhydrous)
A	201.5	181.3	99.5
	213.6	192.6	99.6
B	203.3	181.6	98.8
	206.2	184.2	99.1
C	208.3	187.5	99.9
	206.5	185.2	99.2
	210.6	188.3	99.2
D	202.6	182.0	99.2
	202.7	182.5	99.4
	...	...	99.2[b]

[a] Water contents used for calculation included in Table I. [b] Average of 5 determinations of S.D. ± 0.20.

RESULTS AND DISCUSSION

Series of tests were conducted to determine the stability of terpin hydrate and its TMS derivative at various component temperatures. Results of the investigations showed that terpin hydrate could be chromatographed directly at a temperature not exceeding 175° and terpin hydrate bis-trimethylsilyl ether could be exposed to a temperature of about 300° before thermal decomposition was evidenced. Additional studies produced the instrument parameters established for methods *A* and *B*. Results obtained for commercial samples of terpin hydrate by each method are summarized in Tables I and II, respectively.

The method is specific for the determination of terpin hydrate in the presence of its dehydration products. A sample of terpin hydrate was boiled in dilute sulfuric acid, the mixture steam distilled, the water immiscible portion distilled, and an alcoholic solution of the distillate chromatographed

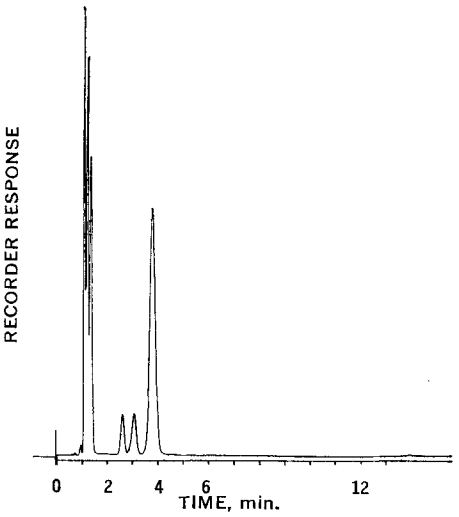


Fig. 3—Chromatogram of dehydroxylated terpin hydrate in alcohol solution on a Carbowax 20M column.

on a Carbowax 20 M column. The resultant chromatogram, shown in Fig. 3, indicates the low retention times for the reaction products which include terpinene, terpineol, and eucalyptol. The distillate also was subjected to etherification using the conditions stated under *Method B* and the chromatogram produced showed the absence of any significant peaks after a retention time of 4 min.

Dodecyl alcohol was selected as an internal standard based on a similarity in molecular weight to terpin hydrate, reactivity to form a trimethylsilyl ether, and ready availability of the material in a high state of purity. General use of the internal standard method has the advantage of minimizing sampling variation as a source of error in gas chromatographic procedures.

Terpin hydrate and dodecyl alcohol exhibited a linear response between the ratios of 0.5 to 3.0 (DDA/TH standard) in the range of 70 mg. to 200 mg. of either compound. An analogous study with the TMS ethers indicated a linear response between essentially the same ratios as the parent compounds. Average values for the relative response factor F were determined to be 1.75 for method A and 0.64 for method B.

REFERENCES

(1) Fernandez, O., and Luengo, N., *Anal. fis. quim.*, **18**, 158(1920); through *Chem. Abstr.*, **15**, 1869(1921).
(2) Mesnard, P., and Bertucat, M., *Bull. Soc. Chim. France*, **1959**, 307; through *Chem. Abstr.*, **53**, 16919(1959).
(3) Mesnard, P., and Bertucat, M., *Boll. Chim. Farm.*, **101**, 519(1962).
(4) Mitchell, J., Jr., and Smith, D. M., "Aquametry," Interscience Publishers, Inc., New York, N. Y., 1948, pp. 267–282.
(5) Domange, L., and Longuevalle, S., *Compt. Rend.*, **247**, 209(1958).
(6) Porcaro, P. J., and Johnston, V. D., *Anal. Chem.*, **33**, 361(1961).

Determination of Terpin Hydrate in Elixirs by Gas Chromatography

By LEON KURLANSIK, CAROLYN DAMON, HANNAH KLEIN, and EDWARD F. SALIM

A previous paper describes the determination of terpin hydrate by gas chromatographic analysis. The method has been extended to the assay of terpin hydrate in official elixirs after isolation of the drug in relatively pure form. The procedure is considered to be an improvement over existing methods in specificity and reproducibility.

TERPIN HYDRATE is used extensively as an ingredient in the preparation of cough suppressing elixirs. The most common formulations are terpin hydrate elixir, terpin hydrate and codeine elixir, and terpin hydrate and dextromethorphan hydrobromide elixir which are official in N.F. XII (1).

Literature reports on determinations of terpin hydrate in elixirs are confined to gravimetric (2–4) and colorimetric (5–7) procedures. Spectrophotometric assays in the visible region are due to the formation of molybdenum blue, a reduction product of the phosphomolybdic acid reagent.

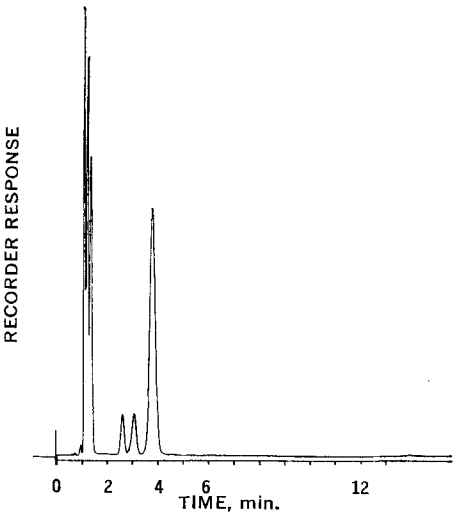
Received April 13, 1967, from the Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037
Accepted for publication May 25, 1967.

A previous paper by the authors deals with gas chromatographic analysis of terpin hydrate by two techniques (8). A method is presented for the isolation of terpin hydrate from the elixirs in relatively pure form and application of the gas chromatographic procedures developed for the drug substance.

EXPERIMENTAL

The gas chromatograph, columns, chromatography conditions, reagents, and terpin hydrate reference standard employed are described in a previous paper (8).

Isolation of Terpin Hydrate—Transfer 10.0 ml. of the elixir into a separator, add 25 ml. of saturated sodium acetate solution, shake for 1 min.,



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Dodecyl alcohol was selected as an internal standard based on a similarity in molecular weight to terpin hydrate, reactivity to form a trimethylsilyl ether, and ready availability of the material in a high state of purity. General use of the internal standard method has the advantage of minimizing sampling variation as a source of error in gas chromatographic procedures.

Terpin hydrate and dodecyl alcohol exhibited a linear response between the ratios of 0.5 to 3.0 (DDA/TH standard) in the range of 70 mg. to 200 mg. of either compound. An analogous study with the TMS ethers indicated a linear response between essentially the same ratios as the parent compounds. Average values for the relative response factor F were determined to be 1.75 for method A and 0.64 for method B.

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(1) Fernandez, O., and Luengo, N., *Anal. fis. quim.*, **18**, 158(1920); through *Chem. Abstr.*, **15**, 1869(1921).
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(4) Mitchell, J., Jr., and Smith, D. M., "Aquametry," Interscience Publishers, Inc., New York, N. Y., 1948, pp. 267–282.
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EXPERIMENTAL

The gas chromatograph, columns, chromatography conditions, reagents, and terpin hydrate reference standard employed are described in a previous paper (8).

Isolation of Terpin Hydrate—Transfer 10.0 ml. of the elixir into a separator, add 25 ml. of saturated sodium acetate solution, shake for 1 min.,

and allow to stand for 30 min. with occasional shaking. Extract with four 25-ml. portions of chloroform, filtering each portion through chloroform-saturated cotton into a beaker. Concentrate the solution to about 10–15 ml. by warming on a steam bath with the aid of a current of air, and evaporate the remaining solvent without heat using a gentle current of air.

Method *A*

Internal Standard Solution—Dissolve about 1 Gm. of dodecyl alcohol, accurately weighed, in 100.0 ml. of absolute alcohol.

Procedure—Dissolve the residue from the elixir extract in about 15 ml. of absolute alcohol and transfer quantitatively to a 50-ml. volumetric flask containing 10.0 ml. of internal standard solution. Dilute to volume with absolute alcohol, and mix. A 0.5-μl. portion of this solution was injected into the chromatograph.

Calculation—The quantity of terpin hydrate N.F. in the elixir, in mg. per ml., is calculated by the formula:

$$\frac{H \times (C \times V) \times F \times 1.104}{10}$$

in which

H = $\frac{\text{peak ht. of terpin hydrate}}{\text{peak ht. of internal std.}}$

C = concentration, in mg. per ml., of internal standard solution,

V = volume, in ml., of internal standard solution taken,

F = relative response of equal weights of dodecyl alcohol (DDA) and terpin hydrate (TH) standard as determined by peak height measurement (DDA/TH),

1.104 = factor to convert anhydrous terpin hydrate to terpin hydrate N.F.

The per cent recovery of terpin hydrate is obtained by the expression

$$\text{terpin hydrate found } \frac{(\text{mg./ml.}) \times 100}{17}$$

in which the denominator represents the theoretical amount of terpin hydrate, in mg. per ml. of official elixirs.

Method *B*

Internal Standard Solution—Dissolve about 2 Gm. of dodecyl alcohol, accurately weighed, in 100.0 ml. of pyridine.

Procedure—Dissolve the residue from the elixir extract in about 15 ml. of pyridine and transfer quantitatively to a 50-ml. volumetric flask containing 10.0 ml. of internal standard solution. Dilute to volume with pyridine, and mix. Transfer 1.0 ml. to a screw-cap vial, add 0.3 ml. of hexamethyldisilazane, 0.2 ml. of trimethylchlorosilane, stopper the vial, and shake vigorously for 1 min. Allow to stand for 1 hr. and inject 0.4 μl. of supernatant liquid into the chromatograph.

Calculation—The formulas given in method *A* may be used for calculation of terpin hydrate recoveries. However, for this procedure H is the ratio of peak heights for the corresponding trimethylsilyl ethers of terpin hydrate and dodecyl alcohol, and F is the relative response determined for the TMS ethers.

Table I—Analysis of Terpin Hydrate in Elixirs

Elixir	Recoveries, % Method *A*	Method *B*
Terpin hydrate[a]	99.7 ± 0.22[c]	99.8 ± 0.27[c]
Terpin hydrate[b]	100.2	102.0
	100.9	101.1
Terpin hydrate and codeine[b]	102.4	103.1
	103.3	103.3
Terpin hydrate and dextromethorphan HBr[b]	102.6	100.9
	103.4	101.4

[a] Prepared in this laboratory according to N.F. XII. [b] Commercial products. [c] Based on 10 determinations.

RESULTS AND DISCUSSION

Isolation of terpin hydrate in the elixirs is achieved by salting the material out of solution and subsequent extraction with chloroform. This has been found to be necessary due to the similarity of terpin hydrate solubility in water and chloroform. Several saturating agents were investigated and sodium acetate was selected on the basis of reproducibility of initial assays. Recoveries obtained for terpin hydrate, terpin hydrate and codeine, and terpin hydrate and dextromethorphan hydrobromide elixirs through the addition of saturated sodium acetate solution are summarized in Table I. Satisfactory results may be obtained by the addition of a saturated magnesium sulfate solution. However, on occasion it has been observed that gel formation occurs during the prescribed waiting period. This effect may be eliminated by adding 1–2 ml. of water and gentle mixing.

Care must be exercised in the evaporation of solvent following extraction of terpin hydrate with chloroform. Terpin hydrate is fairly volatile and can be vaporized under the influence of excess heat or air. The preferred handling is to concentrate the solution and remove the last portion of chloroform without the application of heat.

Chromatograms produced by injections of sample solutions are basically the same as those obtained from previous gas chromatographic analyses of terpin hydrate. One extraneous peak is noted at a retention time of about 2 min. This peak has been assigned to a component in orange oil by preparation and injection of an alcoholic solution of orange oil U.S.P. and does not interfere with the dodecyl alcohol or terpin hydrate peaks which have a much longer retention time.

REFERENCES

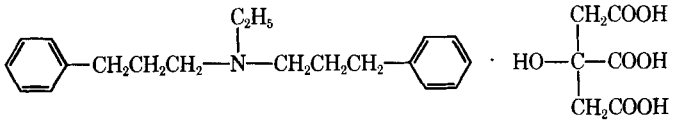
(1) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.
(2) Murray, A. G., *J. Am. Pharm. Assoc.*, 10, 440(1921).
(3) Harrison, C. W., *J. Assoc. Offic. Agr. Chemists*, 11 358(1928).
(4) Carol, J., *ibid.*, 21, 575(1938).
(5) Platt, H., and James, A. E., *J. Am. Pharm. Assoc., Sci. Ed.*, 44, 666(1955).
(6) Milos, C., *J. Assoc. Offic. Agr. Chemists*, 42, 459(1959).
(7) Vadodaria, D. J., Parikh, P. M., and Mukherji, S. P., *Indian J. Pharm.*, 23, 301(1961).
(8) Kurlansik, L., Damon, C., and Salim, E. F., *J. Pharm. Sci.*, 56, 1158(1967).

Qualitative and Quantitative Tests for Alverine Citrate

By EDWARD F. SALIM* and WILLIAM R. EBERT†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

BIS-γ-PHENYLPROPYLETHYLAMINE CITRATE; $C_{26}H_{27}N \cdot C_6H_8O_7$; mol. wt. 473.57. The structural formula of alverine citrate may be represented as:



Physical Properties—Alverine citrate occurs as a white to off-white powder having a sweet odor and a slightly bitter taste, m.p. 100–102° (U.S.P. class I). It is slightly soluble in water and in chloroform, sparingly soluble in alcohol, and very slightly soluble in ether. The pH of a solution of alverine citrate in carbon dioxide-free water (1 in 200) is between 3.5 and 4.5.

Identity Tests—A 1 in 1500 solution of alverine citrate in 0.1 *N* hydrochloric acid exhibits ultraviolet absorbance maxima at about 253 mμ, 258 mμ [absorptivity (*a*) about 0.9], and 267 mμ and absorbance minima at about 233, 255, and 265 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of alverine citrate in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Suspend about 500 mg. of alverine citrate in 10 ml. of water, add a slight excess of sodium hydroxide to liberate alverine base, allow to stand 5 min. with occasional swirling, and filter: the clear filtrate responds to the U.S.P. tests for citrate.

Purity Tests—Dry about 1 Gm. of alverine citrate, accurately weighed, at 80° for 2 hr.: it loses not more than 0.5% of its weight.

Char about 1 Gm. of alverine citrate, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Assay—Transfer about 360 mg. of alverine citrate, accurately weighed, to a separator, add 25 ml. of water, 5 ml. of sodium hydroxide T.S., and extract with four 25-ml. portions of chloroform. Wash the combined extracts with 10 ml. of water and filter the chloroform solution through a layer of chloroform-moistened anhydrous sodium sulfate contained on a pledget of cotton, collecting the filtrate in a titrating beaker. Wash the aqueous phase with 10 ml. of chloroform and filter as before into the beaker. Add methanolic methyl red T.S. and titrate with 0.1 *N* perchloric acid in dioxane. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 47.36 mg. of $C_{26}H_{27}N \cdot C_6H_8O_7$. The amount of alverine citrate found is not less than 99% and not more than 102%.

DOSAGE FORMS OF ALVERINE CITRATE

Alverine Citrate Tablets

Identity Test—Powder a suitable number of tablets and transfer an amount equivalent to about 120 mg. of alverine citrate to a 200-ml. volumetric flask. Add about 100 ml. of 0.1 *N* hydrochloric acid, allow to stand unstoppered until effervescence ceases, stopper and shake for 30 min., dilute to volume with the acid, and mix. Filter a portion of the solution, discarding the first 20 ml. of filtrate. The filtrate exhibits ultraviolet absorbance maxima and minima at the same wavelengths as the bulk drug as shown in Fig. 1.

Assay—Weigh and finely powder not less than 20 alverine citrate tablets. Weigh accurately a portion of the powder, equivalent to about 360 mg. of alverine citrate, and transfer to a separator. Add 5 ml. of water and 5 ml. of sodium hydroxide T.S., swirl occasionally for 5 min., and extract with four 25-ml. portions of chloroform. Wash the combined extracts with 10 ml. of water and filter the chloroform solution through a layer of chloroform-moistened anhydrous sodium sulfate contained on a pledget of cotton, collecting the filtrate in a titrating

Received May 2, 1967, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037

Accepted for publication June 5, 1967.

† Philips Roxane Laboratories, Columbus, OH 43216. Philips Roxane Laboratories has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The authors express their appreciation to Enos E. Vannatta, Philips Roxane Laboratories, and Carolyn Damon and Hannah Klein, the Drug Standards Laboratory, for valuable contributions to the monograph.

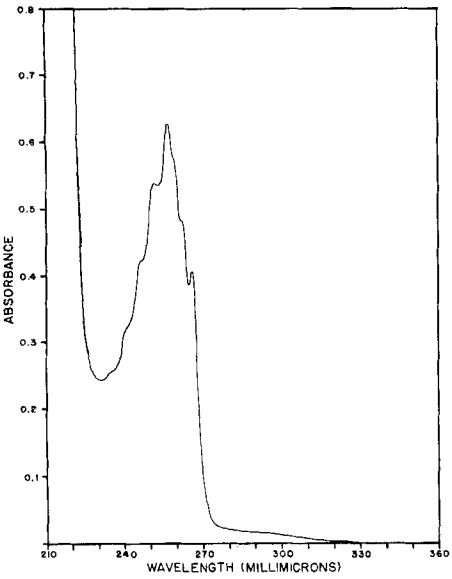


Fig. 1—Ultraviolet absorption spectrum of alverine citrate in 0.1 N hydrochloric acid (700 mcg./ml.); Beckman model DK-2A spectrophotometer.

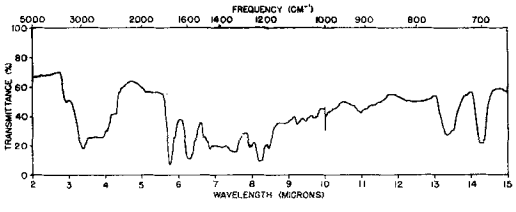


Fig. 2—Infrared spectrum of alverine citrate in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

beaker. Wash the aqueous phase with 10 ml. of chloroform and filter as before into the beaker. Add methanolic methyl red T.S., and titrate with 0.1 *N* perchloric acid in dioxane. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 47.36 mg. of $C_{20}H_{27}N \cdot C_6H_8O_7$. The amount of alverine citrate found is not less than 95% and not more than 105% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Alverine citrate[1] is a non-narcotic, smooth-muscle antispasmodic which is approximately 3 times as potent as papaverine and with a longer duration of action. It is neither an anticholinergic nor a derivative of atropine and adverse reactions commonly associated with anticholinergics are not manifest.

Identity Tests—Alverine citrate may be identified by the preparation of an oxalate salt. The free base is isolated by extraction with ether from basic solution and is reacted with oxalic acid in aqueous solution to form the oxalate. The derivative when dried at 55° in vacuum melts between 139° and 142°.

Quantitative Tests—The nonaqueous titration of the extracted base with perchloric acid gave an average value of 100.4 ± 0.3%.[2] Alternatively, alverine citrate may be dissolved in glacial acetic acid and titrated directly with 0.1 *N* perchloric acid. With crystal violet indicator, the color change from blue to blue-green corresponds to the midpoint of the inflection obtained by simultaneous potentiometric measurement. Montequi *et al.* (1) have assayed alverine citrate by formation of a reineckate and subsequent bromometric determination. The method is reported to be accurate to within 1%. Titrimetric analyses of commercial tablets gave an average value of 97.3 ± 1.0%[2] of the labeled amount of alverine citrate.

REFERENCE

(1) Montequi, R., de Valderrama, E. F., and Collado, M. C., *Anales Real Acad. Farm.*, **30**, 281(1964); through *Chem. Abstr.*, **63**, 434(1965).

[1] Marketed as Spacolin by Philips Roxane Laboratories, Columbus, Ohio.

[2] Maximum deviation from the mean value.

Rheological Estimation of the Spreading Characteristics of Pharmaceutical Semisolids

By JAMES C. BOYLAN

A Ferranti-Shirley cone and plate viscometer was utilized to characterize the spreading properties of white petrolatum U.S.P., white ointment U.S.P., and anhydrous lanolin U.S.P. Data were obtained for structural breakdown during repetitive cycling and for structural breakdown at constant shear. The problem of slippage at the revolving cone/sample interface is discussed. An attempt is made to relate rheological differences and molecular structure.

THE EASE of application of a pharmaceutical semisolid to the skin is an important factor in consumer product acceptance. Realizing that each individual applies ointment-like materials to the skin with a slightly different motion, stroke, and rate, any rheological estimation of this process, however good, involves some degree of error. However, good approximations of the shearing rates involved in spreading can still be made. Kostenbauder *et al.* (1, 2) estimated the shearing rates for a pharmacist compounding an ointment with a spatula and for a consumer spreading ointment on a surface (such as skin) as 200 sec.$^{-1}$ and 120 sec.$^{-1}$, respectively. Interestingly, it was recently shown that for ointments comprised mainly of white petrolatum or white ointment, 120 sec.$^{-1}$ represented the point of greatest resistance to flow at low rates of shear (3). In addition, it was clear from this study that the shearing region of 0 to 250 sec.$^{-1}$ would be important when characterizing the spreading properties of the 13 pharmaceutical semisolids studied.

In recent years significant progress has been made in the area of rheologically defining the spreading and extrusion properties of pharmaceutical ointments and lotions. In particular, Kostenbauder and co-workers (1, 2) and Wood and associates (4–6) have published useful information in this area.

Kostenbauder and Martin (1) after consultation with several dermatologists, grouped pharmaceutical ointments into 3 classes. Ophthalmic ointments, the softest products, constituted class I. The common medicated ointments, such as ammoniated mercury ointment and boric acid ointment, comprised class II. These products are soft, but stiff enough to remain at the point of application. Class III consisted of protective ointments, such as zinc oxide paste, which are hard and stiff enough to remain at the site of moist, ulcerated areas. Rheological evaluation of various bases selected by dermatologists for these classes indicated that the values in Table I were acceptable guideposts.

Wood and co-workers (4–6) used several techniques to measure a variety of properties concerned with the rheology of pharmaceutical semisolids and lotions. They were able to measure the force required to extrude products from a plastic bottle as a function of bottle design, wall thickness, type of orifice, and rheological character of the contents (4). In another study (5), they used a modified Castor-Severs extrusion rheometer to extrude samples from collapsible tubes and to measure the shear rates involved in simulated high speed tube filling. Wood and his associates (6) also evaluated the increase in tackiness that occurs in some lotions and ointments as they dry. An empirical test was devised that directly measured the cohesive-adhesive capability of skin surface during absorption after rub-in.

The Ferranti-Shirley cone and plate viscometer has instrumental advantages that make it worthwhile to evaluate its usefulness in studying the spreading properties of semisolid materials. These advantages are: the direct recording of a continuous, rather than a multipoint-rheogram, the ease of maintaining a constant shearing rate for an indefinite period of time, and a shearing geometry that (*a*) closely duplicates that of ap-

Received April 14, 1967, from Eli Lilly and Co., Indianapoils, IN 46206
Accepted for publication June 7, 1967.
Presented to the Industrial Pharmaceutical Technology Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The author thanks Dr. R. R. Pfeiffer for a constructive review of the manuscript.

TABLE I—AVERAGE CONSISTENCIES OF OINTMENTS AS SELECTED BY PHYSICIANS[a]

	125 sec.$^{-1}$		210 sec.$^{-1}$	
	Class I	Class II	Class I	Class II
dynes/cm.^{2}	800–900	4000–10000	2000–2500	5000–12000
poises	23–31	30–38	14–18	20–26

[a] Adapted from Kostenbauder and Martin (1). Data were not presented for class III ointments.

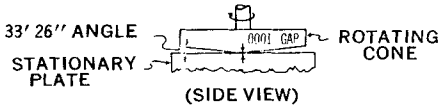


Fig. 1—Schematic representation of the Ferranti-Shirley cone and plate.

plying materials with a circular motion and (*b*) subjects all portions of the sample to uniform shearing stress (see Fig. 1).

It is the purpose of this paper to illustrate the advantages and limitations of the Ferranti-Shirley cone and plate viscometer for the evaluation of pharmaceutical semisolids in the shearing region between 0 and 270 sec.$^{-1}$ and, also, to present rheological procedures for estimating the spreading properties of semisolid materials using this instrument.

EXPERIMENTAL

Materials—The semisolids studied were white petrolatum U.S.P. and anhydrous lanolin U.S.P. These substances were chosen for this study because they are widely used in formulating pharmaceutical and cosmetic products and because they are dissimilar in their spreading characteristics. The white petrolatum was also used to prepare white ointment U.S.P. Storage of all samples was at room temperature.

Rheological Evaluation—The viscometer used in this study was a Ferranti-Shirley cone and plate viscometer[1] equipped with a 400 Gm.-cm. spring, an automatic gap setting device, an *x-y* recorder,[2] and a constant-temperature water bath.[3] The use of this viscometer has been described elsewhere (7). Calibration of the instrument was carried out using N.B.S. standard viscosity oils. The sample to be evaluated (about 1 ml.) was gently put on the plate of the viscometer with a spatula. The plate was immediately raised into position with the cone. The sample then remained undisturbed for 5 min. (to allow temperature equilibration at 30 ± 0.1°) before obtaining the rheogram. All rheograms were obtained using a truncated cone having an angle of 33 min. 26 sec. and a radius of 2 cm.

Procedure A—This procedure was designed to measure the amount of slippage at the cone/sample interface. The instrument was set to achieve a maximum of 100 r.p.m. (1074 sec.$^{-1}$). Using a fresh sample of white petrolatum each time, rheogram upsweep times (0–100 r.p.m.) were 10, 20, 40, 60, 120, 240, 480, and 600 sec. The downsweep time was identical to the upsweep time in each instance.

Procedures B, C, and D—These procedures were designed to measure the amount of structural breakdown at a constant rate of shear (see Table II).

Procedure E—This procedure was designed to measure structural breakdown as a result of repetitive cyclic shearing. The instrument was set to achieve a maximum of 11 r.p.m. (120 sec.$^{-1}$) in 13.3 sec. (instrument sweep time setting of 120 sec.). The downsweep time was identical. Immediately upon completion of the first cycle, the cycle was repeated on the same sample 4 additional times. Then a 6th cycle was immediately run utilizing a maximum r.p.m. of 100 (1074 sec.$^{-1}$).

Procedure F—This procedure was identical to procedure *E* in every respect except one—25 r.p.m. (270 sec.$^{-1}$) was the maximum r.p.m. for the first 5 cycles, instead of 11 r.p.m.

Tackiness—None of the procedures used was intended to measure tackiness. For this, special instrumentation, such as the modified tackmeter described by Wood and Lapham (6), is needed.

RESULTS AND DISCUSSION

Slippage—Evidence of slippage between the rotating member of the viscometer and the sample, resulting in reproducible artifacts, has been observed by several authors working with pharmaceutical substances. To minimize this problem, Kostenbauder and Martin (1) found it necessary to have a grooved bob prepared for the modified Stormer viscometer. Likewise, Wood and coworkers (4) "ribbed" the bobs for their Hercules Hi-Shear viscometer.[4] Wood attributes this artifact to the development of slippage planes at the wall-sample interface (artifact) and compares it with internal sample slippage planes (true yield value). Bruss (8) advocates the use of a star-shaped rotor. A star-shaped rotor is commercially available with the Haake Rotovisco viscometer.[5]

The Ferranti-Shirley viscometer used in this study offers another approach to the solution of this problem. The portions of rheograms shown in Figs. 2 and 3 illustrate the dramatic effect that the speed at which the shearing rate is increased can have on the rheogram. These data were obtained by procedure *A*. A comparison of Figs. 2 and 3 shows also that interpretation of the upcurve suffers more severely than does interpretation of the downcurve. The downcurve patterns show that shearing to 1074 sec.$^{-1}$ has destroyed most of the structure and also the rotating cone has been able to "catch up" and minimize any slippage effect.

It is felt that when using the Ferranti-Shirley cone and plate viscometer with semisolids, proper

[1] Ferranti Electric Co., Plainview, Long Island, N. Y.
[2] Model HR-92, Houston Instrument Co., Houston, Tex.
[3] Haake model F, Brinkmann Instrument Co., Westbury N. Y.
[4] Martinson Machine Co., Kalamazoo, Mich.
[5] Brinkmann Instrument Co., Westbury, N. Y.

TABLE II—RHEOLOGICAL PROCEDURES USED TO MEASURE STRUCTURAL BREAKDOWN AT A CONSTANT RATE OF SHEAR

Procedure	Max. r.p.m.	Shearing Time (0 to max. r.p.m.), sec.	Time Held at Max. r.p.m., sec.	Shearing Time (Max. r.p.m. to 0), sec.
B	11 (120 sec.$^{-1}$)	67[a]	40	67[a]
C	11 (120 sec.$^{-1}$)	1[b]	40	1[b]
D	25 (270 sec.$^{-1}$)	2.5[b]	40	2.5[b]

[a] Sweep setting of 600 sec. [b] Sweep setting of 10 sec.

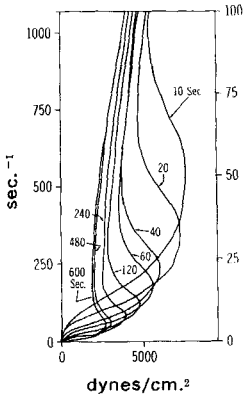


Fig. 2—The effect of sweep time on the ascending portion of the white petrolatum U.S.P. rheogram.

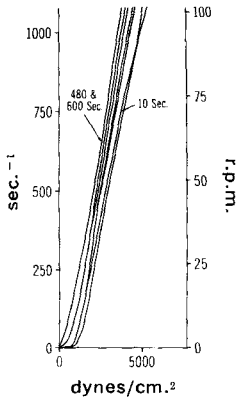


Fig. 3—The effect of sweep time on the descending portion of the white petrolatum U.S.P. rheogram.

sweep time selections can overcome serious slippage artifacts. Accordingly, it was not felt advisable to score the cone used for this study. However, the warning is clear. Each laboratory must recognize that this problem exists and determine how serious a problem it is for their particular instrument and samples, and deal with it accordingly.

Structural Breakdown at Constant Shear—Procedures *B*, *C*, and *D* were used to gather data on structural breakdown at constant shear. Two approaches were tried. Procedure *B* permitted a very slow increase in shearing rate in an attempt to minimize cone/sample slippage during measurement. Procedures *C* and *D* relied on a minimum of time being spent in the interval 0 to 120 sec.$^{-1}$ or 0 to 270 sec.$^{-1}$ to minimize prebreakdown of structure before the 120 sec.$^{-1}$ or 270 sec.$^{-1}$ constant shear rate. It was recognized that procedures *C* and *D* would be subject to cone/sample slippage artifacts, but it was felt that this could be taken into account when interpreting the rheograms. Typical results are shown in Figs. 4 and 5.

Procedure *B* was disappointing as a technique. It was readily apparent from Fig. 4 that when using procedure *B* much structural breakdown had occurred before reaching 120 sec.$^{-1}$.

The information gained from procedures *C* and *D* (Fig. 5) illustrates the importance of maintaining a constant maximum r.p.m. for reproducible and comparative results. Slippage is very evident in this figure. To follow structural breakdown from figures similar to Figs. 4 and 5, while at the same time eliminating slippage artifacts, the reduction in dynes/cm.^{2} was ignored for the constant shear interval 0–10 sec. The data obtained with procedures *C* and *D* are shown in Fig. 6. From Fig. 6 it can be seen that, as shearing continues (10–20 sec., 20–30 sec., 30–40 sec.) breakdown becomes proportionately less (*i.e.*, most structural breakdown occurs early).

When using the 400 Gm.-cm. torque spring, only white petrolatum and white ointment could be measured satisfactorily at 270 sec.$^{-1}$. In Fig. 6 it is interesting to compare the 270 sec.$^{-1}$ and the 120 sec.$^{-1}$ curves for these semisolids. The 270 sec.$^{-1}$ curves do not follow an apparent first-order

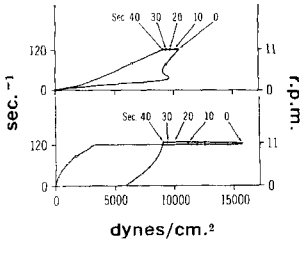


Fig. 4—Typical rheograms of anhydrous lanolin U.S.P. obtained using procedures B (*top*) *and* C (*bottom*).

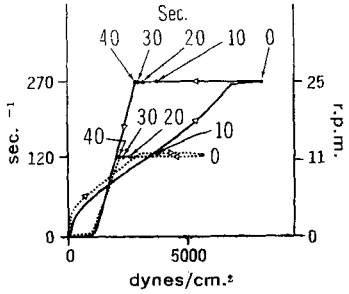


Fig. 5—Typical rheograms of white petrolatum U.S.P. obtained using procedures C (---) *and* D (——).

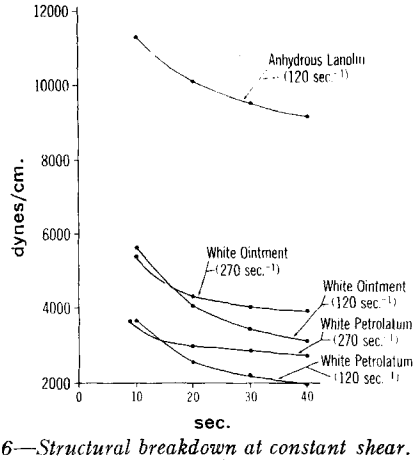


Fig. 6—Structural breakdown at constant shear. Data obtained using procedure C *(120 sec.$^{-1}$ max.) and procedure* D *(270 sec.$^{-1}$ max.)*

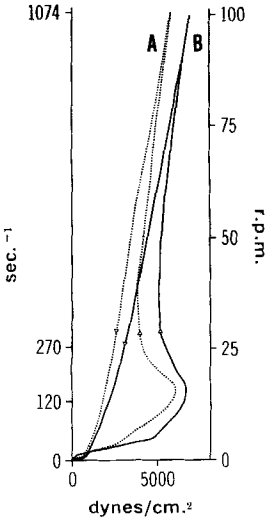


Fig. 7—Rheograms obtained using an upsweep time of 120 sec. Key: A, *white petrolatum U. S. P.;* B, *white ointment U. S. P.*

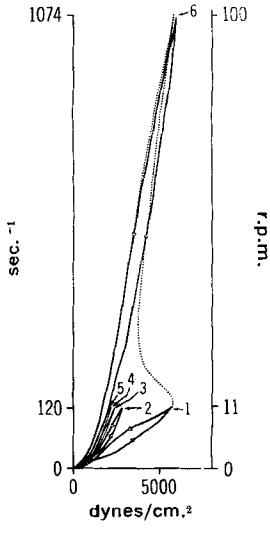


Fig. 8—The effect of repetitive cyclic shearing (procedure E, *6 cycles) on white petrolatum U. S. P. The dotted line is from Fig. 7.*

pattern, whereas the 120 sec.$^{-1}$ curves do. In both instances the white ointment has a greater resistance to flow and in both instances an approximately equal difference is maintained between the white ointment and white petrolatum for the interval 10–40 sec. This can be explained by the fact that while the white wax in the ointment is dispersed throughout the petrolatum it does not contribute to chemical structure, only to stiffness. Furthermore, microscopic examination shows the white wax particles to be unaffected as a result of shearing.

One feature of the curves in Fig. 6 for white ointment and white petrolatum that at first glance appears unusual is that apparently less structural breakdown occurs at 270 sec.$^{-1}$ than at 120 sec.$^{-1}$. Intuitively it would be expected that the higher constant shearing rate would cause a greater breakdown of structure. This is not so and can best be explained by referring to Fig. 7. Here it can be seen that for white petrolatum and white ointment much structural breakdown has already occurred by the time 270 sec.$^{-1}$ has been reached.

Also from Fig. 6 it is readily seen that even after 40 sec. of shearing at 120 sec.$^{-1}$ anhydrous lanolin is still over twice as stiff as white ointment or white petrolatum were after only 10 sec. of shearing.

This type of information regarding structural breakdown is among the most useful data obtainable from a rheological study.

Structural Breakdown by Repetitive Cyclic Shearing—Procedures *E* and *F* were used to gather data on structural breakdown by repetitive cyclic shearing. It was quickly evident that the 25 r.p.m. used in procedure *F* was sufficient to destroy most of the structure on the first cycle. Consequently, the data were collected using procedure *E* (11 r.p.m. maximum). Typical results obtained for white petrolatum are shown in Fig. 8. For all 3 ointment bases studied the trend is similar; namely, the major structural change occurs during the first cycle. Breakdown continues linearly, but very slowly, as cycling continues (Fig. 9). Finally, when the 6th cycle (0–100 r.p.m.) is run, it nearly coincides with portions of the 1 cycle 0–100 r.p.m. rheogram for white petrolatum (Fig. 8, dotted line). Figure 8 is an excellent example of the effect previous sample shearing can have on results and why it is

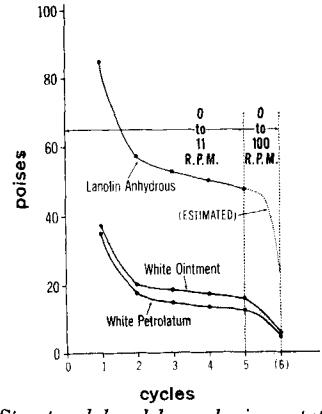


Fig. 9—Structural breakdown during repetitive cyclic shearing. Data obtained using procedure E.

TABLE III—FATTY ACIDS OF LANOLIN ISOLATED BY WEITKAMP (12)

Series	Structural Formula	n
Normal	$CH_3—(CH_2)_{2n}—COOH$	4 to 12 incl.
2-Hydroxyl	$CH_3—(CH_2)_{2n-1}—\underset{\displaystyle OH}{\underset{\vert}{CH}}—COOH$	6, 7
iso	$CH_3—\underset{\displaystyle CH_3}{\underset{\vert}{CH}}—(CH_2)_{2n}—COOH$	3 to 11 incl.
Anteiso	$C_2H_5—\underset{\displaystyle CH_3}{\underset{\vert}{CH}}—(CH_2)_{2n}—COOH$	2 to 13 incl.

essential to run samples that have been equilibrated with respect to structure as well as temperature.

The data obtained using procedure *E* are shown in Fig. 9. This figure clearly shows that although structural breakdown during repeated cycling with anhydrous lanolin occurs at a faster rate than with either white petrolatum or white ointment, it still possesses more than twice the viscosity of either other vehicle. This agrees well with data obtained under constant shear conditions (Fig. 6).

Structural Changes with Shear—*White Petrolatum*—Despite the widespread use of white petrolatum, much remains unknown regarding its composition. Franks (9) attributes this lack of knowledge to the formidable problems of analysis and the general familiarity and satisfaction with the material.

Variation from lot to lot of white petrolatum may be due to several factors. These include (9): (*a*) the type of crude oil started with, (*b*) the extent to which the volatile oils are removed during distillation, (*c*) whether any refining is done before dewaxing, (*d*) the type of process used for separating waxy constituents, (*e*) the viscosity and other characteristics of the oil used to soften the crude petrolatum before refining to U.S.P. specifications by filtration.

Schulte and Kassem (10) separated petrolatum into *n*-, iso-, and cyclic paraffin fractions. Their experiments showed that the rheological properties of petrolatum are determined by the ratio of these constituents to each other, the best pharmaceutical petrolatums containing a low content of *n*-paraffins. During mechanical strain, structural collapse of iso-paraffins is less than *n*-paraffins. Schulte and Kassem feel a principal reason for this is because iso-paraffins form a finer crystallite gel structure. A fine crystallite structure is less susceptible to mechanical strain than is a large crystallite structure. Also, as the crystallite size decreases, the viscosity of the petrolatum increases.

An explanation for the "bulge" in the upcurve of the rheograms of white petrolatum and white ointment and also the breakdown at constant shear rates for these products may be as follows. At rest, the petrolatum structure is probably a three-dimensional gel comprised of *n*-, iso-, and cyclic paraffins attached through random entanglement and chemical bonding. As the shear rate increases the *n*-paraffins tend to align in the direction of shear, whereas the iso and cyclic paraffins cannot align as readily and serve to retain some of the three-dimensional character of the system. As the shear rate further increases, the paraffins become further aligned concurrent with rupture of some of the entangled paraffin chains. At this point, the difference between the upcurve and downcurve becomes minimal.

Anhydrous Lanolin—Since lanolin is a product of animal synthesis, it shows a great deal of variation due to differences in heredity, environment, and age of the animal, time of shearing, age and storage conditions of the greasy wool, and variations in lanolin processing (11).

Purified anhydrous lanolin contains approximately 96% esters, 2% free alcohols, 1% free fatty acids, and 1% hydrocarbons (11). The alcohols that result from saponification of the esters of lanolin, and the free alcohols, can be classified as aliphatic, triterpene, or sterol. The aliphatics comprise about 23% of the total alcohols. Most of these are normal (C_{18}–C_{26}) or branched (C_{17}–C_{26}) chain alcohols. Triterpene alcohols, principally lanosterol and dihydrolanosterol, constitute about another 25%. Sterols (mostly cholesterol) comprise about 30% of the alcohols in lanolin. At present about 20% of the lanolin alcohols remain unclassified (11).

Weitkamp (12) formed the methyl esters of lanolin fatty acids, fractionated them by vacuum distillation, and identified 32 acids. Table III summarizes his results.

As was the case with white petrolatum, it is difficult to visualize what is occurring when anhydrous lanolin is mechanically sheared. There is undoubtedly some alignment of long-chain molecules in the direction of shear and some rupturing of weak chemical bonds at higher shear rates. Any additional speculation is hindered by the unknown constitution and complexity of lanolin.

Recommended Plan of Approach for Evaluating Spreading Properties—On the basis of the findings discussed above, it is apparent that when a user applies white petrolatum or anhydrous lanolin to the epidermis, or a pharmacist levigates these materials, the first few seconds of shearing stress serve to destroy much of the structural integrity, making the use of these materials much easier. This is desirable from a user's acceptance viewpoint. In addition, differences among the common ointment bases studied are great enough to allow a ranking of formulas. The following procedure would appear to be useful when using the Ferranti-Shirley cone and plate viscometer to evaluate the spreading properties of pharmaceutical semisolids.

(*a*) Establish a semisolid material as a standard of acceptable spreadability. This should involve the judgment of several experienced people.

(*b*) Evaluate the rheological characteristics of the standard material utilizing the hysteresis loop and procedures *C* and *E*.

(*c*) Relate the semisolid formulation under study to the standard material using the hysteresis loop and procedures *C* and *E*. It would be wise to make the comparison at several points in the temperature range 20 to 35° to simulate most temperatures of use.

SUMMARY

1. Some of the advantages and limitations of the Ferranti-Shirley cone and plate viscometer for the evaluation of pharmaceutical semisolids in the shearing region between 0 and 270 sec.$^{-1}$ are discussed.

2. Rheological procedures by which the spreading properties of pharmaceutical semisolids can be reproducibly measured are presented.

3. The usefulness of the data in the cases of white petrolatum U.S.P., white ointment U.S.P., and anhydrous lanolin U.S.P. is discussed.

4. The problem of slippage between the revolving cone and the sample is discussed.

5. Structural breakdown at constant shear, structural breakdown during repetitive cycling, and the structural theory possibly involved in some of the observed rheological changes are discussed.

REFERENCES

(1) Kostenbauder, H. B., and Martin, A. N., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 401(1954).
(2) Henderson, N. L., Meer, P. M., and Kostenbauder, H. B., *J. Pharm. Sci.*, **50**, 788(1961).
(3) Boylan, J. C., *ibid.*, **55**, 710(1966).
(4) Wood, J. H., Giles, W. H., and Catacalos, G., *J. Soc. Cosmetic Chemists*, **15**, 565(1964).
(5) Wood, J. H., Catacalos, G., and Lieberman, S. V., *J. Pharm. Sci.*, **52**, 375(1963).
(6) Wood, J. H., and Lapham, E. A., *ibid.*, **53**, 825(1964).
(7) Boylan, J. C., *Bull. Parenteral Drug Assoc.*, **19**, 98 (1965).
(8) Bruss, H., *Parfumerie M. Kosmetik*, **41**, 141(1960).
(9) Franks, A. J., *Soap, Perfumery Cosmetics*, March and April, 1964.
(10) Schulte, K. E., and Kassem, M. A., *Pharm. Acta Helv.*, **38**, 358(1963).
(11) "Amerchol Lanolin Derivatives, "American Cholesterol Products, Inc., Edison, N. J., 1964.
(12) Weitkamp, A. W., *J. Am. Chem. Soc.*, **67**, 447(1945).

Color Stability of Ascorbic Acid Tablets Measured by Light Reflectance

By ROBERT B. WORTZ

The effect of eight commonly used lubricants and glidants on the color stability of ascorbic acid tablets was studied. Light reflectance measurements were used to measure color change after an accelerated aging test. Lubricants low in metallic ion content, such as stearic acid and hydrogenated vegetable oil, conferred maximum color stability to ascorbic acid tablets. The alkaline lubricants such as magnesium and calcium stearate and minerals such as talc and hydrated sodium silico-aluminate were shown to cause excessive color reversion. The importance of humidity control when accelerated test conditions are used to measure the stability of pharmaceutical tablets is shown. Color stability was found to be closely related to chemical stability.

IT IS WELL known that ascorbic acid tablets discolor during storage under normal conditions of temperature and humidity. In fact, tablets stored in closed amber bottles at room temperature will gradually age from a white to a yellowish-brown color. The color stability of ascorbic acid in tablet form is not only affected by conditions of humidity and temperature, but can be influenced by the fillers, lubricants, and binders which are usually found in tablet formulations (1, 2).

Pharmaceutical tablets are often subjected to accelerated aging tests at elevated temperatures and humidities so that color stability may be ascertained rapidly. The end point of most such studies has been visual examination of tablets for color change. However, this type of evaluation is dependent on the judgment of the individual making the observation. No physical measurement is recorded and further deterioration or destruction of sample tablets makes it impossible to compare new samples prepared at a later date.

Light reflectance measurements are widely used by the pulp and paper industry to measure discoloration. The standard brightness test used for evaluating the discoloration of paper measures reflectance in the blue range at 458 mμ (3, 4). This wavelength is considered to give the best measure of yellow color. Such a light reflectance test should be useful in evaluating discoloration of ascorbic acid tablets. Gunsel and Lachman have used light reflectance measurements in comparing formulations containing conventionally processed and spray-dried lactose (5).

The purpose of this study was to determine the effects of lubricants and glidants commonly used in pharmacy on the color stability of ascorbic acid

Received September 13, 1966, from the Avicel Laboratory, FMC Corp., American Viscose Division, Marcus Hook, PA 19061
Accepted for publication June 1, 1967.

(*b*) Evaluate the rheological characteristics of the standard material utilizing the hysteresis loop and procedures *C* and *E*.

(*c*) Relate the semisolid formulation under study to the standard material using the hysteresis loop and procedures *C* and *E*. It would be wise to make the comparison at several points in the temperature range 20 to 35° to simulate most temperatures of use.

SUMMARY

1. Some of the advantages and limitations of the Ferranti-Shirley cone and plate viscometer for the evaluation of pharmaceutical semisolids in the shearing region between 0 and 270 sec.$^{-1}$ are discussed.

2. Rheological procedures by which the spreading properties of pharmaceutical semisolids can be reproducibly measured are presented.

3. The usefulness of the data in the cases of white petrolatum U.S.P., white ointment U.S.P., and anhydrous lanolin U.S.P. is discussed.

4. The problem of slippage between the revolving cone and the sample is discussed.

5. Structural breakdown at constant shear, structural breakdown during repetitive cycling, and the structural theory possibly involved in some of the observed rheological changes are discussed.

REFERENCES

(1) Kostenbauder, H. B., and Martin, A. N., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 401(1954).
(2) Henderson, N. L., Meer, P. M., and Kostenbauder, H. B., *J. Pharm. Sci.*, **50**, 788(1961).
(3) Boylan, J. C., *ibid.*, **55**, 710(1966).
(4) Wood, J. H., Giles, W. H., and Catacalos, G., *J. Soc. Cosmetic Chemists*, **15**, 565(1964).
(5) Wood, J. H., Catacalos, G., and Lieberman, S. V., *J. Pharm. Sci.*, **52**, 375(1963).
(6) Wood, J. H., and Lapham, E. A., *ibid.*, **53**, 825(1964).
(7) Boylan, J. C., *Bull. Parenteral Drug Assoc.*, **19**, 98 (1965).
(8) Bruss, H., *Parfumerie M. Kosmetik*, **41**, 141(1960).
(9) Franks, A. J., *Soap, Perfumery Cosmetics*, March and April, 1964.
(10) Schulte, K. E., and Kassem, M. A., *Pharm. Acta Helv.*, **38**, 358(1963).
(11) "Amerchol Lanolin Derivatives, "American Cholesterol Products, Inc., Edison, N. J., 1964.
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tablets. The filler used in most cases was microcrystalline cellulose.

EXPERIMENTAL

Materials—Ascorbic acid used in this study was either the coated, fine crystal, or fine powder grade.[1] The diluents used included microcrystalline cellulose,[2] cornstarch,[3] and spray-dried lactose.[4] Lubricants included in the study were hydrogenated vegetable oil,[5] stearic acid,[6] magnesium stearate,[6] and calcium stearate.[6] The glidants used were submicroscopic pyrogenic silicas,[7] hydrated sodium silico aluminates,[8] and talc.[9]

Sample Preparation—Mixtures of microcrystalline cellulose and ascorbic acid were dry blended with the various lubricants and glidants. Formulations containing starch and lactose were granulated with a 10% starch paste, air dried, and ground through a 30-mesh screen on a Wiley mill. Fifteen grams of each formulation was pressed into 2.25-in. disks using a Carver[10] test cylinder at a pressure of 20,000 p.s.i. The disks were pressed on a Drake hydraulic press.[11] The 2.25-in. disks were used rather than tablets to obtain the necessary surface area for reflectance measurements.

Storage Conditions—The accelerated test conditions used to store the samples were 2 weeks at 40° and 75% relative humidity. Selected samples were also stored for 16 to 32 weeks at 6 different conditions of humidity and temperature. The various humidity conditions were established by storing the samples in closed desiccators containing the saturated salt solutions listed in Table I (6). The

Table I—Storage Conditions

Saturated Salt Soln.	Temp., °C.	% R.H.
$KC_2H_3O_2$	24	23
$Ca(NO_3)_2$	24	51
NaCl	24	75
KF	40	23
NaBr	40	52
NaCl	40	75

variations in temperature were ±1°. All disks were stored in the absence of light.

Assay of Ascorbic Acid—The ascorbic acid concentrations were determined by the U.S.P. XVI method (7).

Reflectance Measurements—The light reflectance of the disks was measured with a tristimulus reflectometer both before and after the 14-day stability test. A Gardner precision reflectometer exposure head[12] and a Gardner automatic photometric unit were used for the reflectance measurements. Measurements were made using blue, amber, and green light.

[1] Merck & Co., Inc., Rahway, N. J.
[2] Marketed as Avicel PH by FMC Corp., American Viscose Division, Marcus Hook, Pa.
[3] Corn Products Co., New York, N. Y.
[4] Foremost Dairies, Appleton, Wis.
[5] Sterotex, Capitol City Products Co., Columbus, Ohio.
[6] M. W. Parsons, Plymouth Division, New York, N. Y.
[7] Marketed as Cab-O-Sil by Cabot Corp., Boston, Mass.
[8] Zeolex, J. M. Huber Corp., New York, N. Y.
[9] Sierra Talc & Clay Co., Pasadena, Calif.
[10] Fred S. Carver, Inc., Summit, N. J.
[11] Drake Corp., Grand Haven, Mich.
[12] Gardner Laboratories, Bethesda, Md.

RESULTS AND DISCUSSION

The ascorbic acid formulations listed in Table II were pressed into disks and stored for 14 days at 40° and 75% relative humidity. Table III compares the initial and final reflectance values of the disks using green, amber, and blue light. The per cent change in reflectance or the per cent reversion was calculated by the formula:

$$\% \text{ reversion} = \frac{\text{initial reflectance} - \text{final reflectance}}{\text{initial reflectance}} \times 100$$

Calculation of the per cent color reversion enables direct comparison of data without having to compensate for differences in the initial reflectance of each sample.

The color of the disks after the accelerated stability test ranged from off-white to a yellowish-brown color. In all cases the greatest change in reflectance was detected with the blue light. The per cent reversion obtained with the green and amber light was less than one-half the magnitude of that obtained with the blue light. The blue light was also much more sensitive to small color changes. For example, formulation *5* showed a reversion of 8.9% with blue light while reversions of only 2.4 and 1.4% were detected with the green and amber lights. Due to the increased sensitivity of the blue light to color change as the tablets aged, only blue light reflectance values are reported for the remainder of the paper.

The change in reflectance units that could be detected by visual observations was determined. Disks prepared from the same tableting formulation were removed from the oven at close intervals to obtain samples having similar reflectance values. Disks having blue light reflectance values of 89.7, 88.3, 85.9, 84.2, and 82.5 were examined. Five observers arranged the samples in order of decreasing reflectance values. The data demonstrate that a change in reflectance as small as 1.4 units could be detected by visual examination. When this is expressed in terms of per cent color reversion, a change as small as 1.6% could be detected by visual examination. Several observers were able to distinguish between the samples having a reflectance difference as small as 0.8 units. The reproducibility limit of the reflectance measurements was approximately ±0.3 reflectance units.

The data listed in Table III demonstrate a definite relationship between tablet color stability and the lubricants and glidants present in the tablet. Formulations *1*, *2*, *3*, and *4* which contained either magnesium stearate, hydrated sodium silico aluminates, or talc displayed excessive color reversion. However, formulations *5* and *6*, with either stearic acid or hydrogenated vegetable oil as lubricants and pyrogenic silica as a glidant, displayed the same discoloration as the control (formulation *8*).

The formulations described in Table II were assayed for ascorbic acid before and after storage for 2 weeks at 40° and 75% R.H. In Table IV, the assay and the per cent color reversion obtained with blue light are listed. Color stability is shown to be closely related to chemical stability.

To study the separate effect of various lubricants and glidants, blends were made up to contain 69% fine powder ascorbic acid, 30% microcrystalline

TABLE II—ASCORBIC ACID TABLETING FORMULATIONS IN WEIGHT PER CENT

	Formulation							
	1	2	3	4	5	6	7	8
Ascorbic acid fine crystal	60	60	60	60	60	60	60	60
Microcrystalline cellulose	36	37	37	37	37.5	37.5	38.5	40
Stearic acid	..	..	..	..	...	2	...	..
Hydrogenated vegetable oil	..	..	..	2	2	...	...	..
Magnesium stearate	1	2	2	..	...	...	...	..
Hydrated sodium silico aluminates	..	1	..	1	...	...	...	..
Pyrogenic silica	..	..	1	..	0.5	0.5	0.5	..
Talc	3	..	..	..	...	...	...	..
Calcium stearate	..	..	..	..	...	...	1	..

TABLE III—REFLECTANCE VALUES OF ASCORBIC ACID TABLETING FORMULATIONS BEFORE AND AFTER 2 WEEKS' STORAGE AT 40° AND 75% R.H.

Formulation	Green Light			Amber Light			Blue Light		
	Reflectance		%	Reflectance		%	Reflectance		%
	Initial	Final	Reversion	Initial	Final	Reversion	Initial	Final	Reversion
1	94.8	82.5	13.0	96.5	86.8	10.1	89.2	65.5	26.6
2	96.6	70.8	26.7	98.3	76.5	22.2	91.0	50.4	44.6
3	96.1	80.8	15.9	98.0	85.9	12.4	90.7	60.4	33.4
4	95.1	75.5	20.6	97.5	81.3	16.6	89.6	54.2	39.5
5	96.4	94.1	2.4	98.3	96.9	1.4	90.8	82.7	8.9
6	96.6	93.6	3.1	98.4	96.5	1.9	90.9	81.4	10.5
7	96.3	90.7	5.8	98.0	92.2	5.9	91.0	76.4	16.0
8	95.7	92.2	3.7	97.4	94.7	2.8	90.2	81.7	9.4

TABLE IV—CHEMICAL AND COLOR STABILITY OF ASCORBIC ACID FORMULATIONS STORED FOR 2 WEEKS AT 40° AND 75% R.H.

Formulation	Initial Activity, mg./Gm.	Activity After 2 Wk. mg./Gm.	%	% Color Reversion with Blue Light After 2 Wk.
1	270	253	93.7	26.6
2	270	249	92.2	44.6
3	269	255	94.8	33.4
4	270	255	94.4	39.5
5	273	271	99.3	8.9
6	268	266	99.3	10.5
7	269	268	99.6	16.0
8	272	271	99.6	9.4

TABLE V—SEPARATE EFFECTS OF LUBRICANTS AND GLIDANTS ON COLOR REVERSION OF ASCORBIC ACID TABLETS[a]

Sample	Initial Reflectance	Final Reflectance	% Reversion (Blue Light)
Control	94.1	87.5	7.0
Magnesium stearate	94.2	76.0	19.3
Calcium stearate	93.6	81.2	13.2
Stearic acid	92.9	88.8	4.4
Hydrogenated vegetable oil	95.1	88.9	6.5
Hydrated sodium silico aluminates	93.3	57.2	38.7
Talc	94.2	82.4	12.5
Pyrogenic silica	95.1	87.9	7.6

[a] Tablets contained 69% fine powder ascorbic acid, 30% microcrystalline cellulose, and 1% lubricant or glidant. Stored 2 weeks at 40° and 75% R.H.

cellulose, and 1% of the tested material. Data listed in Table V indicate that formulations containing stearic acid, hydrogenated vegetable oil, or pyrogenic silica displayed approximately the same discoloration as the ascorbic acid–microcrystalline cellulose control sample. Discoloration increased markedly over the control when magnesium stearate, calcium stearate, talc, or hydrated sodium silico aluminates were used.

The effect of lubricant concentration on color stability is shown in Table VI. Color reversion increased with lubricant concentration when magnesium stearate and talc were used. However, with stearic acid and hydrogenated vegetable oil, the degree of discoloration was independent of lubricant concentration.

Blaugh, Chakravarty, and Lach (1) have indicated that precoating ascorbic acid with ethylcellulose and granulating with ethylcellulose helped protect against decomposition due to metallic contamination. Coated ascorbic acid is prepared by granulating ascorbic acid with an alcoholic solution of ethylcellulose. To determine if the use of coated ascorbic acid improved color stability in tablets prepared by direct compression, formulations were prepared both with the coated and uncoated ascorbic acid. Various lubricants and glidants were added separately to formulations containing 75 parts ascorbic acid and 33 parts microcrystalline cellulose. Data listed in Table VII demonstrate that, in most cases, no improvement in color stability is gained by using coated ascorbic acid. In fact, formulations with stearic acid or hydrogenated vegetable oil showed greater stability with the regular ascorbic acid. The only significant improvement in color stability with the coated ascorbic acid was in the formulation containing hydrated sodium silico aluminates. There is no apparent explanation for the increased discoloration in the formulations containing coated ascorbic acid.

TABLE VI—EFFECT OF LUBRICANT OR GLIDANT CONCENTRATION ON COLOR STABILITY[a]

Wt. % of Lubricant	% Color Reversion (Blue Light) Magnesium Stearate	Talc	Stearic Acid	Hydrogenated Vegetable Oil
0.0	7.0	7.0	7.0	7.0
0.1	11.0	...	5.4	...
0.3	13.3	...	4.5	...
0.5	17.4	11.4	6.6	6.5
1.0	19.3	12.5	4.4	6.5
1.5	...	...	4.6	...
2.0	...	16.9	...	...

[a] Tablets contained 75 parts fine powder ascorbic acid and 33 parts of microcrystalline cellulose. Stored 2 weeks at 40° and 75% R.H.

TABLE VII—COMPARISON OF COLOR STABILITY OF COATED AND REGULAR ASCORBIC ACID[a]

	% Color Reversion Fine Powder Ascorbic Acid	Coated Ascorbic Acid
Control	7.0	9.0
Magnesium stearate	19.3	21.7
Calcium stearate	13.2	12.3
Stearic acid	4.4	8.9
Hydrogenated vegetable oils	6.5	9.9
Hydrated sodium silico aluminates	38.7	26.3
Talc	21.9	18.0

[a] All blends contained 75 parts of ascorbic acid and 33 parts microcrystalline cellulose. All materials added at a 1% concentration except talc which was present at 3%. Stored 2 weeks at 40° and 75% R.H.

TABLE VIII—COLOR STABILITY OF FORMULATIONS WITH LACTOSE AND STARCH AS FILLERS[a]

	Formulations *9*	*10*	*11*
Ascorbic acid, fine crystal	63.6	82.8	60.0
Microcrystalline cellulose	...	...	37.5
Lactose	17.6	6.4	...
Starch	15.8	6.9	...
Talc	2.9	3.0	...
Magnesium stearate	1.0	1.0	...
Hydrogenated vegetable oil	...	...	2.0
Pyrogenic silica			0.5
	Color Stability		
Initial reflectance	92.3	92.8	92.5
Final reflectance	56.3	58.4	85.8
% reversion	38.3	37.2	7.2

[a] Amount of each ingredient listed as the weight per cent. Stored 2 weeks at 40° and 75% R.H.

Table VIII compares the color stability of tablets containing starch and lactose fillers with those containing microcrystalline cellulose. Since microcrystalline cellulose by itself requires no lubrication in direct compression it is possible to use the less efficient lubricants such as hydrogenated vegetable oil or stearic acid. Formulations *9* and *10* in Table VIII resemble those found in commercial ascorbic acid tablets. The tableting formulation with microcrystalline cellulose as the filler was markedly more stable to color reversion than those containing lactose and starch.

The relationship between the color reversion

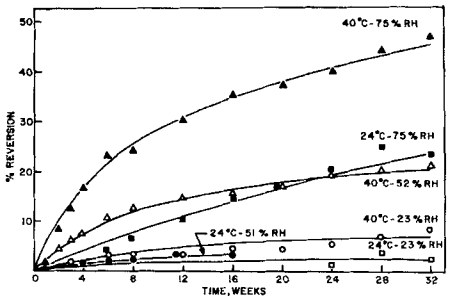

Fig. 1—Per cent color reversion plotted versus *storage time for formulation containing 60% ascorbic acid, 38.5% microcrystalline cellulose, 2% stearic acid, and 0.5% pyrogenic silica.*

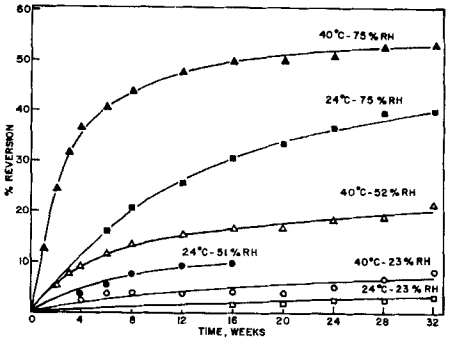

Fig. 2—Per cent color reversion plotted versus *storage time for formulation containing 60% ascorbic acid, 38.5% microcrystalline cellulose, 1% magnesium stearate, 0.5% pyrogenic silica.*

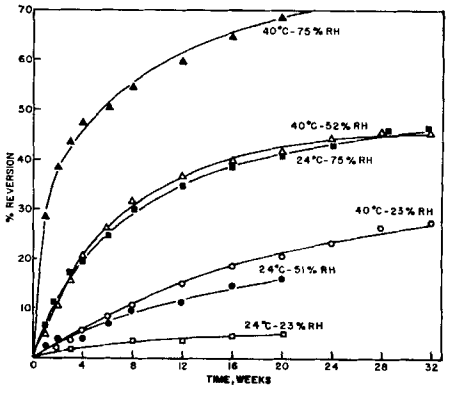

Fig. 3—Per cent color reversion plotted versus *storage time for formulation containing 63% ascorbic acid, 18% lactose, 16% starch, 1% magnesium stearate, 3% talc.*

obtained under accelerated test conditions with that obtained at room temperature was determined. Three formulations that displayed varying degrees of color stability were stored for 16 to 32 weeks under 6 different conditions of temperature and humidity. In Figs. 1, 2, and 3 the per cent color reversion is plotted against storage time. The storage times required for each sample to display a reversion of

TABLE IX—STORAGE TIMES REQUIRED FOR THE FORMULATIONS IN FIGS. 1–3 TO SHOW REVERSIONS OF 10%

Storage Conditions	Storage Time, Days Fig. 1	Fig. 2	Fig. 3
40°, 75% R.H.	5	2	14
40°, 50% R.H.	34	11	42
40°, 23% R.H.	...	50	..
24°, 75% R.H.	25	11	67
24°, 51% R.H.	112	64	..

TABLE X—STORAGE TIMES EQUIVALENT TO 2 WEEKS AT 40° AND 75% R.H.

Storage Conditions	Time, Wk.
40°, 50% R.H.	6–11
40°, 23% R.H.	50
24°, 75% R.H.	8–11
24°, 51% R.H.	45–64

10%, at each condition of temperature and humidity was extracted from the figures and listed in Table IX. These data were used to determine storage times that were equivalent to 2 weeks at 40° and 75% R.H. (See Table X.)

The importance of humidity control, when accelerated test conditions are used to measure color stability, is clearly shown. At a constant temperature, color reversion increased markedly with increasing relative humidity. For example, after 12 weeks' storage at 40°, the formulation listed in Fig. 2 had color reversions of 4, 15, and 47% at relative humidities of 23, 52, and 75%, respectively. The formulations shown in Figs. 1 and 3 displayed approximately the same color reversion when stored at 40° and 52% R.H. or at 24° and 75% R.H. Very little color change was detected in samples stored at 24° and 23% R.H. The accelerated test conditions used, 2 weeks at 40°, and 75% R.H., were shown to be equivalent to approximately 1 year of storage at 24° and 51% R.H.

SUMMARY AND CONCLUSIONS

The light reflectance values of ascorbic acid tablets were measured before and after aging with a tristimulus reflectometer using amber, green, and blue light. The blue light reflectance values were found to be most effective for detecting small color changes in ascorbic acid tablets as they degraded from a white to a yellowish-brown color. The blue light reflectance values correlated closely with color changes detected by visual examination. Color stability was found to be closely related to chemical stability.

The color stability of ascorbic acid tablets was shown to be affected by the lubricants and glidants present in the tablet. Of the seven lubricants and glidants studied, stearic acid, hydrogenated vegetable oil, and pyrogenic silica were found to confer maximum stability to formulations of ascorbic acid. Tablets containing alkaline stearates such as magnesium or calcium stearate and minerals such as talc or hydrated sodium silico aluminate all displayed excessive color reversion. Use of ethylcellulose coated ascorbic acid did not improve color stability of tablets prepared by direct compression.

The importance of humidity control when accelerated stability tests are used to determine tablet stability is clearly shown. At a constant temperature color reversion of ascorbic acid tablets increased dramatically with increasing relative humidities. Two weeks' storage at 40° and 75% R.H. was found to be equivalent to storage for approximately 1 year at 25° and 50% R.H.

The use of reflectance measurements to measure tablet color stability greatly improved the reliability and usefulness of data obtained from accelerated stability tests.

REFERENCES

(1) Blaugh, M. R., Chakravarty, D., and Lach, J. L., *Drug Std.*, **26**, 199(1958).

(2) Wai, K., DeKay, H. G., and Banker, G. S., *J. Pharm. Sci.*, **51**, 1076(1962).

(3) "Standard Method of Test for 45-Deg, 0-Deg Directional Reflectance for Blue Light (Brightness) of Paper," ASTM Designation D985-50, American Society for Testing Materials, Philadelphia, Pa., 1961.

(4) Casey, J. P., "Pulp and Paper," vol. III, Interscience Publishers, Inc., New York, N.Y., 1961.

(5) Gunsel, W. C., and Lachman, L., *J. Pharm. Sci.*, **52**, 178(1963).

(6) Lange, A. N., "Handbook of Chemistry," 9th ed., Handbook Publishers, Inc., Sandusky, Ohio, 1956, p. 1420.

(7) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960.

Effect of Additives and Formulation Techniques on Controlled Release of Drugs from Microcapsules

By LOUIS A. LUZZI* and ROBERT J. GERRAUGHTY

The objectives of this study were to evaluate the effects of polysorbate 20 and a polyelectrolyte dispersing agent on the release of solids from microcapsules and to formulate microcapsules which, when subjected to gastrointestinal fluids, would release the encapsulated material at a predictable rate. Samples of microcapsules were subjected to simulated gastric fluid for 0.5 hr. and transferred to simulated intestinal fluid for an additional 2-hr. period. The filtered gastrointestinal fluids were compared spectrophotometrically to known absorption spectra in order to determine the quantity of solid released after each time interval. Results showed that the additives generally increased the release rate and that a large differential in release rate could be obtained without additives.

PREVIOUS WORK carried out by these authors and reported in *J. Pharm. Sci.* (1, 2) has indicated that the release of materials encapsulated by the process of complex coacervation could be controlled to make the encapsulated material available at a relatively predictable rate.

It was reported (1) that certain surfactants, when added to the system, would decrease the protection offered to the encapsulated substance. In this case, the encapsulated substance was mineral oil and the extracting substance was ethyl ether.

In another work (2) it was reported that solid particles (pentobarbituric acid) had been encapsulated in the same system and that a certain amount of control of the release rate of the solid to gastrointestinal fluids could be attained when conditions used in the encapsulation process were varied. The variables included: starting pH, starting temperature, ratio (dry weight) of solid to encapsulating material, quantity of formaldehyde added, and final pH.

With the above in mind, it became desirable to determine the effect of surfactants on microencapsulation as well as to attempt the formulation of fast and slow release microcapsules.

EXPERIMENTAL

Preparation of Microcapsules—The method of preparation of microcapsules followed that of Green and Schleicher (3, 4), as modified by Luzzi and Gerraughty (1). A previously described method of including solids (2) was incorporated into the process. Changes in pH were brought about by the addition of either 20% sodium hydroxide or diluted hydrochloric acid U.S.P.

Polysorbate 20[1] and a polyelectrolyte dispersing agent[2] were added in various quantities to the aqueous phase prior to the addition of the solid. In order to minimize the degradation of pentobarbituric acid, the temperature at which the capsules were dried was reduced from 50° to 40°.

The conditions incorporated into the formulation of fast release microcapsules are as follows: the starting pH was 5.0, the starting temperature was 34°, the ratio (dry weight) of pentobarbituric acid to encapsulating agents was 20/6; 4 ml. of formaldehyde solution U.S.P. was added for each 100 ml. of 3% gelatin solution, and the final pH was 8.8.

Samples for slow release microcapsules were prepared using the following conditions: the starting pH was 6.0, the starting temperature was 37°, the ratio of pentobarbituric acid to encapsulating agents was 4/6; 8 ml. of formaldehyde solution was added for each 100 ml. of 3% gelatin solution, and the final pH was 6.5.

The conditions accompanying the addition of both polysorbate 20 and the dispersing agent were as follows. The starting pH was 6.0, the starting temperature was 40°, the ratio of pentobarbituric acid to encapsulating agents was 8/6; 10 ml. of formaldehyde solution for each 100 ml. of 3% gelatin solution was added, and the final pH was 7.5. In addition samples of microcapsules containing the dispersing agent were prepeard where the lowest encapsulating pH values were 4.8 and 3.5 rather than the usual 4.0.

Method of Evaluation of Microcapsules—The method used to evaluate the microcapsules formed with the additives and for fast and slow release follows the method outlined by Luzzi and Gerraughty (2). The dried capsules were immersed in simulated gastric fluid and shaken for 0.5 hr. after which they were transferred to intestinal fluid and assayed after 1 and 2-hr. periods.

The assay method consisted of spectrophotometrically comparing the properly diluted, filtered extracts to known absorption spectra.

Photography—Photomicrographs of the wet microcapsules, taken prior to drying, were made to determine any visual peculiarities of the various formulations. The microscope slides were prepared for photography by placing 1 drop of slurry on a slide and covering it with a cover slip. The slurry was taken from the preparation while it was being filtered.

Polysorbate 20 (0.0071%) was added to the system shown in Fig. 1. In this photograph both

Received February 9, 1967, from the College of Pharmacy, University of Rhode Island, Kingston, RI 02881

Accepted for publication June 5, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by L. A. Luzzi to the Graduate School, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.

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[1] Polyoxyethylene sorbitan monolaurate. Marketed as Tween 20 by Atlas Powder Co., Wilmington, Del.

[2] Obtained as Darvan 7 from the R. T. Vanderbilt Co., New York, N.Y.

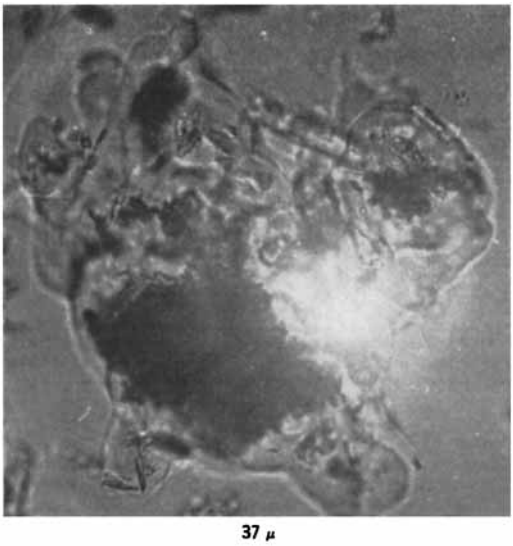


Fig. 1—Photograph of incomplete microencapsulation due to the addition of polysorbate 20.

Fig. 2—Photograph of large groups of microcapsules to which 0.019% of dispersant had been added to the system.

encapsulated and unencapsulated particles are visible. Photographs of a system containing less surfactant were prepared and, although not shown, suggested a progressive interference with encapsulation as the per cent of surfactant was increased.

Figure 2 is a photograph made of capsules containing 0.019% of the dispersing agent. It can be seen that the particles were not well dispersed and that large groups of particles seem to have gathered together to form a large group or groups of capsules. Figure 3 is a photograph prepared from microcapsules to which 0.029% of the dispersing agent had been added and shows elongated capsules filled with solid particles. The elongation of capsules seemed to be typical when moderate quantities of this dispersant were used. A comparison of the shell thickness of the over-all capsules to the shell thickness of capsules with no additive seemed to

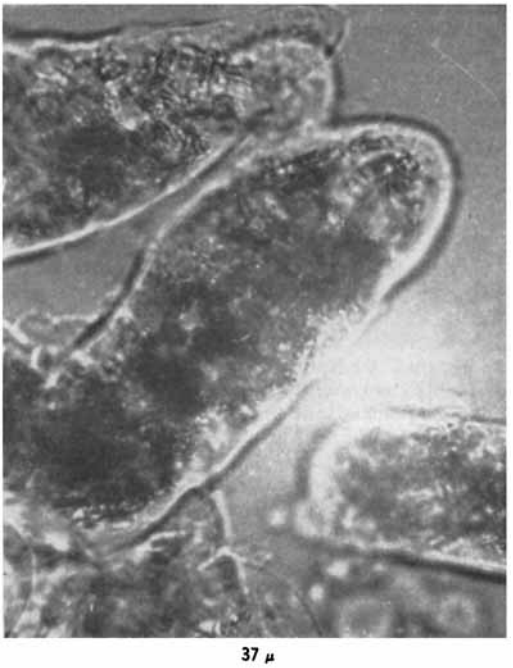


Fig. 3—Photograph of typical elongated groups of microcapsules which were present when 0.029% dispersant was added to the system.

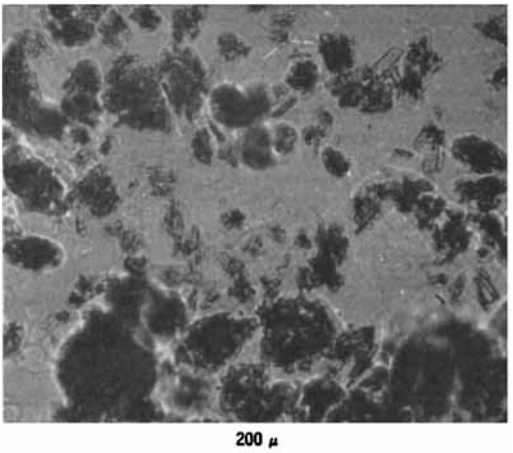


Fig. 4—Photograph of well dispersed microcapsules to which 0.116% of dispersant had been added.

indicate that high concentrations of the dispersant tend to decrease the wall thickness.

Figure 4 is a photograph of microcapsules to which 0.0071% polysorbate 20 had been added and was made at a slightly reduced enlargement. With this as a consideration, it can still be seen that the solids are in smaller groups, that the walls are quite thin, and that some capsules are not filled.

RESULTS

Effect of Polysorbate 20 on Microcapsules—Luzzi and Gerraughty (1) showed that polysorbate 20 in concentrations of 2% completely inhibited the encapsulation of mineral oil; it was thought that the orientation of polysorbate 20 molecules at the oil–water interface acted to prevent shell forming coacervate droplets from becoming oriented at the interface of the oil. The preparation of a series of microcapsules was undertaken in an attempt to establish the effect on microencapsulation of polysorbate 20 in systems employing solid rather than liquid particles.

In this series the per cent of polysorbate 20 included in the aqueous phase was varied from zero to 0.0142%. Table I shows that, under the conditions described, there was no significant change in the per cent of pentobarbituric acid released except at the highest concentration of polysorbate 20.

Effect of Dispersant on Microcapsules—Since the solid particles of pentobarbituric acid tended to settle out of suspension and were at times difficult to disperse, the dispersing agent was added to three separate series. Also, since preliminary work indicated that the dispersant was basic (pH 8.5–9.0) in the concentrations used, it was desirable to determine what effect changing the lowest pH would have on microcapsules as well as on the activity of the dispersant. The three series differed in that the lowest pH's attained during the encapsulating process were 4.0, 4.8, and 3.5, respectively.

The samples of Table II were prepared in an identical manner to those in the polysorbate 20 series (Table I), the only difference being that the dispersing agent rather than polysorbate 20 was added. At the lowest concentrations of the dispersant there seemed to be less acid extracted by the gastrointestinal fluids than in those samples prepared with no dispersant: thereafter, the per cent of acid released became greater with increasing additions of the dispersant.

Where the samples of Table III correspond to the dispersant concentrations of those in Table II, they are identical, with the exception that the lowest pH in the former was 4.8 rather than 4.0. Table III shows that there was a greater release of pentobarbituric acid as the concentration of the dispersant was increased and also that all of the samples of Table III released a greater percentage of acid than the corresponding members of Table II.

All of the samples within the series found in Table IV were also prepared in a manner similar to those of the polysorbate 20 group with the exception that the lowest pH was at 3.5 rather than at 4.0. Table IV shows that samples were not collectable until the second increment of the dispersant had been added; with the addition of the second increment of the dispersant, they become collectable.

TABLE I—EFFECT OF POLYSORBATE 20 ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Polysorbate 20, %	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
...	34.8	19.2	26.3	61.1
0.0001	35.0	17.5	26.8	61.8
0.0002	33.9	18.5	27.0	60.9
0.0006	32.7	20.1	28.3	61.0
0.0071	34.4	19.7	28.2	62.6
0.0142	40.7	20.0	26.1	66.8

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid the samples were transferred to intestinal fluid.

TABLE II—EFFECT OF DISPERSANT ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES (FORMATION pH 4.0) EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Dispersant, %	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
...	34.8	19.2	26.3	61.1
0.0195	30.2	18.4	24.6	54.8
0.0290	35.0	20.1	25.6	60.6
0.0580	39.1	22.1	27.1	66.2
0.1160	34.7	19.7	28.1	62.8
0.1450	43.0	21.5	26.2	67.2
0.1740	38.0	23.1	30.7	68.7

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid the samples were transferred to intestinal fluid.

TABLE III—EFFECT OF DISPERSANT ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES (FORMATION pH 4.8) EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Dispersant, %	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
...	42.2	21.7	25.9	68.8
0.029	43.5	21.2	25.7	69.2
0.116	45.3	16.3	24.7	70.0
0.174	48.5	28.4	30.7	79.2

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid the samples were transferred to intestinal fluid.

TABLE IV—EFFECT OF DISPERSANT ON THE PER CENT OF PENTOBARBITURIC ACID EXTRACTED FROM MICROCAPSULES (FORMATION pH 3.5) EXPOSED TO GASTROINTESTINAL FLUIDS[a]

Dispersant, %	After 0.5 hr. in Gastric Fluid[b]	After 1 hr. in Intestinal Fluid	After 2 hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
.....	Sample not collectable			
0.029	... Sample not collectable ...			
0.058	39.8	24.6	32.5	72.3
0.116	40.4	23.4	30.3	70.7
0.174	41.3	32.1	36.7	78.0
0.232	41.0	22.2	29.9	70.9

[a] All values expressed as per cent of total pentobarbituric acid found in microcapsules. [b] After removal of gastric fluid the samples were transferred to intestinal fluid.

Formulation of Fast and Slow Release Microcapsules—Using the information gathered from a previous work by the authors of this paper (2), an attempt was made to formulate microcapsules which would release all or nearly all of the pentobarbituric acid which they contained. An attempt was also made to formulate microcapsules which would retain more than 50% of this original content of acid after 2.5-hr. exposure to gastrointestinal fluids. Samples of both formulations were prepared, and they were subjected to the gastrointestinal fluids as previously described (2).

The results of assays for fast release microcapsules revealed that 47.3% of the drug was extracted after 0.5-hr. exposure to gastric fluid. After being transferred to intestinal fluid and remaining immersed for 1 hr., an additional 30.1% was extracted and during the second hour in intestinal fluid 1.1% of the original quantity of acid was released; for a total of 78.5% released after 2.5-hr. exposure to gastrointestinal fluids.

The results of assays for slow release microcapsules revealed that 37.7% of the drug was released during 0.5-hr. exposure to gastric fluid. After being transferred to intestinal fluid and remaining immersed for 1 hr., an additional 13.0% was extracted, and during the second hour in intestinal fluid 5.5% of the original quantity of acid was released for a total of 56.2% released after 2.5-hr. exposure to gastrointestinal fluids.

In the fast release samples there was a difference of 1.1% in total per cent acid extracted between 1- and 2-hr. exposure periods to intestinal fluid, whereas there was a difference of 5.5% acid extracted under the same conditions for slow release samples.

Although the per cent of pentobarbituric acid released after the 2.5-hr. assay period fell somewhat short of the goal of this experiment, there seemed to be a significant difference in the per cent of acid released between the two types of samples.

DISCUSSION

Reasoning concerning the effect of starting pH, starting temperature, ratio, and quantity of formaldehyde on the strength or stability of capsule walls when in contact with gastrointestinal fluids has been given elsewhere (1, 2). That is, the starting pH will affect the coacervation already present at low pH values (*i.e.*, 5) or to the salt present (in the case of pentobarbituric acid) at high pH values; the starting temperature will affect the mobility of the micromolecules or the formed coacervate droplets thus limiting encapsulation; the ratio of solid encapsulating agents may affect the shell thickness; the quantity of formaldehyde may be insufficient to cause stable droplets to form or may possibly cause cracking of the shell when present in excess.

Combination of those conditions contributing to optimum shell strength were incorporated into the slow release microcapsules and selected conditions (those where collection of samples could be made) away from the optimum were incorporated into the fast release microcapsules. It seems likely that the reasoning which applied to the individual variables would also apply to the combined conditions.

Polysorbate 20 may have interfered with encapsulation because of its orientation at the solid–liquid interface (1). However, this may not be the case with the dispersing agent. Here it seems likely that the increased release of solid from the capsule was due to an increase in the number of particles which resulted from better dispersion of the solid and thus in more individual capsules having been formed. (See Fig. 4.) With the increased number of particles present, it would seem that the ratio of solid surface to encapsulating agents has been increased. As shown previously, this too would decrease the capsule strength.

Studies are now being carried out in order to determine the degree to which surface orientation and particle dispersion or size affect capsule strength.

CONCLUSIONS

Polysorbate 20, in the higher concentration used, seems to enhance the release of pentobarbituric acid to simulated gastrointestinal fluids.

Photomicrographs indicate that the polyelectrolyte dispersing agent aids in the dispersion of solid containing microcapsules and that at higher concentrations the encapsulations are sometimes incomplete.

It is possible to control the rate of release of pentobarbituric acid to gastrointestinal fluids by varying certain conditions in the encapsulation process.

REFERENCES

(1) Luzzi, L. A., and Gerraughty, R. J., *J. Pharm. Sci.*, **53**, 429(1964).
(2) *Ibid.*, **56**, 634(1967).
(3) Green, B. K., and Schleicher, L., U. S. pat. 2,703,457 (1956).
(4) Green, B. K., U. S. pat. 2,722,507(1955).

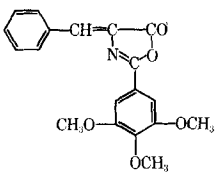
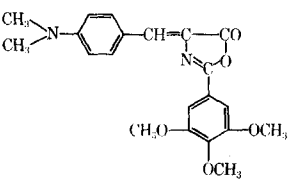
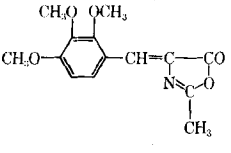
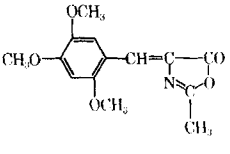
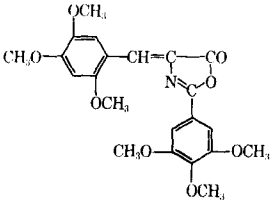
Infrared Studies of Some New Azlactones

By P. K. SHARMA and S. S. DAVE

The infrared spectra of five azlactones possessing the trimethoxy benzene structure are discussed, and an attempt is made to draw some structural inferences from the data reported.

FIVE AZLACTONES having the trimethoxy benzene structure were synthesized previously, and their central nervous system depressant actions have already been reported (1). The present communication deals with the infrared studies (Table I) and interpretation of those azlactones.

TABLE I—CHEMICAL STRUCTURE AND INFRARED ABSORPTION PEAKS OF SOME AZLACTONES

No.	Compd.	Structure	Absorption Peaks, cm.$^{-1}$	Intensity	Structural Inference
I	Azlactone of 3,4,5-trimethoxy benzoyl amino cinnamic acid		1750–1770	Strong	5-Membered cyclic ketone
			1240–1280	Variable	C=O (stretching)
			1650–1680	Variable	C=N (stretching)
			1130–1170	Weak	3,4,5-Trisubstituted aromatic ring
II	Azlactone of 3,4,5-trimethoxy benzoyl α-amino p-dimethyl-amino cinnamic acid		1770	Strong	5-Membered cyclic ketone
			1240	Strong	C=O stretching
			1680–1690	Strong	C=N (stretching)
			1130–1170	Weak	3,4,5-Trisubstituted aromatic ring
			1630	Variable	Dimethylamino group
III	Azlactone of α-acetyl amino 2,3,4-trimethoxy cinnamic acid		1750	Strong	5-Membered cyclic ketone
			1260	Variable	C=O (stretching)
			1260–1670	Variable	C=N (stretching)
			1000–1170	Strong	1,2,3-Trisubstituted aromatic ring
			1350–1370	Strong	C—CH_3 (CH deformation)
IV	Azlactone of α-acetyl amino 2,4,5-trimethoxy cinnamic acid		1750–1770	Strong	5-Membered cyclic ketone
			1260–1280	Variable	C=O (stretching)
			1650–1680	Variable	C=N (stretching)
			1020–1040	Strong	2,4,5-Trisubstituted aromatic ring
			1350	Strong	C—CH_3 (CH deformation)
V	Azlactone of 3,4,5-trimethoxy benzoyl α-amino 2,4,5-trimethoxy cinnamic acid		1730	Strong	5-Membered cyclic ketone
			1250	Variable	C=O (stretching)
			1660	Strong	C=N (stretching)
			1020	Strong	2,4,5-Trisubstituted aromatic ring
			1140	Weak	3,4,5-Trisubstituted aromatic ring

All of the five azlactones give absorption bands of variable intensity in the region of 1750 to 1770 cm.$^{-1}$. This peak suggests the existence of a 5-membered saturated cyclic ketone (2). To lend support to the above fact a C=O stretching mode appears between 1240 and 1280 cm.$^{-1}$ with strong intensity, indicating the presence of a cyclic anhydride type C—O—C structure. Moreover, since all of these compounds are azlactones, the presence of C=N in the structure is essential, as shown by the C=N stretching vibrations of variable intensity at frequencies 1680 and 1690 cm.$^{-1}$.

Received August 4, 1966, from the Department of Physiology and Biochemistry, Medical College, Ajmer, Raj, India. Accepted for publication May 5, 1967.

Compound I gives the two characteristic bands at 1170 cm.$^{-1}$ and 1130 cm.$^{-1}$ of weaker intensity due to the presence of the 3,4,5-trisubstituted aromatic ring. Compound II shows in its spectra two bands at 1170 and 1130 cm.$^{-1}$ of the 3,4,5-trisubstituted aromatic ring, and the peak at 1630 cm.$^{-1}$ of weak intensity is due to the presence of the aromatic tertiary amine structure in the molecule. Compound III, being a 1,2,3-trisubstituted aromatic compound, shows absorption bands at 1000 and 1170 cm.$^{-1}$. Since there is a C-methyl group in the molecule, a peak at 1350 cm.$^{-1}$ due to CH deformation is observed. Compound IV, being a 2,4,5-trisubstituted derivative, gives absorption bands at 1000 and 1150 cm.$^{-1}$ and CH deformation for the C—CH_3 group at 1350 cm.$^{-1}$. With compound V, the bands of absorption at frequencies 1130 and 1000 cm.$^{-1}$ and 1170 to 1130 cm.$^{-1}$ clearly reveal that this compound contains two aromatic nuclei, both of which are trisubstituted.

REFERENCES

(1) Sharma, P. K., Menon, M. K., and Dandiya, P. C., *J. Pharm. Sci.*, **53**, 1055(1964).
(2) Bellamy, L., "The Infrared Spectra of Complex Molecules," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958.

Synthesis and Chemotherapeutic Effects of Ethyl Bis-(2,2-dimethyl)-ethylenamido Phosphate. A Preliminary Report

By Z. F. CHMIELEWICZ, T. J. BARDOS, A. MUNSON, H. L. BABBITT, and J. L. AMBRUS

The title compound, a new analog of the experimental anticancer agent, AB-132, was synthesized and tested against a spectrum of transplanted animal tumors. It was found to have significant chemotherapeutic activity in all of the tumor systems studied. In these assays, the new compound was a much more effective antitumor agent than AB-132.

ETHYL BIS(2,2 - DIMETHYL - 1 - AZIRIDINYL)PHOSPHINYL CARBAMATE (AB-132) was synthesized in this laboratory several years ago (1). This compound, although only moderately effective against various transplanted tumors in rodents, nevertheless showed promising therapeutic activity in the clinical testing against various forms of human cancer, particularly, in conjunction with X-irradiation (2–5). In view of these results, it appeared of interest to synthesize the title compound (I), in which the urethan moiety of AB-132 is replaced by an ethoxy group.

RESULTS AND DISCUSSION

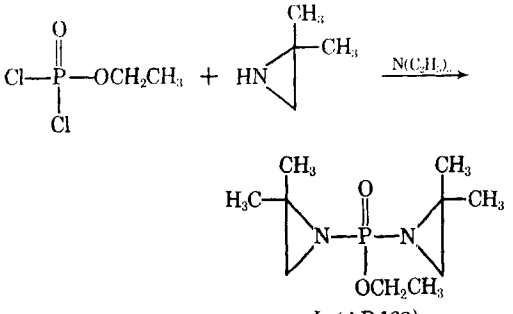
Ethyl bis(2,2-dimethyl)ethylenamido phosphate (I), designated as AB-163, was synthesized by the reaction of ethyl dichlorophosphate with 2,2-dimethylaziridine (in the presence of triethylamine) and purified by distillation under high vacuum, as described under *Experimental*. (Scheme I.)

The compound, a colorless, viscous oil, is readily soluble in water but, like other 2,2-dimethylaziridine derivatives, it undergoes rapid hydrolysis; it gradually decomposes even on exposure to air (humidity and carbon dioxide) and has to be stored in sealed containers. Its NMR spectrum shows the characteristic β-phosphorus splitting of the methylene protons, both in the aziridine rings and the ester group, and a singlet for the methyl substituents of the tertiary ring carbon.

In chemical alkylation studies, using 4-(*p*-nitrobenzyl)pyridine as a model nucleophile (6), AB-163 demonstrated a higher initial rate of alkylation compared to AB-132 (Fig. 1). Its higher chemical reactivity was paralleled by its greater toxicity. The LD_{50} of AB-163 in mice was 159 mg./Kg. (i.p.), in comparison to the 600 mg./Kg. LD_{50} value for AB-132.

The results of some of the antitumor studies are shown in Figs. 2–5. Details of the methods used in the animal assays will be published elsewhere.

Figure 2 shows the effects of various doses of AB-163 against Ehrlich ascites tumor in ICR/Ha mice. Treatment was started 24 hr. after inoculation, and the drug was administered intraperitoneally for 9 consecutive days. The animals were sacrificed on the 10th day and tumor cell counts were taken. Tumor inhibition was expressed as the per cent difference between the mean number of cells in the control group and the test group. At the optimal dose level (30 mg./Kg.), 95% inhibition was observed.



I (AB-163)

Scheme I

Received April 28, 1967, from the Departments of Medicinal Chemistry and Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214, and the Roswell Park Memorial Institute, Buffalo, NY 14203

Accepted for publication June 8, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by grant CA-06695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

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Synthesis and Chemotherapeutic Effects of Ethyl Bis-(2,2-dimethyl)-ethylenamido Phosphate. A Preliminary Report

By Z. F. CHMIELEWICZ, T. J. BARDOS, A. MUNSON, H. L. BABBITT, and J. L. AMBRUS

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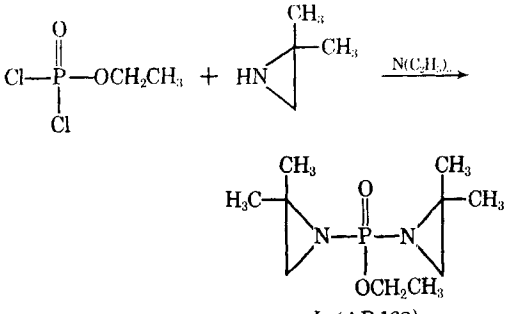
Ethyl bis(2,2-dimethyl)ethylenamido phosphate (I), designated as AB-163, was synthesized by the reaction of ethyl dichlorophosphate with 2,2-dimethylaziridine (in the presence of triethylamine) and purified by distillation under high vacuum, as described under *Experimental*. (Scheme I.)

The compound, a colorless, viscous oil, is readily soluble in water but, like other 2,2-dimethylaziridine derivatives, it undergoes rapid hydrolysis; it gradually decomposes even on exposure to air (humidity and carbon dioxide) and has to be stored in sealed containers. Its NMR spectrum shows the characteristic β-phosphorus splitting of the methylene protons, both in the aziridine rings and the ester group, and a singlet for the methyl substituents of the tertiary ring carbon.

In chemical alkylation studies, using 4-(*p*-nitrobenzyl)pyridine as a model nucleophile (6), AB-163 demonstrated a higher initial rate of alkylation compared to AB-132 (Fig. 1). Its higher chemical reactivity was paralleled by its greater toxicity. The LD_{50} of AB-163 in mice was 159 mg./Kg. (i.p.), in comparison to the 600 mg./Kg. LD_{50} value for AB-132.

The results of some of the antitumor studies are shown in Figs. 2–5. Details of the methods used in the animal assays will be published elsewhere.

Figure 2 shows the effects of various doses of AB-163 against Ehrlich ascites tumor in ICR/Ha mice. Treatment was started 24 hr. after inoculation, and the drug was administered intraperitoneally for 9 consecutive days. The animals were sacrificed on the 10th day and tumor cell counts were taken. Tumor inhibition was expressed as the per cent difference between the mean number of cells in the control group and the test group. At the optimal dose level (30 mg./Kg.), 95% inhibition was observed.



I (AB-163)

Scheme I

Received April 28, 1967, from the Departments of Medicinal Chemistry and Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214, and the Roswell Park Memorial Institute, Buffalo, NY 14203

Accepted for publication June 8, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by grant CA-06695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

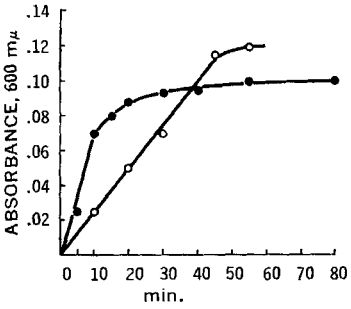


Fig. 1—Comparative reaction rates of ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinyl carbamate (AB-132) and ethyl bis(2,2-dimethyl)ethylenamido phosphate (AB-163) with 4-(p-nitrobenzyl)pyridine, at 80°. Standard initial concentrations: compound, 0.2 μmole/ml.; reagent, 240 μmole/ml. Key: O, AB-132; ●, AB-163.

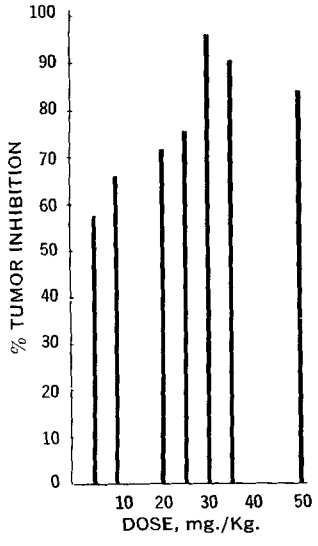


Fig. 2—Effect of AB-163 against Ehrlich ascites tumor (E-2) in ICR/Ha mice.

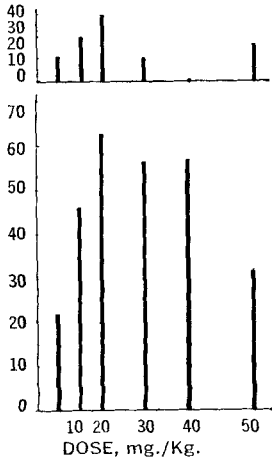


Fig. 3—Effect of AB-163 against sarcoma 180 in ICR/Ha mice. Top: per cent of animals without tumor, at various dose levels indicated on bottom abscissa. Bottom: per cent tumor inhibition in the remaining animals, at each of these dose levels.

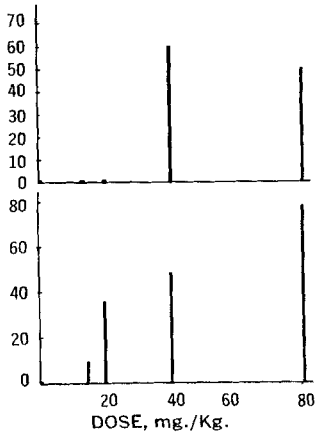


Fig. 4—Effect of AB-163 against adenocarcinoma 755 in 57B16 mice. Top: per cent of animals without tumor, at various dose levels indicated on bottom abscissa. Bottom: per cent tumor inhibition in the remaining animals, at each of these dose levels.

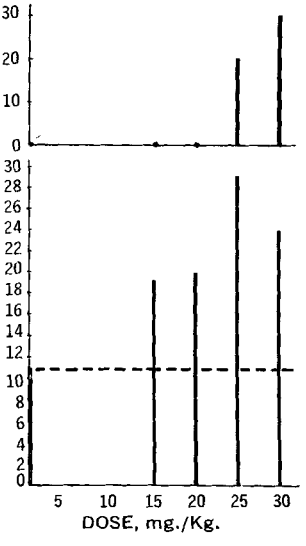


Fig. 5—Effect of AB-163 against leukemia L1210 in BDF1 mice. Top: per cent of animals surviving for 45 days, at various dose levels indicated on bottom abscissa. Bottom: per cent mean survival time of the remaining animals, at each of these dose levels. (Drug administered 1–11 days.)

Figure 3 shows the effect of the compound against sarcoma 180 in ICR/Ha mice. The drug was administered intraperitoneally for 7 consecutive days starting 24 hr. after tumor implantation. At the optimal dose range (15–25 mg./Kg.), 20–30% of the animals did not develop tumors while in the rest of the animals the tumors were 57–63% smaller (by weight) as compared to the controls.

Figure 4 illustrates the results obtained against adenocarcinoma 755 in 57B16 mice. Treatment was continued daily from day 1 to 11 following tumor implantation. At 40–80 mg./Kg. dose levels, 55–60% of the treated animals had no tumors

while the rest of the treated animals showed 48–77% inhibition of tumor growth as compared to the controls.

Figure 5 shows the effect of AB-163 against leukemia L1210 in BDF1 mice. In this experiment treatment was continued daily from day 1 to 11 post-inoculation. At optimal dose range, 25–30 mg./Kg., 20–30% of the animals survived for more than 45 days and are considered "cures." The remaining animals showed an average increase of survival time of 107–170%.

These results demonstrate that AB-163 is a much more effective compound against a spectrum of transplanted tumors than was AB-132(2).

EXPERIMENTAL

Into a 1-L. round-bottom flask equipped with a mechanical stirrer, pressure equalized addition funnel, thermometer, and a calcium chloride drying tube was introduced a solution of 15.6 Gm. (0.22 mole) of 2,2-dimethylaziridine, 22.2 (0.22 mole) of triethylamine in 350 ml. of toluene. The content of the flask was cooled to 0° by immersion in a mechanically refrigerated bath maintained at −3° to 0°, and a solution of 16.3 Gm. (0.1 mole) of ethyl dichlorophosphate in 100 ml. of toluene was slowly added. The reaction mixture was stirred for 24 hr. and then slowly warmed to room temperature and filtered. The precipitate contained nearly the theoretical amount of pure triethylamine hydrochloride. The filtrate was concentrated in a flash evaporator under reduced pressure, and the crude product was obtained as a viscous oil. The material was purified by high vacuum distillation and the analytically pure sample was obtained by collecting the fraction boiling at 70–71°/0.03–0.04 mm.; yield 17.3 Gm. (74%). This distillation had to be conducted with care because of the tendency of the material to decompose on overheating.

Anal.—Calcd. for $C_{10}H_{21}N_2O_2P$: C, 51.72; H, 9.05; N, 12.07. Found: C, 51.60; H, 9.18; N, 11.94.

Infrared absorption bands (Beckman IR-8): $\nu_{max.}^{CHCl_3}$ 2990, 1460 (broad), 1385, 1375, 1340, 1265, 1210 (broad), 1145, 1105, 1035, 960, 840 cm.$^{-1}$.

NMR absorption bands (in CCl_4, with tetramethylsilane as internal standard): 8.78 triplet (ester CH_3); 8.65 singlet (CH_3 ring-substituents); 8.05 doublet, P_H = 14 cps. (ring CH_2); 6.05, multiplet (ester CH_2).

Alkylation Studies—Comparative alkylating activities of I and AB-132 were determined at 80° by a previously described method (6).

REFERENCES

(1) Bardos, T. J., Barua, A. K., Chmielewicz, Z. F., Crevar, G. E., Dailey, J. P., Divald, S., and Papanastassiou, Z. B., *J. Pharm. Sci.*, **54**, 187(1965).
(2) Bardos, T. J., and Ambrus, J. L., in "IIIrd International Congress for Chemotherapy," vol. II, Kummerle, H. P., ed., Hafner Publishing Co., New York, N. Y., 1964, p. 1036.
(3) Ross, C. A., Velasco, H. A., Ambrus, J. L., Stutzman, L., Webster, J., and Bardos, T. J., *Proc. Am. Assoc. Cancer Res.*, **3**, 355(1962).
(4) Ross, C. A., Velasco, H. A., Webster, J. H., Ambrus, J. L., and Sokal, J. E., *ibid.*, **4**, 59(1963).
(5) Velasco, H. V., Ross, C. A., Webster, J. H., Sokal, J. E., Stutzman, L., and Ambrus, J. L., *Cancer*, **17**, 841 (1964).
(6) Bardos, T. J., Datta-Gupta, N., Hebborn, P., and Triggle, D. J., *J. Med. Chem.*, **8**, 167(1965).

Determination of Ephedrine, Phenobarbital, and Theophylline in Tablets by Gas Chromatography

By MILTON ELEFANT, LESTER CHAFETZ, and JOSEPH M. TALMAGE

A gas chromatographic procedure is described for the rapid, specific, and precise determination of ephedrine, phenobarbital, and theophylline in tablets. Ephedrine is converted to benzaldehyde by periodate oxidation prior to chromatography, while the phenobarbital and theophylline are chromatographed directly after simple tablet disintegration in a co-solvent. Adaptation of the method to single tablet analysis is described.

THE PROBLEM of devising a convenient and accurate assay for combinations of ephedrine, a barbiturate, and theophylline or its complexes has become classical in the literature of pharmaceutical analysis. The literature has been reviewed by Connors (1) and, more recently, by Foreman and Blake (2). Most of the solutions described so far require lengthy and tedious liquid–liquid extractions, ion-exchange chromatography, and/or liquid–liquid partition chromatographic separations followed by quantitation of the separated drugs by gravimetry, titrimetry, or spectrophotometry. Since gas chromatography combines separation with quantitation, it appeared to be a singularly attractive means for assay of a tablet declaring ephedrine HCl, phenobarbital, and theophylline.

Gas chromatographic methods have been successfully applied to assay of barbiturates (3–7) and ephedrine (8–11); however, mixtures of ephedrine, phenobarbital, and theophylline present more difficulties in gas chromatography than the assay of the individual drugs because of their differences in functionality, polarity, and dose. The polar, strongly basic amine, ephedrine, tails badly on columns which give good separation and symmetrical peaks for the weakly acidic barbiturate and xanthine drugs. Ephedrine is quantitatively oxidized by periodate, and this observation has been made the basis of quantitative assays of the alkaloid by Wickström (12), who determined the acetaldehyde formed by colorimetry, and by Chafetz (13),

Received March 10, 1967, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950
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while the rest of the treated animals showed 48–77% inhibition of tumor growth as compared to the controls.

Figure 5 shows the effect of AB-163 against leukemia L1210 in BDF1 mice. In this experiment treatment was continued daily from day 1 to 11 post-inoculation. At optimal dose range, 25–30 mg./Kg., 20–30% of the animals survived for more than 45 days and are considered "cures." The remaining animals showed an average increase of survival time of 107–170%.

These results demonstrate that AB-163 is a much more effective compound against a spectrum of transplanted tumors than was AB-132(2).

EXPERIMENTAL

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(1) Bardos, T. J., Barua, A. K., Chmielewicz, Z. F., Crevar, G. E., Dailey, J. P., Divald, S., and Papanastassiou, Z. B., *J. Pharm. Sci.*, **54**, 187(1965).
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By MILTON ELEFANT, LESTER CHAFETZ, and JOSEPH M. TALMAGE

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Table I—Analysis of a Production Lot of Tablets

	Ephedrine HCl	Phenobarbital	Theophylline
Declared, mg./tablet	24.0	8.0	130.0
Found			
Chemical assay	23.7	7.9	130.0
GC assay trial *1*	23.4	7.9	132.0
2	23.6	7.8	135.0
3	23.8	7.8	135.0
4	23.8	7.8	129.0
5	23.6	7.7	133.8
GC av.	23.6	7.8	133.0
Relative S.D., %	±0.72	±0.9	± 1.9

who measured the benzaldehyde produced on oxidation by ultraviolet spectrophotometry.

Gas chromatography of the benzaldehyde produced by oxidation of ephedrine provided sharp peaks suitable for quantitation on a chromatographic column useful for phenobarbital and theophylline. Benzaldehyde conveniently chromatographed at 80° while the ureides are chromatographed at 250°. Moreover, it was difficult to find an oxidation medium for ephedrine which would serve also as a good solvent for eluting phenobarbital and theophylline from tablets. For these reasons, separate sample preparations and separate determinations were performed for ephedrine and the other two drugs. The use of separate chromatograms allowed use of closely resolved internal standards, with an advantage in precision and accuracy over programmed temperature technique. A procedure for assay of the three drugs in individual tablets is described.

EXPERIMENTAL

Instrumental Conditions—A Perkin-Elmer model 801 gas chromatograph equipped with dual flame ionization detectors and a Honeywell-Brown Electronik recorder was used. The column employed was a 6-ft. glass helix, 0.25 in. o.d., packed with 3% HI-EFF 8 BP on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Gas flow rates of 30 ml./min. for hydrogen and 40 ml./min. for helium were used.

Phenobarbital and Theophylline Assay—*Internal Standard*—Use an approximately 0.6 mg./ml. solution of 4,4′-methylene-bis-(*N*,*N*-dimethylaniline) (Eastman Organic) in equal volumes of chloroform and methanol.[1]

Mixed Standard—Dissolve accurately weighed amounts of about 13 mg. of phenobarbital and 200 mg. of anhydrous theophylline in 25.0 ml. of the internal standard solution.

Sample Preparation—Determine the average weight of not less than 10 tablets, and reduce the sample to a fine powder. Weigh accurately the equivalent of one average tablet weight of the composite sample, and transfer it to a 50-ml. glass-stoppered centrifuge tube. Add exactly 15.0 ml. of internal standard solution, stopper the mixture, and shake vigorously for 5 min. Centrifuge the mixture and use the clear supernatant solution for chromatography.

[1] The compound should be stored under nitrogen after opening to protect it from oxidation.

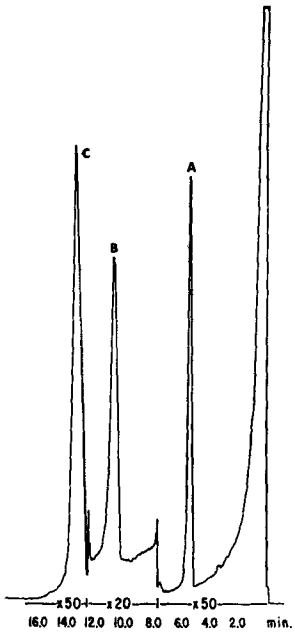


Fig. 1—Key: A, *internal standard;* B, *phenobarbital;* C, *theophylline.*

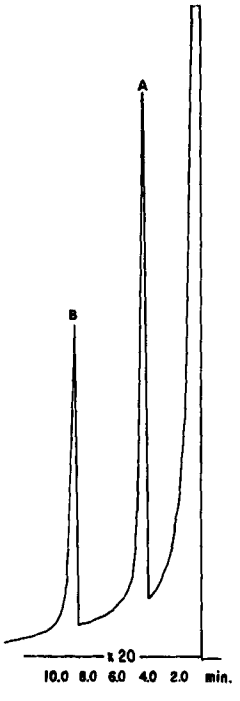


Fig. 2—Key: A, *benzaldehyde;* B, *internal standard.*

Procedure—Set the injector temperature at 325° and the column temperature at 250°. Inject in turn 2-μl. volumes of the sample and standard solutions at preselected attenuation settings.

Ephedrine Determination—*Internal Standard*—Use a solution of 0.06 ml. of benzyl alcohol in 100 ml. of ethyl acetate.

Standard Preparation—Dissolve approximately 65 mg. of ephedrine HCl, accurately weighed, in 25.0 ml. of saturated sodium bicarbonate solution.

TABLE II—ASSAY OF INDIVIDUAL TABLETS

Trial	Ephedrine HCl	Phenobarbital	Theophylline
1	23.6	7.7	127.0
2	23.2	7.6	128.0
3	24.1	7.7	127.0
4	23.9	7.6	127.0
5	23.8	7.7	127.0
6	23.8	7.6	127.0
7	23.6	7.8	130.0
8	23.6	7.8	126.0
9	23.4	7.7	126.0
10	23.8	7.8	126.0
Av.	23.7	7.7	127.0
Relative S.D.	1.1%	1.0%	0.9%

Pipet 10.0 ml. into a glass-stoppered centrifuge tube.

Sample Preparation—Accurately transfer the equivalent of one average tablet weight of composite sample into a glass-stoppered centrifuge tube. Add exactly 10.0 ml. of saturated sodium bicarbonate solution.

Procedure—Add 2.0 ml. of 10% sodium metaperiodate to the sample and standard preparations and shake 5 min. Pipet 10.0 ml. of internal standard solution into each tube, stopper, and shake for 3 min. Centrifuge the mixtures, and use the clear upper phases for chromatography.

Set the injector block temperature at 175° and the column temperature at 80°, and inject approximately 0.5 μl. of sample and standard solutions in turn. Program the column temperature at 6°/min. to a final temperature of 130° immediately after injection of each sample.

Assay of Individual Tablets—The procedures described above were employed with one modification; the single tablet was dispersed in the appropriate solvent by immersing the centrifuge tube in an ultrasonic cleaning bath (Maxomatic Ultrasonic, L. & R. Manufacturing Co., Kearny, N.J.).

Quantitation—Peak heights were employed according to the method described by Celeste and Turczan (14).

Sample—A commercially available tablet,[2] which has a label declaration of 24 mg. ephedrine HCl, 8.0 mg. phenobarbital, and 130 mg. of theophylline, was the only test preparation employed.

[2] Tedral is a registered trademark of Warner-Chilcott Laboratories, Morris Plains, N.J.

RESULTS

A production lot of tablets was analyzed by the gas chromatographic procedure, and the results were compared with values obtained by the conventional quality control methods. The data are presented in Table I. Figures 1 and 2 show typical chromatograms for phenobarbital and theophylline and for ephedrine, respectively.

The method was employed with equal success for the assay of individual tablets. The data obtained from assay of 10 individual tablets are presented in Table II. The average results and the relative standard deviations obtained do not differ appreciably from those obtained from powdered composite tablets.

SUMMARY

A gas chromatographic method has been developed for the determination of ephedrine, phenobarbital, and theophylline, and its application to the assay of composite and individual tablets has been described for a commercial tablet dosage form. Ephedrine is chromatographed after quantitative periodate oxidation to benzaldehyde at a lower temperature than the ureide drugs; thus, the method requires two sample preparations, two chromatograms, and one chromatography column. The procedure provided relative standard deviations of about 1% for each of the components of individual tablets, and it is rapid and selective.

REFERENCES

(1) Connors, K. A., in "Pharmaceutical Analysis," Higuchi, T., and Brochmann-Hanssen, E., eds., Interscience Publishers, New York, N.Y., 1961, pp. 239–240.
(2) Foreman, R., and Blake, M. I., *J. Pharm. Sci.*, **54**, 12(1965).
(3) Brochmann-Hanssen, E., and Svendsen, A. B., *ibid.*, **51**, 318(1962).
(4) Ceplinski, E. W., *Anal. Chem.*, **35**, 256(1963).
(5) Parker, K. D., Fontan, C. R., and Kirk, P. L., *ibid.*, 418(1963).
(6) Gudzinowicz, B. J., and Clark, S. J., *J. Gas Chromatog.*, **May**, 147(1965).
(7) Braddock, L. I., and Morec, N., *ibid.*, **August**, 274 (1965).
(8) Lawless, G. B., Sciarra, J. J., and Monte-Bovi, A., *J. Pharm. Sci.*, **54**, 273(1965).
(9) Brochmann-Hanssen, E., and Svendsen, A. B., *ibid.*, **51**, 938(1962).
(10) Parker, K. D., Fontan, C. R., and Kirk, P. L., *Anal. Chem.*, **34**, 1345(1962).
(11) *Instrument News*, **16**, (3), 1–4(1966), Perkin-Elmer Corp.
(12) Wickström, A., *Ann. Pharm. Franc.*, **8**, 86(1950).
(13) Chafetz, L., *J. Pharm. Sci.*, **52**, 1193(1963).
(14) Celeste, A., and Turczan, J., *J. Assoc. Offic. Agr. Chemists*, **46**, 1055(1963).

Chemical Study of the Latex, Stems, Bracts, and Flowers of "Christmas Flower" (*Euphorbia pulcherrima*) I

By XORGE A. DOMINGUEZ, JESUS GARCIA DELGADO, MA. DE LOURDES MAFFEY, JOSÉ G. MARES, and CARLOS ROMBOLD

The latex, stems, bracts, and flowers of *Euphorbia pulcherrima* Willd were chemically studied. Each part was extracted separately with petroleum ether. All the extracts contained germanicol, β-amyrin, and pseudotaraxasterol. From the latex was obtained a new sterol, $C_{24}H_{40}O$, pulcherrol. From the extracts of the stems were isolated an octaeicosanol and β-sitosterol.

In Mexico and Central America there are many native members of the *Euphorbiaceae*, particularly of the genus *Euphorbia* (which has over 1600 species) (1, 2), among them the *Euphorbia pulcherrima* Willd, called "flor de pascua" (Christmas flower). The bracts of this plant were used as galactopoietic and abortive agents by the Aztecs and they are still used for both purposes in medicine popular in some parts of Mexico (3, 4). Because of its colorful red bracts, the *E. pulcherrima* is actually cultivated in many parts of the world, being used as a flower arrangement at Christmastime, but there are only a few chemical studies of this plant. It has been reported that the ethanolic extracts of its petals have insecticidal properties and that its leaves contain alkaloids (5). Asen (6, 7) has identified by chromatographic and spectrophotometric methods four anthocyanins present in its bracts. The purpose of this work was to look for compounds with possible hormonal activity in different parts of the plant. From the petroleum extracts of the latex, bracts, and stems, β-amyrin, germanicol, and pseudotaraxasterol were isolated. In addition to these compounds, the latex provided a new steroid, $C_{24}H_{40}O$, for which the name, pulcherrol, is proposed. Also, in the extracts of the stems an octaeicosanol and β-sitosterol were identified.

EXPERIMENTAL

Melting points (uncorrected) were determined on a Kofler hot stage. Infrared spectra were taken on a Beckman IR-8 spectrophotometer and NMR spectra were obtained on a Varian A-60 instrument,[1] with tetramethylsilane as an internal standard and deuterochloroform as a solvent. Thin-layer chromatograms (TLC) were run on Silica Gel G (Merck), according to Stahl (8), and the system benzene–chloroform (7:1) (v/v) was used as solvent *A*.

Authentic specimens of triterpenoids were kindly supplied by Dr. Guy Ourisson and Dr. G. Poinsinet, Strasbourg University, and Dr. P. De Mayo, University of Western Ontario, Canada, whose assistance is acknowledged.

Microanalysis was by Dr. A. Bernhardt, Milheim/Ruhr, Germany. Identification methods and reactions were those described by Cheronis (9), and for the triterpenoids the methods used have been described by Steiner (10).

Plant Materials—The aerial part (stems, bracts, and flowers) and latex of *E. pulcherrima* were collected around Monterrey, Nuevo León, Mexico, in December 1963, 1964, and 1965.

Extraction of the Latex—The stems and terminal twigs were cut, and the flowing milky latex was collected. The latex (15 Gm.) was refluxed 12 hr. with methanol (150 ml.) and KOH (6 Gm.). The suspension was evaporated under reduced pressure, and the gummy residue was extracted five times with 50-ml. portions of ethyl ether. To the residue, water (100 ml.) was added, and a rubber-like material (9.8 Gm.) was obtained. The water solution was acidified with 5% HCl and an oily suspension (10.8 Gm.) was obtained; its principal component was palmitic acid which was isolated and identified by its physical constants and the melting point of its anilide (9). The combined ethyl ether solutions were washed with water and dried with Na_2SO_4. On TLC (solvent *A*) seven spots were detected with iodine vapors and also with 20% $SbCl_3$ in chloroform. The residue (2.8 Gm.), obtained on the removal of the ether, was dissolved in hot methanol–chloroform (20:1). On standing overnight, colorless crystals (1.45 Gm.) of crude pulcherrol were obtained, m.p. 110–116°. The mother liquors produced six spots on TLC; then the filtrate was evaporated and the residue (1.0 Gm.) was chromatographed on silica gel (150 Gm.). The substances were eluted successively by benzene, benzene–chloroform, and chloroform. A total of 36 fractions were collected; their composition was monitored by TLC. After rechromatography of several fractions and the corresponding recrystallization of the eluates, germanicol (23.5%), β-amyrin (9%), and pseudotaraxasterol (22%) were obtained.

Identification of the Known Substances (11)—Germanicol recrystallized from methanol, m.p. 178–180°; $[\alpha]_D^{24}$ +8°. [Reported (12) m.p. 180–181°; $[\alpha]_D^{24}$ +7°.] Acetylation with acetic acid anhydride in pyridine (12) produced an acetate, m.p. 276–280°; $[\alpha]_D^{24}$ +11°. [Reported (12) m.p. 279–280°; $[\alpha]_D^{24}$ +18°.] Mixed melting points with authentic germanicol and its acetate showed no depression.

β-Amyrin recrystallized from methanol, m.p. 197–199°; $[\alpha]_D^{24}$ +82°. [Reported (11) m.p. 199–200°; $[\alpha]_D^{24}$ +88°.] Acetylation with acetic acid anhydride in pyridine produced an acetate, m.p. 238–241°; $[\alpha]_D^{24}$ +81°. [Reported (11) m.p. 241°; $[\alpha]_D^{24}$ +81°.] Mixed melting point with an authentic β-amyrin showed no depression.

Received February 27, 1967, from the Institute of Technology of Superior Studies, Monterrey, N. L., Mex.
Accepted for publication June 6, 1967.
This work was supported by a grant from the Research Corporation of New York.
Presented to the Meeting of Plant Phenolics Group of North America, Austin, Tex., April 17, 1966.
[1] Spectra obtained by Dr. Roger Ketcham, School of Pharmacy, University of California, whose assistance is gratefully acknowledged.

Pseudotaraxasterol, recrystallized from ethanol, melted at 220–222°; $[\alpha]_D^{24}$ +46°. [Reported (11) m.p. 220–221°; $[\alpha]_D^{24}$ +50°.] Acetylation with acetic acid anhydride in pyridine (12) produced an acetate, m.p. 244–246°; $[\alpha]_D^{24}$ +50.4°. [Reported (11) m.p. 246–247°; $[\alpha]_D^{24}$ +53°.] Mixed melting point with an authentic pseudotaraxasterol showed no depression.

Purification of Pulcherrol—Pulcherrol was recrystallized from ethanol–chloroform and colorless needles, m.p. 115–116°, were obtained. It produced a single spot on TLC and gave positive results for the Liebermann-Burchard test for steroids and the tetranitromethane test for unsaturation. Negative results were obtained from the selenium dioxide–acetic acid test, for Δ^7 steroids (13); $[\alpha]_D^{24}$ (in $CHCl_3$) +35°. No absorption in the U.V.; infrared absorption: $\nu_{max.}^{CHCl_3}$ 3600, 2950, 2850, 1470, 1370, 1010 cm.$^{-1}$.

Anal.—Calcd. for $C_{24}H_{40}O$: C, 83.65; H, 11.70; O, 4.64. Found: C, 83.73; H, 11.59; O, 4.68.

The NMR spectrum: 5.2 (multiplet), 6.52 τ (singlet), 7.9–9.2 τ (38H, complex with five singlet signals).

Pulcherryl Acetate—Pulcherrol (100 mg.) was mixed with acetic anhydride (5 ml.) and pyridine (1.0 ml.). After it stood at room temperature for 12 hr., the mixture was diluted with water. The crude acetate was collected and recrystallized several times from methanol. The bright plates melted at 228–229°. I.R. spectrum: $\nu_{max.}^{CHCl_3}$ 2950, 2850, 1720, 1465, 1455, 1370, 1250, 1200, 1100, 1020, 980 cm.$^{-1}$. The NMR spectrum: 2.5 τ (multiplet) 7.9 τ (singlet, 3H), 3.1–9.2 τ (38H complex).

Anal.—Calcd. for $C_{26}H_{42}O_2$: C, 80.77; H, 10.95; O, 8.28. Found: C, 80.66; H, 10.74.

Oxidation of Pulcherrol—Pulcherrol (100 mg.) in pyridine (1 ml.) was mixed with 4 ml. of chromic acid–pyridine–acetic acid complex (14), and the temperature was held at 0° for 1 hr. Methanol (2 ml.) was added, then the mixture was diluted with water, and the ketone was recovered as usual. On recrystallization from methanol, needles (80 mg.), m.p. 151–152°, were obtained. I.R. spectrum: $\nu_{max.}^{CHCl_3}$ 2950, 2850, 1700, 1465, 1460, 1380, 1110, 1005 cm.$^{-1}$.

Anal.—Calcd. for $C_{24}H_{38}O$: C, 84.15; H, 11.18; O, 4.67. Found: C, 84.33; H, 11.28.

Epoxide of the Pulcherryl Acetate—A chloroform solution of pulcherryl acetate (50 mg.) was mixed with 50 mg. of *m*-chloroperbenzoic acid and 5 ml. of chloroform. The mixture stood at room temperature for 12 hr. Then, 5% NaOH solution (25 ml.) was added. The chloroform layer was rinsed with water, dried with Na_2SO_4, and evaporated. The residue was recrystallized from methanol–chloroform, m.p. 267–269°.

Anal.—Calcd. for $C_{24}H_{40}O_2$: C, 79.94; H, 11.19; O, 8.88. Found: C, 80.07; H, 11.26; O, 8.69.

Extraction of Stems—Dried stems (1400 Gm.) of *E. pulcherrima* were extracted in a Soxhlet apparatus with petroleum ether (b.p. 30–60°) for 100 hr. The green solution was evaporated and the greenish residue (7.0 Gm., yield 0.5%) was saponified as described for the latex, but the unsaponifiable material was extracted with several portions of chloroform. The chloroform solutions were mixed and added to a column of alumina (Merck). The elutions were obtained in the usual way (10) with benzene, chloroform, ethyl acetate, and ethanol. From the first fraction was obtained an aliphatic alcohol, 326 mg., which after recrystallization from chloroform–methanol melted at 63–65°.

Anal.—Calcd. for $C_{28}H_{58}O$: C, 81.87; H, 14.23; O, 3.90. Found: C, 81.53; H, 14.12; O, 3.86.

Also there were eluted and identified, as mentioned for the latex, germanicol, 370 mg., m.p. 177–179°; β-amyrin, 825 mg., m.p. 198–200°; pseudotaraxasterol, 263 mg., m.p. 237–240°; and β-sitosterol, 108 mg., m.p. 134–136°; $[\alpha]_D^{CHCl_3}$ −37°, from which β-sitosteryl acetate was produced by acetylation, in the usual manner (11); it melted at m.p. 125–127°. There was no depression of the melting point when each compound was mixed with an authentic specimen.

Extraction of Bracts and Flowers—Dried flowers and bracts (1700 Gm.) were extracted with petroleum ether (b.p. 30–60°) in a Soxhlet apparatus during 160 hr. The solution, containing 22.85 Gm. of solids, and showing five spots on TLC, was chromatographed on alumina (Merck). The fractions were eluted by benzene, chloroform, and ethanol. The crude ester mixtures, detected by I.R. spectroscopy on several fractions, were saponified with KOH in methanol as usual (10). The unsaponifiable material was extracted with ethyl ether and the solution was chromatographed on alumina. From the eluates there was obtained and identified germanicol (1.32 Gm.); β-amyrin (0.386 mg.); pseudotaraxasterol (649 mg.); the same aliphatic alcohol of 28 carbons isolated from the stems (64 mg.); and an aliphatic hydrocarbon, melting at 75–77°.

REFERENCES

(1) Lawrence, G. H. M., "Taxonomy of Vascular Plants," The Macmillan Co., New York, N. Y. 1931, p. 569.
(2) Poinsinet, G., and Ourisson, G., *Phytochemistry*, **4**, 799(1965).
(3) Martinez, M., "Las Plantas Medicinales de México," 4th ed., Editorial Botas, México, 1959, p. 137.
(4) Stanley, P., "Trees and Shrubs of Mexico," Carnegie Institute, Washington, D. C., 1924, p. 600.
(5) Rao, D. S., *Econ. Botany*, **10**, 274(1956).
(6) Asen, S., *Plant Physiol.*, **33**, 14(1958).
(7) Asen, S., *J. Chromatog.*, **18**, 602(1965).
(8) Stahl, E., "Dunnschicht Chromatographie," Springer-Verlag, Berlin, Germany, 1964, p. 338.
(9) Cheronis, N., and Entriken, J. B., "Semimicro Qualitative Organic Analysis," 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1957.
(10) Steiner, M., and Holtzem, H., in "Moderne Methoden der Pflanzenanalyse," vol. 3, Paech, K., and Tracy, M. V., eds., Springer-Verlag, Berlin, Germany, 1956, p. 59.
(11) Djerassi, C., and Mills, J. S., *J. Am. Chem. Soc.* **80**, 1241(1958).
(12) Heilborn, I., Cook, A. H., and Bunbury, H. M., "Dictionary of Organic Compounds," 4th ed., Eyre & Spottiswoode, London, England, 1965.
(13) Fieser, L. F., and Fieser, M., "Steroids," Reinhold Publishing, New York, N. Y., 1959.
(14) Stensio, K. E., and Wachtmeister, C. A., *Acta Chem. Scand.*, **18**, 1063(1964).

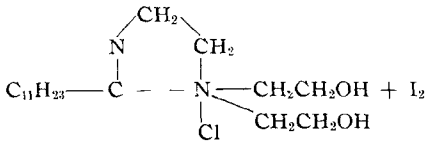
Toxicology of the Iodophor, Imidecyl Iodine

By T. A. FAKOUHI*, C. GRIFFIN†, R. S. McCUTCHEON‡, and J. F. BONE

The iodophor imidecyl iodine is relatively nontoxic in rats, guinea pigs, rabbits, and dogs. In rats, calculated oral LD_{50} for males was 14.0 ± 2.1 mg./Kg., and for females 11.0 ± 1.5 mg./Kg. Dermal, eye, skin, and chronic toxicity studies were essentially negative. Urinary bladder studies in rabbits terminated fatally in two animals given undiluted and 1:2 dilutions in water. Dilutions of from 1:10 to 1:64 were progressively decreasing local irritants. Microscopic lesions in LD_{50} studies included pulmonary emphysema, ascites, distended stomach, hyperemia, and parenchymal damage in the liver and kidneys. Lesions in rabbits were compatible with shock. Lesions in chronic studies were mainly mild kidney damage. Three dogs fed imidecyl iodine for 3 to 5 months were unaffected.

FOR MANY YEARS, iodine has been used as a topical germicide and antiseptic. Although it is highly effective against fungi, bacteria, and viruses, it also has some detrimental attributes in the elemental form (1). It is irritating to body tissues and has objectionable odor and staining properties, and thus has given way to other antiseptics with less offensive side effects.

The combination of iodine with certain detergents greatly reduces objectionable side effects while retaining most of the desirable qualities of iodine (2). One such preparation, the iodophor imidecyl iodine[1] is a germicidally effective combination of iodine with an amphoteric detergent possessing the approximate structural formula:



There appears to be a positive chemical interaction between the detergent and the elemental iodine which results in a nearly complete loss of the caustic, corrosive, staining, and physically unstable properties of the halogen. The toxicity of this compound, however, has not been completely investigated.

This paper deals with the toxicological properties of imidecyl iodine and its effect upon various tissues in several species under various conditions and routes of administration. Specifically the studies involve determination of the LD_{50} in rats, dermal toxicity in rats, intradermal sensitivity reactions in guinea pigs, toxicity to ocular tissues of adult and newborn rabbits, toxicity when instilled into the urinary bladder of adult rabbits, and chronic toxicity feeding trials in dogs.

METHODS

LD_{50} in Rats—The oral LD_{50} was determined for each sex of adult Wistar rats in good physical condition. The animals were starved for 24 hr. and then given graduated solutions of imidecyl iodine *via* stomach tube. One hour after receiving the drug, the animals were allowed food and water *ad libitum*. Controls were run with each group and surviving animals closely observed for 14 days. LD_{50} values were calculated by the method of Reed and Muench (3). Fatalities were examined postmortem and outstanding lesions were noted. Results are shown in Table I.

TABLE I—ORAL LD_{50} OF IMIDECYL IODINE

Species and Sex	Dose, ml./Kg.	Observed Deaths	Calcd. LD_{50}, ml./Kg.
Rat, male	4.0	0/5	14.0 ± 2.1
	10.0	1/5	
	12.5	2/5	
	15.0	3/5	
	25.0	5/5	
Rat, female	4.0	0/5	11.0 ± 1.5
	10.0	2/5	
	15.0	4/5	
	25.0	5/5	

Acute Dermal Toxicity—The abdomens and backs of five Wistar rats of both sexes were shaved and the skin abraded. Specially fitted rubber sleeves were placed over the lesions to prevent the loss of material from the skin areas being tested. Five milliliters of imidecyl iodine in various concentrations, *e.g.*, undiluted, 1:5, and 1:10, was placed under the sleeves, and 5 ml. of saline applied in the same manner served as the control. The animals were observed at 1, 4, 24, and 48-hr. intervals after the application for signs of edema, erythema, vesiculation, and other signs of irritation.

Guinea Pig Sensitization—Five adult female guinea pigs were used to determine sensitivity. Concentrations of undiluted, 1:10, and 1:15 of imidecyl iodine in distilled water were injected intradermally. One animal was kept as control. Each animal received 0.05 ml. of imidecyl iodine intradermally as the first dose, followed by a dose of 0.125 ml. daily for 10 days. The sensitizing injection (0.05 ml.) was given 10 days after the last dose.

Eye Toxicity—*Adult Rabbits*—Twelve adult male domestic rabbits were used to determine if local eye irritation was produced by imidecyl iodine. Four strengths of the drug were employed, *e.g.*, undiluted, 1:10, 1:15, 1:25. One instillation of 0.3 ml. was made in the left eye; the right eye received 0.3 ml. of normal saline and served as control. The corneas, irises, and conjunctivae of each animal were observed for erythema, vesiculation, edema, or other signs of irritation at 1 hr., 24 hr., 96 hr., and 168 hr. after instillation. The degree of toxicity was evaluated by the method of Draize *et al.* (4).

Newborn Rabbits—Three drops of 1:64 dilution of

Received March 20, 1967, from the Department of Veterinary Medicine, and ‡ the College of Pharmacy, Oregon State University, Corvallis, OR 97331
Accepted for publication June 6, 1967.
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TABLE II—ADULT RABBIT EYE TOXICITY

Concn. of Imidecyl Iodine	Area of the Eye	Rabbit 1					Rabbit 2					Rabbit 3				
		Hr. After Imidecyl Iodine Application														
		1	24	48	96	168	1	24	48	96	168	1	24	48	96	168
Undiluted	Cornea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Iris	5	2	0	0	0	5	1	0	0	0	4	3	0	0	0
	Conjunctiva	4	3	1	0	0	4	3	1	0	0	5	5	1	0	0
1:10	Cornea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Iris	5	2	0	0	0	5	1	0	0	0	3	3	1	0	0
	Conjunctiva	2	1	0	0	0	2	0	0	0	0	5	5	5	0	0
1:15	Cornea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	3	2	0	0	0	1	1	0	0	0
	Conjunctiva	2	0	0	0	0	5	5	2	0	0	4	3	0	0	0
1:25	Cornea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	1	2	0	0	0	2	2	0	0	0
	Conjunctiva	0	1	0	0	0	4	4	0	0	0	4	4	1	0	0

imidecyl iodine was instilled in the left eye of five newborn rabbits (7–10 days old) four times daily for 5 days. The right eye served as a control. The corneas, irises, and conjunctivae were observed for erythema, vesiculation, edema, and other signs of irritation during the days of administration and daily thereafter for 2 months. The degree of toxicity was evaluated by the method of Draize *et al.* (4).

Rabbit Bladder Toxicity—Fifteen milliliter solutions of the following strengths of imidecyl iodine, *e.g.*, undiluted, 1:2, 1:10, 1:20, and 1:40 were each used in two animals and a dilution of 1:64 was used in four animals and of 1:128 in two animals. In each case the solutions were placed in an empty bladder of male and female adult domestic rabbits through a urethral catheter. The solutions were kept in the bladder for 30 min., after which the bladder was evacuated by catheter if urination was not spontaneous. Three animals given equal volumes of saline were used as controls. Each animal received three treatments at intervals of 24 hr., and 24 hr. after the last treatment, was killed and examined grossly and microscopically for abnormalities.

Chronic Toxicity—A group of three mature male dogs maintained on a basic diet of Purina laboratory chow (Ralston-Purina, St. Louis, Mo.) were given 1:15 dilutions of imidecyl iodine four times weekly. Dosage level was based on extrapolation of the calculated LD_{50} for rats. During the course of this experiment, the animals were examined daily for appearance, behavior, weight gain, and food consumption (5). After 90 days, one animal was sacrificed. At autopsy the entire carcass was examined grossly and the heart, lung, liver, kidney, spleen, and brain were removed and weighed, and portions of these organs were prepared for histological examination. The two other animals were sacrificed and examined in the same way at the end of 5 months.

RESULTS

The median lethality (LD_{50}) of imidecyl iodine for rats is given in Table I. The deaths were usually very rapid; nearly all fatalities were found within 18 hr. after dosage. No deaths occurred in treated animals after the ninth day. Prior to death, the rats showed depression, piloerection, and a crouched attitude indicative of abdominal pain. Immediately prior to death, each animal went into mild convulsions and regurgitated a small amount of brownish material. The survivors were lethargic for periods of 1 to 3 days after administration of the drug.

On postmortem the viscera contained a fairly uniform pattern of lesions which included mild thoracic and abdominal ascites, pulmonary emphysema, and petchiation, and grossly distended stomachs filled with a mixture of gas, food, and a dark greenish-brown fluid. Other lesions included gassy distention of the intestines, cloudy swelling and congestion of the liver, engorgement of the myocardial vessels, hyperemia, and tubule damage in the kidneys. Scattered and insignificant damage was found in other organs. The pattern of lesions was indicative of a toxic reaction affecting the vascular system. Death probably occurred from a combination of pulmonary lesions and distention of the abdominal viscera resulting in respiratory collapse and suffocation, perhaps partly due to the large volumes used.

The administration of such a large (10 ml.) quantity of solution *via* stomach tube apparently caused pylorospasm since no drug was found in the intestinal tract. Regurgitation, which is not a normal phenomenon in rats, occurred immediately prior to death. This was probably the result of gas pressure in the stomach overcoming the resistance of the distal esophagus to dilation in the moribund animals.

Acute Dermal Toxicity—The different concentrations of imidecyl iodine failed to cause any significant dermal reactions with the exception of reddening which disappeared shortly after removal of the agent.

Guinea Pig Sensitization—The challenging dose of imidecyl iodine failed to cause any abnormal or unique reactions at the time of injection or afterward.

Eye Toxicity—*Adult Rabbits*—The results of instillation of imidecyl iodine in the eyes of adult rabbits are reported in Table II. With all dilutions, the eyes were back to normal by 96 hr. after each instillation of the maximum concentration. There were no signs of damage in the corneas of any of the animals. Sixty days after the tests, the eyes of these animals were still normal.

Newborn Rabbits—Imidecyl iodine concentration of 1:64 failed to produce any clinical evidence of ocular irritation during treatment or within the 2-month period following treatment.

Rabbit Bladder Toxicity—Gross and microscopic examination of the urinary bladders and viscera of rabbits treated with various concentrations of imidecyl iodine indicated that dilutions up to and including 1:64 were tissue irritants. Dilutions stronger than 1:64 were accompanied by effects ranging from mild toxicosis to sudden death. The deaths occurred in the animals given undiluted and 1:2 dilutions of imidecyl iodine in water. At a dilution of 1:128 there were no gross or microscopic lesions that could be attributed to the iodophor. Dilutions of 1:10 or greater produced local lesions in the bladder. The lungs of the animals receiving up to 1:64 dilutions of imidecyl iodine contained congested areas and small areas of emphysema and consolidation. The extent and severity of the lesions dropped markedly between 1:40 and 1:64 dilutions. However, there was no correlation between dosage levels and extent and severity of lesions in the 1:10 through 1:40 dilutions. In the kidneys, glomerular and tubular damage compatible with toxicosis, *e.g.*, nonsupportive nephritis, epithelial and glomerular swelling, and edema were seen in a majority of the animals receiving dilution through 1:64 of the iodophor. Other accompanying lesions ranged through a wide spectrum that had no uniformity and were more compatible with naturally occurring lesions in conventional animals than with iodophor toxicity.

In dilutions up to 1:10, death occurred shortly after injection. The lungs were congested, but the lesions were not sufficient to account for the collapse of the animals. Death was probably the result of shock.

Dilutions of 1:10 through 1:40 produced urinary bladder lesions consisting mainly of edema and local hemorrhage. In these animals, the bladder was empty at the time of examination. There were no other significant lesions except in the lungs, and these could be attributed to the manner in which the animals were killed (*e.g.*, Na pentobarbital intracardially) rather than to the imidecyl iodine. Dilutions of 1:40 were, at least as far as postmortem evidence is concerned, on the borderline toxic level.

Two rabbits receiving the 1:64 dilution were killed by pentobarbital intracardially, and the remaining two by concussion. The two killed by pentobarbital had appreciable pulmonary congestion; one of the other two had inflammatory lesions and the second had minimal congestion. The bladders of all animals were slightly edematous, but the lesions were minimal.

Dilutions of 1:128 produced no visible lesions in the urinary bladder, nor any lesions elsewhere in the body which could be attributed to imidecyl iodine toxicity. At this dilution the drug exercised no observable effect upon the viscera. Lesions present were evaluated as conditions existing prior to the experiment and were not apparently related to the iodophor.

Chronic Toxicity—There was no clinical evidence of adverse effects in the dogs continuously fed imidecyl iodine. Gross and microscopic postmortem examination showed no lesions which could be attributed to imidecyl iodine. All organs and structures were examined grossly. Organs examined microscopically were the heart, lungs, liver, kidneys, and urinary bladder.

SUMMARY

The LD_{50} potential toxic effects of sublethal doses of imidecyl iodine were investigated. Oral lethal dosage in rats, skin sensitization, and dermal reaction in guinea pigs, corneal, and urinary bladder reaction in rabbits, and the effect of chronic sublethal dosage in dogs were studied. LD_{50} values measured by oral administration to male and female Wistar rats were, respectively, 14.0 ± 2.1 and 11.0 ± 1.5 ml./Kg. Corneal reactions, skin sensitization, and dermal reactions were transient or negative.

Dilutions of 1:64 infused into the urinary bladder of rabbits produced edema of the bladder musculature. Dilutions of 1:40 and below were accompanied by lung and kidney damage in addition to the bladder lesions. Full strength and 1:2 dilution produced collapse and death, probably from shock. Dogs fed diets containing imidecyl iodine over a period of 5 months exhibited no toxic lesions or symptoms

REFERENCES

(1) *Am. Perfumer Aromat.*, **13**, 37(1957).
(2) Cantor, A., Most, S., and Shelanski, M. V., *J. Soc. Cosmetic Chemists*, **7**, 419(1956).
(3) Reed, L. J., and Muench, H., *Scanca*, **27**, 493(1937).
(4) Draize, J. H., Woodard, J., and Calvery, H. O., *J. Pharmacol. Exptl. Therap.*, **82**, 377(1944).
(5) Fitzhugh, G. O., "An Appraisal of the Safety of Chemicals in Foods Drugs, and Cosmetics," Association of Food and Drug Officials of the U. S., Austin, Tex., 1959, pp. 46–59.

Mechanism and Stereochemistry of Epoxy Ether Formation and Cleavage IV

By HARSHADKUMAR PATEL* and GILBERT HITE

An independent proof of the mechanism and stereochemistry of epoxy ether formation from (−)-1-methyl-3-benzoyl-3-chloropiperidine[(−)-I] is presented.

IN PREVIOUS PAPERS on this subject (1–5) the anomalous racemization observed in the sequence, (−)-I → (−)-II → (±)-III (1) was rationalized on the basis of an asymmetric induction at the carbonyl carbon followed by halide ejection with concomitant participation of the free electron pair on nitrogen and α-C-symmetrization (*cf.* IV). This requires formation of identical amounts of the two possible diastereoisomeric epoxy ethers, epimeric at the spiro carbon, both arising from IV. The antipodal intermediate formed in the asymmetric induction would give rise to the remaining two diastereoisomeric epoxy ethers, also epimeric at the spiro carbon, but enantiomorphic to the first set. Since the product must then be a unique mixture consisting of identical quantities of erythro and threo epoxy ethers in identical degrees of optical purity, the optical activity of II must be attributed to the ketal carbon and not to the carbon common to both rings.

The *total* optical specificity of the reaction sequence, α-(+)-I $\xrightarrow{O}$ β-(−)-II → β-(+)-III (2,3) in which participation of the electron pair on nitrogen is obviated, has made possible this study. The interjection of an analogous but symmetrical cyano blocking group, following epoxy ether [(−)-II] formation, permitted observation of the opticochemical course of the conversion of (−)-I to (−)-II. (Scheme I.)

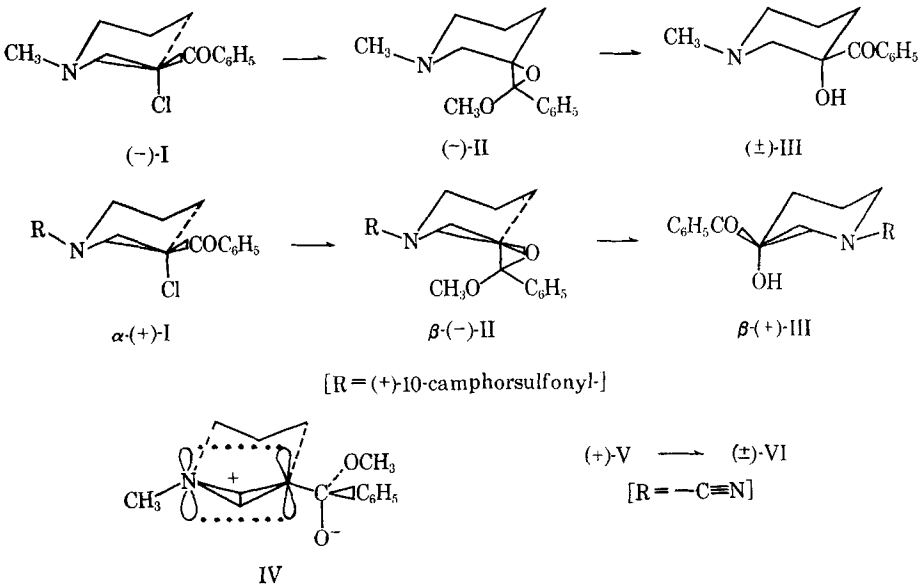
After treatment of (−)-1-methyl-3-benzoyl-3-chloropiperidine [(−)-I] with sodium methoxide in methanol, the total levorotatory mixture containing 2 - methoxy - 2 - phenyl - 5 - methyl - 1 - ox - 5 azaspiro[2.5]octanes (II) was isolated and was allowed to react with cyanogen bromide in order to effect demethylation. The total, dextrorotatory, benzene soluble fraction, containing 2-methoxy-2-phenyl-5-cyano-1-ox-5-azaspiro[2.5]octanes (V), was treated with dilute aqueous acid to give racemic 1-cyano-3-benzoyl-3-hydroxypiperidine (VI).

Since this epoxy ether cleavage is analogous to the totally optically specific transformation of β-(−)-II to β-(+)-III (2, 3), it is clear that the carbon common to both rings in (+)-V, and thus in (−)-II, must be present in both configurations, *equally*. Accordingly, the previously proposed (1) salient features of epoxy ether [(−)-II] formation from (−)-I are unequivocally established.

EXPERIMENTAL[1]

(−) - 2 - Methoxy - 2 - phenyl - 5 - methyl - 1-ox-5-azaspiro[2.5]octanes [(−)-II]—This was prepared, as described earlier (1), from (−)-I, $[\alpha]_D^{25}$ (ethanol) −4.2° (c 15.00).

(+) - 2 - Methoxy - 2 - phenyl - 5 - cyano - 1-ox-5-azaspiro[2.5]octanes [(+)-V]—A solution of 4.67 Gm. (20 mmoles) of (−)-II, $[\alpha]_D^{28}$ (ethanol) −1.28° (c 15.32), in 20 ml. of dry benzene was slowly added to a solution of 8.5 Gm. (60 mmoles) of cyanogen bromide in 30 ml. of dry benzene. The mixture was heated to 50° and stirred for 48 hr. The insoluble salts (0.8 Gm.) were filtered off



Scheme I

Received March 2, 1967, from the College of Pharmaceutical Sciences, Columbia University, New York, NY 10023
Accepted for publication June 22, 1967.
This investigation was supported by grant NB-3593 from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service, Bethesda, Md.

* Present address: Nease Chemical Co., State College, PA 16801

[1] See Footnote 15 in *Reference 1.*

and the benzene and cyanogen bromide were removed under reduced pressure. The residual oil was dissolved in dry benzene and treated with carbon. The mixture was filtered through sintered glass. The solvent was removed under reduced pressure (0.01 mm. Hg/24 hr.) to give a colorless oil, 3.04 Gm. (12.3 mmoles, 61.5%), $[\alpha]_D^{28}$ (benzene) +2.1° (c 3.04), exhibiting a band at 2208 cm.$^{-1}$ (film) (C≡N) in the infrared spectrum. There were no bands in the —OH or C=O region.

Anal.—Calcd. for $C_{14}H_{16}N_2O_2$: C, 68.8; H, 6.6. Found: C, 69.3; H, 6.2.

(±) - 1 - Cyano - 3 - benzoyl - 3 - hydroxypiperidine [(±)-VI]—To 2.44 Gm. (10 mmoles) of (+)-V in 40 ml. of methanol was added 10 ml. of 1 *N* hydrochloric acid. After standing for 24 hr., the methanol was removed under reduced pressure to give, on cooling, 1.87 Gm. (8.1 mmoles, 81%) of product which was optically inactive.[1] There were bands in the infrared spectrum (benzene solution) at 1665 (C=O), 2218 (C≡N), and 3285 cm.$^{-1}$ (OH). The spectrum was identical to that of an authentic sample prepared as previously described (5) melting point and mixed melting point of crystals from methanol, 131.5–132.5°.

REFERENCES

(1) Zalucky, T. B., Malspeis, L., and Hite, G., *J. Org. Chem.*, **29**, 3143(1964).
(2) Patel, H., and Hite, G., *ibid.*, **30**, 4337(1965).
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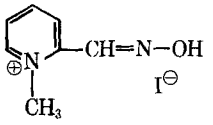
Synthesis of Aldoxime Analogs of Arecoline as Reactivators of Organophosphorus Inhibited Cholinesterase

By J. N. WELLS, J. N. DAVISSON, I. BOIME, D. R. HAUBRICH, and G. K. W. YIM

A series of arecoline-like aldoximes were synthesized and evaluated as potential reactivators of organophosphate-inhibited cholinesterase. These arecoline analogs were patterned after the potent quaternary oximes 2-PAM and TMB-4 and were synthesized by sodium borohydride reduction of the corresponding pyridinium aldoxime. Biological results show that although these aldoximes are less toxic than the quaternary aldoximes, they are much less effective as reactivators. They did exhibit weak muscarinic activity (1/60 that of arecoline) on dog blood pressure and guinea pig ileum.

THE EFFICIENT reactivating property of 2-pyridine aldoxime methiodide (2-PAM) has been attributed to the affinity of its quaternary nitrogen for the anionic site of cholinesterase (1). This affinity could cause the nucleophilic aldoxime oxyanion to be in a favorable position for reactivating phosphorylated cholinesterase.



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The cholinergic agent arecoline contains a tertiary amine. Since the muscarinic activity of arecoline has been shown to be dependent on its protonated cationic nitrogen (2), the arecolinium ion might also possess a marked affinity for the anionic site of cholinesterase. Therefore, aldoxime analogs of arecoline, which should enter the CNS readily, might be expected to provide good reactivation of phosphorylated cholinesterase in the brain.

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Accepted for publication June 5, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

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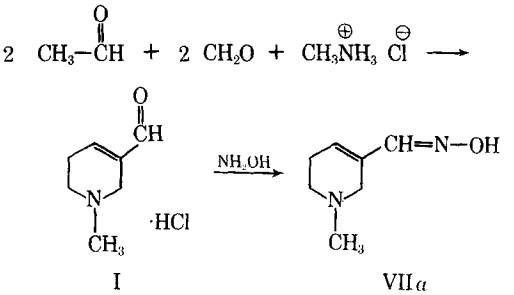
DISCUSSION

The authors have prepared a few 1,2,5,6-tetrahydropyridine aldoximes and report here the chemistry and pharmacological data. 1-Methyl-1,2,5,6-tetrahydropyridine-3-aldoxime (VII*a*) has been prepared by the reaction of formaldehyde, methylamine hydrochloride, and acetaldehyde to give 1-methyl-1,2,5,6 - tetrahydropyridine - 3 - carboxaldehyde (I) which was isolated as the oxime (3). (Scheme I.)



Scheme I

This reaction is quite laborious and gave poor yields of impure VII*a*. Thus, a more general method was sought which would be applicable to 1-alkyl-1,2,5,6-tetrahydro-3- and 4-aldoximes.

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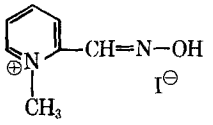

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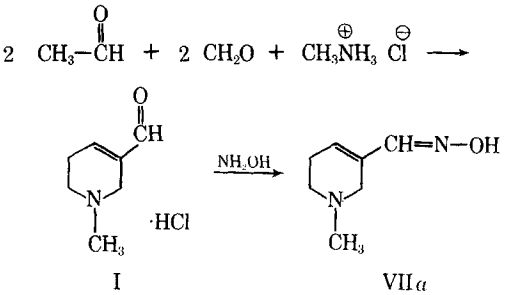

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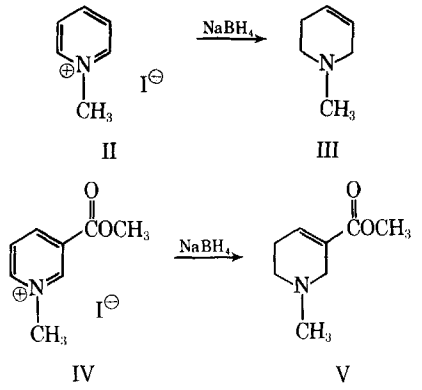
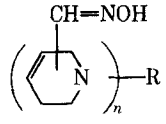
sodium borohydride has been shown to give arecoline (V) (5). The conditions of these reactions seemed applicable to these systems. (Scheme II.)



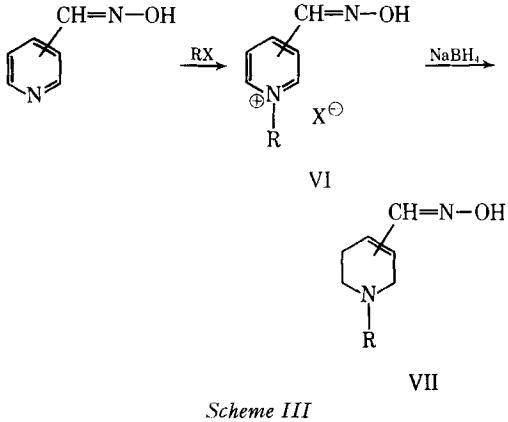
Scheme II

The quaternary pyridine aldoximes (VI) were prepared by reacting the pyridine aldoxime with an appropriate alkyl halide. Reduction of the resulting pyridinium aldoxime with sodium borohydride in methanol or ethanol gave the 1-substituted 1,2,5,6-tetrahydropyridine-3- or 4-aldoxime (VII) in 50–74% yield (Table I). (Scheme III.)

TABLE I—1-SUBSTITUTED-1,2,5,6-TETRAHYDROPYRIDINE-3- OR 4-ALDOXIMES



	Position of Aldoxime	*n*	R	% Yield from Reduction
VII*a*	3	1	CH_3	59
VII*b*	4	1	CH_3	50
VII*c*	4	2	$(CH_2)_3$	74



Scheme III

Elemental analysis of these compounds (VII) indicated that the tetrahydropyridine system had been formed but the position of the double bond had to be established.

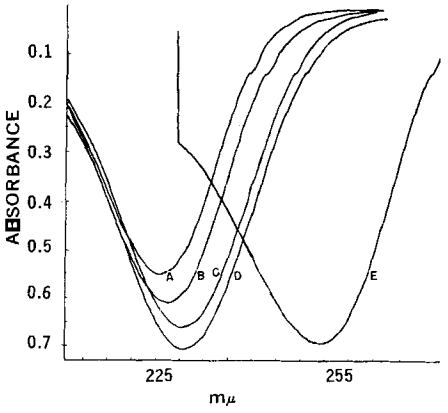


Fig. 1—Ultraviolet absorption spectrum of tetrahydropyridine aldoximes. Key: A, *VII*b *in 0.05* N *HCl;* B, *VII*a *in 0.05* N *HCl;* C, *VII*b *in water;* D, *VII*a *in water;* E, *VII*b *in base (pH 11.5). Bausch & Lomb model 505 spectrophotometer.*

Infrared and NMR spectra were consistent with the Δ-3 system but were not stringent proof. Another point of confusion arose from the fact that Mannich reported the melting point of VII*a* hydrochloride to be 240–242° (3), while the product (VII*a* hydrochloride) from the sodium borohydride reduction melted at 261.5–262°.

The ultraviolet spectrum of compound VII*b* in water showed $\lambda_{max.}$ 226 mμ; ϵ = 17,700. The $\lambda_{max.}$ 226 mμ is consistent with that expected of an α, β-unsaturated aldoxime (6). The molar absorptivity clearly indicates conjugation. Since very little shift was noted when the spectrum was obtained from an acidic solution of any of the compounds (VII*a* or *b*), conjugation with the amino nitrogen may be ruled out in all cases (see Fig. 1). The slight shift to shorter wavelengths in acidic solution must have been due to suppression of ionization of the oxime hydroxyl. The U.V. spectrum of VII*a* in water gave $\lambda_{max.}$ 226 mμ; ϵ = 17,100. Again a conjugated oxime is indicated. Of the two possibilities for location of the double bond, only the 3–4 position should give the same $\lambda_{max.}$ as compound VII*b*. If the double bond of VII*a* were in the 2–3 position one would also see a contribution from the amine electrons which would give rise to absorption at a longer wavelength.

The results of the pharmacological evaluation of compounds VII*a*, *b*, and *c* are summarized in Table II. Only 6–20% reactivation of the TEPP inhibited erythrocyte cholinesterase was obtained with these aldoximes, whereas 60–75% reactivation was observed with even lower concentrations of 2-PAM. In agreement with this weak *in vitro* activity was the lack of protection afforded by the tetrahydropyridine aldoximes against TEPP toxicity in mice. In contrast, 2-PAM afforded 80% protection, and all six mice injected with 20 mg./Kg. TMB-4 survived the TEPP treatment. Only one mouse survived of 22 control mice that were injected with the 1 mg./Kg. dose of TEPP.

Compound VII*a* showed definite muscarinic activity. It caused contractions of the isolated

TABLE II—PHARMACOLOGICAL DATA ON TETRAHYDROPYRIDINE ALDOXIMES STUDIED

Compd.	Reactivation of Phosphoryl-ChE *In Vitro*[a]	LD_{50} in Mice,[b] mg./Kg. i.p.	Effect on TEPP Toxicity in Mice[b,c] (1 mg./Kg. TEPP S.Q.)	Ionization Constants[d] Ring Nitrogen pKa	Ionization Constants[d] Aldoxime pKa	% Ionized at pH 7.4 Ring Nitrogen	% Ionized at pH 7.4 Aldoxime
2 PAM	76.2% at $1.5 \times 10^{-4}M$	...	10/12 survived at 50 mg./Kg.	...	7.77 ± .03	100%	30%
VII*a*	19.5% at $3.0 \times 10^{-3}M$	274 (254–295)	0/12 survived at 100 mg./Kg.	8.04 ± .10	10.84 ± .14	83%	0.03%
VII*b*	6.6% at $3.0 \times 10^{-3}M$	275 (237–319)	0/12 survived at 100 mg./Kg.	8.15 ± .11	10.91 ± .07	85%	0.03%
VII*c*	11.0% at $3.0 \times 10^{-4}M$	78 (62–97)	0/12 survived at 35 mg./Kg.	...	...	...	...
VIII	18.6% at $3.0 \times 10^{-4}M$	91 (72–114)	0/12 survived at 50 mg./Kg.		10.11 ± .02	100%	0.02%
Arecoline	...	...	...	7.78 ± .10	...	70%	...

[a] Bovine erythrocyte ChE, inhibited with $10^{-7}M$ TEPP, was incubated with the aldoximes for 55 min. ChE activity was measured manometrically (Umbreit *et al.*, 1964). [b] Swiss albino mice weighing 25–35 Gm. were used. [c] Aldoximes were given 6–8 min. prior to TEPP; TEPP killed 21 of 22 control mice. [d] Dissociation constants were determined potentiometrically by titration of 0.01 *M* solutions of the compounds with 0.1 *N* KOH according to the method of Albert and Serjeant (1962).

guinea pig ileum, gave depressor responses in the anesthetized cat, and induced salivation, urination, and defecation in mice. These actions were readily antagonized by atropine. When atropine was combined with the aldoxime pretreatment, the mice were still not protected from the lethal effects of TEPP. Thus, it is unlikely that beneficial effects of possible reactivation induced by the aldoximes were masked by the toxic muscarinic effects of the aldoximes.

The ionization data offer a feasible explanation for the low activity of the tetrahydropyridine aldoximes. The pKa values of compounds VII*a* and VII*b* (8.04 and 8.15, respectively) were close to the dissociation constant of the basic ring nitrogen of arecoline, and were therefore attributed to the dissociation of the basic ring nitrogens. Similarly, the pKa values of compounds VII*a* and VII*b* (10.84 and 10.91, respectively) were close to the dissociation constant of the aldoxime group of compound VIII (the methiodide of VII*a*) and were attributed to the dissociation of the aldoxime groups. The data in the last column indicate that, as predicted, the ring nitrogen of the tetrahydropyridine aldoximes would be sufficiently protonated at physiological pH to form the cationic head which could be attracted to the anionic site of the phosphorylated enzyme. The aldoxime groups of compounds VII*a* and VII*b* were almost completely unionized, as contrasted to the 30% ionization of the aldoxime group of 2-PAM. Thus, the weak reactivating activity of the tetrahydropyridine aldoximes can be attributed to insufficient ionization of the oxime group to the active oximate form. The importance of the steric configurations of 2-PAM and the nonaromatic aldoximes could not be ascertained from the present study.

EXPERIMENTAL

1 - Methyl - 1,2,5,6 - tetrahydropyridine - 4-aldoxime (VIIb)—To a solution of pyridine-4-aldoxime methiodide (7) (10.6 Gm., 0.04 mole) in 200 ml. of methanol was added 6.0 Gm. (0.16 mole) of sodium borohydride in small portions. The reaction was stirred continuously and the temperature was maintained between 25–30°. The solution was stirred for an additional 0.5 hr. at room temperature after the addition was completed.

The solution was concentrated *in vacuo* to 50 ml. and diluted with 200 ml. of water. The aqueous solution was saturated with Na_2CO_3 and extracted with ether. The ether solution was dried over K_2CO_3 and then removed *in vacuo* to give 2.8 Gm. (50% yield) of white crystalline solid, m.p. 156–158°.

The hydrochloride was prepared and recrystallized from ethanol, m.p. 241–242°.

Anal.—Calcd. for $C_7H_{12}N_2O \cdot HCl$: C, 47.59; H, 7.42. Found: C, 47.43; H, 7.48.

1 - Methyl - 1,2,5,6 - tetrahydropyridine - 3-aldoxime (VIIa)—Pyridine-3-aldoxime methiodide (7) (8.6 Gm., 0.03 mole) was reduced with 4.7 Gm. (0.12 mole) of $NaBH_4$ using the same conditions as for VII*b* except that ethanol (200 ml.) was used as solvent. Using the work-up described for VII*b*, 2.5 Gm. (59% yield) of VII*a* was obtained. The hydrochloride was prepared and recrystallized from ethanol, m.p. 261.5–262°. [Lit. (3) m.p. 240–242°.]

Anal.—Calcd. for $C_7H_{12}N_2O \cdot HCl$: C, 47.59; H, 7.42. Found: C, 47.73; H, 7.26.

The methiodide (VIII) was prepared by reaction of VII*a* with methyl iodide in acetone, m.p. 181–182°.

1,3 - Bis(4 - formyl - 1,2,5,6 - tetrahydropyridyl)-propane Dioxime (VIIc)—To a solution of 7.0 Gm. (0.02 mole) of 1,3-bis(4-formylpyridinium bromide)-propane dioxime (8), in 200 ml. of methanol, was added portionwise 4.8 Gm. (0.13 mole) of sodium borohydride. A precipitate formed as the reaction proceeded. The solid was collected and dissolved in 20 ml. of 6 *N* hydrochloric acid. Ethanol (100 ml.) was added. It was necessary then to add a small quantity of ether to initiate crystallization. After recrystallization from ethanol, 2.6 Gm. (77% yield) of white crystalline solid was obtained, m.p. 246–247° dec.

Anal.—Calcd. for $C_{15}H_{24}N_4O_2 \cdot HCl$: C, 49.32; H, 7.12. Found: C, 49.08; H, 7.25.

REFERENCES

(1) Wilson, I. B., Ginsburg, S., and Quan, C., *Arch. Biochem. Biophys.*, **77**, 286(1958).
(2) Van Rossum, J. M., *Arch. Intern. Pharmacodyn.*, **140**, 592(1962); Burgen, A. S. V., *J. Pharm. Pharmacol.*, **16**, 638(1964); Burgen, A. S. V., *Brit. J. Pharmacol.*, **25**, 4 (1965).
(3) Mannich, C., *Ber.*, **75B**, 1480(1942).
(4) Panouse, J. T., *Compt. Rend.*, **233**, 260(1951).
(5) Lyle, R. E., Perlowski, E. F., Troscianiec, H. J., and Lyle, G. G., *J. Org. Chem.*, **20**, 1761(1955).
(6) Benson, W. R., and Pohland, A. E., *ibid.*, **30**, 1126 (1965).
(7) Ginsburg, S., and Wilson, I. B., *J. Am. Chem. Soc.*, **79**, 481(1957).
(8) Poziomek, E. J., Hackley, B. E., and Steinberg, G. M., *J. Org. Chem.*, **23**, 714(1958).

Effect of Griseofulvin Upon Lipid Films at the Air-Water Interface

By RONALD P. QUINTANA, ANDREW LASSLO, and SANDRA L. OUSLEY

The effect of griseofulvin upon monomolecular films of stearic acid and cholesterol has been determined by comparing the pressure-area curves obtained when these film-forming materials were spread on a redistilled water substrate and one consisting of a solution of griseofulvin in redistilled water.

IN CONNECTION with studies on the structural requirements and physicochemical characteristics associated with a compound's capacity for anchoring to dermal tissue constituents or for enhancing its localization in the latter, the authors sought to determine the effects of griseofulvin upon monomolecular films of two representative lipid dermal constituents (1), *i.e.*, stearic acid and cholesterol.

EXPERIMENTAL

Materials—Stearic acid was obtained in purity greater than 99%, m.p. 71.1–72.0°.[1] Chromatographically pure cholesterol (99 +%) was procured (Sigma Chemical Co.), m.p. 148.8–149.4°. Griseofulvin was recrystallized from benzene, yielding white needles, m.p. 220.5–221.4°; $[\alpha]_D^{26}$ +331.03° (c 1.015 in acetone); $\lambda_{max.}^{EtOH}$ 215 mμ (ε 23,107), 235 (22,754), 292 (23,636), 330 (5,644); $\lambda_{max.}^{CHCl_3}$ 5.85 μ (C=O), 6.02 (COC=C). Its properties were consistent with those reported in the literature (2, 3).

Anal.[2]—Calcd. for $C_{17}H_{17}ClO_6$: C, 57.88; H, 4.86; Cl, 10.05. Found: C, 57.79; H, 4.78; Cl, 10.30.

The substrate water was obtained by redistillation through a 45-cm. Vigreaux column from aqueous permanganate (4). It had a pH of 6.0, and gave a value of 0.15 p.p.m. in conductometric measurements of ionizable substances expressed as sodium chloride. Solutions of griseofulvin (20 mcg./ml.; $5.7 \times 10^{-5} M$) were prepared by boiling the substrate water to which griseofulvin had been added (5); the absorbance at 295 mμ for the solutions prepared in this manner (5) was 1.35 ± 0.02.

Instrumentation and Methods—The surface balance utilized is a refinement of the commercially available Hydrophil balance (Central Scientific Co.) (see Fig. 1). The trough and all Teflon parts of the apparatus which come into contact with the substrate and monolayer were thoroughly cleaned with reagent grade benzene followed by rinsing with spectro-grade benzene and redistilled water. The equipment was assembled and used within an isolation cabinet (Kewaunee Scientific Equipment). All experiments were carried out at room temperature (substrate temperature, 24 ± 2°).

After the cleanliness of the substrate surface was checked, the compression barrier was positioned (initial area, 490–560 cm.²), and the film-forming material was applied to the surface of the substrate by means of an Agla micrometer-syringe (Burroughs Wellcome & Co.), in spectro-grade benzene (0.125–0.176 μmole/0.05–0.10 ml.). Following a period of 20 min. during which the solvent evaporated, the compression barrier was moved slowly toward the float. Usually 2-mm. decrements were employed, starting about 3 cm. before pressure was first observed; as the pressure increased rapidly with a small decrease in area, 1-mm. decrements were used. In each case, there was a 1-min. interval between the time when the barrier was stopped and that when the reading of surface pressure was made.

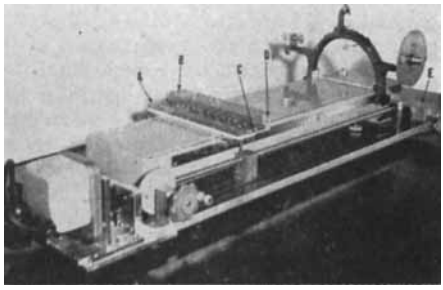


Fig. 1—The modification of the surface balance. The trough is Teflon coated, and the float and end piece systems, patterned after those of Mann and Hansen (6), are constructed of Teflon. Key: A and B, compression barrier units (solid Teflon attached to solid brass) transported at uniform rates by motor-driven operation; C, restraining arm for part B; D, scale pointer; E, switch providing automatic cessation of barrier movement.

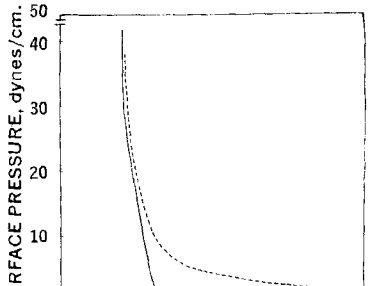


Fig. 2—Surface pressure vs. *area per molecule for stearic acid spread on: redistilled water (———); 5.7 × 10⁻⁵ M solution of griseofulvin in redistilled water (– – – –). In the latter case the corrected surface pressure is shown.*

Received April 12, 1967, from the Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee, Memphis, TN 38103

Accepted for publication June 7, 1967.

This investigation was supported by research contract DA-49-193-MD-2636 from the U. S. Army Medical Research and Development Command, Washington, D. C.

The authors acknowledge valuable discussions with Dr. Herman E. Ries, Jr., American Oil Co., and with Dr. Robert G. Crounse, Johns Hopkins University School of Medicine. The authors also acknowledge the work of Mr. W. A. Frase, who constructed the modifications incorporated in the surface balance, and that of Mr. B. Carpenter, who prepared the Teflon end piece system. The authors thank Dr. K. E. Avis for conductometric measurements, and Dr. R. J. Grabenstetter, Procter and Gamble Co., for the stearic acid, and Mr. G. R. Goetchius, Ayerst Laboratories, for the griseofulvin employed in this investigation.

[1] Melting points are corrected; they were determined with a Büchi melting point apparatus.

[2] Analyses were performed by Drs. G. Weiler and F. B. Strauss, Oxford, England.

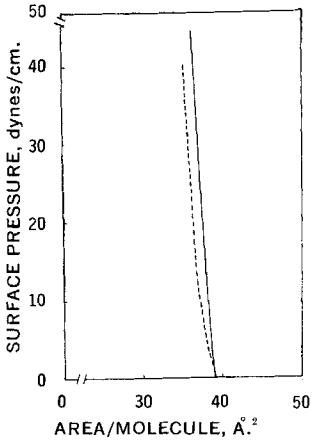


Fig. 3—Surface pressure vs. *area per molecule for cholesterol spread on: redistilled water (———); 5.7 × 10⁻⁵ M solution of griseofulvin in redistilled water (- - - -). In the latter case the corrected surface pressure is shown.*

After the film collapsed, a check was made for monolayer leakage.

RESULTS AND DISCUSSION

Each of the pressure-area isotherms (Figs. 2 and 3) was obtained by averaging the results of three independent experiments; the experimental points deviated from the curve average generally by no more than 0.2 Å.² per molecule, at a given surface pressure. It should be noted that the pressure-area curves for stearic acid and cholesterol spread on water agree well with those reported by Walker and Ries (7) and Taylor and Haydon (8). The surface pressure values for stearic acid and cholesterol, spread on the aqueous solution of griseofulvin, are corrected.[3] This permitted direct comparison with values obtained when the substrate was redistilled water.

The broken line in Fig. 2 indicates that griseofulvin was incorporated into the monolayer, and that, as the pressure increased, most of it was forced out of the film. The collapse pressure for this curve was slightly lower than that for pure stearic acid; this appears to be consistent with the observation (10) that the presence of irregularly shaped molecules in films of regularly oriented molecules renders the film less stable.

[3] The correction factor (8, 9) was determined to be 0.6 dyne/cm. using a du Nouy tensiometer and employing the ring method by procedures previously described (4).

The curve for cholesterol spread on the solution of griseofulvin (Fig. 3) is displaced slightly toward a lower area per molecule compared with that spread on redistilled water. This suggests that griseofulvin either facilitates a closer packing of the cholesterol molecules than that in the film of the pure substance, or that griseofulvin causes some of the cholesterol molecules to leave the area-determining positions in the surface; the latter may be, possibly, due to a process of interfacial dissolution (10).

The data indicate a somewhat limited but unequivocally significant affinity of griseofulvin for stearic acid. This is interesting in the light of evidence (5, 11) that the presence of lipid material in skin tissue may be associated with uptake of griseofulvin. While the authors are aware of the fact that a response to a given compound *in vitro* cannot be interpreted as evidence of the latter's corresponding involvement *in vivo*, even without conclusive causal connection, one should not underestimate the importance of leads of this nature. In the instance of the interactions observed in the cholesterol monolayer, one is tempted to interpret the isotherm also in terms of a type of affinity; it is possible to visualize that some of the cholesterol molecules shifted from their area-determining positions toward the aqueous phase because of their affinity for the griseofulvin.

Studies concerned with the affinity of a series of grisan derivatives for stearic acid and other lipid constituents have been recently completed (12, 13). They are expected to be supplemented with complementary studies.[4]

REFERENCES

(1) Rothman, S., "Physiology and Biochemistry of the Skin," The University of Chicago Press, Chicago, Ill., 1954, pp. 313–318, 322–325, 485–488.
(2) Oxford, A. E., Raistrick, H., and Simonart, P., *Biochem. J.*, **33**, 240(1939).
(3) Stork, G., and Tomaz, M., *J. Am. Chem. Soc.*, **86**, 471(1964).
(4) Quintana, R. P., *J. Pharm. Sci.*, **53**, 1221(1964).
(5) Freedman, M. H., Baxter, R. M., and Walker, G. C., *J. Invest. Dermatol.*, **38**, 199(1962).
(6) Mann, J. A., Jr., and Hansen, R. S., *Rev. Sci. Instr.*, **34**, 702(1963).
(7) Walker, D. C., and Ries, H. E., Jr., *Nature*, **203**, 292(1964).
(8) Taylor, J. L., and Haydon, D. A., *Biochim. Biophys. Acta*, **94**, 488(1965).
(9) Skou, J. C., *Acta Pharmacol. Toxicol.*, **10**, 317(1954).
(10) Shanes, A. M., and Gershfeld, N. L., *J. Gen. Physiol.*, **44**, 345(1960).
(11) Crounse, R. G., personal communication.
(12) Quintana, R. P., Lasslo, A., Boggs, P. P., and Yeaglin, E. D., unpublished data.
(13) Quintana, R. P., Lasslo, A., and Boggs, P. P., unpublished data.

[4] By Dr. Robert G. Crounse, Division of Dermatology, Johns Hopkins University School of Medicine, Baltimore, Md.

Investigations of Philippine Plants for Alkaloids, Antimalarial Agents, and Antineoplastic Agents

By F. D. POPP, J. M. WEFER, G. ROSEN, and A. C. NOBLE

Extracts of 28 different Philippine plants were prepared and the extracts tested for alkaloids. The extracts were also screened, in many cases, for antimalarial and antineoplastic activity. In some cases preliminary fractionation into petroleum ether soluble and ethanol soluble extracts were made.

THE IMPORTANCE of screening plant materials for various chemical constituents of medicinal importance is well established. Plants have provided many drugs which are being used for the prevention or cure of diseases or which have been the basis for synthetic drugs. This work was directed to the search for new drugs which may find application as antineoplastic agents or as antimalarial agents.

TABLE I—ALKALOID TEST RESULTS

No.	Name	Plant Part[a]	Extract[b]	Alkaloid Tests[c] A	B	C	D	E
1	*Ageratum conyzoides* (*Compositae*)	S, L	Alc	−	+	+	+	+
1a			PE	−	−	−	−	−
1b			Al/PE	+	+	+	+	+
2	*Anona squamosa* (*Annonaceae*)	L	Alc	+	+	+	−	−
2a			PE	+	−	−	−	−
2b			Al/PE	+	+	−	−	−
3	*Asclepias curassavica* (*Asclepiadaceae*)	W	Alc	−	+	−	+	+
4	*Blumea balsamifera* (*Compositae*)	L	Alc	+	+	−	+	+
5	*Calophyllum blancoi* (*Guttiferae*)	B	Alc	−	+	−	−	−
5a			PE	−	−	−	−	−
5b			Al/PE	−	+	−	−	−
6	*Calophyllum inophyllum* (*Guttiferae*)	B	Alc	−	+	−	−	−
7	*Cassia siamea* (*Leguminosae*)	L, P	Alc	−	+	−	+	+
8	*Cinnamomum mercadoi vidal* (*Lauraceae*)	L	Alc	−	+	+	−	−
8a			PE	−	−	−	−	−
9	*Cucumis melo* (*Tournaceae*)	L	Alc	−	−	−	−	−
10	*Dioscorea hispida* (*Dioscoreaceae*)	T	Alc	+	+	+	+	+
11	*Dolichos lablab* (*Leguminosae*)	L	Alc	+	+	+	+	+
12	*Eclipta alba* (*Compositae*)	S, L	Alc	−	+	−	+	+
13	*Erythrina variegata* (*Leguminosae*)	L	Alc	−	+	−	+	+
14	*Eugenia jambolana* (*Myrtaceae*)	S	Alc	−	−	−	−	−
15	*Gammatophyllum scriptum* (*Orchidaceae*)	Bl	Alc	−	−	−	−	+
16	*Geodorum nutans* (*Orchidaceae*)	Bl	Alc	−	+	−	+	+
16a			PE	−	−	−	−	−
16b			Al/PE	−	+	−	+	+
17	*Hernandia ovigera* (*Hernandiaceae*)	L	Alc	+	−	−	−	−
18	*Hibiscus rosa-sinensis* (*Malvaceae*)	L	Alc	+	+	+	+	+
19	*Ipomoea pes-caprae* (*Convolvulaceae*)	L	Alc	−	+	−	+	+
20	*Kibatalia gitingensis* (*Apocynaceae*)	B	Alc	+	+	+	+	+
21	*Mitragyna rotundifolia* (*Rubiaceae*)	L	Alc	+	+	+	+	+
22	*Nerium oleander* (*Apocynaceae*)	L	Alc	+	+	−	−	−
23	*Phaseolus aureus* (*Leguminosae*)	L	Alc	−	−	−	−	+
24	*Pseudelephantopus spicatus* (*Compositae*)	S, L	Alc	−	−	−	−	−
25	*Sphaeranthus africanus* (*Compositae*)	W	Alc	−	+	−	−	+
25a			PE	−	−	−	−	−
25b			Al/PE	−	+	−	−	+
26	*Strophanthus cumingii* (*Apocynaceae*)	S	Alc	−	+	+	+	+
26a			PE	−	−	−	−	−
26b			Al/PE	−	+	−	+	+
27	*Tabernaemontana pandacaqui* (*Apocynaceae*)	B	Alc	+	+	+	+	+
28	*Vitex trifolia* (*Verbenaceae*)	L	Alc	+	+	+	+	+
28a			PE	−	−	−	−	−
28b			Al/PE	+	+	+	+	+

[a] S, stems; L, leaves; W, whole plant; B, bark; P, pods; T, tubers; Bl, bulbs. [b] Alc, 80–90% ethanol extract; PE, petroleum ether extract; Al/PE, extract of petroleum ether extracted material with ethanol. [c] A, Hager's reagent (picric acid); B, Sonnenschein's reagent (phosphomolybdic acid); C, Mayer's reagent (mercuric potassium iodide); D, Dragendorff's reagent; E, Scheibler's reagent (phosphotungstic acid).

EXPERIMENTAL

Materials—All plant materials were collected, identified, and supplied in the air-dried state by Dr. Demetrio Mendoza, Philippine National Museum, Manila.

Received April 21, 1967, from the Department of Chemistry, Clarkson College of Technology, Potsdam, NY 13676
Accepted for publication June 12, 1967.
The authors thank Dr. D. Mendoza, the National Museum, Manila, for assistance in obtaining the plant materials and for many helpful suggestions and Dr. L. Rane and Dr. J. L. Hartwell for assistance in having the extracts screened.
This investigation was supported in part by grant T-234 from the American Cancer Society and grant CA-07891 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

TABLE II—MALARIA SCREENING DATA[a]

No.[b]	Dose,[c] mg./Kg.	Survival Time, Days[d] Test	Control	Notes
2a	160	6.4	6.1	Toxic at 640 mg./Kg.
3	640	8.0	6.2	Some toxicity
4	640	7.2	6.4	
6	640	6.8	6.3	
7	1280	8.0	6.8	
8	40	9.6	6.4	10.0 survival with some toxicity at 160 mg./Kg.
8a	160	6.4	6.2	
10	640	8.0	6.4	
13	640	7.0	6.3	Some toxicity
15	640	7.6	7.0	
17	640	6.2	6.1	
21	640	6.2	6.1	
22	640	6.4	6.1	
23	160	7.4	7.2	
27	40	6.8	6.1	

[a] Samples sent to Dr. L. Rane, University of Miami, for screening; results received from Walter Reed Army Medical Center. Screening against *Plasmodium berghei* in mice infected with a lethal dose 3 days prior to administration of the extract. [b] Refer to Table I. [c] At least three different doses were given for each extract, the highest nontoxic dose is recorded here. [d] A compound is considered to be active when the survival time of the test is more than twice the survival time of the control.

Preparation of Extracts—The air-dried plant materials were ground and extracted in a Soxhlet extractor until a fresh extract had relatively little color to it. All plant materials were extracted with 80–90% ethanol and the ethanol extract was concentrated to dryness *in vacuo*. The residue from this concentration was tested for alkaloids and submitted for screening.

In selected cases a fresh batch of air-dried plant materials were extracted first with petroleum ether (naphtha) and then with 80–90% ethanol. These individual extracts were concentrated as noted above.

Screening—Most of the concentrated extracts were submitted to Dr. J. L. Hartwell, Cancer Chemotherapy National Service Center (CCNSC), for anticancer screening under the CCNSC standard protocol, and the anticancer results included in this paper were supplied by the CCNSC. Many extracts were also submitted to Dr. L. Rane, University of Miami, for screening against *Plasmodium berghei* in mice. The antimalarial results included in this paper were supplied by the Walter Reed Army Medical Center.

Alkaloid Tests—The concentrated extract (0.1 Gm.) was dissolved in 10 ml. of 0.5% hydrochloric

TABLE III—CANCER SCREENING DATA[a]

No.[b]	KB Cell Culture[c] ED_{50}, mcg./ml.	Slope	T/C (%)/Dose (mg./Kg.) LE[d]	SA[e]	DL[f]	WM[g]	LL[h]
1a			106/400	...	...	106/25	...
1b	5.4×10^{1}	−1.0	...	...	...	...	...
2	9.4×10^{-2}	−0.2	123/5	75/6	...	100/5	...
2a	2.5×10^{1}	−0.4	110/18	...	...	106/25	...
4	1.7×10^{1}	−0.4	...	...	...	...	...
5	9.6×10^{0}	−0.6	...	...	...	...	...
5b	3.0×10^{0}	−0.7	...	...	...	...	...
6	M1.0 × 10^{2}		...	...	...	...	...
7	M1.0 × 10^{2}		...	...	100/480	...	...
8	3.3×10^{1}	−0.6	...	...	...	...	...
9	M1.0 × 10^{2}		100/400	62/500	...	56/400	...
10	M1.0 × 10^{2}		...	...	...	...	...
11	1.2×10^{2}	−0.5	...	...	...	...	...
12	3.6×10^{1}	−1.0	...	...	...	...	...
13	6.0×10^{1}	−0.6	...	...	...	...	...
14	9.1×10^{1}	−0.7	...	...	100/320	...	...
15	M1.0 × 10^{2}		95/400	98/500	...	100/400	...
16	M1.0 × 10^{2}		93/175	154/500	...	...	93/350
16a	M1.0 × 10^{2}		103/100	122/125	...	117/100	...
16b	7.9×10^{1}	−0.7	94/400	75/500	...	...	61/400
18	M1.0 × 10^{2}		...	...	...	...	...
19	4.2×10^{1}	−0.6	...	...	...	...	...
20			...	...	104/640	...	...
21	M1.0 × 10^{2}	−0.4	92/400	...	...	76/400	...
23	2.7×10^{1}	−1.2	104/100	44/125	...	...	51/100
24			93/500	...	...	102/500	...
25	1.3×10^{1}	−0.4	...	...	107/640	...	...
25a	2.8×10^{0}	−0.3	...	...	...	...	...
25b	M1.0 × 10^{2}		...	...	...	...	...
26			...	...	104/20	...	...
26a	M1.0 × 10^{1}		...	...	...	...	...
26b	2.7×10^{1}	−1.1	...	...	...	...	...
27	4.5×10^{1}	−0.8	100/100	...	...	85/400	...
28			...	...	104/320	...	...
28a	M1.0 × 10^{1}		90/80	62/100	...	...	61/80
28b	1.7×10^{1}	−0.4	92/200	47/500	...	...	135/100

[a] Screening results received from Cancer Chemotherapy National Service Center (CCNSC), National Institutes of Health. All screening done by CCNSC contract screeners according to standard CCNSC screening protocol. [b] Refer to Table I. [c] ED_{50}, dose that inhibits growth to 50% of control growth. Slope, difference in result for a tenfold difference in dose. [d] L-1210 lymphoid leukemia. [e] Sarcoma 180. [f] Dunning leukemia (solid). [g] Walker carcinosarcoma 256 (intramuscular). [h] Lewis lung carcinoma.

acid and 1 ml. of this solution was added to each of the standard alkaloid test reagents used.

RESULTS AND DISCUSSION

Since many substances can interfere with the alkaloid test reagents these should be taken only as an indication of the possible presence of alkaloids. Ten of the 28 plants examined had four or five positive alkaloid tests out of the five run and it would appear that these materials almost certainly contain alkaloids (Table I.). Seven additional plant extracts had three positive tests and thus these could be considered as having a high probability of containing alkaloids. As might be expected, the petroleum ether extracts contained no alkaloids and thus the petroleum ether extract followed by an ethanol extract provides a good preliminary separation of some of the nonalkaloid components from the alkaloids.

Based on the standards of activity set by the Walter Reed Army Medical Center, none of the extracts tested could be considered as being active against *P. berghei* in mice (Table II.). Only 4 of the 13 extracts screened for antimalarial activity extended the survival time more than 1 day beyond the control and the most active, *Cinnamomum mercadoi vidal*, had only about one half the extension in survival time needed to be considered an active compound.

Five (serial No. 2, 4, 5, 23, and 25) of the original ethanol extracts had an activity worthy of note against the KB cell culture (Table III.). In the case of *Sphaeranthus africanus*, this activity was also present in the petroleum ether extract but not in the ethanol extract which followed the petroleum ether extract. With *Anona squamosa* the activity was not present in the petroleum ether extract, while both *Calophyllum blancoi* and *Straphanthus cumingii* had activity in the ethanol extract which followed the petroleum ether extraction. None of the extracts had outstanding activity against any of the animal tumors but five (serial No. 2, 9, 16b, 23, and 28a) had at least moderate activity against two animal systems each. Two (serial No. 21 and 28b) had moderate activity against one system each. It is of interest to note that only two of the compounds active in the cell culture screen had any appreciable activity in animal screens, *A. squamosa* and *Phaseolus aureus*.

SUMMARY

Of 28 Philippine plants studied, between 10 and 17 could be considered to contain alkaloids.

None of the extracts screened had any appreciable antimalarial activity, although one had a slight indication of activity.

Five of the plants were active against KB cell culture, and a number had moderate activity against a variety of animal cancers.

Improvement of the Color Stability of Parenteral Solutions of Papaverine Hydrochloride

By D. E. GRIFFITH

Disodium ethylenediaminetetraacetate (EDTA) 0.005 per cent successfully inhibits color formation in parenteral papaverine hydrochloride solutions.

PAPAVERINE HYDROCHLORIDE, one of the alkaloids of opium, is used primarily as an antispasmodic for smooth muscle, including arterial (1). It has also been prepared synthetically, and is chemically the hydrochloride of 6, 7, 3′, 4′-tetra-methoxy-1-benzylisoquinoline (1).

The chemical stability at room temperature of papaverine hydrochloride injection, as determined by the N.F. XII alkaloid extraction method, is extremely good. There is no detectable loss in potency in 4 years. However, an amber color starts to form within 1 year at 25° and within 1 month at 37°.

Disodium ethylenediaminetetraacetate (EDTA or sodium edetate) is a sequestering agent which has been used in medicine primarily as a binding agent in heavy metal poisonings; the usual dose being 75 mg./Kg. of body weight of a 20% solution (2). Salk poliomyelitis vaccine has been stabilized with sodium edetate, 7 : 20,000.[1]

Received March 22, 1967, from the Research and Development Laboratories, Eli Lilly & Co., Indianapolis, IN 46206

Accepted for publication June 19, 1967.

[1] Eli Lilly and Co.

The use of sodium edetate to prevent discoloration of pharmaceuticals is recorded several times in the literature (3–8). Discoloration has been prevented in morphine, phenylephrine, sodium sulfacetamide, procaine, and ajmaline solutions. Amounts of sodium edetate used vary from 0.005–0.04% in the references cited.

The purpose of this study was to inhibit the color formation of papaverine hydrochloride injection without changing the chemical stability. The approach was through use of sodium edetate while controlling all pertinent variables.

EXPERIMENTAL

The experiments were designed to determine the effect on papaverine hydrochloride color stability of (*a*) various concentrations of sodium edetate, (*b*) the container, (*c*) light and atmosphere, and (*d*) different papaverine hydrochloride raw material lots.

The experimental solutions were formulated with conventional laboratory apparatus, reagent grade chemicals, and water for injection U.S.P. The solutions were sterilized by either autoclaving or sterile filtration by Selas 02 candles. The glass-sealed ampuls and rubber-stoppered vials were flint type I glass. They were washed on conventional ampul washing machines and sterilized by

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The purpose of this study was to inhibit the color formation of papaverine hydrochloride injection without changing the chemical stability. The approach was through use of sodium edetate while controlling all pertinent variables.

EXPERIMENTAL

The experiments were designed to determine the effect on papaverine hydrochloride color stability of (*a*) various concentrations of sodium edetate, (*b*) the container, (*c*) light and atmosphere, and (*d*) different papaverine hydrochloride raw material lots.

The experimental solutions were formulated with conventional laboratory apparatus, reagent grade chemicals, and water for injection U.S.P. The solutions were sterilized by either autoclaving or sterile filtration by Selas 02 candles. The glass-sealed ampuls and rubber-stoppered vials were flint type I glass. They were washed on conventional ampul washing machines and sterilized by

TABLE I—RELATIVE COLOR STABILITY OF PAPAVERINE HYDROCHLORIDE PARENTERAL SOLUTIONS WITH AND WITHOUT SODIUM EDETATE—TIME TO DISCOLOR TO A.P.H.A. COLOR STANDARD 15[a] PAPAVERINE HYDROCHLORIDE WITH NITROGEN ATMOSPHERE

Temp., °C.	No EDTA	0.005% EDTA
50	2 wk.	5 mo.
37	1 mo.	9 mo.
25	12 mo.	Colorless to date (2 yr.)

[a] Standards of American Public Health Association using solutions of platinum chloride in water. A.P.H.A. 15 is a pale yellow color.

dry heat. The glass-sealed ampuls were sealed by pull-type seals. The rubber-stoppered vials were sealed with West natural rubber stoppers and aluminum seals. Aging tests were conducted at room temperature and in ovens at 37° and 50°. Light exposure consisted of exposure to direct sunlight in a window facing south, and also by exposure on a shelf to fluorescent lights approximately 10 ft. away. Color was noted visually by observing against a white background. The N.F. XII assay procedure for papaverine hydrochloride was used in all cases. Samples of papaverine hydrochloride containing 0%, 0.005%, 0.01%, and 0.02% sodium edetate were formulated.

Samples of papaverine hydrochloride with 0.005% and no sodium edetate were stored in glass-seal ampuls and rubber-stoppered vials. Two lots of papaverine hydrochloride raw material were used for the experiments. Some ampuls of each section were exposed to light. Some contained a nitrogen atmosphere. All sections were exposed to 25, 37, and 50° temperatures. The samples were observed weekly to 2 months, then monthly to the point of discoloration.

DATA AND RESULTS

Oxygen in the air and light detract from color stability of papaverine hydrochloride solutions. The effect, however, is considerably less for sodium edetate formulations. Temperature has less effect on discoloration with the sodium edetate formulations, but still is a significant factor. It was found that color inhibition was maximum at 0.005% sodium edetate and was not improved by increasing the concentration of sodium edetate. The discoloration noted in glass-seal ampuls and rubber-stoppered vials was the same, indicating no effect from the package. No difference was seen between either of the lots of papaverine hydrochloride raw material. The type of sterilization (filtration *versus* autoclave) did not affect color formation. The amount of improvement of color stability can be seen in Table I.

CONCLUSIONS

Disodium ethylenediaminetetraacetate (sodium edetate or EDTA) successfully inhibits color formation in injectable solutions of papaverine hydrochloride at a concentration of 0.005%. Protection from light and oxygen offer additional protection. Chemical potency is still at 100% at 37° at 18 months. It is believed that the mechanism of protection is through sequestering trace quantities of heavy metals present, such as iron, which may react with chloride ions in the solution at the low pH (3.0–4.0) of these solutions. That this is so was determined by adding a tiny piece of iron oxide to each of several vials of papaverine hydrochloride which turned dark amber soon after.

REFERENCES

(1) Osol, A., Farrar, G. E., and Pratt, R., "The Dispensatory of the United States of America," J. B. Lippincott Co., Philadelphia, Pa., 1960, vols. 1 and 2, pp. 968–970.
(2) Spring, W. C., "New and Nonofficial Drugs," J. B. Lippincott Co., Philadelphia, Pa., 1963, pp. 609–611.
(3) Barkman, R., and Ortengren, R., *J. Mondial Pharm.*, **315**, 26(1959).
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(5) Anderson, R. A., and Maudson, J. W., *Australasian J. Pharm.*, **44**, S24(1963).
(6) Parafinska, Z., *Acta Polon. Pharm.*, **20**, 395(1963).
(7) Coombe, R. G., and Roxon, J. J., *Australasian J. Pharm.*, **44**, S34(1963).
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New Compounds: *N*-Substituted Tetrahydroisoquinolines

By JOSEPH SAM*

The Mannich, sodium borohydride reduction, and esterification reactions were utilized for the preparation of several *N*-substituted tetrahydroisoquinolines. Aminolysis of ethyl cyanoacetate with tetrahydroisoquinoline provided the corresponding cyanoacetamide.

SEVERAL *N*-SUBSTITUTED tetrahydroisoquinolines were prepared and screened for biological activity. The Mannich reaction with *p*-chloro- and *p*-methoxyacetophenone yielded two tetrahydroisoquinolino-ketones (I). Mannich and Lammering (1) earlier had described the preparation of unsubstituted I. The sodium borohydride reduction of I resulted in II; treatment of II*a* with 3,4,5-trimethoxybenzoyl chloride provided the corresponding ester.

The aminolysis of ethyl cyanoacetate with tetra-

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Accepted for publication June 7, 1967.
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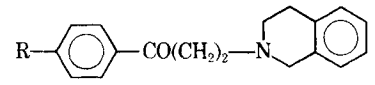
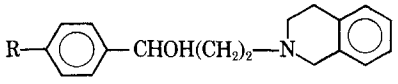
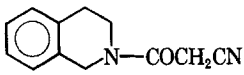


Ia, R = CH_3O
b, R = Cl



IIa, R = CH_3O
b, R = Cl

hydroisoquinoline gave a good yield (65%) of the corresponding amide III.



III

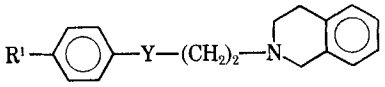
Preliminary pharmacological evaluation did not indicate any pronounced biological activity in the compounds.

and Brown (3) was followed. To a mixture of 5 Gm. of sodium borohydride and 50 ml. of methanol was added dropwise with stirring a solution of 0.1 mole of carbonyl compound. Thereafter, the mixture was refluxed for 2 hr. The methanol was distilled *in vacuo*. The residual material was treated with 200 ml. of water, extracted with ether, and dried over anhydrous sodium sulfate. The product was isolated as the base or converted to the hydrochloride in the usual manner.

α - (p - Methoxyphenyl) - γ - [3,4 - dihydro-2(1) - isoquinolyl]propyl - 3,4,5 - trimethoxybenzoate (Table I, Method C)—A solution of 6.0 Gm. (0.02 mole) of the alcohol in 150 ml. of chloroform was treated with 5.0 Gm. of 3,4,5-trimethoxybenzoyl chloride and an excess of potassium carbonate. The resulting mixture was allowed to remain at room temperature for 18 hr. Water was added to the mixture and the chloroform layer separated and dried over anhydrous potassium carbonate. A hydrochloride was prepared in the usual manner and recrystallized from a mixture of methyl ethyl ketone and ether, m.p. 163–164°.

2 - Cyanoacetyl - 1,2,3,4 - tetrahydroisoquinoline

TABLE I—*N*-SUBSTITUTED TETRAHYDROISOQUINOLINES



R'	Y	Recrystn. Solvent[a]	Method	% Yield	B.p., °C. (mm.) M.p., °C.	Molecular Formula	Anal. Calcd. C	Calcd. H	Found C	Found H
Cl	C=O	M	*A*	66	200–202	$C_{18}H_{19}Cl_2NO$[b]	64.3	5.69	64.5	5.79
CH_3O	CHOH	M–MIK	*B*	60	155–156	$C_{19}H_{24}ClNO_2$[b]	69.0	7.37	68.5	7.38
CH_3O	3,4,5-tri$CH_3OC_6H_2$-CO_2CH	E-MEK	*C*	28	163–164	$C_{29}H_{34}ClNO_6$[b]	66.0	6.49	66.2	6.62
CH_3O	C=O	...	*A*	61	81–82	$C_{19}H_{21}NO_2$	...	...	...	...
CH_3O	C=O	M–MEK	..	90[c]	184–186	$C_{19}H_{22}ClNO_2$[b]	68.8	6.68	69.2	6.85
Cl	CHOH	E–H	*B*	80	194–200 (0.4 mm.) 99–100	$C_{18}H_{20}ClNO$	71.6	6.68	71.7	6.78
Cl	CHOH	M–E	..	95[c]	184–186	$C_{18}H_{21}Cl_2NO$[b]	63.9	6.26	64.3	6.60

[a] M, methanol; MIK, methyl isobutyl ketone; E, ether; MEK, methyl ethyl ketone; H, *n*-hexane. [b] Hydrochloride. [c] From the parent amine.

EXPERIMENTAL[1]

Mannich Reaction Products (Table I, Method A)—The procedures described by Mannich and Lammering (1) and Mosettig and May (2) were followed. To a mixture of 10 ml. of concentrated hydrochloric acid were added 0.1 mole of substituted acetophenone and 10 ml. of 37% formaldehyde. The resulting mixture was heated on a steam bath with stirring for 2 hr. The hydrochloride was removed by filtration and recrystallized from a suitable solvent (Table I).

Sodium Borohydride Reduction Products (Table I, Method B)—The procedure described by Chaiken

[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

—A mixture of 13 Gm. (0.1 mole) of 1,2,3,4-tetrahydroisoquinoline and 12 Gm. (0.1 mole) of ethyl cyanoacetate was refluxed for 2 hr. and then allowed to stay at room temperature for 12 hr. The reaction mixture was treated with ether. The solid (13 Gm., 65%) was removed by filtration and recrystallized from a mixture of methanol and ether, m.p. 105–106°.

Anal.—Calcd. for $C_{12}H_{12}N_2O$: C, 72.0; H, 6.04. Found: C, 72.2; H, 6.13.

REFERENCES

(1) Mannich, C., and Lammering, D., *Ber.*, **55B**, 3510 (1922).
(2) Mosettig, E., and May, E., *J. Org. Chem.*, **5**, 528 (1940).
(3) Chaiken, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, **71**, 122(1949).

New Compounds: Mannich Bases from 2-Phenylindolizines II. 3-Dialkylaminomethyl Derivatives

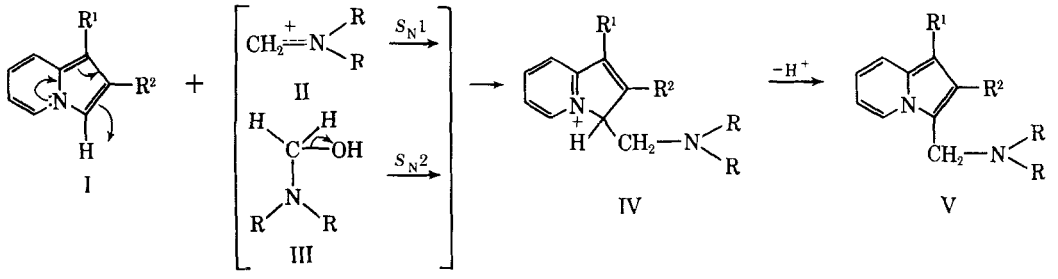
By WILLIAM B. HARRELL* and ROBERT F. DOERGE

In continuing the search for new therapeutic agents in the indolizine series, eight new Mannich bases derived from 1,2-diphenylindolizine have been synthesized. In addition, 1-(2-hydroxyethyl)-2-phenylindolizine, a new indolizine, and its 3-pyrrolidinomethyl derivative have been synthesized. The mechanism by which 1,2-disubstituted indolizines participate in the Mannich reaction has been discussed and alternate S_N1 and S_N2 routes have been proposed.

IN A PREVIOUS PAPER (1) the authors described the synthesis of a series of Mannich bases with the dialkylaminomethyl substituent at the C-1 position of the indolizine ring. The purpose of this investigation was to continue the search for useful therapeutic agents by synthesizing a series of 3-dialkylaminomethyl-1,2-diphenylindolizines. Eight new compounds were prepared in this series. In addition, a new indolizine, 1-(2-hydroxyethyl)-2-phenylindolizine, was synthesized and its 3-pyrrolidinomethyl derivative was prepared by the Mannich reaction (2). These compounds are currently being evaluated for possible activity on the central nervous system. If interesting activity is found, a study of the relationship of chemical structure with activity will be conducted.

DISCUSSION

The successful preparation of Mannich bases derived from indolizines in which the dialkylaminomethyl substituent occurs at the C-3 position has not been reported previously in the literature. In part I of this study the authors proposed a mechanism by which indolizines, based on their eneamine nature (3), participate in the Mannich reaction to form the 1-dialkylaminomethyl derivatives. Scheme I illustrates how this mechanism can be expanded to include the preparation of Mannich bases with the dialkylaminomethyl substituent occurring at the C-3 position. Alternate S_N1 and S_N2 routes are proposed.



Scheme I

EXPERIMENTAL

All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were provided by Weiler and Strauss Microanalytical Laboratory, Oxford, England. Results of pharmacological screening will be reported elsewhere.

Materials—The starting indolizines were prepared by the Chichibabin synthesis (4) which involved condensing phenacyl bromide with the appropriate 2-substituted pyridine and heating the resulting pyridinium bromide in aqueous sodium bicarbonate. The preparation of 1,2-diphenylindolizine (I) has been reported previously (5).

Preparation of Mannich Bases from 1,2-Diphenylindolizine—The following general procedure was employed in the synthesis of these compounds. A mixture containing formaldehyde (0.05 mole), the secondary amine (0.05 mole), and 20–30 ml. of *N,N*-dimethylformamide (DMF) was stirred with a magnetic stirrer at room temperature for 30 min. The indolizine (0.01 mole) was then dissolved in the mixture and stirring was continued for 2 to 3 hr. In all cases except one, the product crystallized out during the stirring process. In the case of the 3-morpholinomethyl derivative, crystallization occurred after allowing to stand at room temperature for 24 hr. The preparation of 3-diethylaminomethyl-1,2-diphenylindolizine is described in the following section and is typical of the experimental procedures employed.

3 - Diethylaminomethyl - 1,2 - diphenylindolizine—To a 125-ml. conical flask equipped with a Teflon coated magnetic stirrer was added 4 ml. of 37% aqueous formaldehyde (0.05 mole), 3.7 Gm. diethylamine (0.05 mole), and 20 ml. DMF, and the mixture was stirred at room temperature for 30 min. To the mixture was added 2.7 Gm. 1,2-diphenylindolizine (0.01 mole) and stirring was continued for 3 hr. The product began to crystallize about 1 hr. after the addition of the indolizine.

Received April 14, 1967, from the School of Pharmacy, Oregon State University, Corvallis, OR 97331

Accepted for publication June 5, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted from a thesis submitted by William B. Harrell to the Graduate School, Oregon State University, Corvallis, in partial fulfillment of Doctor of Philosophy degree requirements.

Previous paper: Harrell, W. B., and Doerge, R. F., *J. Pharm. Sci.*, **56**, 225(1967).

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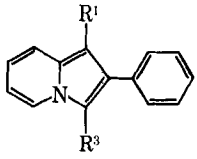
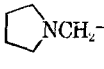
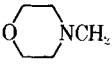
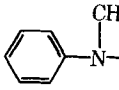
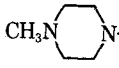
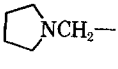
TABLE I—MANNICH BASES PREPARED



No.	Empirical Formula	R^1	R^3	M.p., °C.	Yield, %	Recrystn. Solvent	Anal., % Calcd.	Found
I*a*	$C_{23}H_{22}N_2$		$(CH_3)_2NCH_2$—	140–141	95	Ethanol	C, 84.63 H, 6.79 N, 8.58	84.09 7.45 8.58
I*b*	$C_{25}H_{26}N_2$		$(C_2H_5)_2NCH_2$—	114.5–115.5	87.5	Acetone–water	C, 84.71 H, 7.39 N, 7.90	84.32 7.35 7.82
I*c*	$C_{27}H_{30}N_2$		$[(CH_3)_2CH]_2NCH_2$—	123–124	58	Acetone–water	C, 84.77 H, 7.90 N, 7.32	84.43 7.85 7.22
I*d*	$C_{25}H_{24}N_2$			143–144	85	Acetone–water	C, 85.19 H, 6.86 N, 7.95	85.81 6.92 6.81
I*e*	$C_{26}H_{26}N_2$			128–129	90	Acetone–water	C, 85.21 H, 7.15 N, 7.64	85.18 7.17 7.55
I*f*	$C_{25}H_{24}N_2O$			141–142	78.5	Acetone–water	C, 81.49 H, 6.57 N, 7.60	81.20 6.54 7.45
I*g*	$C_{28}H_{24}N_2$			162–163	85	DMF–water	C, 86.56 H, 6.23 N, 7.21	85.80 6.25 7.98
I*h*	$C_{26}H_{27}N_3$			36–137	84	Ethyl acetate	C, 81.85 H, 7.13 N, 11.01	82.00 7.09 10.70
II*a*	$C_{21}H_{24}N_2O$	$HOCH_2CH_2$—		122–124	62	DMF–water	C, 78.72 H, 7.55 N, 8.74	78.65 7.54 8.72

Upon completion of the crystallization, the flask was placed in the refrigerator overnight. The product was filtered, washed with 25% ethanol, recrystallized from hot ethanol, and yielded 3.1 Gm. (87.5%), m.p. 114.5–115.5°.

Anal.—Calcd. for $C_{25}H_{26}N_2$: C, 84.71; H, 7.39; N, 7.90. Found: C, 84.32; H, 7.35; N, 7.82.

2 - (3 - Hydroxypropyl) - 1 - phenacylpyridinium Bromide—To a 1-L. conical flask, equipped with a magnetic stirrer and cooled in an ice bath was added 99.5 Gm. phenacyl bromide (0.5 mole) dissolved in 300 ml. ether. To the well-stirred ethereal solution was added dropwise 110 Gm. (0.8 mole) of 3-(2-pyridyl)-1-propanol over a 10-min. period. The temperature of the surrounding ice water bath was allowed to rise gradually to room temperature and stirring was continued for an additional 10 to 12 hr. The crystalline product was collected and washed with ether. Recrystallization from ethanol yielded 131 Gm. (78%), m.p. 138–140°. The identity of this new intermediate was confirmed by the successful ring closure to 1-(2-hydroxyethyl)-2-phenylindolizine in the Chichibabin reaction.

1 - (2 - Hydroxyethyl)-2-phenylindolizine—In a 2-L. beaker was placed 79 Gm. (0.24 moles) of 2 - (3 - hydroxypropyl) - 1 - phenacylpyridinium bromide and 1200 ml. of water. The beaker was placed in a water bath on a magnetic stirrer hot plate. The temperature of the reaction mixture was raised to about 60°, and 120 Gm. sodium bicarbonate was then added in small portions over a 10-min. period. After the addition, the temperature of the reaction mixture was gradually raised and maintained in the range of 80–85° with constant stirring for 2 hr. Foaming occurred during the reaction and was controlled by supplemental manual stirring with a glass rod. At the end of the reaction period, the crystalline product was filtered off while

hot by suction and washed with warm water. The air-dried product was immediately recrystallized from hot ethanol and yielded 51 Gm. (92%), m.p. 101.5–102.5°.

Anal.—Calcd. for $C_{16}H_{15}NO$: C, 80.98; H, 6.37; N, 5.90. Found: C, 81.10; H, 6.40; N, 5.81.

1 - (2 - Hydroxyethyl) - 2 - phenyl - 3 - pyrrolidinomethylindolizine—Six milliliters of 37% aqueous formaldehyde (0.075 mole) and 5.34 Gm. of pyrrolidine (0.075 mole) were combined with 30 ml. of dioxane and allowed to stand for 15 min. at room temperature. 1-(2-Hydroxyethyl)-2-phenylindolizine, 3.57 Gm. (0.015 mole), was dissolved in the mixture which was then allowed to stand at room temperature for 24 hr. The reaction mixture was transferred to an evaporating dish and the solvent was removed by blowing cold air over the surface. Scratching with a glass rod during evaporation failed to induce crystallization and a viscous oil was obtained. The oil was transferred to a 50-ml. conical flask with the aid of about 3 ml. of ethanol and placed in the freezer compartment of the refrigerator. The product crystallized after 1 week in the refrigerator. The crystals were removed by filtration, washed with 50% ethanol, and recrystallized from hot DMF–water. The yield was 2.9 Gm. (62%), m.p. 122–124°.

Anal.—Calcd. for $C_{21}H_{24}N_2O$: C, 78.72; H, 7.55; N, 8.74. Found: C, 78.65; H, 7.54; N, 8.72.

REFERENCES

(1) Harrell, W. B., and Doerge, R. F., *J. Pharm. Sci.*, **56**, 225(1967).
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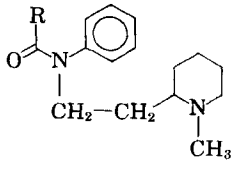
New Compounds: Amides Derived from 2-(2-Pyridyl)ethylamines

By JOSEPH SAM*

Basic amides containing the pyridylethyl- and piperidylethylamino groups have been prepared either from 2-vinylpyridines, 2-(2-aminoethyl)pyridines, or from substituted 2-(2-aminoethyl)piperidines. The amides possess some of the structural characteristics of the phenothiazine tranquilizers.

Amides, in general, exhibit a depressant effect on the central nervous system. Phenacetin, an analgesic amide, and barbital, an hypnotic amide derivative, were forerunners of many other biologically active amides (1).

Based on (*a*) the general CNS depressant properties of amides, (*b*) the presence of the 1,3-propylenediamine entity in many biologically active compounds, and (*c*) the similarity of the reactions of the NH of the phenothiazine ring to the NH of amides, it was of interest to prepare some amides (I) possessing structural characteristics of the phenothiazine tranquilizers (II).



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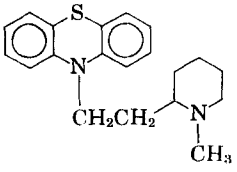

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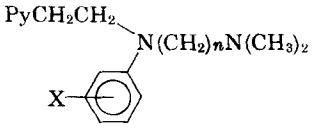

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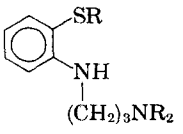

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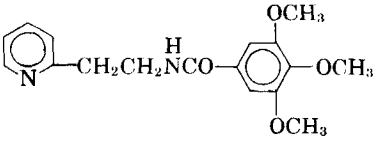

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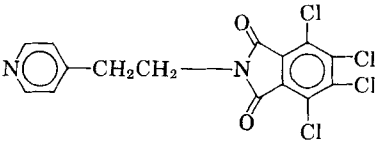

VI

Shapiro and associates (2) prepared some similar compounds (III) which were related to the antihistamine, tripelennamine [*N*-benzyl-*N'*,*N'*-dimethyl-*N*-(2-pyridyl)ethylenediamine] and to the phenothiazine tranquilizers. Nieforth (3) prepared

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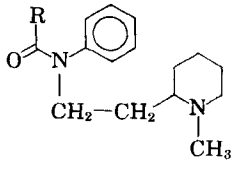

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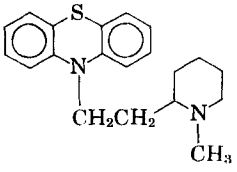

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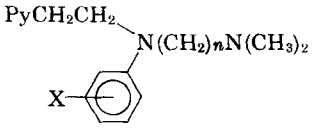

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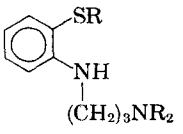

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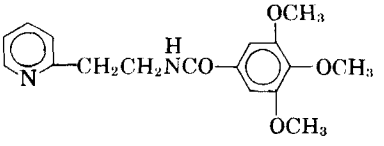

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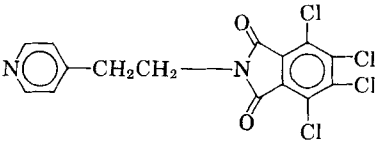

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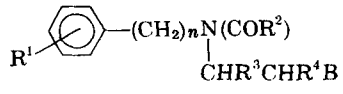
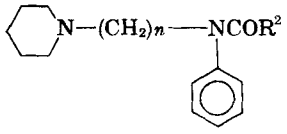


VII



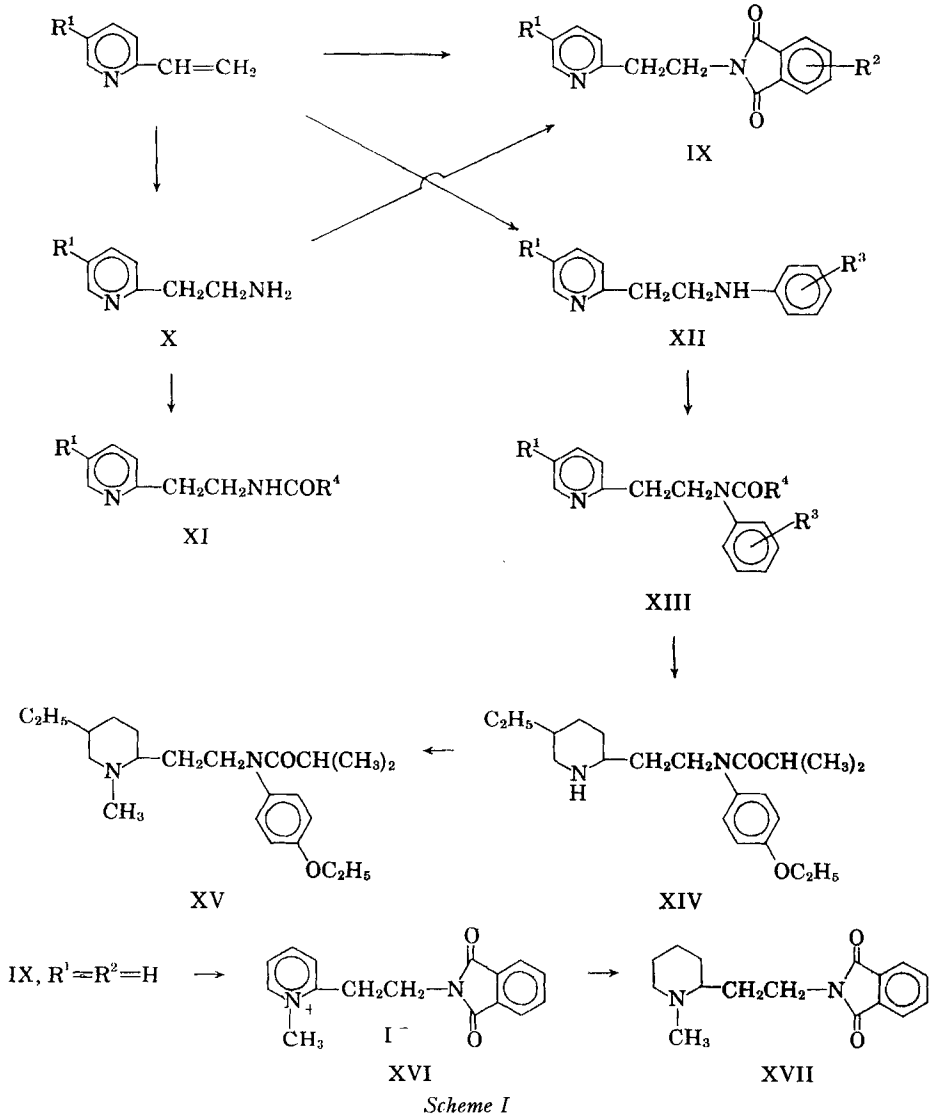
VIII

some 2-aminobenzenethiol derivatives (IV) related to the phenothiazine tranquilizers and to reserpine. Perron and Sam (4) noted tranquilizing properties in the 3,4,5-trimethoxybenzamide (V) of 2-(2-pyridyl)ethylamine; Lewis (5) observed analgesic properties with the phthalimide (IX) $R^1 = R^2 = H$; and VI was prepared as an intermediate to bis-quaternary hypotensive agents (6). Compounds possessing structure I are closely related to potent analgesic amides (VII and VIII, respectively) described by Fancher and associates (7) and Carabateas and co-workers (8).

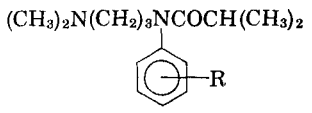
The procedures described by Reich and Levine (9) and Shapiro and associates (2) were employed for the preparation of the phthalimides (IX) and pyridylethylanilines (XII). The preparation of the *N*-methylpiperidine derivative (XV) was accomplished *via* hydrogenation of XIII followed by methylation, whereas XVII was obtained from IX *via* the methiodide (XVI) followed by reduction. The amides (XI) were prepared by reacting X with the appropriate acid chloride. (Scheme I.)

Several related compounds possessing structures XVIII and XIX were prepared for comparison purposes. The latter compounds are related to analgesic anilides described by Wright and Brabander (10).

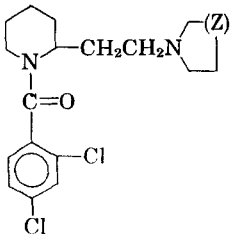
Preliminary pharmacological evaluation did not indicate any pronounced biological activity in the compounds that were studied.



Scheme I



XVIII



XIX (Z = CH_2, CH_2O)

EXPERIMENTAL[1]

2-(2-Aminoethyl)-5-ethylpyridine—The procedure described by Magnus and Levine (12) for the preparation of 2-(2-aminoethyl)pyridine was followed. A mixture of 214 Gm. (4 moles) of ammonium chloride, 266 Gm. (2 moles) of 5-ethyl-2-vinylpyridine, 300 ml. of water, and 400 ml. of methanol was refluxed for 3 days. The methanol was distilled *in vacuo*. The aqueous solution was cooled, poured into ice water, and neutralized with excess 40% sodium hydroxide. The oily layer was extracted with chloroform and dried over anhydrous potassium carbonate. Distillation of the solvent and then the residual oil gave 192 Gm. (64%) of distillate, b.p. 90–95° (2 mm.).

Anal.—Calcd. for $C_9H_{14}N_2$: C, 71.9; H, 9.38; N, 18.76. Found: C, 72.1; H, 9.56; N, 18.55.

Amides (Table I)—*Method A*—A mixture of 0.2 mole of amine, 40.4 Gm. (0.4 mole) of triethylamine, 0.4 mole of acid chloride, and 600 ml. of dry benzene was refluxed on a steam bath for 18–24 hr. Thereafter, the mixture was cooled, washed with water, and 10% sodium hydroxide, respectively, and dried over anhydrous potassium carbonate. The benzene solution was distilled and the residual material either recrystallized or distilled.

Method B—A mixture of 0.5 mole of the desired vinylpyridine and 73.6 Gm. (0.5 mole) of phthalimide was treated with 0.5 mole of a surfactant[2] and heated slowly and carefully to 175–180° and kept at this temperature for 3 hr. The oil, consisting of a single phase, was cooled and treated with 100 ml. of chloroform and filtered. The filtrate was concentrated and the residual oil crystallized.

Method C—A mixture of 24.1 Gm. (0.2 mole) of 2-(2-aminoethyl)pyridine (11) and 0.2 mole of anhydride was heated at 170–180° for 2 hr. and then cooled. The residual solid was recrystallized from a suitable solvent.

Method D—A mixture of 24.5 Gm. (0.2 mole) of 2-(2-aminoethyl)pyridine and 0.2 mole of ester was heated on a steam bath for 20 hr. and then vacuum distilled.

1 - (2,4 - Dichlorobenzoyl) - 5 - ethyl - 2 - (2 - morpholinoethyl)piperidine—The procedure described under *Method A* was followed. From 22.6 Gm. (0.1 mole) of 5-ethyl-2-(2-morpholinoethyl)-piperidine and 21 Gm. (0.1 mole) of 2,4-dichlorobenzoyl chloride there was obtained 31 Gm. (77%) of product, b.p. 234–236° (0.7 mm.) (13).

A methiodide was prepared in 87% yield as described under *Method E* and was recrystallized from methanol, m.p. 235–236° (13).

1 - (2,4 - Dichlorobenzoyl) - 5 - ethyl - 2 - (2 - pyrrolidinoethyl)piperidine—The procedure described under *Method A* was followed. From 19 Gm. (0.09 mole) of 5-ethyl-2-(2-pyrrolidinoethyl)-piperidine and 21 Gm. (0.1 mole) of 2,4-dichlorobenzoyl chloride there was obtained 28 Gm. (81%) of product, b.p. 218–220° (1.0 mm.) (13).

A methiodide was prepared in 98% yield as described under *Method A* and recrystallized from methanol, m.p. 205–207° (13).

Quaternary Ammonium Iodides—*Method E*—A solution of 0.02 mole of the requisite tertiary amine in 50 ml. of acetonitrile was treated with 14 Gm. (0.1 mole) of methyl iodide and refluxed on a steam bath for 4 hr. The solution was either filtered to remove the product or concentrated to dryness and then recrystallized from a suitable solvent.

2 - [2 - (p - Ethoxyanilino)ethyl] - 5 - ethylpyridine—The procedure described by Reich and Levine (9) was followed using 133 Gm. (1.0 mole) of 5-ethyl-2-vinylpyridine, 137 Gm. (1.0 mole) of *p*-phenetidine, 60 Gm. (1.0 mole) of acetic acid, and 300 ml. of methanol. One hundred and ninety grams of product, boiling at 168–176° (0.1 mm.), was obtained.

Anal.—Calcd. for $C_{17}H_{22}N_2O$: C, 75.51; H, 8.20. Found: C, 75.70; H, 8.38.

N - [2 - (1 - Methyl - 2 - piperidyl)ethyl]phthalimide Hydriodide—A mixture of 39 Gm. (0.1 mole) of 1-methyl-2-(2-phthalimidoethyl)pyridinium iodide, 200 ml. of methanol, and 0.4 Gm. of platinum oxide catalyst was hydrogenated at 60 p.s.i. at 50° for 18 hr. The resulting mixture was treated with 800 ml. of methanol, heated to reflux, filtered, and cooled. The product (30 Gm., 75%) was removed by filtration, m.p. 230–231°.

Anal.—Calcd. for $C_{16}H_{21}IN_2O_2$: C, 48.01; H, 5.29. Found: C, 48.20; H, 5.29.

N - (p - Ethoxyphenyl) - N - [2 - (5 - ethyl - 2-piperidyl)ethyl]isobutyramide—A solution of 40 Gm. (0.12 mole) of *N*-(*p*-ethoxyphenyl)-*N*-[2-(5-ethyl-2-pyridyl)ethyl]isobutyramide in 150 ml. of glacial acetic acid was hydrogenated at 58 p.s.i. in the presence of 0.4 Gm. platinum oxide catalyst for 30 hr. The catalyst was removed by filtration and the filtrate concentrated under reduced pressure. The residual material was treated with 100 ml. of concentrated sodium hydroxide solution. The oily layer was extracted with ether and dried over anhydrous potassium carbonate. Distillation of the ether solution gave 36 Gm. (88%) of product, b.p. 200–205° (1.0 mm.); n_D^{26} 1.5161.

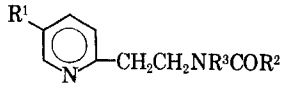
Anal.—Calcd. for $C_{21}H_{34}N_2O_2$: C, 72.79; H, 9.89. Found: C, 72.80; H, 9.70.

N - (p - Ethoxyphenyl) - N - [2 - (5 - ethyl - 1-methylpiperidyl)ethyl]isobutyramide—To 25 Gm. (0.07 mole) of *N*-(*p*-ethoxyphenyl)-*N*-[2-(5-ethyl-2-piperidyl)ethyl]isobutyramide, cooled in an ice bath, was added 7.8 Gm. (0.17 mole) of 98% formic acid. The mixture was allowed to warm slightly and then cooled while 7 ml. of 37% formaldehyde

[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Boiling points are uncorrected.

[2] Triton-B, Rohm and Haas, Philadelphia, Pa.

TABLE I—*N*-(2-PYRIDYLETHYL)AMIDES



R¹	R²	R³	Method	Recrystn. Solvent[a]	% Yield	M.p., °C. (B.p./mm.)	Molecular Formula	Anal., % Calcd. C	Calcd. H	Found C	Found H
H	$(C_6H_5)_2CH$	H	*A*	A–H	63	114–116	$C_{21}H_{20}N_2O$	79.7	6.37	79.9	6.53
C_2H_5	$(C_6H_5)_2CH$	H	*A*	M–W	74	116–117	$C_{23}H_{24}N_2O$	80.2	7.02	80.5	6.96
H	$NCCH_2$	H	*D*	M–E	58	(190–200/0.07) 118–120	$C_{10}H_{11}N_3O$	63.5	5.86	63.2	5.62
C_2H_5	3,4,5-$(CH_3O)_3C_6H_2$	H	*A*	E	73	98–99	$C_{19}H_{24}N_2O_4$	66.3	7.02	66.3	7.14
C_2H_5	CH_3	*p*-$C_2H_5OC_6H_4$	*A*	...	40	(197–199/0.5)	$C_{19}H_{24}N_2O_2$	73.1	7.74	73.1	7.85
C_2H_5	$(CH_3)_2CH$	*p*-$C_2H_5OC_6H_4$	*A*	...	89	(187–189/0.2)	$C_{21}H_{28}N_2O_2$	74.1	8.29	73.9	8.10
C_2H_5	*p*-$CH_3OC_6H_4$	*p*-$C_2H_5OC_6H_4$	*A*	E–H	71	68–69	$C_{25}H_{28}N_2O_3$	74.2	6.98	74.1	6.93
C_2H_5	$(CH_3)_2CH$	*m*-ClC_6H_4	*A*	...	56	(188–193/1.1)	$C_{19}H_{23}ClN_2O$	69.0	6.72	69.1	6.88
C_2H_5	$C_6H_4(CO)_2$[b]		*B*	C	43	94–97	$C_{17}H_{16}N_2O_2$	72.8	5.75	73.0	5.80
H	$C_6Cl_4(CO)_2$[c]		*C*	DMF	58	167–170	$C_{15}H_8Cl_4N_2O_2$[d]	46.2	2.07	46.4	2.27
H	$C_6H_4(CO)_2$[b]		*B*, *C*	C	80	96–98	$C_{15}H_{12}N_2O_2$[e]				
H	$C_6H_4(CO)_2$[b]		*E*	W	91	261–263	$C_{16}H_{15}IN_2O_2$[f,g]	48.7	3.83	48.8	3.71

[a] A, acetone; H, hexane; M, methanol; W, water; E, ether; C, cyclohexane. [b] NR^3COR^2, phthalimido. [c] NR^3COR^2, tetrachlorophthalimido. [d] Calcd. for Cl: 36.4. Found: Cl, 36.0. [e] *Reference 11.* [f] Methiodide. [g] Calcd. for I: 32.2. Found: I, 31.8.

solution was added. The resulting mixture was heated on a steam bath for 4 hr., cooled, and treated with 25 ml. of concentrated hydrochloric acid, and then concentrated under reduced pressure on a steam bath. The residual oil was neutralized with 15% sodium hydroxide solution, extracted with ether, and dried over anhydrous potassium carbonate. Distillation of the ether solution gave 20 Gm. (80%) of product, b.p. 187–190° (0.4 mm.); n_D^{26} 1.5141.

Anal.—Calcd. for $C_{22}H_{36}N_2O_2$: C, 73.29; H, 10.07. Found: C, 73.50; H, 10.02.

N - (3 - Dimethylaminopropyl) - N - (m - chlorophenyl)isobutyramide—Method *A* for the preparation of amides was followed using 25 Gm. (0.12 mole) of *N,N*-dimethyl-*N'*-(*m*-chlorophenyl)-1,3-propylenediamine (2); 28 Gm. (88%) of product was obtained, b.p. 132–135° (0.3 mm.); n_D^{26} 1.5157.

Anal.—Calcd. for $C_{15}H_{23}ClN_2O$: C, 63.70; H, 8.20. Found: C, 63.75; H, 8.20.

N - (3 - Dimethylaminopropyl) - N - (p - ethoxyphenyl)isobutyramide—The procedure described under *Method A* for the preparation of amides was followed, using 21 Gm. (0.1 mole) of *N,N*-dimethyl-*N'*-(*p*-ethoxyphenyl)-1,3-propylenediamine; 21 Gm. (72%) of product was obtained, b.p. 148–150° (0.4 mm.).

Anal.—Calcd. for $C_{17}H_{28}N_2O_2$: C, 69.82; H, 9.65. Found: C, 69.50; H, 9.35.

REFERENCES

(1) Burger, A., "Medicinal Chemistry," 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1960, chap. 6.
(2) Shapiro, S., Soloway, H., Chodos, E., and Freedman, L., *J. Pharm. Sci.*, **50**, 1035(1961).
(3) Nieforth, K. A., *ibid.*, **52**, 1136(1963).
(4) Perron, Y. G., and Sam, J., U. S. pat. 2,870,156 (January 20, 1959); through *Chem. Abstr.*, **53**, 10264(1959).
(5) Lewis, J. R., *Arch. Intern. Pharmacodyn.*, **88**, 142 (1951); through *Biol. Abstr.*, **27**, 9727(1953); through *Chem. Abstr.*, **46**, 2680(1952).
(6) Irwin Neisler and Co., Brit. pat. 842,995 (August 4, 1960).
(7) Fancher, O. E., Hayoa, S., Strycher, W. G., and Sancilio, L. F., *J. Med. Chem.*, **7**, 721(1964).
(8) Carabateas, P. M., Wetterau, W. F., and Grumbach, L., *ibid.*, **6**, 355(1963).
(9) Reich, H. E., and Levine, R., *J. Am. Chem. Soc.*, **77**, 4913, 5434(1955).
(10) Wright, W. B., and Brabander, H. J., U. S. pat. 3,016,382(January 9, 1962); through *Chem. Abstr.*, **57**, 16485 (1962).
(11) Kirchner, F. K., McCormick, J .R., Cavallito, C. J., and Miller, L. C., *J. Org. Chem.*, **14**, 388(1949).
(12) Magnus, G., and Levine, R., *J. Am. Chem. Soc.*, **78**, 4127(1956).
(13) Sam, J., U. S. pat. 2,922,786(January 26, 1960).

Quantitative Determination of Sympathomimetic Amines Using Interference Refractometer

Sir:

Interference refractometry, the phenomenon of light interference which is used to measure differences in the refractive index of liquids (and gases), has been used as a means for quantitative analysis of solutions, and a method for the determination of concentration of protein in blood serum has been established (1) using this technique.

In the present investigation, quantitative determination of some sympathomimetic amines has been carried out using a Carl Zeiss laboratory interferometer and a 80-mm. cell.

Very dilute solutions of the amines in various solvents were prepared, and the measuring process followed directly after the determination of the zero point in the instrument. This means that the zero point must be determined at the beginning of the experiment and before each individual series. For this purpose the solvent to be used for the solution under measurement is placed in the twin-cell, and the zero reading is taken. The reference substance is then removed from one half of the cell and the test sample is poured into it and allowed to stand until the temperature has become equalized (approximately 20 min.), because at this stage only the lower interference band can be seen, the upper band of the band system having been displaced laterally due to the filling cell.

The actual measurement process is to determine the amount of this displacement. The reading, less the zero point reading, is the required result.

The purity of the amines used was detected, before use, by thin-layer chromatography, melting point determination, and infrared spectra analysis.

Using solutions of amphetamine sulfate (National Biochemical Corp.) in water, a linear relationship between the various solutions of the amine and the interference refractive index was observed, and this relationship was found to be concentration dependent (Fig. 1). An unknown amount of the amine, measured under the same conditions, can be then estimated.

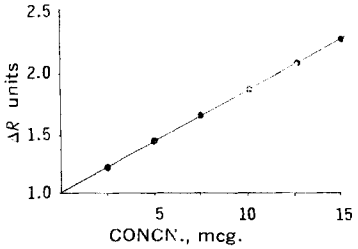


Fig. 1—Relation between differential refractive index (ΔR) and concentration for amphetamine sulfate in water solution.

Similar results were obtained using aqueous solutions of nordefrin hydrochloride (Mann Research Laboratories, Inc.) and phenylephrine hydrochloride (K & K Laboratories, Inc.).

Due to the high precision of the instrument (with monochromatic light, an accuracy of $\pm 1 \times 10^{-7}$ to $\pm 1 \times 10^{-8}$ can be obtained), the method is regarded as being very accurate even for microgram quantities of the amines.

A complete picture of the results, including all amines and solvents used, and a full discussion of the method will be cited in a definite publication.

(1) Hirsch, P., "Handbook der Biologischen Arbeitsmethoden," 1926, p. 761.

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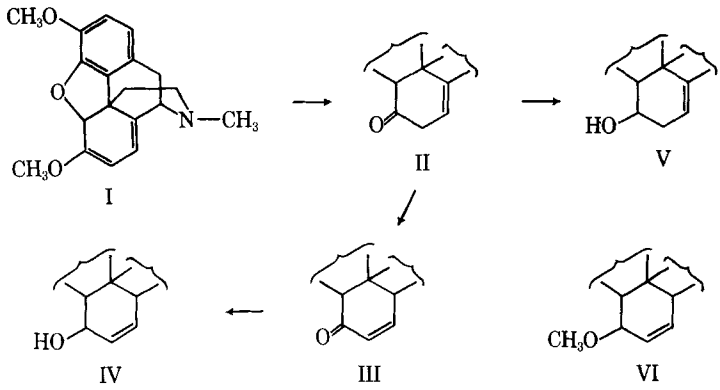
Received May 4, 1967.
Accepted for publication July 5, 1967.

Biosynthetic Conversion of Thebaine to Codeine and Role of Codeine Methyl Ether

Sir:

The mechanism by which thebaine (I) is converted to codeine in the opium poppy has been postulated to involve demethylation to the ketone neopinone (II), rearrangement to codeinone (III), followed by reduction to codeine (IV) (1–3). This scheme also explains the presence in the plant of the alkaloid neopine (V) as a reduction product of neopinone. In 1965 codeine methyl ether (VI) was isolated from the crude mother liquor obtained during the commercial production of morphine (4).



The suggestion was made that this substance might conceivably be a biosynthetic intermediate between thebaine and codeine if the reduction step preceded demethylation. It was therefore decided to test these hypotheses by feeding experiments with radioactively labeled codeinone and codeine methyl ether and by demonstrating whether these two substances exist as genuine alkaloids in the fresh plant. This investigation had been completed when Blaschke, Parker, and Rapoport (5) described experiments with generally labeled materials of a similar nature and with similar results. Our work with specifically labeled codeinone, which provides independent evidence for the role of this compound in the biosynethetic conversion of thebaine to codeine, is published elsewhere (6). However, we want to report here on our experiments with codeine methyl ether and the conclusions which may be drawn from these.

Codeine *N*-oxide (3-$^{14}CH_3$) was prepared as described by Chang *et al.* (7). It was methylated with dimethyl sulfate, and reduced with sulfur dioxide to codeine (3-$^{14}CH_3$) methyl ether which was purified by preparative thin-layer chromatography and crystallization from ether–petroleum ether, m.p. 142° (micro K), specific activity 1.35 mc./mmole. The substance was shown to be chromatographically pure by thin-layer and gas chromatography. Autoradiograms (7 days' exposure) from thin-layer chromatograms with two solvent systems (8) revealed only one radioactive spot corresponding to codeine methyl ether. In July 1965, 56 mg. (0.241 mc.) of this substance was administered to 5 opium poppies by a technique described before (9). The plants were harvested 3 days later, extracted with methanol, and the nonphenolic alkaloid fraction isolated. An autoradiogram of this fraction showed radioactive codeine and codeine methyl ether, while thebaine was inactive. Preparative thin-layer chromatography on silica gel GF (benzene–ethanol, 8 : 2, triple development) gave codeine (41 mg.), codeine methyl ether (48 mg.), and thebaine (56 mg.). Codeine was purified by repeated crystallization, first as the picrate, then as the free base, until the activity remained constant (liquid scintillation counting). Total incorporation of radioactivity from the methyl ether was 2.5%. A Zeisel *O*-demethylation showed that all activity resided in the *O*-methyl group. Although these results were encouraging, it was felt that the amount of precursor which had been fed was too large for the number of plants, possibly resulting in a swamping of the biosynthetic pool. Second, the feeding time of 3 days might have been too short for efficient incorporation since most of the methyl ether was recovered unchanged. The feeding experiments were, therefore, repeated in May 1966 with 5 mg. of labeled codeine methyl ether for 10 plants and a 7-day feeding period. This resulted in an incorporation into codeine of 15.8% of the total radioactivity. Thus, it is clear that

the opium poppy is capable of demethylating codeine methyl ether. However, this did not prove that the methyl ether is a required intermediate. It would be necessary to show (*a*) that this substance is present in the fresh plant, and (*b*) that it is formed directly from thebaine. The biosynthetic pathway leading from reticuline to thebaine is now well established (10–12). Radioactively labeled (±)-reticuline-3-^{14}C (0.045 mc.) (10) was administered to 9 opium poppies. The plants were harvested 2 weeks later, and 100 mg. of radioinactive codeine methyl ether was added to the plant mash during extraction. It was recovered by isolation and separation of the nonphenolic alkaloids and was found to be practically inactive (0.0004% incorporation). This suggested that codeine methyl ether is not produced in the plant by way of thebaine, nor is it derived from codeine. The latter point was also confirmed by addition of the unlabeled methyl ether to an extract from 20 opium poppies which had been fed 2,6-^{3}H-codeine (0.108 mc.) (6). Again the isolated codeine methyl ether was essentially inactive (0.0003% incorporation). We, therefore, conclude that codeine methyl ether is not a genuine opium alkaloid, at least not in the varieties of *P. somniferum* which we have used.

When the isolation of codeine methyl ether was reported, the possibility of this substance being an artifact was considered (4). During large-scale production of morphine, reducing agents such as sulfur dioxide, bisulfite, or hydrosulfite are usually added as antioxidants. It now appears reasonable to believe that the small amounts of codeine methyl ether present in the mother liquors were actually produced by reduction of thebaine during the isolation and purification of morphine.

(1) Barton, D. H. R., and Cohen, T., "Festschrift für A. Stoll," Birkhäuser Verlag, Basel, Switzerland, 1957, p. 117.
(2) Battersby, A. R., *Proc. Chem. Soc.*, **1963**, 189.
(3) Barton, D. H. R., *ibid.*, **1963**, 293.
(4) Brochmann-Hanssen, E., and Nielsen, B., *J. Pharm. Sci.*, **54**, 1393(1965).
(5) Blaschke, G., Parker, H. I., and Rapoport, H., *J. Am. Chem. Soc.*, **89**, 1540(1967).
(6) Battersby, A. R., Brochmann-Hanssen, E., and Martin, J. A., *Chem. Commun.*, **1967**, 483; Battersby, A. R., Brochmann-Hanssen, E., and Martin, J. A., *J. Chem. Soc.*, to be published.
(7) Chang, F. N. H., Oneto, J. F., Sah, P. P. T., Tolbert, B. M., and Rapoport, H., *J. Org. Chem.*, **15**, 634(1950).
(8) Mary, N. Y., and Brochmann-Hanssen, E., *Lloydia*, **26**, 223(1963).
(9) Battersby, A. R., and Harper, B. J. T., *J. Chem. Soc.*, **1962**, 3526.
(10) Battersby, A. R., Binks, R., Francis, R. J., McCaldin, D. J., and Ramuz, H., *ibid.*, **1964**, 3600.
(11) Battersby, A. R., Foulkes, D. M., and Binks, R., *ibid.*, **1965**, 3323.
(12) Barton, D. H. R., Kirby, G. W., Steglich, W., Thomas, G. M., Battersby, A. R., Dobson, T. A., and Ramuz, H., *ibid.*, **1965**, 2423.

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Received June 9, 1967
Accepted for publication July 25, 1967.

This investigation was supported by research grant MH-03487 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JOURNAL OF Pharmaceutical Sciences

October 1967 volume 56, number 10

Review Article

Drug-Induced Cardiomyopathies

By DUANE G. WENZEL

WHILE FREQUENT REFERENCE is made to the toxicity of drugs for the myocardium, and at least one major symposium has dealt with the subject of cardiomyopathies (1), there has been no general review of the drug-induced condition. The lack of well-defined and universally-employed criteria for cardiomyopathies necessitated some latitude when interpreting reports of myocardial drug toxicity. For the purpose of this review the terms degeneration, fatty degeneration, necrosis, primary myocardial disease, myocardial injury or lesions, calcification, fibrosis, or myocarditis were accepted as being synonymous with a cardiomyopathy. Obviously, some of these are not necessarily related to a true cardiomyopathy. Myocarditis, for example, while frequently preceding, accompanying, or following degeneration, is essentially an inflammatory rather than a degenerative process. While drugs acting primarily on the coronary vasculature were included in this review, the mechanism was indicated whenever it was apparent. Alterations in biochemical, electrical, or hemodynamic functions may accompany, or even precede, morphologic evidence of injury to the myocardium, but such changes were not included as cardiomyopathies unless accompanied by evidence of physical alterations.

Degeneration of the heart muscle can result from a wide variety of causes. In this country the most common causes are cardiovascular diseases which result in acute or chronic impairment of coronary circulation. These include coronary artery disease, hypertension, heart-lung disease (*corpulmonale*), valvular heart disease, and congenital heart disease. As practically all adult males in the United States are believed to have some degree of coronary artery disease, the incidence of minimal myocardial degeneration must be considerable. For this reason an assessment of drugs in human degenerative heart disease must include the possibility of an interaction with impaired coronary circulation. While it is possible to experimentally distinguish between drugs producing primary toxic or allergic lesions of the myocardium and those producing similar end effects through alterations in coronary blood flow, such differentiation is often not possible on the basis of clinical reports. In fact, some drugs, of which the catecholamines are an outstanding example, probably produce their degenerative effects through both mechanisms.

Primary myocardial disease may also result from a variety of infections, nutritional and metabolic disturbances, and allergies, and in some cases may have a genetic component. Once again, superimposing the effect of a drug upon such underlying conditions would be expected to intensify the response. Most drugs which produce primary cardiomyopathies are believed to interfere with the metabolic activity of the cell, probably by poisoning enzyme systems. The remaining drugs act through an allergic mechanism, either by a direct effect on the myocardium or secondary to coronary vasculitis. In addition to the possibility that some drug cardiomyopathies seen in the human are related to predisposing

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factors, it may be argued that myocardial lesions may occur with almost any drug if administered in a large enough dose and for a sufficient length of time. Despite the use of unphysiologic doses in many of the reported animal experiments, these data were included in order to present a complete picture. Drugs having a low order of intrinsic activity on the normal heart would be expected to demonstrate their toxicity on hearts already damaged or otherwise rendered susceptible.

SYMPATHOMIMETIC AMINES

The earliest evidence relating the sympathomimetic amines to necrotic lesions of the myocardium as cause and effect was in a report on epinephrine by Josué in 1907 (2). Since then this observation has been repeated many times with epinephrine and other sympathomimetic amines. A variety of mechanisms have been postulated to explain the pathogenesis of these lesions. Proposed mechanisms include: a direct oxygen wasting action (3–29); altered permeability of the myocardial cell membrane through elevation of plasma nonesterified fatty acids (30); impairment of venous drainage of the heart (31); potassium depletion (32); constriction of the coronary arteries (33); dilatation of precapillary shunts between coronary arteries and veins (34); damage to the coronary endothelial cells (35); increased myocardial oxygen requirements plus alterations of systemic circulation (36); and combinations of three different effects including myogenic, physical, and metabolic mechanisms (37).

Raab and his co-workers were the earliest and have been the principal proponents of a mechanism by which the amines increase myocardial oxygen consumption and cardiac work without a corresponding increase in energy production (3–29). It has been repeatedly demonstrated that following administration of the catecholamines the oxygen consumption of the heart greatly exceeds the normal energy requirements (38–46). Raab not only proposed a mechanism by which endogenous neurohormones or administered sympathomimetic amines produce myocardial degeneration, but he seriously challenged the classical concept of occlusive coronary artery disease as *the* cause of myocardial degeneration. It is notable that even in defining the term infarction, as applied to the myocardium, recognition is now given to the fact that arterial obstruction or insufficiency is not the sole cause (47). Since the early study of Herrick (48), demonstrating the effects of coronary occlusion, and the subsequent emphasis by Keefer and Resnik (49) on the role of myocardial oxygen deprivation in the symptoms of angina pectoris, relatively little attention has been given to the nonocclusive mechanisms. While there can be little doubt that coronary occlusion is a major factor in the production of myocardial necrosis, it is also probable that the primary cardiomyopathies constitute a significant factor.

Of the known mechanisms by which the sympathomimetic amines may act, oxygen wastage is at least of theoretical importance. This mechanism appears to be related to mitochondrial function. Following the admnistration of catecholamines the mitochondria become swollen (50) and undergo uncoupling of oxidative phosphorylation (51). This effect is apparently unrelated to hypoxia, congestive heart failure, or the accumulation of free fatty acids, and does not occur when catecholamines are added to the mitochondria *in vitro* (51). The lack of an *in vitro* action suggests a preliminary *in vivo* effect. Possible *in vivo* mechanisms include the reduction of myocardial potassium by the catecholamines (52, 53) because potassium has an essential role in mitochondrial metabolism (54). Still another possible explanation is the *in vivo* conversion of catecholamines to adrenochrome, a metabolite with a demonstrated capacity to uncouple oxidative phosphorylation in mitochondria (55). Some investigators have concluded that the catecholamine-induced increase in myocardial oxygen consumption is the result of hemodynamic changes rather than of a direct metabolic action (56). To reach this conclusion, the heart was arrested by potassium, which in addition to preventing hemodynamic changes would also modify any potassium-depleting effect of the catecholamines. In the isolated rat heart it was shown, however, that the increase in oxygen uptake produced by epinephrine still occurred following arrest by potassium (57), citrate, or procainamide (57, 58). These results suggest that if uncoupling of oxidative phosphorylation is the cause of the observed increase in oxygen consumption, the uncoupling is not the result of potassium depletion. Hauge and Øye (58) concluded that part of the effect of epinephrine on the arrested heart was due to an increased utilization of ATP by several separate reaction systems. They found that this increase in oxygen consumption was blocked by a β-adrenergic receptor blocking agent, propranolol, but not by phentolamine, an α-adrenergic receptor blocking agent.

While the relative importance of the various mechanisms is still unresolved, there is direct evidence that the adrenergic receptors responsible for the myocardial toxicity of the catecholamines are alpha in character rather than beta. The intravenous administration of epinephrine to dogs has been shown to produce a fatty degeneration of the

myocardium associated with elevated serum levels of several myocardial enzymes (59–61). These effects are prevented by the administration of an α- receptor blocking agent (62–64). In a study with rats, it was found that the myocardial damaging effects of epinephrine, norepinephrine, or phenylephrine were prevented by the α-adrenergic receptor blocking agent, phentolamine, but the β-adrenergic receptor blocking agent, pronethalol, did not add to the effectiveness of the phentolamine, nor was it effective alone (65). In fact, with low doses of isoproterenol and with high doses of epinephrine the pronethalol added to the toxicity. The β-receptor blocking agents prevent phosphorylase activation and hyperglycemia (66) without affecting hyperkalemia (67). Alpha-receptor blocking agents, on the other hand, prevent the loss of myocardial potassium without appreciably affecting hyperglycemia (68). As previously suggested, potassium depletion is a likely causal mechanism by which the catecholamines are toxic to the heart. Among the known consequences of potassium depletion is interference with mitochondrial metabolism (54, 69). It has been suggested that this effect is based upon the importance of potassium in transphosphorylation reactions (70) as shown by the potassium dependence of enzymes involving phosphate transfer, such as fructokinase (71, 72), pyruvic phosphoferase (73–75), phosphotransacetylase (76), and acetate activating enzyme (77). Still other potential potassium-related effects are the requirements for high concentrations of this element in protein synthesis (78) and the fall in muscle pH produced by potassium deficiency (79). Numerous studies have demonstrated the production of myocardial necrosis by potassium depletion as well as the loss of potassium following myocardial injury (80–85). The extent to which hemodynamic effects are responsible for the myocardial lesions produced by the sympathomimetic amines is still conjectural. To summarize the vascular effects of the three principal catecholamines: isoproterenol is almost entirely a vasodilator; epinephrine produces both vasoconstriction and vasodilation; while norepinephrine is almost entirely a vasoconstrictor. It has been suggested that at least part of the toxicity of isoproterenol for the myocardium is related to the marked decrease in systemic blood pressure (86, 87). Just as a marked fall in peripheral vascular resistance may result in embarrassment to the myocardial circulation, an intense rise in peripheral pressure produced by the pressor amines would be expected to produce an increase in the myocardial work load.

The role of the coronary vessels in the production of myocardial necrosis by the sympathomimetic amines may be significant yet variable. The cardiovascular effects of the catecholamines are usually accompanied by marked compensatory dilatation of the coronary vessels, provided that they are not sclerotic (88, 89). The nature of the response appears to depend upon vessel size and the amount of catecholamine. Norepinephrine was found to produce good relaxation of the small coronary arteries, but somewhat less relaxation resulted from equal doses of epinephrine (90). This dilatation was blocked by pronethalol. Larger coronary vessels tend to be contracted by the catecholamines, and this effect is blocked by phenoxybenzamine hydrochloride[1] (90). The over-all myocardial circulation, as measured by coronary flow, is improved by epinephrine or norepinephrine, while after β-adrenergic receptor blockade these same sympathomimetic amines produce coronary constriction (91). The predominant effects of low doses of catecholamines are seen upon the myocardium and coronary circulation, while large doses produce greater peripheral effects (92). Furthermore, isoproterenol in large doses actually produces a pressor response which is blocked by α-adrenergic receptor blocking agents (93).

A factor of great importance in the action of sympathomimetic amines as myocardial necrotizing agents is their interaction, either as drugs or hormones, with other agents capable of producing cardiomyopathies. Because such unplanned interactions are probably common in the human, results obtained with sympathomimetic amines in normal animals may give an unrealistic impression of a low order of toxicity.

Epinephrine—Myocardial injury ranging from edema to massive necrosis can be produced by the injection of epinephrine. The nature of these effects and their severity are dependent upon the dose of epinephrine and the species tested. Cats appear to be most susceptible, demonstrating necrosis after the intravenous administration of moderate doses (94, 95). Larger doses are usually necessary to produce equivalent lesions in dogs (60, 61, 96, 99), rabbits (100), and rats (65, 86, 101–103). The importance of interacting factors is demonstrated by the fact that a dose of epinephrine producing no lesions alone (104, 105) consistently causes myocarditis when combined with low doses of caffeine (104–108), sparteine, or strophanthidin (105–106). A similar interaction with caffeine has been observed with guinea pigs (109) and dogs (104, 110). While the sensitivity of the human

[1] Trademarked as Dibenzylamine by Smith Kline & French Laboratories, Philadelphia, Pa.

myocardium is not known, large doses of epinephrine have been reported to produce functional evidence of lesions (111).

Norepinephrine—High mortality rates resulting from the use of norepinephrine in the treatment of cardiogenic shock or shock-like states prompted the initial studies of its myocardial toxicity (112). It was found that patients dying after treatment with norepinephrine or those with pheochromocytoma presented evidence of morphologic changes in the myocardium similar to those obtained by treating dogs with therapeutic doses of norepinephrine (113, 114). Lesions observed ranged from myocarditis to necrosis and/or focal fibrosis. It has been suggested that the mortality in cardiogenic shock would be reduced if lower doses of norepinephrine were employed (115). A hazard in the use of norepinephrine was demonstrated by the marked cardiac toxicity resulting from its administration to patients receiving iproniazid (116).

Focal myocarditis, patchy, fatty, degenerative changes, necrosis, and hemorrhages of the myocardium were produced in dogs by the intravenous administration of therapeutic doses of norepinephrine (60, 61, 112, 113). Rats were similarly susceptible to single or repeated subcutaneous injections (65, 86, 117–123). Doses producing minimal lesions when administered alone resulted in severe lesions when combined with dihydrotachysterol and calcium acetate (120), with a low potassium diet (121) or with cold stress (117).

Isoproterenol—In terms of the extent of its use, isoproterenol has become the reference standard for producing experimental myocardial necrosis. The rat has generally been the species of choice. Since the first studies (87, 124) using isoproterenol to produce lesions resembling those resulting from human myocardial infarction, a wide variety of factors have been studied in order to determine the pathogenesis of cardiopathies in general. The significance of such factors has been reviewed (125). Different strains of rats, as well as animals from the same strain but from different colonies, have significantly altered sensitivities to isoproterenol (126). Large animals were found to be more susceptible than small ones, while age and sex were not significant factors in this study as long as body weights were equal (126). Treatment of rats with conjugated equine estrogen or progesterone (125), ACTH (127), or glucocorticoids (127) failed to influence the cardiotoxicity of isoproterenol. Factors increasing isoproterenol-induced necrosis were: estrone (125), testosterone (125), hyperthyroidism (128), mineralocorticoids (127), a low-potassium diet (52), a high-sodium diet (52), isolation or cold stress (129), increased body fat (130), and nicotine (131). Factors providing some protection were: hypothyroidism (128), a high potassium diet (52), a low sodium diet (52), starvation (125), and, contrary to a report indicating a potentiation of the effects of norepinephrine in the human (116), monoamine oxidase inhibitors (132–134).

The hamster has also been used in studies to determine the mechanism by which isoproterenol affects the heart (33, 34). From these studies it was initially concluded that isoproterenol produced its effects by dilating the precapillary shunts of the coronary arteries (34); however, it was later concluded that the effects were those of ischemia resulting from coronary vasoconstriction (33).

Other studies of isoproterenol to follow were performed with rats unless otherwise indicated. These studies demonstrated: histochemical and electron microscopic changes (50); the relationship of the lesions to the loss of myocardial aspartate aminotransferase (135); the effect of adrenergic blocking agents on isoproterenol-induced biochemical changes in the myocardium (65, 136, 137); the similarity of isoproterenol-induced myocardial lesions to those occurring naturally in breeder rats (138); hemodynamic changes during the acute (139) and chronic (140) phases of the myocardial damage; the presence of impaired venous drainage as a possible cause of myocardial injury (31); the rate at which necrotic areas heal (36); a correlation of the lesions with hemodynamic and electrocardiographic changes in the dog (141); comparative effects with other sympathomimetic amines (86); a correlation between the disappearance of myocardial glycogen and potassium (142); detailed histological effects (143, 144); lack of protection from cardiac glycosides, monoamine oxidase inhibitors (145), or adrenergic blocking agents (65, 145); and a series of studies in which hemodynamic and metabolic effects were correlated with the histopathologic responses of rats to several sympathomimetic amines including isoproterenol (30, 37, 146).

Other Sympathomimetic Amines—Surprisingly few studies have been performed to evaluate the potential of other sympathomimetic amines in the production of cardiomyopathies. In early studies the oral administration of ephedrine each day for 1 to 2 years produced foci of calcification in the myocardium of guinea pigs (147). Following treatment with xanthine compounds in rabbits, ephedrine was found to produce myocarditis (106). Obviously ephedrine is much less potent in terms of its capacity to produce myocardial necrosis than are the catecholamines. The amount of ephedrine required to produce minimal

gross myocardial lesions in the rat is approximately 1,000 times the amount of epinephrine required, 700 times that of norepinephrine, and 4,400 times that of isoproterenol (86). Ephedrine also failed to produce myocardial injury when it was repeatedly injected in chickens and rabbits, both with and without the administration of sparteine (148).

Tyramine has also been shown to produce myocarditis in rabbits following administration of xanthine alkaloids (106). Methamphetamine administered daily for long periods to mice or rabbits resulted in myocardial degeneration (149). Phenylephrine, a sympathomimetic amine usually regarded as a specific stimulant for α-adrenergic receptors, produced myocardial necrosis, accompanied by loss of myocardial aspartate aminotransferase activity in rats, equivalent to the changes produced by epinephrine or norepinephrine when given in approximately twice the dose (65).

Nicotine—Nicotine has notable pharmacodynamic activities other than those of an indirect-acting sympathomimetic amine, but its principal effects upon the heart are probably mediated through stimulation of the sympathetic nervous system and the release of epinephrine and norepinephrine. Tobacco has long been suspect as a major etiologic factor in the increased mortality from cardiovascular disease of smokers, and nicotine is probably the active agent (150). Chronic administration of nicotine to rats (151–155), or rabbits (156, 157) did not produce myocardial lesions when used by some workers, yet others have produced ischemic changes (158) and necrotic areas of the myocardium (159) with rats. As large doses of nicotine produce death by respiratory arrest, it is not expected that myocardial necrosis would be seen with acute nicotine poisoning, yet myocardial effects have been seen in humans (160, 161) and horses (162). As was suggested from evidence with the catecholamines, however, there are indications that nicotine may owe much of its potential myocardial toxicity to interactions with other agents. It was observed, for example, that while nicotine or bovine serum administered daily to rabbits did not produce myocarditis, this effect resulted when the two agents were combined (163–167). Similarly, nicotine administered to the rat in doses approximating the intake of an average smoker does not produce lesions of the myocardium, yet it significantly intensifies the lesions produced by isoproterenol (131) or corticosteroid–electrolyte-stress treatment (53). Furthermore, when nicotine, ergometrine, and a hypercholesterolemic diet were administered to rabbits, as separate treatments or as any pair of treatments, there was no evidence of morphologic damage to the myocardium. When the three treatments were combined, fatty degeneration of the myocardium accompanied by thickening and fibrosis of the coronaries resulted (168, 169). Because of the multiplicity of the direct and indirect effects of nicotine on the myocardium, the nature of its interacting mechanism(s) can only be speculative at this time.

CORTICOSTEROIDS

In 1942 Miller and Darrow administered repeated doses of desoxycorticosterone to rats and produced necrosis of the myocardium which was indistinguishable from that produced by a low potassium diet (170). These effects were preventable by the administration of potassium chloride in the drinking water. Darrow later observed that a low potassium diet intensified the necrosis produced by this steroid (171). Others have reproduced these effects with desoxycorticosterone (172, 173), and have further observed that the coadministration of sodium chloride potentiates its damaging action (174–184). It was also demonstrated that the myocardial lesions were intensified by experimental hypertension (180, 182), uninephrectomy (177, 181, 183, 184), adrenalectomy (185), growth hormone (176), methandriol (186), digitalis in patients with congestive heart failure (187), hydrocortisone and calciferol (183), and cortisone or hydrocortisone (188). The lesions produced by desoxycorticosterone were reported to be reduced or prevented by promethazine (174), adrenalectomy (185), corticotropin or cortisone (177), or a sodium-free diet (182). Contrary to previous reports concerning the potentiation of the effect of desoxycorticosterone by sodium chloride, the administration of sodium chloride has also been reported to prevent the lesions in unilaterally nephrectomized and adrenalectomized rats (189). A further conflicting report is that the coadministration of cortisone does not influence the response to desoxycorticosterone (190). The guinea pig was also shown to be sensitive to the steroid-induced necrosis (191) as were humans treated for arthritis (192).

Not only are necrotic lesions of the myocardium produced by injections of desoxycorticosterone, but by corticotrophin (193–197, 206), cortisone (188, 194, 198–203), hydrocortisone (195, 201, 204, 205), corticosterone (206), and prednisone (207). The toxic effects of corticotropin for the myocardium were reported in the treatment of asthma (193, 194) and when used in conjunction with tetanus antitoxin (197). Prednisone produced myocardial infarctions in two of 63 tuber-

culous patients treated with average doses of prednisone and antitubercular drugs (207). The toxicity of the glucocorticoids, like that of desoxycorticosterone, was potentiated in rats by sodium chloride in the drinking water (199–201), by a low potassium diet (202), and by coadministration with desoxycorticosterone in doses which produced no effects alone (188). It is interesting that infarcts produced in dogs by ligating a branch of a coronary artery were made worse by the administration of desoxycorticosterone and improved over the controls by the administration of hydrocortisone (208). However, corticosteroid therapy of acute myocardial infarctions of the human has proven to be ineffective (209).

In 1957 Selye and Renaud (210) tested a new corticosteroid for its effectiveness in the production of cardiomyopathies. This compound, 2α-methyl-9α-chlorocortisol, when combined with the oral administration of sodium phosphate, was most effective in producing lesions in the rat heart. Since then a number of similar halogen-substituted steroids have been tested with a variety of other agents and treatments (118, 119, 210–228). Details of the speculations concerning the nature of the numerous interactions between corticosteroids, salts, stressors, and miscellaneous compounds can be found in books by Selye (229, 230). The mechanism of the toxicity has been the subject of considerable speculation and some disagreement. These lesions referred to as "electrolyte-steroid-cardiopathy with necrosis" (ESCN) by Selye's group were prevented by treatment with potassium (32). Their causation was said to be related to an undefined interaction between so-called "activating" anions, specifically phosphate, sulfate, and perchlorate, and certain halogenated corticosteroids. The answer to the question of mechanism begins with the clearly demonstrated activity of either mineralocorticoids or glucocorticoids in producing myocardial necrosis. Such activity has been shown to be intensified by the combination of glucocorticoid and mineralocorticoid activities whether as two separate compounds (14, 19, 53) or as a single highly active halogenated corticosteroid with both effects in a single molecule. In those few cases where an investigator has been unable to produce cardiac necrosis in the rat with desoxycorticosterone (231, 232), it might be concluded that doses, animals, or treatments were not optimal. In an attempt to resolve this seemingly confused picture, Nickerson and co-workers (84) studied the mechanisms of corticosteroid and the so-called "activating" anion. They determined that the "activating" anions were simply producing potassium-depletion through what they felt was catharsis, coupled with the potassium depleting effect of the halogenated steroid. The hypothesis of simple potassium depletion as the causative mechanism was later rejected by Prioreschi (231, 232) because he was unable to produce necrosis with desoxycorticosterone alone, because he seemingly protected the animals by the administration of chlorides other than potassium, and because not all animals with a low cardiac potassium developed necrosis. These apparent discrepancies in the low potassium hypothesis were later examined by Tucker and co-workers (233). They found that necrosis of the heart muscle was associated with a high metabolic alkalosis, an increase in intracellular sodium, and a decrease in skeletal muscle potassium. It was concluded that sodium phosphate as well as the other "activating" anions increased the rate of potassium excretion by their action on the kidney rather than by catharsis. Furthermore, the apparent protection by chlorides described by Prioreschi was interpreted as lack of potentiation rather than protection. The lack of a clear relationship between depletion of myocardial potassium and necrosis was found to result from the protective effect of potassium in skeletal muscle. Lack of a positive effect of desoxycorticosterone, in Prioreschi's studies, was related to the use of liberal amounts of potassium in the diet he fed his rats.

VASOCONSTRICTORS

Although the sympathomimetics include many agents with vasoconstrictor activity, they were treated separately because of their multiple actions and their over-all significance in terms of cardiomyopathies. As is the case with drugs classified as antihypertensives or vasodilators, the vasoconstrictors are assumed to produce myocardial injury through hemodynamic changes rather than direct myocardial toxicity.

The ergot alkaloids produce severe anginal symptoms in the presence of hyperthyroidism, syphilitic aortitis, deformities of the aortic valve, or stenosis of the coronary ostia (234, 235). Electrocardiographic changes, coronary thrombosis, and/or myocardial infarction have also been shown to follow therapy with ergot derivatives (236–238). The effect of the ergot alkaloids on the coronary vessels is seen in the electrocardiographic alterations during the ergonovine (ergometrine) stress test (239). Such ECG alterations occur when the drug is administered in the presence of pathologic changes of the coronary arteries (240, 241). In addition to being employed clinically, this test has been used to obtain evidence of restricted coronary flow in the living animal (242–244). The coronary constriction produced by

ergometrine in the rabbit receiving a hypercholesterolemic diet and chronic nicotine in the drinking water was sufficient to produce electrocardiographic alterations, thickening and fibrosis of the small branches of the coronaries, and myocardial necrosis (245, 246).

Vasopressin is another major vasoconstrictor drug shown to produce myocardial lesions. The earliest indication of its effects on the myocardium were reports by Scheps in 1934 (247, 248). He treated rabbits and guinea pigs with intramuscular injections of vasopressin and observed myocardial ischemia, hyperemia, edema, and necrosis. Others have repeated this observation using rabbits (249), dogs (250, 251), rats (252, 253), and cats (254), with effects ranging from hemorrhage to necrosis. Several cases of infarction and sudden death have been reported from the use of vasopressin in the human (255, 256). The marked reduction of coronary flow by vasopressin in normal, noninfarcted hearts (257) with no evidence of direct toxicity leaves ischemia as the probable mechanism. Vasoconstriction produced by posterior pituitary[2] has been combined with altered blood coagulability (thrombin administration) and/or coronary atherosclerosis (hypercholesterolemic diet) in rabbits (258). Infarction resulted from the combination of posterior pituitary and thrombin, while more severe necrosis was produced by the addition of hypercholesterolemia to the regimen.

VASODILATORS AND ANTIHYPERTENSIVES

Vasodilators or antihypertensives are not usually considered to be precipitating factors in myocardial degeneration, although they may induce ischemia through severe hypotension accompanied by prolonged tachycardia. A shortened diastolic interval coupled with diminished venous return in the presence of aortic stenosis or coronary sclerosis may be sufficient to produce localized ischemia to the point of necrosis. In the absence of evidence suggesting a direct toxic effect, one must assume a hemodynamic mechanism for such drugs.

Ganglionic blocking agents have been found to produce myocardial lesions when employed for the treatment of hypertension (259–266). Specific agents have been: tetraethylammonium chloride (259–261), hexamethonium chloride (264), pentamethonium bromide (263), pentolinium tartrate (262), and mecamylamine hydrochloride (266). In all cases the basis for the development of infarction appeared to be the administration of single or repeated hypotensive doses of the drug to arteriosclerotic patients. In one series, infarctions were produced in 7 hypertensive patients following the daily aministration of antihypertensive agents for periods of 4 days to 9 months (265). It was concluded that these drugs should not be used for elderly patients with systolic hypertension and especially not in the presence of angina pectoris. Other drugs reported to produce myocardial lesions when used for the treatment of hypertension include reserpine (267–269) and hydralazine (270, 271).

While the nitrites are used to treat the symptoms of angina pectoris, they have been reported to increase the incidence of infarction when administered chronically in large doses to patients with arteriosclerosis (272). Degenerative changes of the rat myocardium have been produced by the oral administration of erythritol tetranitrate (273).

THYROID HORMONE

While the term "thyroid hormone," is used throughout this section the actual substances are crude thyroid extract, thyroxin, or thyroid-stimulating hormone. In 1913 Farrant (274) reported that the administration of large doses of desiccated thyroid to the rat produced scattered areas of necrosis in the myocardium. Many others have since repeated his observations on experimental animals (103, 275–294) which have included cats (285, 288, 289, 293), rabbits (280–284, 286, 287, 290), guinea pigs (278), rats and pigeons (291, 292). Histologic changes in the various studies ranged from minor fatty degeneration to frank necrosis of the muscle fibers with exudative cellular infiltration. In some cases fibroblasts were described as replacing myocardial bundles. Not all investigators were able to demonstrate specific myocardial lesions in animals with experimental hyperthyroidism (295–298). Thyroid hormone alone is relatively ineffective in producing focal myocardial lesions in the cat, but when it is combined with otherwise nontoxic doses of digitalis, potentiation results (293).

Treatment with various stressors has failed to produce myocardial necrosis in rats pretreated with thyroid hormone (229). Although clinicians have reported histopathological changes in the hearts of patients with hyperthyroidism (299–305), and it is common to find swollen myocardial fibers in patients with hyperthyroidism, myocarditis or myocardial necrosis are said to be comparatively rare (306).

The mechanism by which thyroid hormone can produce lesions of the myocardium is not known.

[2] Trademarked as Pituitrin by Parke Davis and Co., Detroit, Mich.

It has been observed that, when administered to young growing rats, it produces an increase in the magnesium requirements accompanied by a decreased efficiency of oxidative-phosphorylation by the cardiac mitochondria (307). A similar impairment of oxidative phosphorylation by cardiac mitochondria is observed following magnesium deprivation (308).

CARDIAC GLYCOSIDES

The fact that the cardiac glycosides are capable of producing injury to the myocardium in addition to their positive inotropic action has been observed clinically and experimentally, yet this phenomenon has received relatively little attention. Perhaps the earliest report of this toxic effect was in the doctoral thesis of Lewitzky (309). In his studies with rabbits, dogs, and cats he observed that large doses of digitalis extract produced myocardial necrosis followed by inflammation. Since this study, other investigators and clinicians have confirmed and extended his data with various cardiac glycosides including: crude digitalis (310–318), convallaria (315), digitoxin (319–327), digitalin (319), gitalin (319, 321), lanatoside-C (328, 329), oleandrin (324, 326), ouabain (319), and strophanthin (330, 331).

Myocardial lesions have been reported following the administration of therapeutic (311, 317, 318) or toxic doses (310, 322) to humans. In one case death occurred from a combination of cortisone, corticotropin, antibiotics, meralluride, ammonium chloride, and digitalis (316). Most investigators studying the toxicity of the cardiac glycosides have employed the cat because of its sensitivity (312, 313, 315, 319–321, 323–328, 331, 332). In one comparative study, cats were found to develop focal myocardial necrosis from a single intravenous injection of digitoxin while dogs or rabbits required repeated injections (320). Other studies have confirmed the relative resistance of the dog (314, 328, 329). The rabbit when bled was found to be more susceptible to the cardiac glycosides (330). It is probable that the effect of bleeding acted as a stressor.

While the mechanism by which the cardiac glycosides produce lesions of the myocardium is not known, the evidence is in favor of potassium depletion. The positive correlation between depletion of myocardial potassium and digitalis toxicity is well documented (333–335). The problem is complicated by conjoint factors which increase the potassium loss. Congestive heart failure, the condition for which digitalis is usually employed, results in increased secretion of aldosterone with an accompanying increase in sodium retention and potassium excretion. Potent diuretics, used either for the treatment of congestive heart failure or hypertension, may aggravate myocardial toxicity through potassium depletion. The importance of interacting factors is demonstrated by the production of myocardial lesions with otherwise nontoxic doses of digitalis when administered with thyroid hormone (293).

EMETINE

The therapeutic value of emetine in the treatment of amebic dysentery is limited by the considerable toxicity of the drug. This toxicity has been demonstrated upon the heart, skeletal muscle, and intestine (336), although reports of polyneuritis and nervous involvement are not unusual (337). Evidence of its toxicity for the myocardium in humans treated for amebiasis has been frequently reported (338–348). In addition to the myocardial lesions resulting from emetine, alterations in the electrocardiogram (349), ventricular fibrillation (350), diminished systolic force (351), and tachycardia (352) have been observed following its therapeutic use.

A number of investigators have noted the production of fatty degeneration and necrosis of the myocardium following administration of emetine to rabbits (353–356), dogs (356), and pigeons (357). Its toxic effects for the myocardium have been said to be lessened by the administration of inositol or pyridoxine (358) and intensified by physical exercise (359, 360) or psychological stress (361).

A number of studies have been conducted to determine the biochemical mechanism by which emetine produces cardiomyopathies (361–369). It has been suggested that emetine may act to interfere with the enzyme system by which the heart converts glycogen to useful energy (370). A single dose of 10 to 20 mg./Kg. was found to cause a rapid fall of liver glycogen in the rat (362). In testing this theory Diamant (367) concluded that the synthesis, rather than the utilization, of glycogen was significantly depressed by emetine. He found that emetine depressed the activities of both aldolase and phosphorylase and suggested these effects as causes of the reduced glycogen synthesis. Emetine was shown to have no effect on the synthesis of cocarboxylase by the rat liver (366), and its effects on the heart were not blocked by the administration of nicotinamide adenine dinucleotide (368). The metabolism of vitamin A, riboflavin, nicotinic acid, or biotin were unaffected by the administration of emetine while smaller amounts of folic acid and thiamine were stored in the livers of pair-fed rats (364).

In studies with heart homogenate or mitochondrial preparations, emetine decreased the oxygen uptake in the presence of glucose, pyruvate, malate, fumarate, or without added substrates (365), and with butyrate or β-hydroxybutyrate (371). Oxygen uptake was augmented by succinate (365). Evidence was presented that the enhancement of succinate oxidation may have resulted from inhibition of malic dehydrogenase (369). As liver homogenates from emetine-poisoned rats did not demonstrate an impaired ability to oxidize any of the previously mentioned substrates (372), and as the liver accumulates a far higher concentration of emetine than does the heart (373), it was apparent that the toxicity to the heart was the result of a susceptibility of one or more still unidentified metabolic processes in this organ. The similarity of the effects of emetine to those of thyroxin on heart homogenates and to the toxicity of thyroxin for the myocardium has been noted (369).

METALLIC COMPOUNDS

Arsenic—Toxic interstitial myocarditis and related symptoms have been reported during the use of arsenicals in the treatment of syphilis (374, 375) and in acute or chronic arsenic poisoning (376–378).

Antimony—The usual cause of antimonial toxicity to the myocardium is its use in the treatment of schistosomiasis. Electrocardiographic abnormalities are common and morphologic alterations of the heart are frequently seen when sudden death ensues. These alterations are similar to the findings with arsenic poisoning. The myocardial toxicity of both the older and newer forms of antimonials has been the subject of several reports (379–381).

Other Metallic Compounds—Bismuth has been reported to produce a toxic myocarditis similar to that observed with arsenic or antimony (382, 383). Myocardial toxicity from the mercurials has produced sudden death from ventricular fibrillation or severe abnormalities of the electrocardiogram (384–388). Similar effects with morphologic changes of the myocardium have been demonstrated with animals (389–391).

While the mechanism by which the metallic compounds induce cardiopathies is not known, the protection against their over-all toxicity by dimercaprol suggests inhibition of sulfhydryl enzymes as a general mechanism (392).

ANTIMALARIALS

The earliest indication that the antimalarials may produce myocardial damage was in a report by Chopra and Wahed in 1934 (393). In treating malarial patients with a combination of pamaquine and quinacrine they observed evidence of myocardial injury, including tachycardia, palpitation, ECG changes, and signs of heart failure. It was apparent that the combination was considerably more toxic than either drug alone. It has since been found that quinacrine interferes with the metabolic degradation of the 8-aminoquinolines thus increasing their concentration in the plasma from five to tenfold (394). Quinine has been reported to produce myocardial damage when it was injected intravenously each day for the treatment of malaria (395).

Studies with rats have demonstrated a severe myocardial toxicity for the experimental antimalarial, plasmocid 8-(3-diethylaminopropylamino)-6-methoxyquinoline (117–119, 121, 396). Quinacrine has produced necrosis and fibrosis of the myocardium of rats, mice, or hamsters when administered as a single LD_{50} (397). Dogs, however, appeared to be refractory to this effect. The addition of small amounts of chloroquine to the diet of rats for periods up to 2 years resulted in fibrosis of the myocardium (398).

PSYCHOTHERAPEUTIC AGENTS

The different classes of psychotherapeutic agents are grouped for convenience of reference rather than to imply a common mechanism. Their potential myocardial toxicity was first reported following the intravenous administration of large doses of chlorpromazine to mental patients (399, 400). A deleterious cardiac action of promethazine was also suggested when it was employed for the production of "deep sleep" therapy for infarction (401). While promethazine, combined with chlorpromazine and meperidine, was said to be useful for suppressing the symptoms of myocardial infarction, heart damage was observed when it was used to treat patients in a state of severe collapse or older patients (402). Further indication of the phenothiazine's myocardial toxicity was suggested from the marked quinidine-like effect of thioridazine (403). In these cases the electrocardiographic changes resembled those of severe hypokalemia. With large doses, circulatory collapse and ventricular tachycardia had occurred, terminating in death. At necropsy, however, nonspecific changes were observed in the myocardium. In another instance, 30% of a group of patients under therapy with chlorpromazine or perphenazine had significant ECG abnormalities (404). About this time a number of unexpected deaths associated with chlorpromazine therapy were reported (405–407). In one 2,000-

bed neuropsychiatric hospital at least two cases of sudden, unexplained deaths had occurred each year since 1957 (408). A detailed histochemical analysis of the hearts from 12 such patients who had received phenothiazine therapy revealed lesions in the arterioles and myocardial muscle which were not present in a control group. Focal lesions of the myocardium, although less extensive, were observed in 70% of tranquilized patients dying from other causes. This effect of the phenothiazines was later demonstrated by the same authors using experimental animals subjected to chronic physical stress.

Evidence that imipramine may possess myocardial toxicity is seen in a report of infarction during its use for the treatment of depression (409) and evidence of cardiac complications during treatment with and poisoning by the drug (410).

Isoniazid (411) and iproniazid (412) have been reported to produce myocardial infarction in patients being treated for tuberculosis. Iproniazid has also been thought to damage the myocardium in patients to whom the drug was being administered for angina pectoris (413).

ALCOHOL

The toxicity of alcohol for the myocardium is infrequently noted, possibly because of the more obvious liver damage. Nevertheless, as early as 1873 an association was observed between alcohol and heart disease (414). These effects were described as..."a localized form of cirrhosis occurring in the myocardial wall and *trabeculae carneae* in the absence of impaired coronary circulation." The term "Munchen Bierherz" was introduced in 1884 to describe the cardiac dilatation and hypertrophy observed in Munich brewery workers who consumed excessive amounts of beer (415). This subject was recently revived following the deaths of 17 people in the U. S. and 20 in Canada from what authorities have called "Beer Drinkers Heart Disease" (416). In 1895 alcoholic myocarditis was observed in alcoholics with liver disease (417) and in 1902 the term "Alcoholic Heart Disease" was used to describe the general cardiac symptoms associated with chronic alcoholism (418). The similarity of alcoholic heart disease to beriberi was observed as early as 1906 (419). While later investigators also noted the similarity between these conditions (420), some reported that patients with alcoholic heart disease responded poorly to thiamine therapy (421). A number of investigators have documented the electrocardiographic abnormalities in chronic alcoholics and often noted that these alterations were reversible when alcohol was withheld for a sufficiently long period (422–424). Still others have observed low cardiac output, gallop rhythm, left ventricular failure, and a gradual refractoriness to thiamine therapy (425–427). Pathological changes ranging from microscopic areas of fibrosis to large confluent areas of fibrosis have been reported (428). A detailed study of 50 very heavy drinkers over a 10-year period revealed the usual functional changes of the heart with 10 deaths (429). At postmortem examination macroscopic fibrosis was found in the left ventricles with scattered areas of muscle degeneration and some fibrosis in all chambers. The lesions were said to resemble those produced by hypokalemia. It was suggested that they may have resulted from magnesium depletion, an effect known to occur with heavy drinking (430). The possibility that excessive alcohol consumption may have triggered an autoimmune reaction was also considered. In 10 patients examined for this purpose there were none of the usual reactions such as persistent fever, serositis, raised ESR, or arthropathy, but two of the 10 demonstrated anti-heart antibodies (429). Myocardial lesions have been reported to occur as a consequence of acute intoxication, although there was no evidence that chronic alcoholism was not a factor (431). An attempt has been made to correlate hemodynamic and biochemical changes in a group of hospitalized alcoholics (432). Hemodynamic changes were accompanied by the leakage of myocardial enzymes into the blood suggesting biochemical and physical alterations of the myocardium.

Electron microscope studies have demonstrated swelling of the mitochondria, a decrease in intramitochondrial enzymes, and deposition of lipid in myocardial cells from animals treated with alcohol (433). In chronic studies with alcoholic rats, impaired functioning of the citric acid cycle was observed with increased levels of pyruvate and lactate (434). Such changes were thought to be related to the myocardial lesions of chronic alcoholics (432). Dogs, to whom alcohol was administered for 12 weeks, demonstrated a marked and sustained increase in triglycerides, lipid phosphorus, and total cholesterol with a decrease in free fatty acids (435). The significance of these changes was related to the organ damage produced during chronic alcoholism. Recently, the question of whether chronic alcoholism produces myocardial damage through nutritional deficiencies was studied with pair-fed rats (436). Rats to whom alcohol was administered suffered from functional changes of the heart including arrhythmias following either epinephrine or mechanical

stress. These effects did not occur in the pair-fed animals, nor were they prevented by vitamin supplements. It was concluded that the myocardial toxicity of alcohol was not the result of nutritional deficiencies and it was speculated that alcohol may produce its effect through a reserpine-like action on the heart, an effect which has been previously reported (437, 438). The degeneration observed in the sympathetic neurons of chronic alcoholics (439) may also relate to catecholamine depletion.

DRUGS PRODUCING HYPERSENSITIVITY REACTIONS

It is not always clear whether a hypersensitivity reaction of the myocardium to drugs is the result of a direct injury to the myocardial cells or whether it is secondary to vasculitis. Interstitial and occasionally granulomatous myocarditis are the usual pathological lesions of the heart in these cases, although occasionally necrosis has resulted.

The sulfanamides are prone to produce such injury. The clinical administration of sulfanilamide was first demonstrated to produce myocarditis in 1939 (440). Since then this myocardial hypersensitivity has been reported again clinically (441–443) and been demonstrated using mice, rats, or rabbits (444, 445). The effect is not limited to sulfanilamide (440, 442, 444), but has been reported to occur with sulfapyridine (442, 444), sulfathiazole (441–445), sulfadiazine (441), and sulfasuxidine (442).

Myocarditis as a result of hypersensitivity to other drugs has been noted with chloramphenicol (446–449), chlortetracycline (450), streptomycin (451), carbutamide (452), and phenylbutazone (453). Penicillin sensitivity has been found to result in cardiomyopathies. Since 1950 a number of cases of minor myocardial reactions (454–460) and infarctions (461–466) have been reported.

Allergic myocardial reactions to vaccines, sera, and toxins have also been observed. The nature of the electrocardiographic changes produced by such agents was studied by Criep in 1931 (467). He postulated that the disturbances were the result of anoxia. Diphtheria (468–472) or typhoid (473) toxins were shown to produce fatty degeneration and necrosis of the myocardium in rabbits (469, 470, 473), guinea pigs (468, 470, 472, 473), rats (473), cats (471), or mice (473). Myocarditis and/or myocardial infarction have since been reported to result from the therapeutic administration of tetanus antitoxin (474, 475), rabies vaccine (476), and smallpox vaccine (477, 478).

ANDROGENS

A number of androgens were tested and found to be relatively nontoxic to the myocardium in rats (479) and guinea pigs (191) sensitized with monobasic sodium phosphate. When the rats were further sensitized by uninephrectomy and a high salt diet, or with other treatments, a greater degree of myocardial degeneration was produced (181, 480, 481). Adrenalectomy protected against such treatments, suggesting that at least part of the effect was related to the release of catecholamines and/or corticosteroids (482).

ANTICOAGULANTS

Anticoagulants continue to be a principal form of therapy for myocardial infarction, but there have been reports suggesting that they aggravate the condition (483, 484), retard healing of infarcts (485), and even produce fatal myocarditis due to a sensitivity reaction (486). Prolonged administration of bishydroxycoumarin in the diet of mice resulted in myocardial fibrosis, necrosis, and hemorrhage (487). These lesions were identical in appearance to those produced by the addition of sulfaguanidine to the diets of rats and were prevented by the administration of vitamin K.

HISTAMINE

While histamine is seldom used medicinally, its release from endogenous stores plays an important role in a number of pathological states. Following injury, locally released histamine is responsible for the initiation of an inflammatory response (488, 489). The administration of polymyxin B, a histamine-releaser, has been shown to produce severe myocardial necrosis in rats (490). A number of investigators have produced degenerative lesions in the hearts of experimental animals by the administration of histamine (94, 491–494). The pharmacodynamic and pathologic effects of histamine have been reviewed (495).

INSULIN

The administration of large doses of insulin to experimental animals has produced myocardial changes ranging from edema to disseminated necrotic patches (496–498). Other workers administering insulin for prolonged periods have been unable to demonstrate such lesions (499, 500). It has been suggested that the release of catecholamines and corticosteroids by insulin overdosage is the probable mechanism of toxicity (501). Insulin has been found to produce myocardial infarction during its routine use in the treatment of

diabetes (502–508) or when employed for shock therapy in schizophrenia (509, 510).

LOCAL ANESTHETICS

Large doses of local anesthetics have occasionally been observed to produce myocardial necrosis. The nature of this action has not been studied although, in most instances, it is probable that a marked fall in blood pressure was responsible. In one instance infarction followed procaine infiltration of the stellate ganglion (511). In other cases, injury was reported during the instillation of tetracaine into the urethra (512). Procaine–epinephrine has produced fatal infarction following its administration (513). The production of myocardial injury during spinal anesthesia has been reviewed by Gross *et al.* (514).

VITAMIN D

The administration of large doses of vitamin D has been shown to injure the myocardium of rachitic children (515) and of parathyroid patients (516). Myocardial calcium deposits were observed in patients using excessive amounts of vitamin D in conjunction with alkaline inorganic salts (517). Such effects have since been amply demonstrated in experimental animals (117–121, 183, 225, 518–523). The fact that such effects are difficult to produce except when vitamin D is combined with a potassium-depleting regimen strongly implicates low-potassium as the causative mechanism. In fact, a hypercalcemic nephropathy is produced by large doses of vitamin D, which in turn impair renal conservation of potassium (524).

REFERENCES

(1) "Cardiomyopathies," Wolstenholme, G. E. W., and O'Connor, M., eds., Ciba Foundation Symposium, Little, Bown and Co., Boston, Mass., 1964.
(2) Josué, O., *Compt. Rend. Soc. Biol.*, **63**, 285(1907).
(3) Raab, W., *Z. Exptl. Med.*, **105**, 657(1939).
(4) Raab, W., *Exptl. Med. Surg.*, **1**, 188(1943).
(5) Raab, W., *Biochem. J.*, **37**, 470(1943).
(6) Raab, W., *Exptl. Med. Surg.*, **2**, 252(1944).
(7) Raab, W., and Maes, J. P., *Am. J. Physiol.*, **148**, 470(1947).
(8) Raab, W., *ibid.*, **152**, 324(1948).
(9) Raab, W., *Ann. Internal Med.*, **28**, 1010(1948).
(10) Raab, W., and Gigee, W., *Proc. Soc. Exptl. Biol. Med.*, **76**, 97(1951).
(11) Raab, W., and Gigee, W., *Circulation Res.*, **3**, 553 (1955).
(12) Raab, W., "Hormonal and Neurogenic Cardiovascular Disorders," Williams and Wilkins, Baltimore, Md., 1953.
(13) Raab, W., and Supplee, G. C., *Exptl. Med. Surg.*, **2**, 152(1944).
(14) Raab, W., *J. Lab. Clin. Med.*, **29**, 715(1944).
(15) Raab, W., and Gigee, W., *Circulation*, **11**, 593(1955).
(16) Raab, W., Lepeschkin, E., Starcheska, Y. K., and Gigee, W., *ibid.*, **14**, 614(1956).
(17) Raab, W., "Advances in Cardiology," vol. 1, S. Karger, Basel, Switzerland, New York, N.Y., 1965, p. 65.
(18) Raab, W., and Gigee, W., *Arch. Intern. Pharmacodyn.*, **112**, 297(1957).
(19) Raab, W., *Arch. Internal Med.*, **101**, 194(1958).
(20) Raab, W., Silva, P., de Paula, E., and Starcheska, Y. K., *Cardiologia*, **33**, 350(1958).
(21) Raab, W., de Paula, E., Silva, P., Marchet, H., Kimura, E., and Starcheska, Y. K., *Am. J. Cardiol.*, **5**, 300(1960).
(22) Raab, W., *ibid.*, **5**, 571(1960).
(23) Raab, W., Stark, E., MacMillan, W. H., and Gigee, W. R., *ibid.*, **8**, 203(1961).
(24) Raab, W., *Cardiologia*, **41**, 129(1962).
(25) Raab, W., Van Lith, P., Lepeschkin, E., and Herrlich, H. C., *Am. J. Cardiol.*, **9**, 455(1962).
(26) Raab, W., *Ann. Internal Med.*, **54**, 1191(1961).
(27) Raab, W., *Am. Heart J.*, **66**, 685(1963).
(28) Raab, W., Marchet, H., and Deming, H., *Exptl. Med. Surg.*, **18**, 128(1960).
(29) Raab, W., Chaplin, J. P., and Bajusz, E., *Proc. Soc. Exptl. Biol. Med.*, **116**, 665(1964).
(30) Rosenblum, I., Wohl, A., and Stein, A. A., *Toxicol. Appl. Pharmacol.*, **1**, 344(1965).
(31) Jasmin, G., *Can. J. Physiol. Pharmacol.*, **44**, 367 (1965).
(32) Bajusz, E., *Arzneimittel-Forsch.*, **14**, 1115(1964).
(33) Handforth, C. P., *Med. Serv. J. Can.*, **18**, 506(1962).
(34) Handforth, C. P., *Arch. Pathol.*, **73**, 83(1961).
(35) Shimamoto, T., and Sunaga, T., *Japan Heart J.*, **3**, 581(1962).
(36) Rona, G., Kahn, D. S., and Chappel, C. I., *Am. J. Pathol.*, **39**, 473(1961).
(37) Rosenblum, I., Wohl, A., and Stein, A. A., *Toxicol. Appl. Pharmacol.*, **7**, 9(1965).
(38) Gollwitzer-Meier, K., Kramer, K., and Krüger, E., *Arch. Physiol.*, **1936**, 237.
(39) Gollwitzer-Meier, K., and Kroetz, C., *Klin. Wochschr.*, **616**, 580(1940).
(40) Gremels, H., *Arch. Exptl. Pathol. Pharmakol.*, **182**, 1(1936).
(41) Garcia-Ramos, J., and de Hrellano, J. R., *Arch. Inst. Cardiol.*, **21**, 205(1951).
(42) Eckstein, R. W., Stroud, M., Eckel, R., Dowling, C. V., and Pritchard, W. P., *Am. J. Physiol.*, **163**, 539(1950).
(43) Katz, L. N., Williams, F. L., Laurent, D., Bolene-Williams, C., and Feinberg, H., *Federation Proc.*, **15**, 106 (1956).
(44) Kaindl, F., Lindner, A., London, M., and Werner, G., *Wien. Klin. Wochschr.*, **64**, 718(1952).
(45) Whalen, W. J., *Circulation Res.*, **5**, 556(1957).
(46) Marchetti, G., Maccari, M., and Merlo, L., *Cardiologia*, **42**, 1(1963).
(47) Wartmen, W. B., in "The Etiology of Myocardial Infarction," James, T. N., and Keyes, J. W., eds., Little, Brown and Co., Boston, Mass., 1963, p. 115.
(48) Herrick, J. B., *J. Am. Med. Assoc.*, **70**, 67(1918).
(49) Keefer, C. S., and Resnik, W. H., *Arch. Internal Med.*, **41**, 769(1928).
(50) Ferrans, V. J., Hibbs, R. G., Black, W. C., and Weilbaecher, D. G., *Am. Heart J.*, **68**, 71(1964).
(51) Sobol, B., Jequier, E., Sjoerdsma, A., and Lovenberg, W., *Circulation Res.*, **19**, 1050(1966).
(52) Rona, G., Chappel, C. I., and Gaudry, R., *Lab. Invest.*, **10**, 892(1961).
(53) Wenzel, D. G., and Stark, L. G., *Am. Heart J.*, **69**, 780(1965).
(54) von Korff, R. W., McPherson, E. H., and Glaman, G. V., *J. Biol. Chem.*, **209**, 151(1954).
(55) Park, J. H., Meriwether, B. P., Park, C. R., Mudd, S. H., and Lipmann, F., *Biochim. Biophys. Acta*, **22**, 403 (1956).
(56) Klocke, F. J., Kaiser, G. A., Ross, J., Jr., and Braunwald, E., *Am. J. Physiol.*, **209**, 913(1965).
(57) Challoner, D. R., and Steinberg, D., *Nature*, **205**, 602(1965).
(58) Hauge, A., and Øye, I., *Acta Physiol. Scand.*, **68**, 295(1966).
(59) Maling, H. M., Highman, B., and Thompson, E. C., *Am. J. Cardiol.*, **5**, 628(1960).
(60) Highman, B., Maling, H. M., and Thompson, E. C., *Am. J. Physiol.*, **196**, 436(1959).
(61) Maling, H. M., and Highman, B., *ibid.*, **194**, 590 (1958).
(62) Maling, H. M., Williams, M. A., and Highman, B., *Arch. Exptl. Pathol.*, **248**, 54(1964).
(63) Luduena, F. P., O'Malley, E., and Oyen, I., *Arch. Intern. Pharmacodyn.*, **122**, 111(1959).
(64) Raab, W., and Humphreys, R. J., *J. Pharmacol. Exptl. Therap.*, **88**, 268(1946).
(65) Wenzel, D. G., and Chau, R. Y. P., *Toxicol. Appl. Pharmacol.*, **9**, 514(1966).
(66) Mayer, S., and Moran, N. C., *J. Pharmacol. Exptl. Therap.*, **129**, 271(1960).
(67) Kvan, D. C., Riggilo, D. A., and Lish, P. M., *ibid.*, **149**, 183(1965).
(68) Ellis, S., and Beckett, S. D., *ibid.*, **142**, 318(1963).
(69) Pressman, B. C., and Lardy, A. A., *Biochim. Biophys. Acta*, **18**, 482(1955).
(70) Paradise, R. R., Troop, R. C., and Woodbury, R. A., *Life Sci.*, **3**, 1235(1964).
(71) Hers, H. G., *Biochim. Biophys. Acta*, **8**, 416(1952).
(72) *Ibid.*, **8**, 424(1952).
(73) Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 673(1942).
(74) *Ibid.*, **149**, 529(1943).
(75) Kachman, J. F., and Boyer, P. D., *ibid.*, **200**, 669(1953).
(76) Stadtman, E. R., *ibid.*, **196**, 527(1952).
(77) von Korff, R. W., *ibid.*, **203**, 265(1953).

(78) Lubin, M., *Federation Proc.*, **23**, 994(1964).
(79) Sanslone, W. R., and Muntwyler, E., *Proc. Soc. Exptl. Biol. Med.*, **122**, 900(1966).
(80) Molnar, Z., Larsen, K., and Spargo, B., *Arch. Pathol.*, **74**, 339(1962).
(81) Jennings, R. B., Crout, J. R., and Smetters, G. W., *ibid.*, **63**, 586(1957).
(82) Laks, M. M., and Elek, S. R., *Diseases Chest*, **46**, 609(1964).
(83) Jennings, R. B., Sommers, H. M., Kaltenbach, J. P., and West, J. J., *Circulation Res.*, **14**, 260(1964).
(84) Nickerson, M., Karr, G. W., and Dresel, P. E., *ibid.*, **9**, 209(1961).
(85) Anichkov, S. V., and Vedeneyeva, Z. I., *Acta Physiol. Acad. Sci. Hung.*, **19**, 9(1961).
(86) Chappel, C. I., Rona, G., Balazs, T., and Gaudry, R., *Can. J. Biochem. Physiol.*, **37**, 35(1959).
(87) Chappel, C. I., Rona, G., Balazs, T., and Gaudry, R., *Arch. Intern. Pharmacodyn.*, **122**, 123(1959).
(88) Katz, L. N., and Feinberg, H., *Circulation Res.*, **6**, 656(1958).
(89) Mercker, H., Lochner, W., and Bretschneider, H. J., *Verhandl. Deut. Ges. Kreislaufforsch.*, **22**, 300(1956).
(90) Zuberbuhler, R. C., and Bohr, D. F., *Circulation Res.*, **16**, 431(1965).
(91) Gaal, P. G., Kattus, A. A., Kolin, A., and Ross, G., *Brit. J. Pharmacol.*, **26**, 713(1966).
(92) Benedixen, H. H., Osgood, P. F., Hall, K. V., and Laver, M. B., *J. Pharmacol. Exptl. Therap.*, **145**, 299(1964).
(93) Butterworth, K. R., *Brit. J. Pharmacol.*, **21**, 378 (1963).
(94) Veith, G., *Arch. Kreislaufforsch.*, **6**, 335(1940).
(95) Danilova, K. M., *Arkh. Patol.*, **23**, 11(1961).
(96) Visscher, M. B., and Henschel, A., *Am. Heart J.*, **30**, 592(1945).
(97) Scholz, D. E., Schultz, J. H., Pleune, F. G., Fink, K., Steadman, L. T., and Warren, S. L., *J. Clin. Invest.*, **24**, 154(1945).
(98) Waters, L. L., and de Sütö-Nagy, G. I., *Science*, **111**, 634(1950).
(99) Waters, L. L., and de Sütö-Nagy, G. I., *Yale J. Biol. Med.* **22**, 751(1950).
(100) Shimamoto, T., Fujita, T., Shimura, H., Yamazaki, H., and Iwahara, S., *Am. Heart J.*, **57**, 273(1959).
(101) McJunkin, F. A., Rall, R. R., and Singer, P. L., *Endocrinology*, **16**, 635(1932).
(102) Vishnevskaia, O. P., *Byul. Eksperim. Biol. Med.*, **38**, (No. 10), 29(1954).
(103) Highman, B., Altland, P. D., and Garbus, J., *Arch. Pathol.*, **80**, 332(1965).
(104) Fleisher, M. S., and Loeb, L., *Arch. Internal Med.*, **6**, 427(1910).
(105) Christian, H. A., Smith, R. M., and Walker, I. C., *ibid.*, **8**, 468(1911).
(106) Gruber, C. M., Olch, I. Y., and Blades, B., *J. Pharmacol. Exptl. Therap.*, **49**, 306(1933).
(107) Van Esveld, L. W., *Acta Brev. Neerland. Physiol.*, **7**, 33(1937).
(108) Frolkis, V. V., *Vys. Nerv. Deiat. Kort. Visc.*, **1955**, 137.
(109) Mekes, G., Szekeres, L., Kovascsics, J., and Varga, F., *Acta Physiol. Acad. Sci. Hung.*, **6**, 113(1954).
(110) Feigen, M. B., *Trud. Inst. Fiziol. Im. Pavlova*, **3**, 419(1954).
(111) Lukl, P., *Prakt. Lek.*, **32**, 330(1952).
(112) Szakacs, J. E., and Mehlman, B., Research Report, Project No. MN 71-01-21.3, U. S. Naval Medical School, National Naval Medical Center, Bethesda, Md., 1959.
(113) Szakacs, J. E., and Cannon, A., *Am. J. Clin. Pathol.*, **30**, 425(1958).
(114) Szakacs, J. E., Dimmette, R. M., and Cowart, E. C. R., Jr., Research Report, Project No. MN 71-01-21.3, U.S. Naval Medical School, National Naval Medical Center, Bethesda, Md., 1959.
(115) Littler, T. R., and McKendrick, C. S., *Lancet*, **2**, 825(1957).
(116) Mond, E., and Mack, I., *Am. Heart J.*, **59**, 134 (1960).
(117) Selye, H., and Bajusz, E., *Arch. Intern. Physiol.*, **67**, 78(1959).
(118) Selye, H., and Bajusz, E., *J. Gerontol.*, **14**, 164(1959).
(119) Selye, H., and Bajusz, E., *Angiology*, **10**, 412(1959).
(120) Selye, H., Bajusz, E., Renaud, S., and Lemire, Y., *Am. Heart J.*, **57**, 88(1959).
(121) Selye, H., and Bajusz, E., *Am. J. Pathol.*, **35**, 525 (1959).
(122) Gunther, D., and Schmidt, H., *Z. Kreislaufforsch.*, **48**, 237(1959).
(123) Kinikoski, A., Pylkäs, S., and Näätänen, E., *Ann. Med. Exptl. Fenn.*, **38**, 387(1960).
(124) Rona, G., Chappel, C. I., Balazs, T., and Gaudry, R., *Arch. Pathol.*, **67**, 443(1959).
(125) Rona, G., Chappel, C. I., and Kahn, D. S., *Am. Heart J.*, **66**, 389(1963).
(126) Rona, G., Chappel, C. I., Balazs, T., and Gaudry, R., *J. Gerontol.*, **14**, 169(1959).
(127) Chappel, C. I., Rona, G., and Gaudry, R., *Acta Endocrinol.*, **32**, 419(1959).
(128) Chappel, C. I., Rona, G., and Gaudry, R., *Endocrinology*, **65**, 208(1959).
(129) Balazs, T., Murphy, J. B., and Grice, H. C., *J. Pharm. Pharmacol.*, **14**, 750(1962).
(130) Balazs, T., Sahasrabudhe, M. R., and Grice, H. C., *Toxicol. Appl. Pharmacol.*, **4**, 613(1962).
(131) Wenzel, D. G., and Stark, L. G., *Am. Heart J.*, **71**, 368(1965).
(132) Zbinden, G., *ibid.*, **60**, 450(1960).
(133) Zbinden, G., *Am. J. Cardiol.*, **6**, 1121(1960).
(134) Zbinden, G., *Federation Proc.*, **20**, 128(1961).
(135) Wenzel, D. G., and Chau, R. Y. P., *Toxicol. Appl. Pharmacol.*, **8**, 460(1966).
(136) Lehr, D., Krukowski, M., and Colon, R., *Federation Proc.*, **24**, 561(1965).
(137) Kako, K., *Can. J. Physiol. Pharmacol.*, **44**, 678(1966).
(138) Wexler, B. C., and Kittinger, G. W., *Circulation Res.*, **13**, 159(1963).
(139) Beznak, M., *Can. J. Biochem. Physiol.*, **40**, 25(1962).
(140) Beznak, M., and Hacker, P., *ibid.*, **42**, 270(1964).
(141) Rona, G., Zsoter, T., Chappel, C., and Gaudry, R., *Rev. Can. Biol.*, **18**, 83(1959).
(142) Korb, G., *Arch. Pathol. Anat.*, **339**, 136(1965).
(143) Anshelevich, I. V., Amelin, A. Z., and Melzobs, M. I., *Kardiologiia*, **3**, 11(1963).
(144) Amelin, A. Z., Anshelevich, I. V., and Melzobs, M. I., *Arkh. Patol.*, **25**, 25(1963).
(145) Strubelt, O., and Bruning, H., *Arzneimittel-Forsch.*, **14**, 1196(1964).
(146) Rosenblum, I., Wohl, A., and Stein, A. A., *Toxicol. Appl. Pharmacol.*, **7**, 1(1965).
(147) Schulze, M., *Munch. Med. Wochschr.*, **78**, 1826(1931).
(148) Di Mattei, P., *Arch. Intern. Pharmacodyn.*, **40**, 379 (1931).
(149) Kawamura, M., *Folia Pharmacol. Japon.*, **49**, 382 (1953).
(150) Wenzel, D. G., in "The Etiology of Myocardial Infarction," James, T. N., and Keyes, J. W., eds., Little, Brown and Co., Boston, Mass., 1963, p. 499.
(151) Staemmler, M., *Arch. Pathol. Anat.*, **295**, 336(1935).
(152) Staemmler, M., *Klin. Wochschr.*, **15**, 404(1936).
(153) Thienes, C. H., Behrendt, A., Butt, E. M., Messenger, T. T., and Rollins, E., *J. Pharmacol. Exptl. Therap.*, **60**, 121(1937).
(154) Thienes, C. H., and Butt, E. M., *Am. J. Med. Sci.*, **195**, 522(1938).
(155) Wilson, R. H., McNaught, J. B., and De Eds, F., *Ind. Hyg. Toxicol.*, **20**, 468(1938).
(156) Esser, J., *Arch. Exptl. Pathol.*, **49**, 190(1903).
(157) von Otto, C., *Arch. Pathol. Anat.*, **205**, 384(1911).
(158) Hueper, W. C., *A.M.A. Arch. Pathol.*, **35**, 846(1943).
(159) Erbacher, K., Grumbrecht, P., and Loeser, A., *Arch. Exptl. Pathol.*, **195**, 121(1940).
(160) Scott, W., *Med. Mirror*, **1**, 781(1864).
(161) Fellinger, K., *Wien. Klin. Wochschr.*, **60**, 231(1948).
(162) Andrä, I., *Deut. Tierärztl. Wschr.*, **49**, 409(1941).
(163) Fukuda, Y., *Nippon J. Angio-Cardiol.*, **14**, 42(1950).
(164) *Ibid.*, **14**, 202(1950).
(165) *Ibid.*, **15**, 239(1951).
(166) *Ibid.*, **15**, 28(1951).
(167) *Ibid.*, **15**, 185(1951).
(168) Wenzel, D. G., Turner, J. A., and Kissil, D., *Circulation Res.*, **7**, 256(1959).
(169) Wenzel, D. G., Turner, J. A., Jordan, S. W., and Singh, J., *ibid.*, **9**, 694(1961).
(170) Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, **21**, 601(1942).
(171) Darrow, D. C., *Proc. Soc. Exptl. Biol. Med.*, **55**, 13(1944).
(172) Timiras, P. S., Faribault, C., and Selye, H., *Geriatrics*, **4**, 225(1949).
(173) Telo, W., and Pierangnoli, E., *Arch. Sci. Med.*, **100**, 227(1955).
(174) Guillemin, R., and Fortier, C., *Endocrinology*, **48**, 617(1951).
(175) Peschel, E., Black-Schaffer, B., and Schlayer, C., *ibid.*, **48**, 399(1951).
(176) Selye, H., *Proc. Soc. Exptl. Biol. Med.*, **76**, 510(1951).
(177) Rosenberg, C. A., Woodbury, D. M., and Sayers, G., *J. Clin. Endocrinol.*, **12**, 666(1952).
(178) Masson, G. M. C., Lewis, L. A., Corcoran, A. C., and Page, I. H., *ibid.*, **13**, 300(1953).
(179) Justin-Besancon, L., Rubens-Duval, J., Villaumey, J., and Hazard, J., *Semaine Hop. Paris*, **29**, 2079(1953).
(180) Mucio, G., Andreozzi, G., and Gesi, V., *Boll. Soc. Ital. Biol. Sper.*, **30**, 933(1954).
(181) Skelton, F. R., *Arch. Pathol.*, **60**, 190(1955).
(182) Mucio, G., Luciani, A., Andreozzi, G., and Gesi, V., *Boll. Soc. Ital. Biol. Sper.*, **31**, 247(1955).
(183) Selye, H., *Munch. Med. Wochschr.*, **98**, 1015(1956).
(184) Hall, C. E., and Hall, O., *Am. J. Physiol.*, **194**, 236(1958).
(185) Heroux, P. O., and Dugal, L. P., *Can. J. Biochem. Physiol.*, **29**, 164(1951).
(186) Salgado, E., and Selye, H., *Arch. Intern. Physiol.*, **62**, 352(1954).
(187) Lown, B., Salzberg, H., Enselberg, C. D., and Weston, R. E., *Proc. Soc. Exptl. Biol. Med.*, **76**, 797(1951).
(188) Masson, G. M. C., Corcoran, A. C., Dustan, H., and Page, I. H., *Am. J. Med. Sci.*, **224**, 175(1952).
(189) Bohle, A., Hieronymi, G., and Hartman, F., *Z. Kreislaufforsch.*, **40**, 161(1951).
(190) Lanza, B., and Frassineti, A., *Arch. Maragliano, Patol. Clin.*, **6**, 1011(1951).
(191) Notario, A., *Arch. Sci. Med.*, **103**, 452(1959).
(192) Silverman, J. J., and Schroeder, C. T. H., *N. Y. State J. Med.*, **51**, 1427(1951).

(193) Morelli, A., and Mazella di Bosco, M., *Rif. Med.*, **65** 365(1951).
(194) Bricaire, H., *Semaine Hop. Paris*, **28**, 620(1952).
(195) Sparks, L. L., Rosenau, W., Macalpin, R. N., Daane, T. A., and Li, C. H., *Nature*, **176**, 503(1955).
(196) Salgado, E. D., *Circulation Res.*, **5**, 191(1957).
(197) Casterline, R. L., *Ann. Internal Med.*, **48**, 1121(1958).
(198) Rawls, W. B., and Ancona, V. C., *N. Y. State J. Med.*, **51**, 1625(1951).
(199) Herlant, M., and Toussaint, C., *J. Urol. Med. Chir.*, **57**, 599(1951).
(200) Toussaint, C., *Compt. Rend. Soc. Biol.*, **145**, 1427 (1951).
(201) Telo, W., and Pieragnoli, E., *Arch. Sci. Med.*, **100** 159(1955).
(202) *Ibid.*, **100**, 227(1955).
(203) Andreichev, A. I., *Kardiologiia*, **3**, 33(1963).
(204) Lostroh, A., and Li, C. H., *Nature*, **176**, 504(1955).
(205) Selye, H., *Circulation Res.*, **6**, 168(1958).
(206) Bois, P., and Jasmin, G., *Rev. Can. Biol.*, **18**, 358 (1959).
(207) Gervasoni, L. A., *Schweiz. Med. Wochschr.*, **89**, 713 (1959).
(208) Usvatova, I. Y., *Kardiologiia*, **3**, 16(1963).
(209) Sievers, J., Johansson, B. W., and Nilsson, S. E., *Cardiologia*, **45**, 65(1965).
(210) Selye, H., and Renaud, S., *Proc. Soc. Exptl. Biol. Med.*, **96**, 512(1957).
(211) Selye, H., and Bois, P., *Endocrinology*, **59**, 646(1956).
(212) Selye, H., and Renaud, S., *Exptl. Med. Surg.*, **15**, 335(1957).
(213) Ventura, J., *Rev. Can. Biol.*, **15**, 320(1957).
(214) Ventura, J., and Selye, H., *Am. J. Physiol.*, **189**, 412(1957).
(215) Selye, H., *Brit. Med. J.*, **1**, 599(1958).
(216) Selye, H., and Renaud, S., *Am. J. Cardiol.*, **1**, 208 (1958).
(217) Selye, H., *Angiology*, **9**, 262(1958).
(218) Selye, H., *Z. Kreislaufforsch.*, **47**, 318(1958).
(219) Bajusz, E., *Z. Exptl. Med.*, **130**, 13(1958).
(220) Selye, H., *Can. J. Biochem. Physiol.*, **36**, 717(1958).
(221) Selye, H., and DeSalcedo, I., *Arch. Internal Med.*, **102**, 551(1958).
(222) Selye, H., *Proc. Soc. Exptl. Biol. Med.*, **98**, 61(1958).
(223) Selye, H., Renaud, S., and Nadasi, M., *J. Pharmacol. Exptl. Therap.*, **122**, 504(1958).
(224) Selye, H., *Z. Kreislaufforsch.*, **47**, 318(1958).
(225) Selye, H., and Bajusz, E., *Lab. Invest.*, **8**, 1499(1959).
(226) Zsoter, T., Pinter, I., and Nadasi, M., *Cardiologia*, **34**, 218(1959).
(227) Selye, H., Prioreschi, P., and Jean, P., *Proc. Soc. Exptl. Biol. Med.*, **104**, 68(1960).
(228) Hall, C. E., and Hall, O., *Tex. Rept. Biol. Med.*, **19**, 774(1961).
(229) Selye, H., "The Chemical Prevention of Cardiac Necrosis," The Ronald Press Co., New York, N. Y., 1958.
(230) Selye, H., "The Pluricausal Cardiopathies," Charles C Thomas, Springfield, Ill., 1961.
(231) Prioreschi, P., *Circulation Res.*, **10**, 782(1962).
(232) *Ibid.*, **12**, 55(1963).
(233) Tucker, V. L., Hanna, H., Kaiser, C. J., and Darrow, D. C., *ibid.*, **13**, 420(1963).
(234) Labbé, M., Justin-Besancon, L., and Gouyen, J., *Bull. Mém. Soc. Med. Hop. Paris*, **53**, 429(1929).
(235) Zimmerman, O., *Klin. Wochschr.*, **14**, 500(1935).
(236) Scherf, D., and Schlachman, M., *Am. J. Med. Sci.*, **216**, 673(1948).
(237) Fuchs, M., and Blumenthal, L. C., *J. Am. Med. Assoc.*, **143**, 1462(1950).
(238) Goldfischer, J. D., *New Engl. J. Med.*, **262**, 830 (1960).
(239) Stein, I., *Am. Heart J.*, **37**, 36(1949).
(240) Stein, I., and Weinstein, J., *J. Lab. Clin. Med.*, **36**, 66(1950).
(241) Stein, I., *Angiology*, **14**, 23(1963).
(242) Rinzler, S. H., Travell, J., and Karp, D., *Science*, **121**, 900(1955).
(243) Karp, D., Travell, J., and Rinzler, S. H., "Abstracts of 20th International Physiological Congress," Brussels, Belgium, 1956, p. 482.
(244) Rinzler, S. H., Travell, J., Karp, D., and Charleson, D., *Am. J. Physiol.*, **184**, 605(1956).
(245) Wenzel, D. G., Turner, J. A., and Kissil, D., *Circulation Res.*, **7**, 256(1959).
(246) Wenzel, D. G., Turner, J. A., Jordan, S. W., and Singh, J. S., *ibid.*, **9**, 694(1961).
(247) Scheps, M., *Klin. Wochschr.*, **13**, 1151(1934).
(248) Scheps, M., *Compt. Rend. Soc. Biol.*, **115**, 1367 (1934).
(249) Dill, L. V., and Isenhour, C. E., *Endrocrinology*, **26**, 863(1940).
(250) Rolshoven, H., *Z. Exptl. Med.*, **90**, 225(1933).
(251) Visscher, M. B., and Henschel, A., *Am. Heart J.*, **30**, 592(1945).
(252) Selye, H., and Bajusz, E., *Gerontol.*, **14**, 164(1959).
(253) Selye, H., and Bajusz, E., *Am. J. Pathol.*, **35**, 525 (1959).
(254) Dearing, W. H., Barnes, A. R., and Essex, H. E., *Am. Heart J.*, **27**, 96(1944).
(255) Mills, M. D., Burchell, H. B., Parker, R. L., and Kirklin, B.R., *Proc. Staff Meetings Mayo Clinic*, **24**, 254(1949).
(256) Slotnik, I. L., and Teigland, J. D., *J. Am. Med. Assoc.*, **146**, 1126(1951).
(257) Lange, G., *Arch. Exptl. Pathol. Pharmakol.*, **246**, 240(1963).
(258) Myasnikov, A. L., Kipschidze, N. N., and Tchazov, E. I., *Am. Heart J.*, **61**, 76(1961).
(259) Lindgren, I., and Frisk, A. R., *Acta Med. Scand.*, **131**, 581(1948).
(260) Livingstone, D. J., *Brit. Med. J.*, **2**, 713(1950).
(261) McLemore, G. A., Jr., Green, H. D., and Lide, T. N., *Circulation*, **4**, 47(1951).
(262) Koszalka, M. F., and Walker, H. C., *Lancet*, **75**, 368(1955).
(263) Ripka, O., and Tauer, E., *Vnitr. Lek.*, **1**, 767(1955).
(264) Judson, W. E., Hollander, W., and Wilkins, R. W., *Circulation*, **13**, 553(1956).
(265) Storstein, O., *Tskr. Norske Laegeforen.*, **76**, 799 (1956).
(266) Cottier, P. T., Weller, J. M., and Hoobler, S. W., *J. Lab. Clin. Med.*, **56**, 199(1957).
(267) Matusova, A. P., *Kazansk. Med. Zh.*, **40**, (No. 6), 86(1959).
(268) Koltova, N. N., Kucherenko, E. M., and Chuberkis, T. P., *Sovet. Med.*, **23**, (No. 10), 112(1959).
(269) Chamberlin, R. T., and Putnam, W. S., *Am. J. Med.*, **35**, 396(1963).
(270) Stunkard, A. J., Eurman, G. H., Wachspress, M., and Wertheim, A. R., *ibid.*, **17**, 712(1954).
(271) Grob, D., *J. Chronic Diseases*, **1**, 546(1955).
(272) Obergassner, H., *Med. Wochschr.*, **6**, 646(1952).
(273) Hueper, W. C., and Landsberg, J. W., *Arch. Pathol.*, **29**, 633(1940).
(274) Farrant, R., *Brit. Med. J.*, **2**, 1363(1913).
(275) Hashimoto, H., *Endocrinology*, **5**, 579(1921).
(276) Goodpasture, E. W., *J. Exptl. Med.*, **34**, 407(1921).
(277) Takane, K., *Arch. Pathol. Anat.*, **259**, 737(1926).
(278) Menne, F. R., Keane, R. H., Henry, R. T., and Jones, N. W., *Am. Heart J.*, **8**, 75(1932).
(279) Mattonet, C., *Z. Exptl. Med.*, **90**, 237(1933).
(280) Boyksen, D., *Arch. Pathol. Anat.*, **293**, 342(1934).
(281) Menne, F. R., Jones, O. N., and Jones, N. W., *Arch. Pathol.*, **17**, 333(1934).
(282) von Zalka, E., *Beitr. Pathol. Anat. Allgem. Pathol.*, **95**, 590(1935).
(283) Gruber, C. M., Moon, V. H., and Sufrin, E., *Endocrinology*, **19**, 447(1935).
(284) Manzini, C., *Boll. Soc. Ital. Biol. Sper.*, **10**, 597 (1935).
(285) Dieckhoff, J., *Arch. Exptl. Pathol. Pharmakol.*, **182**, 293(1936).
(286) Zeus, L., *Arch. Kreislaufforsch.*, **2**, 165(1938).
(287) Vizer, T., and Haban, G., *ibid.*, **5**, 49(1939).
(288) Bauer, H., *Arch. Exptl. Pathol. Pharmakol.*, **193**, 622(1939).
(289) Hilbing, R., *ibid.*, **196**, 310(1940).
(290) Nora, E. D., and Flaxman, N., *J. Lab. Clin. Med.*, **28**, 797(1943).
(291) Sarzana, G., and Cascio, G., *Arch. Fisiol.*, **49**, 238(1949).
(292) *Ibid.*, **49**, 32(1949).
(293) Dearing, W. H., Barnes, A. R., and Essex, H. E., *Circulation*, **1**, 394(1950).
(294) Selye, H., and Bajusz, E., *Am. J. Pathol.*, **35**, 525 (1959).
(295) Lewis, J. K., and McEachern, D., *Bull. Johns Hopkins Hosp.*, **48**, 228(1931).
(296) Rake, G., and McEachern, D., *J. Exptl. Med.*, **54**, 23(1931).
(297) Lewis, J. K., and McEachern, D., *Am. Heart J.*, **8**, 19(1932).
(298) David, C., *Z. Exptl. Med.*, **104**, 397(1938).
(299) von Fahr, T., *Zentr. Pathol. Pathol.-Anat.*, **27**, 1(1916).
(300) Goodpasture, E. W., *J. Am. Med. Assoc.*, **76**, 1545 (1921).
(301) Fahr, T., and Kuhle, J., *Arch. Pathol. Anat.*, **233**, 286(1921).
(302) Goodall, J. S., and Rogers, L., *Lancet*, **1**,486(1927).
(303) Bjerkelund, C. J., *Nord. Med.*, **47**, 827(1952).
(304) Gorzkowski, T., *Pol. Tyg. Lek.*, **10**, (No. 20), 647 (1955).
(305) Wallach, E. E., Lubash, G. D., Cohen, B. D., and Rubin, A. L., *J. Am. Med. Assoc.*, **167**, 1921(1958).
(306) Willius, F. A., and Boothby, W. M., *Med. Clin. North Am.*, **7**, 189(1923–1924).
(307) Vitale, J. J., Hegsted, D. M., Nakamura, M., and Conners, P., *J. Biol. Chem.*, **226**, 597(1957).
(308) Hegsted, D. M., *ibid.*, **228**, 573(1957).
(309) Lewitzky, A., abstract in *Zentralbl. Pathol.*, **16**, 532 (1905).
(310) Schellong, F., *Z. Exptl. Med.*, **78**, 1(1931).
(311) Kisch, F., *Klin. Wochschr.*, **11**, 1875(1932).
(312) Büchner, F., *Med. Klin.*, **29**, 591(1933).
(313) Hueper, W. C., and Ichniowski, C. T., *J. Lab. Clin. Med.*, **26**, 1565(1941).
(314) Kyser, F. A., Ginsberg, H., and Gilbert, N. C., *Am. Heart J.*, **31**, 451(1946).
(315) Adamski, R., *Farm. Pol.*, **8**, (No. 12), 454(1952).
(316) Shnider, B. I., and Cardi, E. R., *Bull. Georgetown Univ. Med. Cent.*, **6**, 54(1953).
(317) Bachman, M., *Z. Inn. Med.*, **9**, 71(1954).
(318) Klonowicz, M., *Pol. Tyg. Lek.*, **10**, 716(1955).
(319) Bauer, H., *Arch. Exptl. Pathol. Pharmakol.*, **176**, 74(1934).
(320) Weese, H., and Dieckhoff, J., *ibid.*, **176**, 274(1934).

(321) Büchner, F., *ibid.*, **176,** 59(1934).
(322) Tomaszewski, W., and Lapa, W., *Arch. Mal. Coeur,* **29,** 108(1936).
(323) Korth, C., and Spang, K., *Arch. Expil. Pathol. Pharmakol.*, **184,** 349(1937).
(324) Lindner, W., *ibid.*, **190,** 210(1938).
(325) Bauer, H., and Reindell, H., *ibid.*, **191,** 311(1938–1939).
(326) Lindner, W., *ibid.*, **192,** 155(1939).
(327) Hilbing, R., *Arch. Kreislauffosch.*, **5,** 292(1939)
(328) La Due, J. S., *Proc. Soc. Expil. Biol. Med.*, **46,** 513 (1941).
(329) La Due, J. S., *J. Pharmacol. Exptl. Therap.*, **76,** 126(1942).
(330) Remê, H., *Arch. Expil. Pathol. Pharmakol.*, **178,** 672(1935).
(331) Abendroth, H., *Klin. Wochschr.*, **31,** 496(1953).
(332) Dearing, W. H., Barnes, A. R., and Essex, H. E., *Am. Heart J.*, **25,** 665(1943).
(333) Lown, B., and Levine, S. A., "Current Concepts of Digitalis Therapy," Little, Brown and Co., Boston, Mass., 1954, pp. 49, 63.
(334) Lown, B., and Levine, H. D., "Atrial Arrhythmias, Digitalis and Potassium," Landsberger Medical Books, Inc., New York, N. Y., 1958, p. 45.
(335) Lown, B., Black, H., and Moore, F. D., *Am. J. Cardiol.*, **6,** 309(1960).
(336) Klatskin, G., and Friedman, H., *Ann. Internal Med.*, **28,** 892(1948).
(337) Marino, A., *Science,* **133,** 385(1961).
(338) Anderson, H. H., and Reed, A. C., *Am. J. Trop. Med.*, **14,** 269(1934).
(339) Chopra, R. N., and Sen, B., *Indian Med. Gaz.*, **69** 262(1934).
(340) Kattwinkel, E. E., *New Engl. J. Med.*, **240,** 995 (1949).
(341) Gonzalez de Cossio, A., *Am. Heart J.*, **43,** 456(1952).
(342) Taboada Millas, C., Felipe Jay, L., and Edelstein Tuchman, J., *Rev. Cubana Cardiol.*, **14,** 89(1953).
(343) Brem, T. H., and Konwaler, B. E., *Am. Heart J.*, **50,** 476(1955).
(344) Cera, L. J., *Circulation,* **14,** 33(1956).
(345) Dzambas, D., *Lijecn. Vjesn.*, **82,** 609(1960).
(346) Martin, M., and Peuchot, G., *Med. Trop.*, **22,** 531 (1962).
(347) Fromantin, M., and Azais, J. L., *Bull. Soc. Med. Milil. Franc.*, **57,** 170(1963).
(348) Chopra, R. N., Ghosh, H., and De, M., *Indian Med. Gaz.*, **59,** 338(1924).
(349) Turner, P. P., *Brit. Heart J.*, **25,** 81(1963).
(350) Levy, R. L., and Rountree, L. G., *Arch. Internal Med.*, **17,** 420(1916).
(351) Niccolini, P. M., *Arch. Intern. Pharmacodyn.*, **26,** 375(1922).
(352) Berman, P., and Leake, W. H., *Calif. West. Med.*, **28,** 772(1928).
(353) Rinehart, J. F., and Anderson, H. H., *Arch. Pathol.*, **11,** 546(1931).
(354) McGinn, S., and Sprague, H. B., *New Engl. J. Med.*, **208,** 691, 747, 789(1933).
(355) Hein, G., and Vannotti, H., *Z. Exptl. Med.*, **104,** 629(1939).
(356) Taboaka Millas, C., Pérez Mesa, C., Arias Léon, L., Felipe Jay, L., and Edelstein Tuchman, J., *Rev. Cubana Cardiol.*, **12,** 33(1951).
(357) di Mattei, P., and Paroli, E., *Arch. Ital. Sci. Farmacol.*, **4,** 285(1954).
(358) Marino, A., and Miele, E., *Boll. Soc. Ital. Biol. Sper.*, **35,** 749(1959).
(359) Marino, A., and Russo, E., *ibid.*, **35,** 1244(1959).
(360) Marino, A., *Experientia,* **17,** 116(1961).
(361) *Ibid.*, **17,** 117(1961).
(362) Brueckman, G., and Wertheimer, E., *Acta Med. Orient.*, **4,** 291(1945).
(363) Guggenheim, K., and Halevy, S., *J. Nutr.*, **53,** 129(1954).
(364) Diamant, E. J., Halevy, S., and Guggenheim, K., *ibid.*, **55,** 241(1955).
(365) Deitrich, R. A., and Heim, H. C., *J. Am. Pharm. Assoc., Sci. Ed.*, **45,** 562(1956).
(366) Paroli, E., *Arch. Ital. Sci. Med. Trop. Parassitol.*, **38,** 611(1957).
(367) Diamant, E. J., *J. Pharmacol. Exptl. Therap.*, **122,** 465(1958).
(368) Marino, A., *Folia Med.*, **42,** 564(1959).
(369) Heim, H. C., Froede, N. C., and Erwin, V. G., *J. Pharmacol. Exptl. Therap.*, **137,** 107(1962).
(370) Parmer, L. G., and Cottrill, C., *J. Lab. Clin. Med.*, **34,** 818(1949).
(371) Appelt, G. D., and Heim, H. C., *J. Pharm. Sci.*, **53,** 1080(1964).
(372) *Ibid.*, **54,** 1621(1965).
(373) Gimble, A. I., Davison, C., and Smith, P. K., *J. Pharmacol. Exptl. Therap.*, **94,** 431(1948).
(374) Brown, E. C., and McNamara, D. H., *Arch. Derm. Syph.*, **42,** 312(1940).
(375) Edge, J. R., *Lancet,* **II,** 675(1946).
(376) Leschke, E., *Munch. Med. Wochschr.*, **79,** 57(1932).
(377) Gousios, A. G., and Adelson, L., *Am. J. Med.*, **27,** 659(1959).
(378) McKinstry, W. J., and Hickes, J. M., *Arch. Internal Med.*, **100,** 34(1957).
(379) Honey, M., *Brit. Heart J.*, **22,** 601(1960).
(380) Davis, A., *ibid.*, **23,** 291(1961).
(381) Somers, K., and Rosanelli, J. D., *ibid.*, **24,** 187(1962).
(382) Masson, G. A., *J. Pharmacol. Expil. Therap.*, **30,** 121(1926).
(383) Wachstein, M., *Am. J. Clin. Pathol.*, **14,** 392(1944).
(384) Brown, G., Friedfeld, L., Kissin, M., Modell, W., and Sussman, R. M., *J. Am. Med. Assoc.*, **119,** 1005(1942).
(385) Volini, I. F., Levitt, R. O., and Martin, R., *ibid.*, **128,** 12(1945).
(386) Wexler, J., and Ellis, L. B., *Am. Heart J.*, **27,** 86 (1944).
(387) Kaufman, R. E., *Ann. Internal Med.*, **28,** 1040(1948).
(388) Falck, I., *Z. Inn. Med.*, **11,** 756(1956).
(389) De Graff, A. C., and Lehman, R. A., *J. Am. Med. Assoc.*, **119,** 998(1942).
(390) Farah, A., and Maresh, G., *J. Pharmacol. Expil. Therap.*, **92,** 73(1948).
(391) Lechner, E., Nunez, H., Nemir, P., Jr., and Bellet, S., *Am. J. Med. Sci.*, **228,** 282(1954).
(392) Larson, E., *Confinia Neurologica,* **10,** 108(1950).
(393) Chopra, R. N., and Wahed, A. K. M. A., *Indian Med. Gaz.*, **69,** 213(1934).
(394) Rollo, I. M., in "The Pharmacological Basis of Therapeutics," Goodman, L. S., and Gilman, A., eds., The Macmillan Co., New York, N. Y., 1965, p. 1102.
(395) Heilig, R., and Visveswar, S. K., *Indian Med. Gaz.*, **79,** 514(1944).
(396) Hicks, S. P., *Arch. Pathol.*, **49,** 111(1950).
(397) Siegle, H., and Mushett, C. W., *ibid.*, **38,** 63(1944).
(398) Nelson, A. A., and Fitzhugh, O. G., *ibid.*, **45,** 454 (1948).
(399) Pauly, R., *J. Med. Bordeaux,* **131,** 587(1954).
(400) Mosca, A., *Minerva Anest.*, **23,** 98(1957).
(401) Heinecker, R., *Klin. Wochschr.*, **32,** 803(1954).
(402) Böckel, P., *Arzneimettel Wochschr.*, **10,** 802(1955).
(403) Kelly, G. H., Fay, J. E., and Laverty, S. G., *Can. Med. Assoc. J.*, **89,** 546(1963).
(404) Teitelbaum, I., *Lancet,* **I,** 115(1963).
(405) Reinert, R. E., and Hermann, C. G., *J. Nervous Mental Diseases,* **131,** 435(1960).
(406) Hollister, L. E., and Kosek, J. C., *J. Am. Med. Assoc.*, **192,** 93(1965).
(407) Greiner, A. C., and Nicolson, G. A., *Can. Med. Assoc. J.*, **91,** 627(1964).
(408) Richardson, H. L., Graupner, K. I., and Richardson, M. E., *J. Am. Med. Assoc.*, **195,** 254(1966).
(409) Sloman, L., *Can. Med. Assoc. J.*, **82,** 20(1960).
(410) Arnott, G., and Bouckson, G., *Lille Med.*, **7,** 1036 (1962).
(411) Horak, J., *Lek. Listy,* **7,** 599(1952).
(412) Lederbogen, K., and Rausch, F., *Munch. Med. Wochschr.*, **101,** 1490(1959).
(413) Schweizer, W., and von Planta, P., *Schweiz. Med. Wochschr.*, **88,** 882(1958).
(414) Walshe, W. H., "Diseases of the Heart and Great Vessels," 4th ed., London, England, 1876, cited by Wendt, V. E., Wu, C., Balcon, R., Doty, G., and Bing, R. J., *Am. J. Cardiol.*, **15,** 183(1965).
(415) Bollinger, O., *Deut. Med. Wochschr.*, **10,** 180(1884).
(416) *A. M. A. News,* August 1, 1966.
(417) Aufrecht, A., *Deut. Arch. Klin. Med.*, **54,** 615 (1895).
(418) Mackenzie, J., "The Study of the Pulse," Edinburgh, Scotland, and London, England, 1902, p. 237.
(419) Steell, G., "Textbook on Disease of the Heart," Macmillan, Manchester, England, 1902, p. 18.
(420) Weiss, S., and Wilkins, R. W., *Ann. Internal Med.*, **11,** 104(1937).
(421) Blankenhorn, M. A., Vilter, C. F., Scheinker, I. M., and Rustin, R. S., *J. Am. Med. Assoc.*, **13,** 717(1946).
(422) Eliaser, M. J., and Giansiracusa, F., in "Premier Congres Mondial de Cardiologie Communications," vol. II, J. B. Balliere et Fils, Paris, France, 1952, p. 307.
(423) Eliaser, M., Jr., and Giansiracusa, F. J., *Calif. Med.*, **84,** 234(1956).
(424) Evans, W., *Brit. Heart J.*, **21,** 445(1959).
(425) Benchimol, A. B., and Schlesinger, P., *Am. Heart J.*, **46,** 245(1953).
(426) Brigden, W., *Lancet,* **II,** 1179, 1243(1957).
(427) Burch, G. E., and Walsh, J. J., *Am. J. Cardiol.*, **6,** 864(1960).
(428) Evans, W., *Am. Heart J.*, **61,** 556(1961).
(429) Brigden, W., and Robinson, J., *Brit. Med. J.*, **5420,** 1283(1964).
(430) Heaton, F. W., Pyrah, L. N., Beresford, C. C., Bryson, R. W., and Martin, D. F., *Lancet,* **II,** 802(1962).
(431) Smolik, R., and Zukowski, W., *Pol. Arch. Med. Wewnet.*, **31,** 577(1961).
(432) Wendt, V. E., Wu, C., Balcon, R., Doty, G., and Bing, R. J., *Am. J., Cardiol.*, **14,** 175(1965).
(433) Ferrans, V. J., Hibbs, R. G., and Weilbaccher, P. G., *ibid.*, **13,** 106(1964).
(434) Dajani, R. M., and Orten, J. M., *J. Nutr.*, **76,** 135 (1962).
(435) Beard, J. D., and Barboriak, J. J., *Proc. Soc. Expil. Biol. Med.*, **118,** 1151(1965).
(436) Maines, J. E., and Aldinger, E. E., *Am. Heart J.*, **73,** 55(1967).
(437) Kalant, H., *Quart. J. Studies Alc., Suppl. 1,* **1963,** 1.
(438) Ariton, A. H., *Clin. Pharmacol. Therap.*, **6,** 462 (1965).

(439) Appenzeller, O., and Richardson, E. P., Jr., *Neurology*, **16**, 1205(1966).
(440) Günther, G. W., *Z. Klin. Med.*, **135**, 247(1939).
(441) Black-Schaffer, B., *Arch. Pathol.*, **39**, 301(1945).
(442) More, R. H., McMillan, G. C., and Duff, G. L., *Am. J. Pathol.*, **22**, 703(1946).
(443) Czarniecki, W., Soszka, A. E., and Sliwowski, A., *Pol. Arch. Med. Wewnet.*, **32**, 621(1962).
(444) French, A. J., and Weller, C. V., *Am. J. Pathol.*, **18**, 895(1942).
(445) Varela Nuñez, R., *Rev. Clin. Espan.*, **52**, 407(1954).
(446) Hloucal, L., *Cas. Lek. Ces.*, **90**, 887(1951).
(447) Patrna, H., *ibid.*, **91**, 368(1952).
(448) Gullberg, S., *Nord. Med.*, **48**, 1558(1952).
(449) Runstukova, Schreiber, and Sel, *Lek. Listy*, **8**, 466(1953).
(450) Kline, I. K., Kline, T. S., and Saphir, O., *Am. Heart J.*, **65**, 446(1963).
(451) Chatterjee, S. S., and Thakre, N. W., *Tubercle*, **39**, 240(1958).
(452) Field, J. B., and Federman, B. D., *Diabetes*, **6**, 67(1957).
(453) Hodge, P. R., and Lawrence, J. R., *Med. J. Australia*, **1**, 640(1957).
(454) Binder, M. J., Gunderson, H. J., Cannon, J., and Rosove, L., *Am. Heart J.*, **40**, 940(1950).
(455) Felder, S. L., and Felder, L., *J. Am. Med. Assoc.*, **143**, 361(1950).
(456) Lilienfeld, A., Hochstein, E., and Weiss, W., *Circulation*, **1**, 1060(1950).
(457) Foster, R. F., and Layman, J. D., *J. Am. Med. Assoc.*, **148**, 203(1952).
(458) Glotzer, S. L., *Am. Heart J.*, **47**, 3000(1954).
(459) Bernreiter, M., *J. Am. Med. Assoc.*, **170**, 1628(1959).
(460) Haden, R. F., and Langsjoen, P. H., *Am. J. Cardiol.*, **8**, 420(1961).
(461) Pfister, C. W., and Plice, S. G., *Am. Heart J.*, **40**, 945(1950).
(462) McManus, J. F., and Lawlor, J. J., *New Engl. J. Med.*, **242**, 17(1950).
(463) Krcilek, A., Krcilkova, M., and Zoulek, D., *Cas. Lek. Ces.*, **90**, 706(1951).
(464) Ermoléva, Z. V., *Trud. Akad. Med. Nauk. SSSR*, **22**, (No. 1), 107(1952).
(465) Waugh, D., *Am. J. Pathol.*, **28**, 437(1952).
(466) Zolotukhin, S. I., *Farmakol. Toksikol.*, **20**, (No. 6), 80(1957).
(467) Criep, L. H., *Arch. Internal Med.*, **48**, 19, 1098(1931).
(468) Thaddea, S., *Deut. Med. Wochschr.*, **62**, 1117, 1171 (1936).
(469) Dieckhoff, J., and Laurentius, P., *Z. Exptl. Med.*, **99**, 473(1936).
(470) Gukelberger, M., *ibid.*, **100**, 294(1936–1937).
(471) Dieckhoff, J., and Schulze, E., *Arch. Exptl. Pathol. Pharmakol.*, **185**, 418(1937).
(472) Schwab, R., *Z. Exptl. Med.*, **103**, 1(1938).
(473) Dennis, E. W., *Proc. Soc. Exptl. Biol. Med.*, **42**, 553(1939).
(474) McManus, J. F., and Lawlor, J. J., *New Engl. J. Med.*, **242**, 17(1950).
(475) Casterline, R. L., *Ann. Internal Med.*, **48**, 1121 (1958).
(476) Lyon, E., *Cardiologia*, **12**, 29(1947).
(477) Mant, A. K., *Ann. Med. Leg. (Paris)*, **43**, 49(1963).
(478) Caldera, R., Sarrut, S., Mallet, R., and Rossier, A., *Ann. Pediat.*, **37**, 217(1961).
(479) Selye, H., "The Chemical Prevention of Cardiac Necroses," The Ronald Press Co., New York, N. Y., 1958, pp. 66–77.
(480) Salgado, E., and Selye, H., *J. Lab. Clin. Med.*, **45**, 237(1955).
(481) Selye, H., Stone, H., Nielson, K., and LeBlond, C. P., *Can. Med. Assoc. J.*, **52**, 571(1945).
(482) Salgado, E., and Selye, H., *Endocrinology*, **55**, 550 (1954).
(483) Schmid, J., *Wien. Z. Inn. Med.*, **30**, 137(1949).
(484) Smirnov, V. A., *Zh. Nevropat. Psikhiat.*, **57**, 1001 (1957).
(485) Deutsch, E., *Wien. Med. Wochschr.*, **109**, 611(1959).
(486) Kermin, A. J., *Can. Med. Assoc. J.*, **90**, 33(1964).
(487) Dessau, F. I., Klein, S., Lipchuck, L., O'Grady, J., and Tsalikis, J., *J. Pharmacol. Exptl. Therap.*, **124**, 182 (1958).
(488) Wilhelm, D. L., *Pharmacol. Rev.*, **14**, 251(1962).
(489) Spectro, W. G., and Willoughby, D. A., *Bacteriol. Rev.*, **27**, 117(1963).
(490) Franco-Browder, S., Gorodezky, M., Acenes, S., and Guerrero, M., *Am. Heart J.*, **60**, 545(1960).
(491) Meesen, J., *Med. Klin.*, **33**, 555(1937).
(492) Meesen, J., *Munch. Med. Wochschr.*, **84**, 954(1937).
(493) Hueper, W. C., and Ichniowski, C. T., *Am. J. Pathol.*, **20**, 211(1944).
(494) Franco-Browder, S., Guerrero, M., Gorodezky, M., Bravo, L. M., and Acenes, S., *Arch. Inst. Cardiol. Mex.*, **30**, 720(1960).
(495) Craver, B. N., *Ann. N. Y. Acad. Sci.*, **50**, 1029(1950)
(496) Tannenberg, J., *Am. J. Pathol.*, **15**, 25(1939).
(497) Meesen, H., *Arch. Kreislaufforsch.*, **6**, 361(1940).
(498) Negri, A., *Z. Exptl. Med.*, **111**, 69(1942).
(499) Hadorn, W., *Z. Klin. Med.*, **130**, 643(1936).
(500) Hadorn, W., *Arch. Kreislaufforsch.*, **2**, 1(1937).
(501) Raab, W., "Hormonal and Neurogenic Cardiovascular Disorders," The Williams and Wilkins Co., Baltimore, Md., 1953, p. 50.
(502) Schönbrunner, E., *Med. Klin.*, **31**, 1571(1935).
(503) Hammerschlag, E., *Wien. Klin. Wochschr.*, **50**, 1692(1937).
(504) Hegglin, R., *Deut. Med. Wochschr.*, **65**, 16(1939).
(505) Hegglin, R., *Helv. Med. Acta*, **6**, 89(1939–1940).
(506) Sendrail, M., *J. Med. Bordeaux*, **128**, 136(1951).
(507) Gandevia, B., *Med. J. Australia*, **1**, 33(1954).
(508) Sachsse, B., *Arzneimettel-Wochschr.*, **12**, 312(1957).
(509) Luginbühl, R., *Z. Kreislaufforsch.*, **40**, 174(1951).
(510) Lossius, O. P., *Nord. Med.*, **53**, 58(1955).
(511) Albano, T., and de Matteis, F., *Folia Cardiol. Suppl.*, **12**, 242(1954).
(512) Struve, G., *Deut. Med. Wochschr.*, **72**, 712(1947).
(513) Hartl, F., *ibid.*, **74**, 1306(1949).
(514) Gross, H., Stein, P., and Buchberg, A. S., *Current Res. Anesth.*, **30**, 250(1951).
(515) Kielhorn, E., and Rubenhagen, H., *Deut. Med. Wochschr.*, **64**, 472(1938).
(516) Jelke, H., *Acta Med. Scand.*, **132**, 339(1948).
(517) Mulligan, R. M., *Am. J. Pathol.*, **22**, 1293(1946).
(518) Hahn, F., *Arch. Exptl. Pathol. Pharmakol.*, **1938**, 190.
(519) Nichele, G., Seganti, A., and Contadini, F., *Folia Cardiol. Suppl.*, **10**, 355(1952).
(520) Selye, H., and Renaud, S., *Presse Med.*, **66**, 99(1958).
(521) Wilgram, G. F., *J. Exptl. Med.*, **109**, 293(1959).
(522) Selye, H., Bajusz, E., and Renaud, S., *Z. Kreislaufforsch.*, **48**, 237(1959).
(523) Selye, H., and Bajusz, E., *Am. J. Cardiol.*, **4**, 102 (1959).
(524) Ferris, T. F., Levitin, H., Phillips, E. T., and Epstein, F. H., *J. Clin. Invest.*, **41**, 1222(1962).

Drug Transport Through Model Membranes and Its Correlation with Solubility Parameters

By SAID A. KHALIL* and ALFRED N. MARTIN†

The kinetics of drug transport through lipoidal barriers was studied using a series of nonpolar liquids as models of living membranes. The nonpolar liquids were selected to represent a range of different solubility parameters (Hildebrand δ values), which varied between 7.1 and 10.7. Carboxyl salicylic acid-^{14}C was used as the drug molecule, the solubility parameter of which was found to have a δ value of approximately 10.8. The rate of drug transfer was studied from layer *a*, an aqueous buffer solution of pH 2, representing the stomach, through layer *b*, a liquid lipoidal barrier representing the gastric mucosal barrier, to layer *c*, an aqueous solution buffered at pH 7.4, representing the blood serum. The rate constants for the drug transport were obtained using an analog computer. The distribution of the drug between the three layers can be represented by a mathematical expression from which drug concentrations in the layers at various times can be calculated. It was found that the closer the δ value of the drug molecule to that of the lipoidal barrier, the faster was the disappearance of the drug from layer *a* and the slower its appearance in layer *c*. The results provided an approach for estimating the desired chemical characteristics of drug molecules required for optimum membrane penetration.

THE PASSAGE of drug molecules across living membranes has been the subject of many studies. Mullins (2), in his review of the physical mechanisms involved in narcosis, postulated that the membrane acceptance of a narcotic molecule depends upon its having a cohesive energy density (Hildebrand solubility parameter) appropriate to that of the membrane, and that the membrane acceptance is related to the ideal partition coefficient of the narcotic drug. Brodie and his associates (3–10) reported that the absorption of drug molecules from the gastrointestinal tract depends on their ability to penetrate a lipoidal barrier, and that the transport of most drugs is accomplished by the passive diffusion of their unionized species. The importance of the physicochemical characteristics of drug molecules, their solubility as well as partition coefficients between aqueous media and lipoidal barriers, has received much attention since the start of this century (2, 9–20).

Hansch and his co-workers (21), employed a mathematical treatment to correlate the molecular structure of a series of chemically related compounds with biological activity. They considered the partition coefficient to be an important factor in the equation. Recently, Swintosky and his associates (22–24) reviewed the importance of partition coefficients in biological and pharmaceutical systems. They studied drug partitioning between aqueous solutions of different pH values representing the gastrointestinal contents, a nonpolar solvent representing the lipoidal barrier, and a buffer solution of pH 7.4, corresponding to the blood serum. They used a rocking Y-tube apparatus for their studies. The various possibilities of drug transfer between the three compartments were also reported. Schulman (25) had previously suggested a cell of different design for such studies, and Perrin (26) recently reported on the application of this apparatus for the study of drug absorption.

Factors such as pKa, pH (3–6), binding to plasma proteins (6, 27–32), and the existence of active transport mechanisms (33) have been investigated. No attempts, however, have reportedly been made to evaluate the characteristics of the different lipoidal barriers and to correlate the solubility properties of various drug molecules with the characteristics of lipoidal membranes. Since the early work of Meyer and Overton (11), the partition coefficient of drug molecules between water and immiscible nonpolar liquids has been determined. Yet, the selection of the specific nonpolar liquid in

Received April 14, 1967, from the School of Pharmacy, Purdue University, Lafayette, IN 47907, and the † Laboratory of Physical Medicinal Chemistry, School of Pharmacy, Medical College of Virginia, Richmond, VA 23219
Accepted for publication June 21, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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such studies has not usually been made on a rational basis.

INTRODUCTION

In the present investigation, the Hildebrand solubility theory (1) is used to describe in a quantitative way a series of nonpolar liquids representing models of biological membranes. The values obtained can then be employed to correlate the structures and solubilities of drug molecules with ability to pass through different membranes. Work along similar lines has been reported by Mullins (2, 34) in his studies on narcosis and olfaction. Dikstein (35, 36) also used the solubility parameter as an index of drug action. It is proposed that Mullins' approach might serve as the primary step toward characterizing biological membranes relative to drug penetrability.

In vitro experiments were carried out to correlate the Hildebrand solubility parameters (δ values) of selected lipoidal barriers with the δ values of drug molecules passing through the barriers. This *in vitro* method was used to simulate *in vivo* transport of drugs through biological membranes. It was accomplished by the use of an apparatus essentially the same as that described by Doluisio and Swintosky (23), which consisted of a slow rocking bar to which a set of inverted Y-shaped tubes were fixed. The two arms of each Y-tube contained aqueous layers, and the two aqueous layers were separated by a nonpolar liquid that was immiscible with them. The liquid interface expanded and contracted with the rocking motion of the tubes, simulating movement of the gastrointestinal tract. A series of nonpolar liquids, having δ values which varied between 7.1 and 10.7, were employed. Carboxyl salicylic acid-^{14}C was used to represent the drug molecule, the δ value of which was determined using previously established techniques (37, 38). The rate of drug transfer, as well as equilibrium drug distribution in the different lipoidal barriers, was determined by measuring the radioactivity of the drug in samples of each of the three layers at various time intervals, using a liquid scintillation counter. Plots of the radioactivity *versus* time were made. The equilibrium concentrations in the three phases were read from the graphs, and the rate constants for the transfer of salicylic acid through the various lipoidal barriers were determined from the graphs using an analog computer.

The present kinetic method, developed by Doluisio and Swintosky (23) for determining the partitioning of drugs between various phases, is a more informative technique than usual partition coefficient measurements. Rate constants of both forward and reverse transfer, as well as partition coefficients, can be obtained by this method. Furthermore, a system consisting of two aqueous phases of definite pH and an intervening lipid phase more nearly simulates absorption conditions in some biological processes. Since absorption and drug action depend upon penetration of lipoidal barriers, this *in vitro* model appears to be suitable for studies on transport and action of drugs and has been adopted for the present study.

This report describes the rate of drug transfer through different lipoidal barriers having variable solubility parameters (δ values). An attempt was made to correlate the rate of drug transfer with the Hildebrand δ values of both the drug and the liquid lipoidal barrier, through which it passed. From the preliminary *in vitro* results, as well as those under investigation, it is hoped to correlate *in vitro* results with *in vivo* findings reported in the literature, using a specific drug molecule. By such a means, it should be possible to specify roughly the region of drug penetration and rate of transport. The site of absorption of the gastrointestinal tract and other biological barriers could then be assigned δ values, or similar parameters, which measure the polarity of the membranes.

The correlation of solubility properties of nonaqueous systems with a single parameter such as the δ value or the dielectric constant has been criticized, because it assumes the solvent phase to be a continuum; more emphasis should be placed on such factors as the solute–solvent–cosolvent interactions (39). Furthermore, biological membranes are complex structures, and it is not likely that a single parameter, adapted to express the polar nature of solvents, could be highly successful in characterizing a living membrane. The Hildebrand δ value is used here because it has a precise meaning and can be expressed in a quantitative manner. At a later point in this program, it is planned to introduce modified parameters or combinations of parameters to express in a more satisfactory way the polar character of complex living membranes.

THEORY AND CALCULATION

The system tested may be represented by the model:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \qquad \text{(Eq. 1)}$$

The kinetics of this system is expressed by the following equations:

$$V_a \frac{dA}{dt} = -V_a k_1 A + V_b k_{-1} B \qquad \text{(Eq. 2)}$$

$$V_b \frac{dB}{dt} = V_a k_1 A - V_b k_{-1} B - V_b k_2 B + V_c k_{-2} C \qquad \text{(Eq. 3)}$$

$$V_c \frac{dC}{dt} = V_b k_2 B - V_c k_{-2} C \qquad \text{(Eq. 4)}$$

Where V_a, V_b, V_c, are the volumes and A, B, C, are the concentrations of the drug at any instant in the three layers a, b, and c, respectively. The symbols k_1 and k_2 represent the apparent rate constants for forward drug transfer from a to b and from b to c; and k_{-1} and k_{-2} represent the apparent rate constants of the reverse drug transfer from b to a, and from c to b, respectively. A_0 is the initial concentration present in layer a at time t = 0, and:

$$A_0 = A + B + C \qquad \text{(Eq. 5)}$$

The volumes V_a, V_b, and V_c were in the ratios of 1:2:1. (See under *Experimental.*) In order to keep this ratio constant throughout the experiment, the sample volume withdrawn from layer b was double the volumes withdrawn from layers a

and c. The volume terms may therefore be neglected and Eqs. 2, 3, and 4 reduce to Eqs. 6, 7, and 8:

$$\frac{dA}{dt} = -k_1A + k_{-1}B \quad \text{(Eq. 6)}$$

$$\frac{dB}{dt} = k_1A - k_{-1}B - k_2B + k_{-2}C \quad \text{(Eq. 7)}$$

$$\frac{dC}{dt} = k_2B - k_{-2}C \quad \text{(Eq. 8)}$$

To calculate the true transfer rate constants, pH-partition effects must be taken into consideration by assuming that only the unionized portion of the drug molecule passes through the lipoidal membrane. The true rate constant k_1' is related to the apparent rate constant reported in this study, k_1, for the forward step from a to b of Eq. 1 by:

$$k_1' = \frac{k_1}{(1 - \alpha)} \quad \text{(Eq. 9)}$$

where α is the degree of ionization $[I/(U + I)]$ of the drug at the specified pH. The true rate constant may be defined (23) as:

$$k_1' = \frac{(U + I)}{U} k_1 \quad \text{(Eq. 10)}$$

where U and I are the unionized and ionized species of the drug molecule, respectively. In the present study, only the apparent rate constant is considered.

EXPERIMENTAL

Reagents—The solvents and their sources are given as follows: *n*-pentane, technical grade (b.p. 36.1°), *n*-hexane (b.p. 68.5–69°), and *n*-hexyl alcohol (b.p. 157–158°) from Eastman Organic Chemicals; *n*-octyl alcohol from Matheson Coleman & Bell (b.p. 193.5–195.1°); benzene from Baker & Adamson (b.p. 79.8–80.2°); toluene, Baker analyzed reagent (b.p. 110.1–110.7°); cyclohexane, Phillips (b.p. 80.5–82.0°). The reagents were redistilled, and the first and the last portions of the distillate were rejected. Distillation was continued until gas chromatography indicated that they were pure.

Karl Fischer reagent was obtained from Mallinckrodt Chemical Works; naphthalene, *p*-dioxane, and 2-ethoxyethanol were obtained from Eastman Organic Chemicals; 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) were products of the Packard Instrument Co.

Carboxyl salicylic acid-^{14}C (17.5 μc./mg.) was synthesized in the laboratories of the Bionucleonics Department, Purdue University, Lafayette, Ind.[1] Its chemical purity was established by thin-layer chromatography. A sample of salicylic acid, Fischer certified reagent grade, which has been recrystallized from chloroform, was used as a reference standard. The radio-purity of carboxyl salicylic acid-^{14}C was determined by autoradiography.

Potassium chloride, monobasic sodium phosphate, sodium hydroxide, and hydrochloric acid were all of reagent grade.

Apparatus—The rocking device and Y-shaped tubes described by Doluisio and Swintosky (23) were used for the model membrane penetration experiment; a Beckman model 72 pH meter; Autoprep, model A 700 Aerograph; Wilkens-Anderson Lo-Temp constant-temperature bath; Packard Tri-Carb liquid scintillation spectrometer model 3003; and A. D. analog computer model 2-24 PB (Applied Dynamics, Inc.) were also employed in the study.

Procedure—Experiments were first conducted to determine the solubility of water (from the buffer solutions) in the nonpolar liquid phase. The drug, salicylic acid, was not added to the acid solution in these preliminary experiments. Inverted Y-shaped Pyrex glass cells were filled with the appropriate liquid phases. Into one arm was introduced 40.0 ml. of a KCl–HCl buffer solution of pH 2, and into the other arm 40.0 ml. of a phosphate buffer of pH 7.4. Then 80.0 ml. of a nonpolar liquid was carefully introduced into the cell over the two aqueous layers. A series of nonpolar liquids having different Hildebrand δ values (1) were used. These included *n*-pentane (δ = 7.1), *n*-hexane (δ = 7.3), cyclohexane (δ = 8.2), toluene (δ = 8.9), benzene (δ = 9.2), *n*-octyl alcohol (δ = 10.3), and *n*-hexyl alcohol (δ = 10.7). The rocking apparatus with its tubes was submerged in a thermostatically controlled water bath at 25 ± 0.1°. The apparatus was slowly rocked at a rate of one stroke per minute for a period of 24 hr. Then samples from the nonpolar liquids were taken and left in a separator for 24 hr. to allow any suspended water droplets to separate. The supernatant was analyzed for its water content using the Karl Fischer method.

The experiments were repeated substituting an aqueous pH 2 buffer solution of carboxyl salicylic acid-^{14}C (33.25×10^{-5} moles/L.) for the pH 2 buffer solution alone. The nonpolar liquids, *n*-octyl alcohol and *n*-hexyl alcohol, were presaturated with the aqueous buffer layers for 24 hr. as described above. The other solvents did not dissolve water sufficiently to require presaturation. On introducing the nonpolar liquid over the aqueous layers in each tube, care was taken to minimize any turbulence or mixing. The apparatus was rocked as described above, and samples were withdrawn from each of the three layers in each Y-tube at various intervals. Each sample was injected into a liquid scintillation spectrometer vial containing 15.0 ml. of liquid scintillator. The liquid scintillator consisted of 8.0% w/v naphthalene, 1.0% w/v of 2,5-diphenyloxazole (PPO), and 0.05% w/v of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP), in a mixture of toluene, *p*-dioxane, and 2-ethoxyethanol in the ratio of 1:3:3 parts by volume.

A blank for each experiment was carried out at the time, using the same procedure, but without the radioactive salicylic acid. The activity obtained from each sample of the blank experiment was considered as the background activity for its respective sample, and was subtracted from its activity when recording the results. The samples

[1] The authors wish to acknowledge the assistance of Dr. Wayne Kessler and Dr. A. Noujaim, Department of Bionucleonics, Purdue University, for their assistance in the radioactive analysis conducted in this study. Dr. A. M. Knevel, Department of Medicinal Chemistry, assisted in determining the identity and purity of the solvents.

TABLE I—APPARENT RATE CONSTANTS FOR THE TRANSFER OF SALICYLIC ACID THROUGH THE DIFFERENT LIPOIDAL BARRIERS

Nonpolar Liquid Used as Barrier	δ Value of Liquid Barrier	Adjusted δ Value of Barrier	∇[a]	Apparent Rate Constants (as Obtained from Analog Computer) × 10^4 sec.$^{-1}$ k_1	k_{-1}	k_2	k_{-2}
n-Pentane	7.1	7.1	3.7	0.024	0.003	0.650	0.003
n-Hexane	7.3	7.4	3.4	0.032	0.028	0.570	0.002
Cyclohexane	8.2	8.3	2.5	0.018	0.107	0.250	0.002
Toluene	8.9	8.9	1.9	0.126	0.008	0.335	0.000
Benzene	9.2	9.2	1.6	0.107	0.019	0.330	0.000
n-Octyl alcohol	10.3	10.8	0.0	0.230	0.003	0.019	0.000
n-Hexyl alcohol	10.7	11.4	0.6	0.452	0.005	0.042	0.008

[a] $\nabla = |\delta_D - \delta_B|$, where δ_D is the δ value of salicylic acid taken as 10.8, and δ_B is the δ value of the liquid lipoidal barrier.

were counted in a model 3003 Tri-Carb liquid scintillation spectrometer. Five 1-min. counts were taken and their average recorded.

An internal standard, carboxyl benzoic acid-^{14}C standard solution, was added to each sample and the sample was recounted so that the efficiency of the counting instrument, as well as the existence of any quenching effect due to the different samples in the liquid scintillator, could be determined.

The solubility parameter of the salicylic acid was determined using already established techniques (37, 38).

RESULTS AND DISCUSSION

From the Karl Fischer titration results, it was obvious that under the condition of the experiment the nonpolar layer became saturated with the aqueous layers, which resulted in a change in the δ values in some cases. From the estimation of water content, the δ values of the resulting nonpolar layers were calculated as described by Chertkoff and Martin (37). The results are summarized in Table I.

The water saturation effect was quite apparent in the case of *n*-octyl and *n*-hexyl alcohol resulting in an increase in their δ values of 0.5 and 0.7 unit, respectively. Because of this fact, it was important to presaturate these two liquids before the rate of drug transfer through them was determined. Presaturation was not required for the other liquids, as their water saturation capacity was very small, and their δ values were not changed significantly by the addition of water.

The solubility of the nonpolar phase in both aqueous layers no doubt affects the rate constant to a small extent. Only apparent rate constants are reported. It is more probable that this effect does not significantly alter the conclusion drawn from a consideration of the δ value of the lipoidal phase in relation to drug transport, which is the main concern.

The δ value of salicylic acid was found to be approximately 10.8. This value agrees with that given by Paruta *et al.* (40). They reported a dielectric constant requirement for salicylic acid of 15, possibly another of 25. The equation relating the dielectric constant ε and the delta value δ in pharmaceutical solvents, *i.e.*, alcohols, glycols, and water, reported also by Paruta *et al.* (41) is:

$$\delta = 0.22\,\epsilon + 7.5$$

The δ value for salicylic acid can thus be calculated:

$$\delta = 0.22 \times 15 + 7.5 = 10.8$$

Typical results obtained by determining the concentration of radioactive salicylic acid in the three different layers at various time intervals are shown in Figs. 1–4, where drug concentrations in each of the three layers as measured by radioactivity in c.p.m. *versus* time were plotted.

A consecutive first-order rate of transfer was expected from the results obtained. Curves were drawn to fit the experimental points using an *x–y* recorder attached to an analog computer. The analog computer was programmed to represent a reversible consecutive first-order transfer rate, as shown in Fig. 5. This model was quite suitable for the cases studied as shown by the fit of the computed curves to the experimental points. Apparent rate constants were obtained from the computer potentiometer settings for the best fitting curves, as shown in Table I.[2] From Table I, it is observed that a relation exists between the apparent rate of transfer of salicylic acid from a buffer solution of pH 2, layer *a* to the liquid lipoidal barrier, layer *b*. The results suggest that the closer the δ value of the liquid lipoidal barrier to the δ value of the drug molecule, the greater the rate of drug transfer or disappearance from layer *a*. This fact could also be shown if the logarithm of the concentration of salicylic acid in layer *a* was plotted *versus* time for all cases studied. Such curves for the different nonpolar liquids are found in Fig. 6. Straight lines (in most cases) with negative slopes were obtained. It was possible to relate the slopes of the linear portions of the lines to the δ values of the liquid lipoidal barriers used. The slopes increased as the δ values of the liquid lipoidal barriers increased.

Some of the results did not coincide exactly with those expected from the hypothesis connecting the slope or the apparent rate of transfer with the δ value of the nonpolar liquid layers, and these discrepancies are not easily accounted for. Cyclohexane has a δ value higher than *n*-pentane and *n*-hexane, and it should have produced a slope greater than the slopes for *n*-pentane and *n*-hexane. Yet it has the smallest slope of all tested nonpolar liquids. The reverse transfer rate constant of salicylic acid from *b* to *a* is significant in this case, namely 0.107 ×

[2] The dependability of the analog computer results as well as the *x–y* recorder were checked with an IBM 7094 digital computer. The rate constants calculated by use of the analog computer were fed to a digital computer to give the theoretically expected curves. The curves obtained by use of the digital computer fitted those produced on the *x–y* recorder by the analog computer. The digital program is not reported here, but may be obtained by contacting one of the authors.

10^{-3} sec.$^{-1}$. This finding has been reported previously in the literature (24). Similarly the rate of transfer of salicylic acid through benzene should be higher than through toluene, but the reverse was found. Again the reverse specific rate of transfer from *b* to *a* for benzene is higher than for toluene,

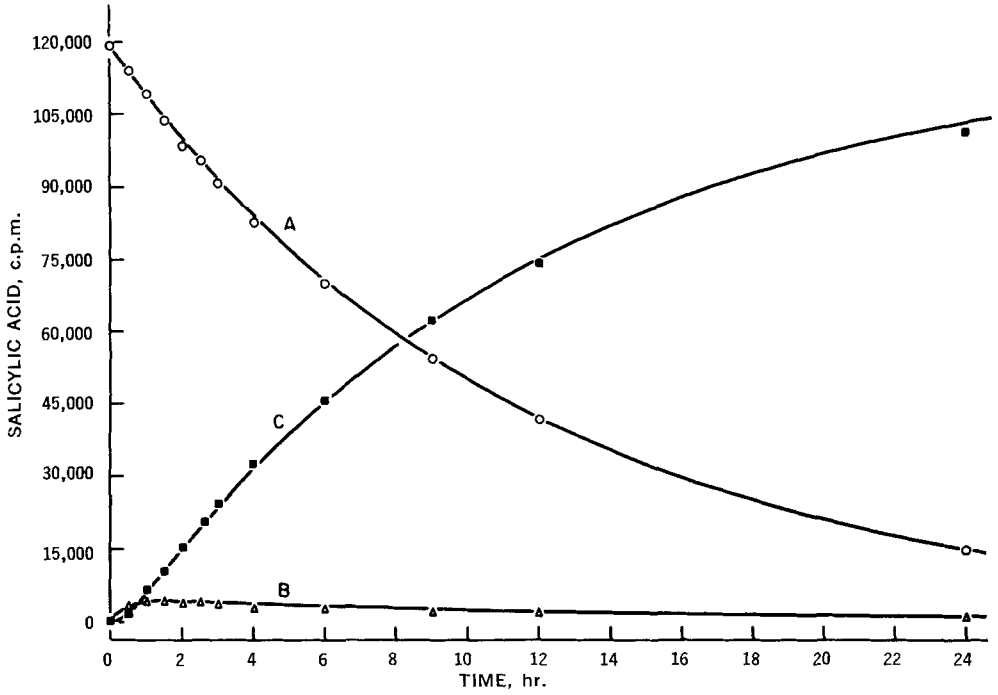

Fig. 1—The transfer of salicylic acid from an aqueous pH 2.0 buffer (layer a*) through* n*-pentane barrier (layer* b*) to an aqueous pH 7.4 buffer (layer* c*). The points are experimental and the lines are theoretical. Key:* O, *pH 2.0 layer* a; Δ, n*-pentane layer* b; ■, *pH 7.4 layer* c.

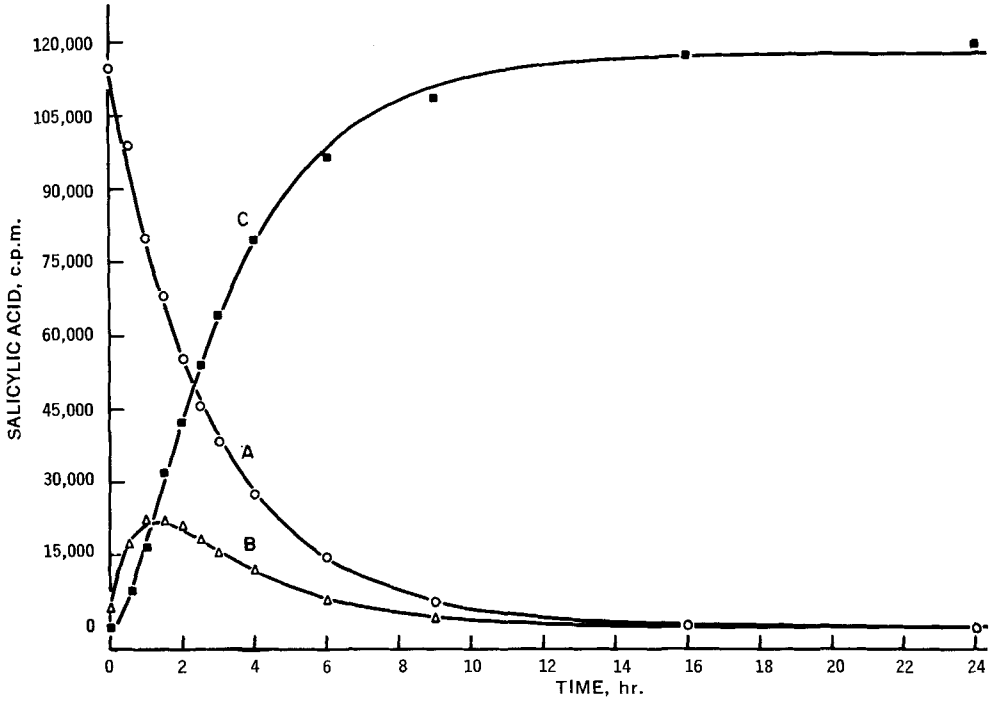

Fig. 2—The transfer of salicylic acid from an aqueous pH 2.0 buffer (layer a*) through a benzene barrier (layer* b*) to an aqueous pH 7.4 buffer (layer* c*). The points are experimental and the lines are theoretical. Key:* O, *pH 2.0 layer* a; Δ, *benzene layer* b; ■, *pH 7.4 layer* c.

as shown in Table I. All other cases followed the expected correlation between the rate of drug transfer and the δ value of the lipoidal barrier through which the drug is transferred. It should be noted from Fig. 6 that the rate of transfer of salicylic acid through the rather polar liquids,

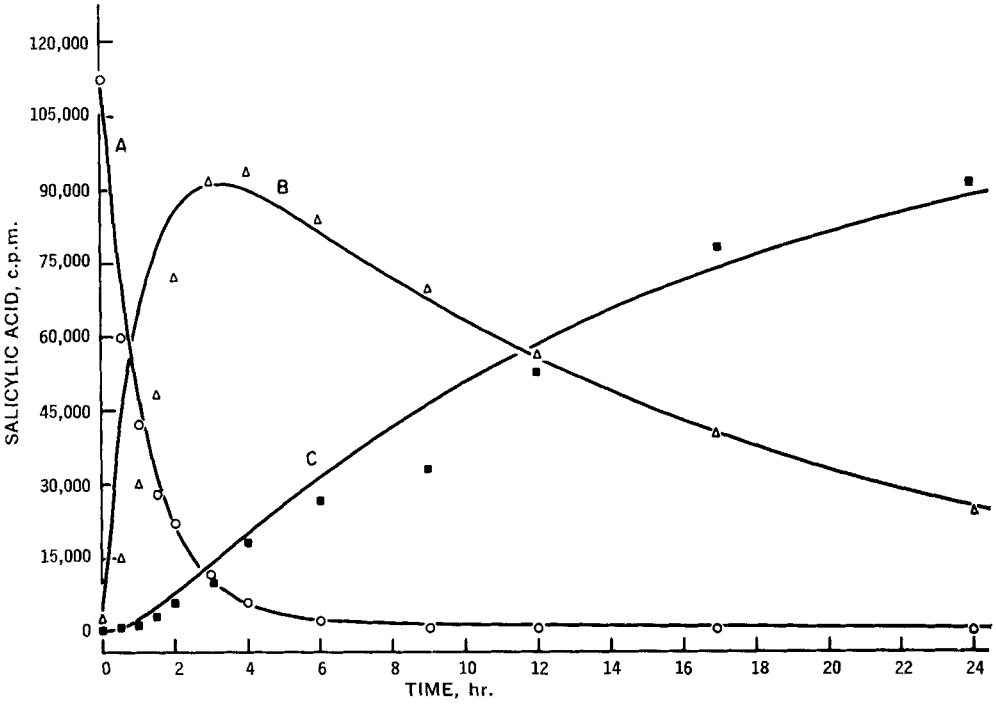

Fig. 3—The transfer of salicylic acid from an aqueous pH 2.0 buffer (layer a) *through a* n-*octyl alcohol barrier (layer* b) *to an aqueous pH 7.4 buffer (layer* c). *The points are experimental and the lines are theoretical. Key:* O, *pH 2.0 layer* a; Δ, n-*octyl alcohol layer* b; ■, *pH 7.4 layer* c.

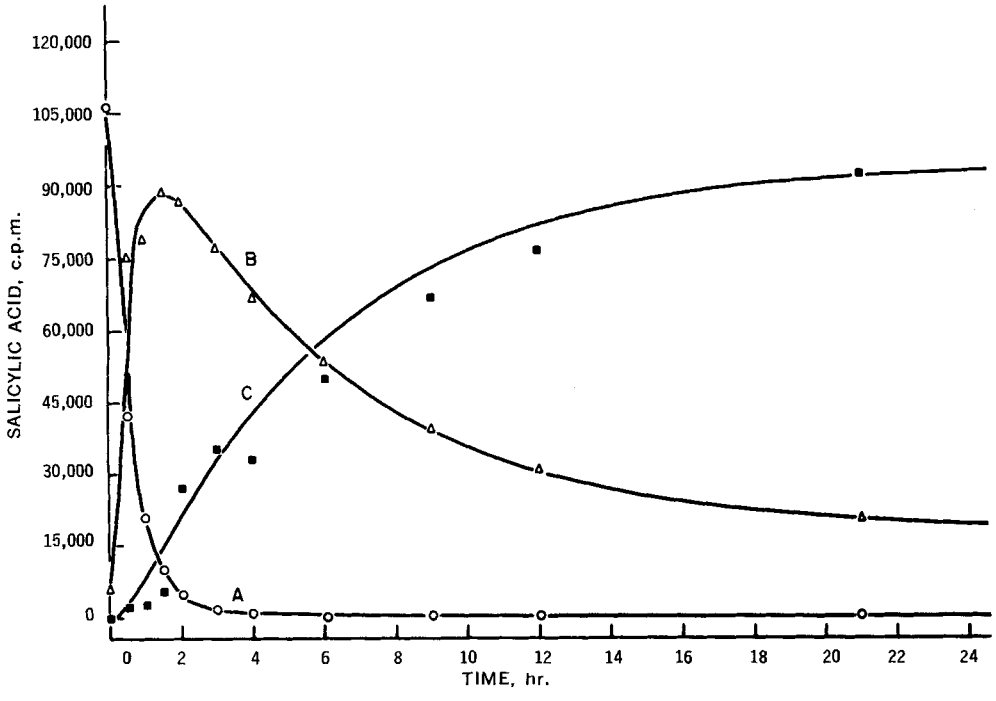

Fig. 4—The transfer of salicylic acid from an aqueous pH 2.0 buffer (layer a) *through a* n-*hexyl alcohol barrier (layer* b) *to an aqueous pH 7.4 buffer (layer* c). *The points are experimental and the lines are theoretical. Key:* O, *pH 2.0 layer* a; Δ, n-*hexyl alcohol layer* b; ■, *pH 7.4 layer* c.

n-octyl alcohol and *n*-hexyl alcohol, followed a first-order transfer in the first 3 to 4 hr., then began to disappear from *a* at a reduced rate, as can be seen by their curvilinear plots. This is probably due to the rapid onset of equilibrium between layers *a* and *b*. Such equilibrium requires longer periods of time to be reached in the relatively nonpolar barriers used.

In the case of *n*-octyl alcohol (Fig. 3) the analog computer curves representing layer *a* and *c* fitted the experimental data quite well, while in layer *b* the sample concentrations withdrawn in the early hours of the experiment were less than the expected theoretical concentrations. This is explained by the high rate of transfer from *a* to *b* with insufficient time for equilibration in layer *b*, and the concentration of the salicylic acid in the interface during the time that samples were taken from the bulk of the nonpolar layer. The same reasoning could explain the relatively lower concentration of salicylic acid in layer *c* as compared with the expected value when hexyl alcohol was used as the lipoidal barrier, as seen in Fig. 4. Doluisio and Swintosky (24) suggested that deviations in solvents, such as octyl alcohol, are possibly due to hydrogen bonding.

The second observation which can be made from Table I is that the apparent rate constant, k_2, of transfer of salicylic acid from the liquid lipoidal barrier, layer *b*, to layer *c*, decreased with the increase in the δ value of the liquid lipoidal barrier. The reverse rates of transfer k_{-1} and k_{-2} are considered to be small in most cases and their effects are negligible. Consequently, a plot of the forward rate constants, k_1 and k_2, can be made *versus* the δ value of the liquid lipoidal barrier, as shown in Fig. 7. Although the points do not lie well along the lines, it is observed that k_1 increases and k_2 declines with an increase in the δ values of the organic solvents. Theoretically, k_1 should reach a maximum and then fall off, whereas k_2 should reach a minimum and then rise. The expected maximum and minimum values should be reached at a δ value of about 10.8, the δ value of salicylic acid. Such a maximum or minimum could not be obtained experimentally in this work due to the failure to find a nonpolar liquid that was immiscible with water and also had a δ value higher than 10.7.

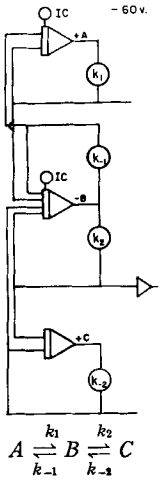

Fig. 5—Analog computer program for determining the rate constants for the system A ⇌ B ⇌ C *by obtaining the best fitting line to the experimental points.*

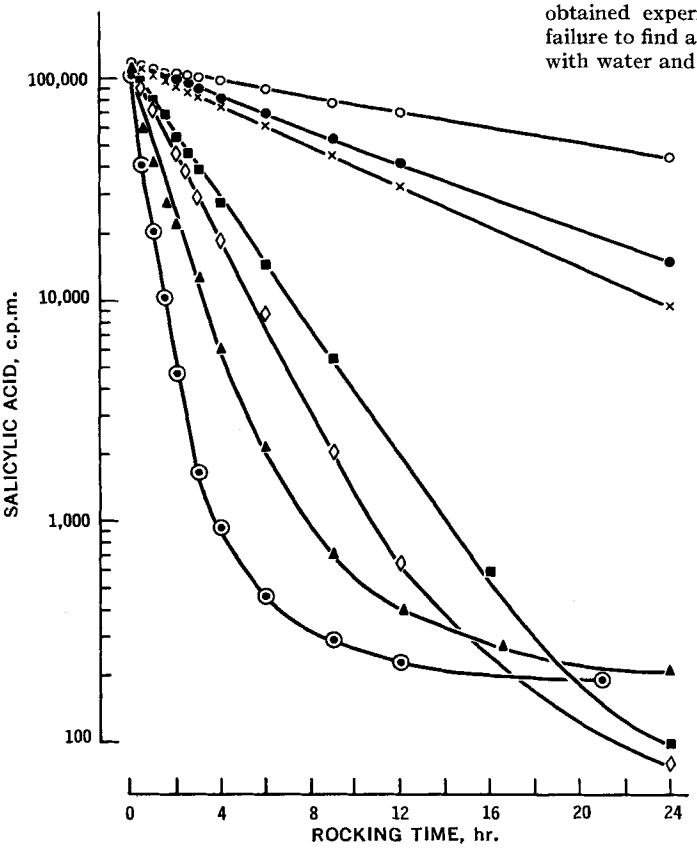

Fig. 6—A semilogarithmic plot showing the rate of salicylic acid disappearance from layer a *to different liquid lipoidal barriers (layer* b*). Key:* ○, *cyclohexane;* ●, n-*pentane;* ×, n-*hexane;* ■, *benzene;* ◇, *toluene;* ▲, n-*octyl alcohol;* ⊙, n-*hexyl alcohol.*

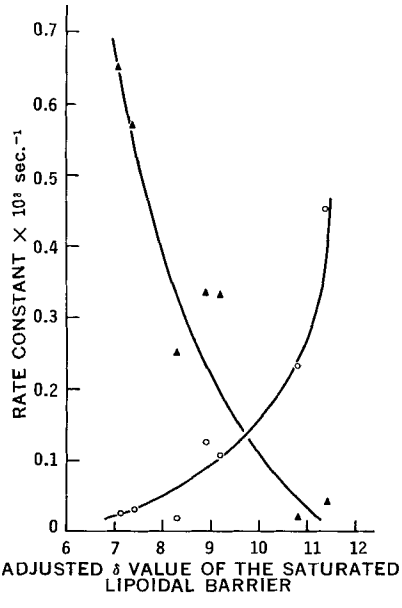

Fig. 7—Apparent rate constants for the transfer of salicylic acid from layer a *to layer* b, k_1 *and from layer* b *to layer* c, k_2, vs. *the adjusted* δ *values of the liquid lipoidal barrier layer* b. *Key:* ○, *rate of transfer from* a *to* b, k_1; ▲, *rate of transfer from* b *to* c, k_2.

If liquids could be found to provide a broader spectrum of δ values, and at the same time immiscible with the buffer solution, a maximum might be reached in the rate of drug transfer from *a* to *b* and a minimum from *b* to *c*. These points should then correspond to the δ value of the drug used.

In general, although only one compound, salicylic acid, was used as a solute in this work, the results suggested that the apparent rate constant, k_1, of drug disappearance from layer *a* to the liquid lipoidal barrier *b*, would increase with a decrease of the difference, ∇, in the δ values between the drug and the lipoidal barrier:

$$\nabla = |\delta_D - \delta_B|$$

where δ_D is the δ value of the drug molecule and δ_B is the δ value of the lipoidal barrier. The apparent rate constant, k_2, of drug release from the lipoidal layer to the aqueous layer *c* should decrease with the decrease in ∇.

The trend of ∇ values with k_1 and k_2 is observed in Table I. Thus, it might be said that as the δ value of the drug molecule approaches that of the lipoidal barrier, the rate of transfer of the drug to the lipoidal barrier increases and the rate of release of the drug from the lipoidal barrier decreases. The lipoidal barrier would tend to act as a storage compartment for the drug molecules with a prolonged release time. An optimum rate of passage of the drug from one aqueous compartment to another through a lipoidal barrier should occur when the δ value of the drug was somewhat different from that of the barrier. As seen in Fig. 7, the curves for k_1 and k_2 intersect at δ = 9.7. This should be the optimum δ value of the simulated membrane for the most rapid penetration of a drug with a δ value of 10.8. The principle might also be applied to the sustained release of drugs from tissue depots. Here one would desire a δ value of the drug relative to that of the tissue such that the drug penetrates rapidly into the tissue yet is released at a slow rate over a prolonged period of time.

The kinetics of the transfer of salicylic acid through the different lipoidal barriers studied can be an *in vitro* model for some of the theoretically possible kinetics cases suggested by Doluisio and Swintosky (24). From the values of the apparent rate constants found in Table I, as well as the concentration of drug at various times as seen in Figs. 1 through 4, the following cases are observed.

The transfer of the drug through *n*-pentane can be considered to represent kinetics of the type $a \rightarrow c$. This is possible because the concentration of the drug in layer *b* is relatively small and does not accumulate (see Fig. 1), and the reverse transfer rates, k_{-1} and k_{-2}, are also small and can be considered negligible (see Table I). Using cyclohexane as the lipoidal barrier, the process can be simulated by kinetics of the type $a \rightleftharpoons b \rightarrow c$. This is due to the relatively large rate of reverse transfer from *b* to *a*, and the relatively fast rate of forward drug transfer from *b* to *c*. Drug transfer through *n*-octyl alcohol can represent the kinetics of the type $a \rightarrow b \rightarrow c$. The drug builds up in layer *b*, and the reverse rate constants are very small and can be considered negligible. The results of the present study are summarized in Fig. 8, where the length of the arrows represent the relative rates of forward and reverse processes.

The concept of using δ values as one of the parameters that may explain the relative rates of drug transport across biological membranes and subsequent equilibrium concentrations can be applied to some of the experimental data reported by Brodie *et al.* (6). They reported that salicylic acid and barbital exist mainly in the stomach in their unionized form; yet the absorption of salicylic acid was quite high, while that of barbital was very poor. This fact can perhaps be explained by saying that the δ value of salicylic acid (δ = 10.8) is closer to the supposed δ value of the gastric lipoidal barrier (δ = 10) than that of barbital with a δ value of about 13.5. The δ values of salicylic acid and barbital were obtained by the method of Chertkoff and Martin (37), and the results are shown in Fig. 9.

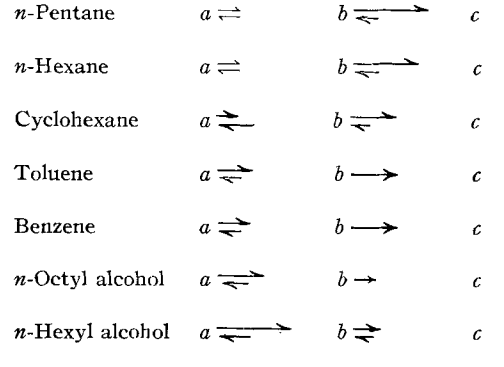

Fig. 8—Representation of the system A ⇌ B ⇌ C, *where the length of the line signifies the relative magnitudes of the rate constants.*

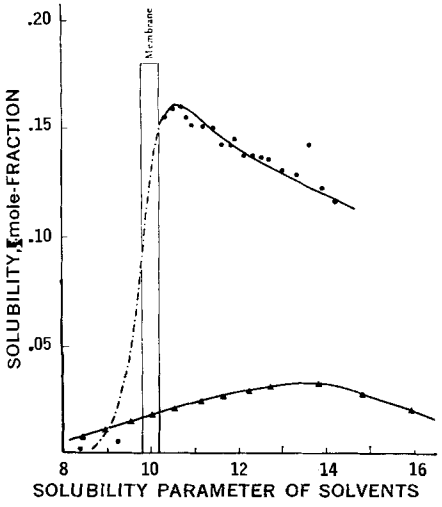

Fig. 9—Determination of the solubility parameter of salicylic acid and barbital at 25°. Key: ●, salicylic acid; ▲, barbital.

One observes from Fig. 9 that the suggested δ value for optimum penetration into the lipoidal membrane is about 10. Salicylic acid with a δ value of 10.8 being close to the δ value of the membrane will easily cross the lipoidal barrier. Barbital has a δ value of about 13.5,[8] well beyond the δ value of the membrane and would not be expected to show marked membrane penetrability.

SUMMARY AND CONCLUSIONS

The rate of transfer of carboxyl salicylic acid-^{14}C through different liquid lipoidal barriers was studied and the rate constants were determined using an analog computer. The liquid lipoidal barriers used represented a range of different solubility parameters expressed as Hildebrand δ values. A correlation between the δ value of the liquid lipoidal barrier, the δ value of the drug model, salicylic acid, and the rate of transfer of the drug through the lipoidal barrier has been presented. It is hypothesized that the smaller the difference between the δ value of a drug molecule that exhibits significant lipid solubility, and the δ value of the liquid lipoidal barrier, the faster would be the transfer across the barrier and into the lipoidal layer, and the slower the drug would be released from the lipoidal layer into the aqueous buffer phase. This suggests that a quantity ∇ could perhaps be used to express the optimum difference in δ values of the drug and membrane for transport and penetration by the drug. The results of the study seem to bear out this principle, but a number of drugs and liquids of varying δ values must be tested before the hypothesis can be accepted.

The δ value concept may also be valuable in relating partition coefficients to drug action. The proper choice of the nonpolar liquid in partition coefficient experiments may be arrived at through a knowledge of δ values, and the authors intend to present the results of a study dealing with this suggestion at a later time.

The goal is to be able to correlate *in vitro* with *in vivo* results and suggest molecular structures which can facilitate drug transfer across membranes, and drug release from tissue depots in the body. The region of absorption and drug release at the lipoidal barriers in the gastrointestinal tract and other membranes and tissues could be assigned δ values, or more acceptable polarity parameters, which would account for the complex nature of these biological structures.

[8] Barbital has no sharp maximum point of solubility in the solvents studied, and it is difficult to assign it a definite δ value. The solubility of barbital over the whole range of δ values from 7 to 16 is seen to be low and accordingly the drug is not expected to show marked solubility in the simulated membranes.

REFERENCES

(1) Hildebrand, J. H., and Scott, R. L., "The Solubility of Nonelectrolytes," 3rd ed., American Chemical Society Monograph No. 17, Reinhold Publishing Corp., New York, N. Y., 1950; Hildebrand, J. H., and Scott, R. L., "Regular Solutions," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1962.
(2) Mullins, L. J., *Chem. Rev.*, **45**, 289(1954).
(3) Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **119**, 361(1957).
(4) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **120**, 528(1957).
(5) Hogben, C. A. M., Schanker, L. S., Tocco, D. J., and Brodie, B. B., *ibid.*, **120**, 540(1957).
(6) Brodie, B. B., and Hogben, C. A. M., *J. Pharm. Pharmacol.*, **9**, 345(1957).
(7) Schanker, L. S., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **122**, 66A(1958).
(8) Schanker, L. S., Tocco, D. J., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **123**, 81(1958).
(9) Hogben, C. A. M., Tocco, D. J., Brodie, B. B., and Schanker, L. S., *ibid.*, **125**, 275(1959).
(10) Schanker, L. S., *ibid.*, **126**, 283(1959).
(11) Meyer, H., *Arch. Exptl. Pathol. Pharmacol.*, **42**, 109(1899); Overton, E., *Vierteljahrsschr. Naturforsch. Ges. Zürich*, **44**, 88(1899).
(12) Ferguson, J., *Proc. Royal Soc. (London)*, **B127**, 387 (1939).
(13) Butler, T. C., *J. Pharmacol. Exptl. Therap.*, **74**, 118(1942).
(14) Gladtke, E., and Liss, E., *Biochem. Z.*, **331**, 65(1959).
(15) Schanker, L. S., *J. Med. Pharm. Chem.*, **2**, 343(1960).
(16) McMahon, R. E., *ibid.*, **4**, 67(1961).
(17) Schanker, L. S., *Pharmacol. Rev.*, **14**, 501(1962).
(18) Rall, D. P., and Zubrod, C. G., *Ann. Rev. Pharmacol.*, **2**, 109(1962).
(19) Quintana, R. P., *J. Pharm. Sci.*, **54**, 462(1965).
(20) Katz, M., and Shaikh, Z. I., *ibid.*, **54**, 591(1965).
(21) Hansch, C., Muir, R. M., Fujita, T., Maloney, P. P., Geiger, F., and Streich, M., *J. Am. Chem. Soc.*, **85**, 2817 (1963).
(22) Reese, D. R., Irwin, G. M., Dittert, L. W., Chong, C. W., and Swintosky, J. V., *J. Pharm. Sci.*, **53**, 591(1964).
(23) Doluisio, J. T., and Swintosky, J. V., *ibid.*, **53**, 597(1964).
(24) *Ibid.*, **54**, 1594(1965).
(25) Rosano, H. L., Duby, P., and Schulman, J. H., *J. Phys. Chem.*, **65**, 1704(1961).
(26) Perrin, J., *J. Pharm. Pharmacol.*, **19**, 25(1967).
(27) Goldstein, A., *Pharmacol. Rev.*, **1**, 102(1949).
(28) Goldbaum, L. R., and Smith, P. K., *J. Pharmacol. Exptl. Therap.*, **111**, 197(1954).
(29) Lindenbaum, A., and Schubert, J., *J. Phys. Chem.*, **60**, 1663(1956).
(30) Anton, A. H., *J. Pharmacol. Exptl. Therap.*, **129**, 282(1960).
(31) Eichman, M. L., Guttman, D. E., Van Winkle, Q., and Guth, E. P., *J. Pharm. Sci.*, **51**, 66(1962).
(32) Klotz, I. M., Urquhart, J. M., and Weber, W. W., *Arch. Biochem.*, **26**, 420(1950).
(33) Schanker, L. S., and Tocco, D. J., *J. Pharmacol. Exptl. Therap.*, **128**, 115(1960).
(34) Mullins, L. J., *Ann. N. Y. Acad. Sci.*, **62**, 247(1955).
(35) Dikstein, S., "Quantitative Methods in Pharmacology," de Jonge, H., ed., Interscience Publishers, Inc., New York, N. Y., 1961, p. 312.
(36) Dikstein, S., "Biophysics of Physiological and Pharmacological Actions," Shanes, A. M., ed., American Association for the Advancement of Science, Washington, D. C., 1961, p. 49.
(37) Chertkoff, M. J., and Martin, A. N., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 444(1960).
(38) Restaino, F. A., and Martin, A. N., *J. Pharm. Sci.*, **53**, 636(1964).
(39) Higuchi, T., Finger, K. F., and Higuchi, W. I., "Annual Reports Medicinal Chemistry," Cain, C. K., ed., Academic Press Inc., New York, N. Y., 1965, p. 331.
(40) Paruta, A. N., Sciarrone, B. J., and Lordi, N. G., *J. Pharm. Sci.*, **53**, 1349(1964).
(41) *Ibid.*, **51**, 704(1962).

Effect of Deuterium Oxide on the Saprophytic Culture of *Claviceps* II

Alkaloid Production

By R. G. MRTEK*, H. L. CRESPI, M. I. BLAKE, and J. J. KATZ

The effect of growth conditions on alkaloid production by *Claviceps* in a deuterated environment was explored. Alkaloid formation was strongly inhibited in fully deuterated organisms. Consequently, a replacement culture technique was developed for the biosynthesis of highly deuterated alkaloids. Cultures grown in an ordinary hydrogen environment were transferred to a deuterated medium containing various substrates such as tryptophan, mevalonic acid, phenylalanine, succinic acid, and sugars, either as hydrogen or deuterium compounds. Multimilligram amounts of alkaloids were obtained from replacement cultures containing phenylalanine and deuterio-succinic acid as substrates. The alkaloid fraction, consisting of elymoclavine with minor amounts of agroclavine, was isolated by solvent extraction. The alkaloids were identified by thin-layer chromatography, and the incorporation of deuterated substrate into the alkaloids by the replacement culture technique was verified by NMR spectroscopy.

In the previous paper (1) of this series the nutritional factors involved in optimal growth of *Claviceps* (strain 47A-Tyler) in high concentrations of heavy water were investigated. The successful maintenance of a fully deuterated organism in saprophytic culture was achieved on a medium containing fully deuterated mixed monosaccharides (2%), deuterio-succinic acid (0.5%), and traces of vitamins in a low phosphate salt medium. However, in high concentrations of D_2O the formation of alkaloids was found to be strongly impaired. Consequently, a replacement culture technique was used to induce the elaboration of deuterated alkaloids from water-grown fungal tissue that was resuspended in a medium containing more than 98% D_2O.

EXPERIMENTAL

General Culturing Techniques—The organism used for all experiments in this investigation was a high alkaloid-yielding strain of *Claviceps purpurea*, strain 47A. Conditions for growth, culturing techniques, and adaptation studies in D_2O are described in the previous paper (1).

Replacement Culture Method—The mycelia of 1-L. sterile cultures were used as the "raw tissue" for preparative replacement cultures. This tissue was transferred aseptically from 3-L. conical growth flasks to 1-L. polypropylene bottles, which had been previously sealed with aluminum foil and then autoclaved. The bottles were then centrifuged for 15 min. at 1600 r.p.m. in an International centrifuge, size 2, model V. The supernatant nutrient was decanted and 600 ml. of sterile D_2O was added to the packed mycelia in each bottle. The contents were stirred thoroughly with a sterile glass rod and the bottles were again centrifuged. This process was repeated with 400 ml. of sterile D_2O. Then the mycelia were washed twice with 500-ml. portions of sterile 0.1 *M* phosphate buffer in D_2O, pD 7.1. Finally, 800 ml. of 0.1 *M* phosphate D_2O buffer solution plus any substrates, as indicated in the particular experiment, was added to each bottle. This constituted the replacement medium. The mycelia were dispersed in the medium and the contents were transferred to a clean sterilized 3-L. culture flask. The bottle was rinsed with 200 ml. of sterile replacement medium, and this solution was also added to the culture flask. A 5-ml. aliquot of the culture was withdrawn aseptically, centrifuged, and the supernatant solution analyzed for deuterium content. The culture flask was returned to the shaker for incubation. All manipulations of exposed cultures were performed in a bacteriological hood under sterile conditions.

In developing the optimum conditions for alkaloid production on a preparative scale and in evaluating the effect of substrates, preliminary replacement cultures were prepared using smaller volumes of medium. The tissue from 50 ml. of a dense aqueous fungal suspension was centrifuged, washed, and transferred to two replacement cultures of 50-ml. volume each in 125-ml. conical flasks, resulting in a twofold dilution of the semisolid fungal culture. All replacement culture flasks were incubated at 25° on a rotary shaker. Deuterium oxide and alkaloid analyses were performed periodically throughout the culturing period.

Addition of Substrates—A variety of metabolites was examined by the replacement culture technique. Phenylalanine and succinic acid were incorporated directly into the medium prior to autoclaving. Heat labile components, such as mevalonic acid and vitamin mixtures, were sterilized by filtration of a concentrate through a Millipore filter into the sterile medium.

Received April 14, 1967, from the Chemistry Division, Argonne National Laboratory, Argonne, IL 60439

Accepted for publication June 19, 1967.

Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a dissertation presented by R. G. Mrtek to the Graduate College, University of Illinois at the Medical Center, Chicago, in partial fulfillment of Doctor of Philosophy degree requirements.

This study was performed under the auspices of the U.S. Atomic Energy Commission, Washington, D.C.

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Previous paper: Mrtek, R. G., Crespi, H. L., Blake, M. I., and Katz, J. J., *J. Pharm. Sci.*, **54**, 1450(1965).

Mevalonic acid was obtained from a commercial source[1] as a high purity dibenzylethylenediamine salt. The free acid was prepared by passing an aqueous solution of the salt through an ion-exchange resin column,[2] 1 × 5 cm. The free acid was eluted from the column with water until the pH was neutral, and the eluate was then concentrated to a small volume with a rotary evaporator. Ten milliliters of phosphate buffer was added, and the solution was then lyophilized. The solids were dissolved in 25 ml. of D_2O and the solution was lyophilized again. Finally, the solids were dissolved in 10 ml. of D_2O and the solution was added to the culture medium by filtration through a Millipore filter.

Tryptophan, which is relatively insoluble and heat labile, was incorporated directly into the replacement medium. The solution was then filtered through a Millipore filter. The filtrate was collected in a sterile 2-L. flask to which was attached a short length of cotton-plugged sterile pressure tubing.

All replacement cultures were prepared in duplicate and were incubated on a rotary shaker. Deuterium analyses of the nutrient were performed at the start of an experiment and periodically throughout the course of the study. Periodic alkaloid determinations were performed during the incubation period.

Alkaloid Analysis—Total alkaloids were determined by a modification of the method of Michelon and Kelleher (2). A sample of medium (5 to 10 ml.) was withdrawn aseptically and clarified by centrifugation. An aliquot of the supernatant solution containing between 5 and 50 mcg. of total alkaloid was transferred to a 25-ml. Squibb funnel. The solution was made basic with a few drops of ammonium hydroxide and extracted with two 7-ml. portions of chloroform. The chloroform extracts were placed in a separator containing 2.0 ml. of water. Exactly 2.0 ml. of 0.1% *p*-dimethylaminobenzaldehyde in 50% (v/v) H_2SO_4 was pipeted into the separator which was then stoppered and shaken vigorously for 1 min. The aqueous layer was transferred quantitatively to a 15-ml. centrifuge tube and was allowed to stand until 15 min. had elapsed from the time that the acidic reagent had been added to the separator. Then 0.10 ml. of a 0.1% aqueous sodium nitrite solution was added to the centrifuge tube. A blue color developed upon gentle agitation of the tube. The tube was centrifuged for 1 min. and the clear supernatant solution was transferred to a 1-cm. cell by a dropping pipet. Absorbance measurements were made at 585 mμ with a Cary-14 spectrophotometer. Total alkaloid calculated as elymoclavine was determined from a standard curve prepared with authentic elymoclavine.

Deuterium Analysis—The deuterium oxide content of the nutrient was determined by the infrared absorption method described by Crespi and Katz (3).

Isolation of Alkaloids—The mycelia in the replacement cultures were separated from the liquid by filtration through a coarse sintered-glass funnel and the filtrate was clarified by passage through a bed of diatomaceous earth.[3] After the volume was reduced to 300 ml. on a rotary evaporator at 30°, the solution was rendered basic with 25 ml. of ammonium hydroxide and extracted with 400 ml. of chloroform in a 1-L. separator. Three extractions with 200-ml. portions of chloroform were carried out and the combined chloroform extracts were concentrated to 200 ml. in a rotatory evaporator at 30°. The concentrate was washed three times with 50-ml. portions of 1 *N* ammonium hydroxide, and the ammoniacal washings were discarded after showing a negative alkaloid test with the modified Ehrlich's reagent. The chloroform solution was then extracted with 20-ml. portions of 2% tartaric acid solution until the extracting solution gave a negative alkaloid test.

The tartaric acid solution was cooled in an ice bath, adjusted to pH 8.5 with ammonium hydroxide, and extracted three times with 15-ml. portions of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate, concentrated to a small volume in a rotary evaporator at 25°, and transferred quantitatively to a tared glass tube sealed at one end. The solution then was evaporated to dryness at room temperature under a stream of nitrogen gas. Last traces of solvent were removed by evacuation in a vacuum line for 30 min., and the yield of alkaloid determined by weighing.

Thin-Layer Chromatography—Chromatographic analysis of alkaloid fractions was performed on Silica Gel G plates. Plates were spread to a uniform 250-μ layer with a Desaga variable thickness spreader, dried at room temperature overnight, and stored in a desiccator until used. Immediately before use, the plates were activated by heating for 20 min. at 110°. After the plates had cooled in a desiccator, spots containing 5 to 20 μl. of sample in chloroform were applied.

The chromatogram was developed in an oversaturated chamber containing ethyl acetate–acetone–diethylamine (10:10:2). The solvent front was marked, and the chromatogram was allowed to dry thoroughly at room temperature. The dried chromatogram was sprayed with von Urk's reagent prepared by dissolving 0.5 Gm. *p*-dimethylaminobenzaldehyde in a mixture of 50 ml. of 25% HCl and 50 ml. of 95% ethanol. After the chromatogram was sprayed, it was heated at 110° for 5 min. The plate was cooled and first sprayed lightly with a fresh solution of 0.05% $NaNO_2$ in 50% ethanol and then sprayed with a mixture of equal volumes of 95% ethanol and 25% HCl. The spots containing the indole alkaloids became intensely blue. R_f values were measured from densitometric scans prepared when the color of the spots was at maximum intensity.

RESULTS AND DISCUSSION

In cultures grown under fully deuterated conditions (1, 4) alkaloids were never detected in the medium in greater than trace amounts. Inocula size, aeration, light, and varying culture volumes had little, if any, effect in stimulating the production of alkaloids, even after incubation periods as long as 3 months. Fully deuterated cultures incubated in closed cabinets containing a D_2O saturated atmosphere were observed for periods up to 6

[1] Calbiochem, Los Angeles, Calif.

[2] The column contained AG 50W-X8 in the hydrogen form, Bio-Rad Laboratories, Richmond, Calif.

[3] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

months. Tissue growth appeared to cease after about 2 months. The deuterio-culture became pigmented, as in the case of ordinary cultures, but no alkaloids formed. Thin-layer chromatograms failed to reveal the presence of even trace amounts of alkaloids in any culture medium obtained from fully deuterated *Claviceps*.

Decantation of the medium from a deuterated culture, followed by replacement with fresh complete D_2O medium, caused additional tissue growth and elaboration of pigment, but no alkaloid synthesis. Essentially the same observations were made when the deuterated cultures were supplemented with added deuterio-monosaccharides and vitamins. Addition of (D_2O-exchanged) protio-phenylalanine or protio-tryptophan also failed to stimulate alkaloid production. Different stock slants of *Claviceps* were adapted to growth in D_2O, but the resultant cultures all failed to produce alkaloids. The protio-ancestor of each deuterium experiment was carried through successive H_2O transfers to test for strain regression. Such "control" protio-cultures were still capable of producing clavine alkaloids at the conclusion of the deuterium experiments.

Thus, the prospect of obtaining alkaloids from a culture of *Claviceps* grown under fully deuterated conditions is not good. The absence of alkaloids may be the result of (*a*) an incomplete medium, or (*b*) a primary repression of one or more enzymatic mechanisms involved in alkaloid synthesis. As for the first possibility, the development of a defined medium for any biologic clone is a highly empirical task. Apart from the obvious additions of selected substrates and variation in phosphate concentration which is known to have a strong effect on alkaloid production in water, there are no obvious nutritional factor or factors that may be invoked to allow *Claviceps* to produce clavine alkaloids. To locate the specific sites of the deuterium isotope effect in the biochemical mechanisms of the fungus requires a much more thorough understanding of individual enzyme systems responsible for alkaloid production than is now available for the organism under normal conditions.

Based on the experiments described above, a new set of experimental conditions was devised to study alkaloid production in *Claviceps* at high levels of D_2O. These experiments were designed to require the organism to utilize deuterium under physiologic conditions in which alkaloid synthesis is known to occur.

Replacement culture techniques have been employed by other investigators in studies involving the saprophytic culturing of strains of *Claviceps* in aqueous media. Thus, Brady and Tyler (5) investigated the effect of certain precursors on the production of clavine alkaloids, while Groger and Erge (6) reported the effects of mannitol solutions in supporting alkaloid production by the replacement technique. Abe *et al.* (7) used a surface replacement culture technique in their studies of alkaloid production. Suitable choice of replacement media allows alkaloid production to be studied under conditions independent of growth. Thus, replacement studies permit the separate analysis of the effects of deuterium isotope substitution on vegetative growth and on alkaloid production.

In these experiments the organism was allowed to mature on an aqueous medium containing 10% mannitol and 0.5% protio-succinic acid added to a stock solution of Tyler's salt mixture (8). The organism was then introduced into a D_2O medium containing only deuterium exchanged phosphate buffer salts and selected substrates. Growth was virtually stopped by selecting a buffer of high ionic strength and a pH far removed from the growth optimum. No trace elements or other substrates normally required in a complete growth medium were added. Under such conditions the clone may either undergo autolysis or it may supply its own respiratory requirements by whatever metabolic routes are still available to it. It is recourse to the latter alternative that usually results in the elaboration of metabolites which are the end products of normally nonobligatory pathways.

In preliminary studies performed on replacement cultures a number of compounds, singly and in combination, were tested for their ability to stimulate alkaloid production. Alanine, 5-fluorotryptophan, *p*-fluorophenylalanine, and anthranilic acid were not found to induce alkaloid synthesis. The amino acid fraction isolated from fully deuterated algae (9) likewise had no demonstrable effect on alkaloid production. Although fully deuterated aspartic acid, glutamic acid, alanine, and glycine are present in large amounts in this mixture, no alkaloids are formed. In accordance with conclusions arrived at by studies in H_2O medium, only phenylalanine and tryptophan of the amino acids tested are capable of serving as substrates for clavine alkaloid synthesis in D_2O replacement cultures.

Figure 1 demonstrates the effect of various substrates on alkaloid production in a replacement culture. These include 0.1% tryptophan, 0.2% mevalonic acid, 2.0% protio-glucose, and 2.0% deuterio-sugars (glucose–mannose, 1:1), obtained from fully deuterated algae (9). In most instances a vitamin mixture supplement was added (1). In the absence of additives no alkaloid production took place (curve F). Mevalonic acid alone was a poor substrate (curve E), but tryptophan alone did serve as an adequate substrate (curve D). However, in the presence of mevalonic acid and vitamins, alkaloid production increased somewhat (curve C). Maximum alkaloid production occurred when tryptophan, mevalonic acid, sugars, and vitamins were included in the medium. Protio and deuterio-sugars produced essentially the same effect as can be seen in curves A and B.

In another series of experiments (Fig. 2) protio-phenylalanine and protio-succinic acid were utilized as substrates in replacement cultures. Phenylalanine alone at a concentration of 0.5% was the most effective in stimulating alkaloid formation (curve E). When succinic acid was added, alkaloid production diminished (curve D). Severe inhibition resulted when the succinic acid concentration was increased tenfold and the phenylalanine concentration was reduced to one-fifth (curve C). After 20 days not even a trace of alkaloid was detected in the medium. The addition of protio-glucose suppressed alkaloid synthesis (curve A). This may be a result of the utilization of the substrates for growth rather than for alkaloid production. When all substrates were omitted the organism failed to produce alkaloids in any measurable quantity.

Multimilligram amounts of deuterated alkaloids

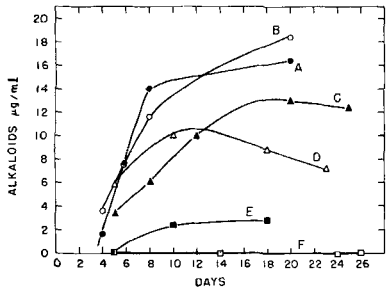

Fig. 1—Effect of substrates on alkaloid production in D_2O replacement cultures. Key: A, *0.1% tryptophan, 0.2% mevalonic acid, 2.0% protio-glucose, and vitamins in trace amounts;* B, *tryptophan, mevalonic acid, deuterio-sugars, and vitamins;* C, *tryptophan, mevalonic acid, and vitamins;* D, *tryptophan;* E, *mevalonic acid;* F, *control.*

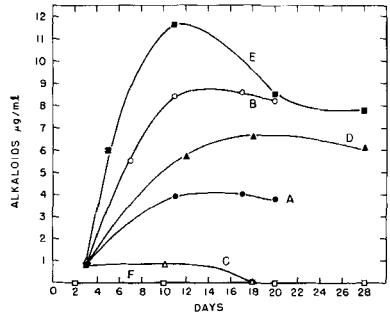

Fig. 2—Alkaloid production in D_2O replacement cultures in the presence of certain substrates. Key: A, *0.5% phenylalanine, 2.5% succinic acid, 2.0% protio-glucose;* B, *0.5% phenylalanine, 2.5% succinic acid;* C, *0.1% phenylalanine, 2.5% succinic acid;* D, *0.5% phenylalanine, 0.2% succinic acid;* E, *0.5% phenylalanine;* F, *control.*

were obtained from large-scale preparative replacement cultures in D_2O. For this purpose the replacement medium contained 0.5% protio-phenylalanine and 0.5% protio-succinic acid dissolved in 99% D_2O. The phenylalanine and succinic acid were exchanged[4] with D_2O prior to incorporation into the medium. In a second experiment protio-phenylalanine and succinic acid-d_6, both of which were exchanged with D_2O, were used as substrates in a D_2O medium. In the first culture, the only source of deuterium accessible to the organism was the solvent D_2O used in the medium. In the second culture the organism was required to utilize deuterium from both the solvent and the nonexchangeable positions of the deuterio-succinic acid. Table I gives the yield of deuterated alkaloids obtained from these cultures. A total of 24.8 mg. was obtained in the first experiment and 9.0 mg. in the second.

Thin-layer chromatography of the purified deuterated alkaloid fraction indicated that only elymoclavine was formed in the highly deuterated cultures. No agroclavine could be detected in the alkaloid fraction. With the chromatographic solvent system employed in this study protio-elymoclavine and protio-agroclavine had R_f values of 0.29 and 0.59, respectively. Deuterated elymoclavine had a distinctly lower R_f value of 0.27, as observed previously for other deuterated compounds (9).

[4] Deuterium atoms exchange only for the hydrogen of the carboxyl and amino groups.

TABLE I—YIELD OF DEUTERATED ALKALOIDS BY REPLACEMENT CULTURE METHOD

Substrate	D_2O Content,[a] %	Incubation Period, Days	Vol. at Harvest, ml.	Total Alkaloids, mg.
Protio-phenylalanine (0.5%) Protio-succinic acid (0.5%)	99.0	26	1900	24.8
Protio-phenylalanine (0.5%) Deuterio-succinate (0.5%)	98.8	24	1920	9.0

[a] Determined at harvest.

A complete nuclear magnetic resonance (NMR) analysis was carried out on authentic samples of protio-clavine alkaloids and on deuterated elymoclavine. Spectra were obtained with a Varian HA-100 spectrometer using alkaloid solutions which were 0.1 *M* in pyridine-d_5. From these data it was possible to deduce the H/D ratio at the various hydrogen positions in the molecule. The elymoclavine obtained from replacement cultures in 99% D_2O with added protio-succinate and protio-phenylalanine was highly deuterated at all positions. The hydrogens at positions 10 and 17 were completely replaced by deuterium and the hydrogens at positions 4, 5, 6 (CH_3—N), 7, and 9 contained from 68 to 86% deuterium. These data indicate extensive labilization of carbon-bound hydrogen during the biosynthesis of the alkaloid. In a similar replacement culture containing deuterio-succinate in place of protio-succinate (Table I) the over-all level of deuteration was not significantly different. The complete details and analysis of these experiments are to be published in a subsequent paper.

When deuterated tissue was used in a replacement culture experiment, no alkaloids were elaborated. In addition, when deuterated tissue was introduced into an H_2O replacement medium, no alkaloid synthesis occurred. The phenomenon of alkaloid production is probably nonessential and organisms which elaborate this class of compounds do so only under specific and often nonobligatory conditions.

It appears that a primary effect of deuterium in biological systems is the suppression of nonessential metabolism. The stress imposed on *Claviceps* by the introduction of large amounts of deuterium results in the utilization of available metabolites to maintain the nutrition of the isotopically altered fungus at the expense of alkaloid synthesis.

REFERENCES

(1) Mrtek, R. G., Crespi, H. L., Blake, M. I., and Katz J. J., *J. Pharm. Sci.*, **54**, 1450(1965).
(2) Michelon, L., and Kelleher, W. J., *Lloydia*, **26**, 192 (1963).
(3) Crespi, H. L., and Katz, J. J., *Anal. Biochem.*, **2**, 274 (1961).
(4) Mrtek, R. G., Ph.D. Thesis, University of Illinois at the Medical Center, Chicago, Ill., 1967.
(5) Brady, L. R., and Tyler, V. E., *Planta Med.*, **7**, 225 (1959).
(6) Groger, D., and Erge, D., "Continuous Cultivation of

Microorganisms," Malek, I., Beran, K., and Hospodka, J., eds., Academic Press Inc., New York, N. Y., 1964, p. 193.
(7) Abe, M., Yamano, T., and Kusumoto, M., *J. Agr. Chem. Soc. Japan*, **27**, 613(1953).
(8) Brady, L. R., and Tyler, V. E., *Lloydia*, **23**, 8(1960).
(9) Blake, M. I., Crespi, H. L., Mohan, V. S., and Katz, J. J., *J. Pharm. Sci.*, **50**, 425(1961).

Establishment of Sink Conditions in Dissolution Rate Determinations

Theoretical Considerations and Application to Nondisintegrating Dosage Forms

By MILO GIBALDI and STUART FELDMAN

The importance of approximating perfect sink conditions for the determination of dissolution rates which may be correlated with *in vivo* results is pointed out. A theoretical treatment of zero and first-order dissolution under perfect sink and nonsink conditions is presented. It is shown that unless sink conditions are maintained, *in vitro* results will bear little relationship to *in vivo* observations. The use of an organic solvent phase which can function as a reservoir for dissolved drug and thereby maintain sink conditions in the aqueous dissolution fluid is demonstrated for a model nondisintegrating tablet exhibiting zero-order drug release. The use of an organic solvent reservoir for the determination of first-order dissolution rates is also discussed.

One of the most important as well as most difficult approaches in the biopharmaceutic evaluation of a drug is the quantitative correlation of absorption and *in vitro* dissolution kinetics. Levy *et al.* (1) have recently reported the development of a single *in vitro* dissolution rate test which correlates quantitatively with the gastrointestinal absorption, in man, of aspirin from three markedly different types of dosage forms. The basic method, however, is limited to the study of drugs which are relatively water soluble.

Dissolution rate-limited absorption implies that there is no build-up of drug concentration in the gastrointestinal fluids, *i.e.*, the fluids function as a perfect sink. Unless this condition is embodied in the design of the *in vitro* test (*i.e.*, drug concentration in the dissolution fluids does not exceed 10 to 20% of solubility), *in vitro* results will bear little relationship to *in vivo* observations (2).

As noted recently by Levy (2), those drugs which represent the greatest dissolution problem are also those which are least soluble and give the greatest difficulty with respect to maintenance of perfect sink conditions. Frequently, it is necessary to use exceedingly large volumes of solvent for this purpose and to adopt very sensitive analytical procedures. At times, it may be very difficult to follow the dissolution of more than a small fraction of the drug contained in the dosage form. Under these conditions one must assume a uniform pattern of release rate. Wood (3), however, has pointed out that in practice there are many cases in which one point in time or a rate constant does not characterize the dissolution process and a full dissolution curve (based on the total drug content of the dosage form) is required for *in vitro* to *in vivo* correlation.

A definite need exists for the development of methodology to maintain sink conditions during dissolution rate determinations of poorly soluble drugs. Levy (2) has suggested two possibilities: the use of an upper organic phase which can act as a reservoir for the dissolved drug or the addition of adsorbents to the aqueous medium. The ability of adsorbents to maintain "sink" conditions had been demonstrated previously by Wurster and Polli (4). Both approaches involve the same principle, *i.e.*, removal of dissolved drug from the dissolution medium and prevention of accumulation. The removal of drug from the dissolution fluid is analogous to removal of drug from the gastrointestinal fluids by the absorption process in dissolution rate-limited absorption.

The present report concerns the theory and application of the use of an upper organic solvent

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Accepted for publication June 22, 1967.

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phase for the maintenance of perfect sink conditions during dissolution rate determinations of nondisintegrating solids in aqueous systems.

EXPERIMENTAL

Model Dosage Form—Nondisintegrating flat-faced tablets of pure drug were prepared by compressing 300 mg. of benzoic acid or 600 mg. of salicylic acid by means of a Carver model B hydraulic press equipped with a 0.5-in. diameter punch and die. The compression pressure was 10,000 p.s.i.

Dissolution Assembly—The assembly consisted of a 500-ml. three-neck round-bottom flask immersed in a constant-temperature bath adjusted to 37° ± 0.1°. A 3-in. Teflon stirring blade and shaft attached to a stirring motor was inserted in the center neck. The motor was controlled by a Servodyne constant torque unit which also served to provide a direct read-out of speed of rotation.

Dissolution Procedure in 0.1 N HCl—One hundred and fifty milliliters of 0.1 *N* HCl was placed in the flask and permitted to equilibrate to 37°. The stirring blade was immersed in the dissolution medium to a depth of 20 mm. and accurately centered by means of a guide. The stirrer was rotated at a speed of 25, 50, or 100 r.p.m. and the benzoic acid or salicylic acid tablet was placed in the medium by dropping it through the side neck, along the side of the flask. The tablet remained at the bottom of the flask throughout each determination.

One-milliliter samples of the solution were taken at appropriate intervals, diluted, and assayed spectrophotometrically using a Beckman model DB-G spectrophotometer. A volume of 0.1 *N* HCl (maintained at 37°) equal to the sample volume was added immediately after each sampling. Absorbances were measured at 226 and 302 mμ for benzoic acid and salicylic acid, respectively. By appropriate calculations (5), the total amount of drug in solution as a function of time was determined.

The dissolution of benzoic acid was followed under conditions approximating a perfect sink, *i.e.*, benzoic acid concentration in the dissolution fluid never exceeded 12% of solubility. The dissolution of salicylic acid was followed well beyond sink conditions. At the end of the determination the salicylic acid concentration in the dissolution medium approximated 80% of solubility.

Dissolution Procedure in 0.1 N HCl with Upper Organic Solvent Phase—After addition of the 150 ml. of 0.1 *N* HCl and positioning of the stirrer, the tablet was dropped into the aqueous phase but remained unstirred while 150 ml. of organic solvent was slowly added to the flask. Stirring was initiated after addition of the organic solvent. Dissolution of benzoic acid was studied in 0.1 *N* HCl layered with a 1:1 mixture of cyclohexane and 1-octanol, while dissolution of salicylic acid was studied in 0.1 *N* HCl layered with a 1:1 mixture of cyclohexane and 2-octanol.

The selection of the upper organic solvent phase is dictated solely by a consideration of the partition coefficient of the drug between the aqueous dissolution medium and the organic sink. The total dose of drug to be studied must represent less than 20% of its solubility in the selected volume of organic solvent.

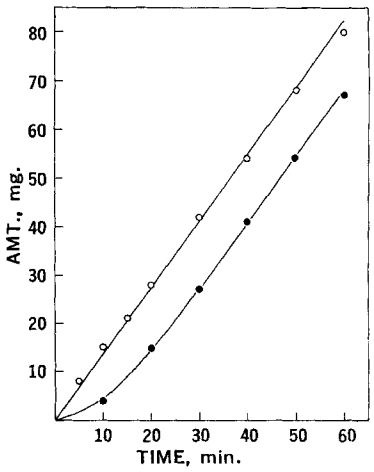

Fig. 1—Rate of appearance of benzoic acid from constant surface tablets in 0.1 N *HCl* (○) *and in cyclohexane–1-octanol layer above a 0.1* N *HCl phase* (●).

At various intervals after initiation of stirring, 1-ml. samples of the organic phase were taken, diluted with methanol, and assayed spectrophotometrically. The volume of the organic phase was maintained constant by replenishing with solvent after each sampling. By appropriate calculations, the total amount of drug appearing in the organic solvent phase as a function of time was determined.

Determination of Rate of Partitioning—The rate of partitioning of benzoic acid from water to 1:1 cyclohexane–1-octanol was determined in the dissolution assembly at 37° and 25 r.p.m. At time zero the aqueous phase contained 0.3 mg./ml. benzoic acid in 0.1 *N* HCl. The organic solvent phase was sampled at various intervals after addition and the quantity of benzoic acid present at each interval was determined.

RESULTS AND DISCUSSION

The dissolution of benzoic acid in 0.1 *N* HCl followed apparent zero-order kinetics over the time period studied. The appearance of benzoic acid as a function of time at 50 r.p.m. is illustrated in Fig. 1. The apparent zero-order dissolution rate constants determined at 25, 50, and 100 r.p.m. are listed in Table I. A plot of log dissolution rate *versus* log stirring rate is shown in Fig. 2. According to the equations developed by Cooper and Kingery (6), which relate the diffusion controlled dissolution rate from the surface of a rotating disk to the velocity of rotation (stirring rate), a plot of the logarithm of dissolution rate (expressed here in mg./min./total surface area) *versus* the logarithm of stirring rate (expressed in r.p.m.) should yield a straight line with a slope of 0.5. As indicated in Fig. 2, the slope of the line fitted to the benzoic acid data (0.494) is quite close to the theoretical value. Thus, the described relationship between dissolution rate and stirring rate holds for the methodology used in the present study.

The rate of appearance of benzoic acid in the cyclohexane–octanol phase during the dissolution process also followed zero-order kinetics, after an initial lag period. A representative plot is included.

TABLE I—KINETIC DATA FOR THE DISSOLUTION OF BENZOIC ACID FROM CONSTANT SURFACE TABLETS IN 0.1 *N* HCl LAYERED WITH CYCLOHEXANE–1-OCTANOL

Stirring Rate, r.p.m.	k_0,[a] mg./min.	k_0[b], mg./min.	B[c], mg.	k_p,[d] min.$^{-1}$
25	1.00	0.87	12.2	0.071 (0.067)[e]
50	1.36	1.35	12.1	0.111
100	2.00	2.00	5.2	0.385

[a] Zero-order dissolution rate constant of drug in 0.1 *N* HCl, for exposed surface area of 9.0 cm.^{2}. [b] Zero-order rate constant for appearance of drug in organic solvent phase. [c] Calculated steady-state amount of drug in 0.1 *N* HCl layered with solvent. [d] Calculated first-order partition rate constant (× interfacial area) of drug from 0.1 *N* HCl to cyclohexane–1-octanol. [e] Experimentally determined partition rate constant (× interfacial area).

in Fig. 1. The zero-order rate constants for appearance of drug in the organic phase at the various stirring speeds studied are listed in Table I. There is excellent agreement between the zero-order dissolution rate constants and the zero-order appearance rate constants at both 50 and 100 r.p.m. At 25 r.p.m. the rate of appearance of drug in the organic phase adhered to zero-order kinetics but the rate constant was somewhat lower than the dissolution rate constant found in 0.1 *N* HCl at 25 r.p.m. The reason for this phenomenon appears to be related to the effective stirring rate, *i.e.*, although the paddle rotated at 25 r.p.m., the flow of aqueous fluids about the tablet was dampened by the physical presence of the organic layer. An estimate of the effective stirring rate in the two-phase system at a paddle speed of 25 r.p.m. was obtained by extrapolating the data shown in Fig. 2 (assuming that appearance rate corresponded to dissolution rate). The experimental appearance rate was found to correspond to an effective stirring rate of 20 r.p.m. The dampening effect appeared to be totally absent at 50 and 100 r.p.m.

The rate of appearance of drug in the organic phase may be related to the dissolution rate in the aqueous phase by means of the following kinetic model:

$$A \xrightarrow{k_0} B \xrightarrow{k_p} C \qquad \text{(Eq. 1)}$$

where A is the amount of drug in the tablet, B is the amount of drug in the aqueous phase, C is amount of drug in the organic layer, k_0 is the zero-order dissolution rate constant, and k_p is the first-order rate constant for partitioning of drug from the aqueous phase to the organic solvent phase. The rates of change of B and C may be expressed by the following differential equations:

$$dB/dt = k_0 - k_pB \qquad \text{(Eq. 2)}$$

and

$$dC/dt = k_pB \qquad \text{(Eq. 3)}$$

Integrating Eqs. 2 and 3 yields, respectively:

$$B = \frac{k_0}{k_p}(1 - e^{-k_pt}) \qquad \text{(Eq. 4)}$$

and

$$C = k_0t - \frac{k_0}{k_p}(1 - e^{-k_pt}) \qquad \text{(Eq. 5)}$$

At some finite time t, $e^{-k_pt} \rightarrow 0$ and Eqs. 4 and 5 reduce to Eqs. 6 and 7:

$$B = \frac{k_0}{k_p} = \text{constant} \qquad \text{(Eq. 6)}$$

and

$$C = k_0t - \frac{k_0}{k_p} \qquad \text{(Eq. 7)}$$

The more rapid the rate of partitioning, the shorter the time period required for the attainment of true steady-state kinetics, *i.e.*, B = constant, $dB/dt = 0$. After attainment of steady-state, a plot of C *versus* time should yield a straight line with a slope of k_0.

The experimental data permitted calculation of k_p by means of Eq. 7 and B at the steady state by means of Eq. 6. These calculated values are listed in Table I. The k_p value of 0.071 calculated from the data at 25 r.p.m. was in agreement with the experimentally determined value of 0.067.

The data in Table I indicate a positive correlation between k_p and stirring rate. The reason for this relationship is that the k_p values are actually a product of the first-order rate constant for diffusion across the interface and the interfacial area between the water and solvent phases. As stirring rate is increased, the turbulence at the interface increases causing a degree of mixing and "emulsification" in the interfacial region. This increase in the "thickness" of the interface results in a marked increase in the mean interfacial area. It is likely that the increased interfacial area is primarily responsible for the increased values of k_p.

The steady-state values listed in the table represent only 0.8 to 1.8% of the solubility of benzoic acid in 0.1 *N* HCl. This is an excellent indication of the efficiency of the organic solvent in maintaining near-perfect sink conditions in the aqueous phase.

The salicylic acid studies were designed to

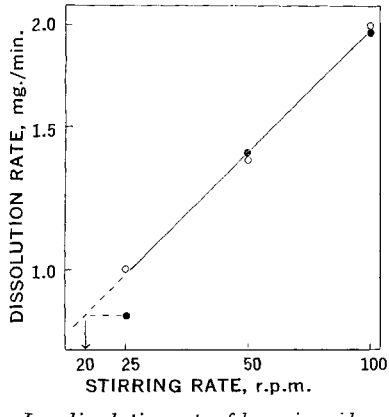

Fig. 2—Log dissolution rate of benzoic acid versus *log stirring rate. Dissolution rate determined in 0.1* N *HCl* (○) *and in 0.1* N *HCl layered with cyclohexane–1-octanol* (●).

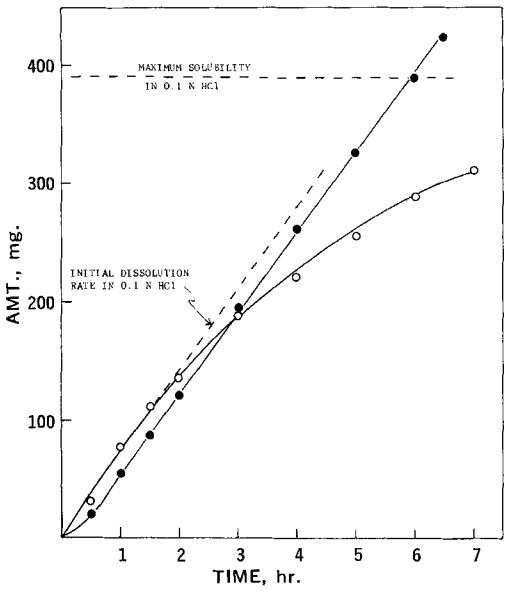

Fig. 3—Rate of appearance of salicylic acid from constant surface tablets in 0.1 N HCl (○) under nonsink conditions and in cyclohexane–2-octanol layer above a 0.1 N HCl phase (●) where perfect sink conditions are approximated. The broken horizontal line indicates the total solubility of drug in 150 ml. of 0.1 N HCl.

illustrate the different dissolution kinetics which result from lack of sink conditions and to demonstrate the resolution of these differences by means of an organic solvent reservoir. The model dosage form of salicylic acid was anticipated to retain a constant surface area during the release of a significant amount of drug. Hence, the gastric absorption of a large percentage of a dose of salicylate from this constant surface dosage form would be expected to follow zero-order kinetics. For any meaningful *in vivo–in vitro* correlation the dissolution rate method should at least demonstrate the same order of kinetics as the absorption process. The data in Fig. 3 show that this is clearly not the case when dissolution is followed under nonsink conditions.

The appearance of salicylic acid in 0.1 *N* HCl at 50 r.p.m. followed apparent zero-order kinetics up to about 25% of solubility. When the salicylate levels exceeded 30% of solubility, marked deviations from zero-order kinetics were noted and in fact, dissolution appeared to be first order. The apparent zero-order rate constant calculable from the initial dissolution data was 1.15 mg./min./total surface area.

When the dissolution of salicylic acid was followed in 0.1 *N* HCl layered with an organic solvent reservoir a totally different kinetic picture emerged. (See Fig. 3.) After an initial lag phase (required to establish steady state conditions), the rate of appearance of drug in the cyclohexane–octanol phase followed apparent zero-order kinetics. By means of the organic reservoir it was possible to demonstrate that the release rate from the model dosage form of a quantity of drug *in excess* of its total solubility in 0.1 *N* HCl adhered to apparent zero-order kinetics. In the absence of the reservoir it appeared that the release rate of a quantity of drug equal to only 25% of its solubility followed zero-order kinetics.

The zero-order rate constant for appearance of salicylic acid in the organic phase after attainment of steady state, *i.e.*, the apparent zero-order dissolution rate constant, was calculated to be 1.14 mg./min. This value is in agreement with the apparent initial zero-order rate constant of 1.15 mg./min. determined under nonsink conditions. The amount of drug in the aqueous phase after attainment of steady state was calculated by means of Eqs. 6 and 7 and found to be 17.1 mg. Thus, the maximum accumulation of drug in the aqueous phase in the presence of the organic reservoir represented less than 5% of solubility.

The reason for the different dissolution kinetics under nonsink conditions is readily explained by the Noyes-Whitney equation (7). The following example is illustrative. The dissolution of a quantity of drug A_0, in a dosage form designed to provide zero-order release, is followed in a quantity of solvent which is just sufficient to dissolve the total amount of drug in the dosage form, *i.e.*, $X_s = A_0$, where X_s is the total solubility of the drug in the given volume of solvent. Under these conditions, the rate of loss of drug from the dosage form may be expressed as:

$$-dA/dt = k(X_s - X) \qquad \text{(Eq. 8)}$$

where X is the amount of drug in solution at time t. Assuming dissolution rate-limited absorption *in vivo*, or sink conditions *in vitro*, Eq. 8 reduces to a zero-order equation, *i.e.*,

$$-dA/dt = k\,(X_s) = \text{constant} \qquad \text{(Eq. 9)}$$

and

$$A = A_0 - (k \cdot X_s)t \qquad \text{(Eq. 10)}$$

However, under the nonsink conditions in this particular example, Eq. 8 may be expressed as:

$$-dA/dt = k[A_0 - (A_0 - A)] \qquad \text{(Eq. 11)}$$

or

$$-dA/dt = kA \qquad \text{(Eq. 12)}$$

and

$$A = A_0 e^{-kt} \qquad \text{(Eq. 13)}$$

Therefore, a drug which may be absorbed, or dissolve, under sink conditions in a zero-order fashion may demonstrate first-order dissolution kinetics under nonsink conditions.

APPLICATION TO FIRST-ORDER DISSOLUTION

A drug which dissolves from a given dosage form in an apparent first-order fashion (1, 2) will also show marked differences in *in vitro* dissolution kinetics depending on the conditions employed for dissolution rate determination. Using an example similar to the one cited above, one may illustrate these phenomena. The dissolution of a quantity of drug A_0 contained in a dosage form from which first-order release occurs is followed in a quantity of solvent which is just sufficient to dissolve the total amount of drug, *i.e.*, $X_s = A_0$, where X_s is the total solubility of the drug in the given volume of solvent.

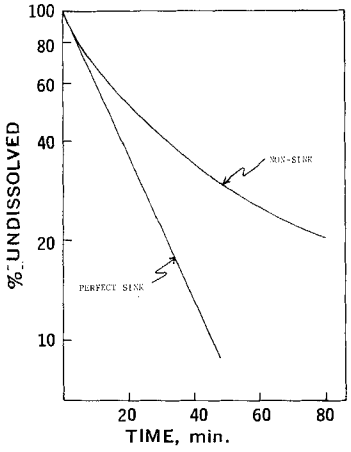

Fig. 4—Plot of per cent drug undissolved from hypothetic dosage form releasing drug in a first-order fashion under perfect sink and nonsink conditions.

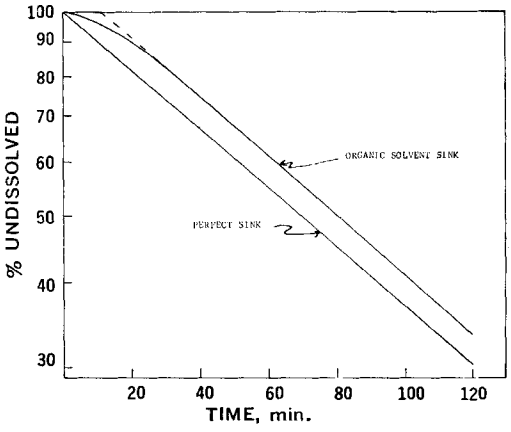

Fig. 5—Plot of per cent drug undissolved from hypothetic dosage form releasing drug in a first-order fashion under perfect sink conditions and under conditions where a perfect sink is approximated by the presence of an organic solvent reservoir.

Under these conditions the rate of loss of drug from the dosage form may be expressed as:[1]

$$-dA/dt = k(X_s - X)A \qquad \text{(Eq. 14)}$$

where X is the amount of drug in solution at time t. Assuming dissolution rate-limited absorption *in vivo* or sink conditions *in vitro*, Eq. 8 reduces to a first-order equation, *i.e.*,

$$-dA/dt = (k\,X_s)A = k'A \qquad \text{(Eq. 15)}$$

and

$$A = A_0 e^{-k't} \qquad \text{(Eq. 16)}$$

However, under the nonsink conditions cited in this example, Eq. 14 may be expressed as:

$$-dA/dt = k[A_0 - (A_0 - A)]A$$

or

$$-dA/dt = kA^2 \qquad \text{(Eq. 17)}$$

Therefore,

$$A = \frac{A_0}{1 + (kA_0)t} = \frac{A_0}{1 + k't} \qquad \text{(Eq. 18)}$$

which represents an integrated form of a *second-order* rate equation.

Figure 4 is a hypothetic plot of first-order loss of drug from a dosage form under perfect sink and nonsink conditions. The marked difference in results clearly reinforces the hypothesis that unless sink conditions are maintained in dissolution rate determinations, *in vitro* results may bear little relationship to *in vivo* observations. The difference between the two plots diminishes as $A_0/X_s \to 0$.

An organic solvent reservoir which functions to maintain approximate sink conditions is also applicable to the determination of first-order dissolution rates. The rate of appearance of drug in the organic phase may be related to the first-order dissolution rate in the aqueous phase by means of the following kinetic model:

$$A \xrightarrow{k'} B \xrightarrow{k_p} C \qquad \text{(Eq. 19)}$$

which is analogous to the model presented in Eq. 1 for zero-order dissolution. If the choice of organic solvent and other experimental conditions are such that $k_p/k' \geqslant 10$, then within a short period of time after initiation of dissolution the appearance of drug in the organic solvent phase may be approximated by the following equation:

$$\ln (A_0 - C) \cong \ln A_0 - k't = \ln A \qquad \text{(Eq. 20)}$$

A hypothetic plot of per cent drug undissolved from a dosage form releasing drug in a first-order fashion (calculated by determining the amount of drug in an organic solvent reservoir phase and subtracting this value from A_0) is illustrated in Fig. 5. This curve is shown in contrast to a curve for the same dissolution process occurring under theoretically perfect sink conditions. The hypothetic conditions were selected such that $k' = 0.01$ min.$^{-1}$ and $k_p = 0.1$ min.$^{-1}$. After an initial lag phase, the curve determined with the use of an organic solvent reservoir parallels exactly the theoretic line. The larger the value of k'/k_p, the shorter will be the lag time before Eq. 20 becomes valid.

Present studies in these laboratories are concerned with the development of methodology for the determination of dissolution rates of poorly water-soluble drugs from disintegrating tablets and capsule dosage forms by means of an organic solvent reservoir for maintenance of sink conditions.

REFERENCES

(1) Levy, G., Leonards, J. R., and Procknal, J. A., *J. Pharm. Sci.*, **54**, 1719(1965).
(2) Levy, G., "Papers Presented Before the Industrial Pharmacy Section," American Pharmaceutical Association, Dallas meeting, April 1966, pp. 233–252.
(3) Wood, J. H., *ibid.*, pp. 181–200.
(4) Wurster, D. E., and Polli, G. P., *J. Pharm. Sci.*, **50**, 403(1961).
(5) Bates, T. R., Gibaldi, M., and Kanig, J. L., *Nature*, **210**, 1331(1966).
(6) Cooper, A. R., Jr., and Kingery, W. D., *J. Phys. Chem.*, **66**, 665(1962).
(7) Noyes, A. A., and Whitney, W. R., *J. Am. Chem. Soc.*, **19**, 930(1897).

[1] Equation 14 represents a modification of the Noyes-Whitney equation. Since dissolution is apparent first order, it follows that $S \cong k'A$ where S is the surface area.

Deaggregation Behavior of a Relatively Insoluble Substituted Benzoic Acid and Its Sodium Salt

By ARMANDO J. AGUIAR, JOYCE E. ZELMER, and ARLYN W. KINKEL

The deaggregation rates of a relatively insoluble benzoic acid derivative and its soluble sodium salt were studied. Based on the postulate that the deaggregation rate follows a first-order process, equations were developed to measure the rates quantitatively. A spectrophotometric procedure, which is simple and convenient for use routinely, is described. The surface area of the aggregates at the final state was estimated from a correlation between the absorbance measurements and the surface area determined with a Coulter counter. It was seen that encapsulating or tableting these compounds decreased the deaggregation rates. The addition of a nonionic polyol surfactant or the sodium salt of polymerized alkyl naphthalene sulfonic acid enhanced the rates. A relationship between the deaggregation rate and rate of solution of the acid in simulated intestinal fluid T.S. (U.S.P. XVI) is demonstrated. The biopharmaceutical significance of the deaggregation rate is discussed.

THE IMPORTANCE of particle size reduction of relatively insoluble drugs to enhance their gastric absorption has been emphasized during the past decade (1–4). The emphasis is based on the premise that the smaller the size, the faster the dissolution rate of the drug due to the increased surface area presented to the dissolving medium. This premise holds as long as the effective species undergoing dissolution are the primary particles. However, when dealing with insoluble drugs which are also difficult to wet and disperse, the effective species are generally not the primary particles but more often their aggregates.

The situation is further aggravated when these drugs are encapsulated or tableted. Very often the aggregates formed during the preparation of the dosage forms are difficult to disperse. The deaggregation or dispersion rate can, in these instances, be the rate-limiting step in the absorption sequence particularly in the initial phases.

The importance of deaggregation or dispersion in pharmaceutical systems has been recognized for a number of years (5) and recently re-emphasized (6–10). However, there has not been a systematic study carried out to evaluate the rate of deaggregation in pharmaceutical systems, except for two recent studies carried out by Lemberger and Mourad (11, 12). Their studies have dealt with the influence of a number of variables on the deaggregation behavior of oil and water emulsions.

A limited number of deaggregation studies were carried out in nonpharmaceutical systems. These have dealt with rates of systems at equilibrium. For instance, Hiemenz and Vold (13) studied the rates of flocculation and deflocculation of carbon black in hydrocarbon liquids, and Reich and Vold (14) of carbon black and ferric oxide in water. Gillespie (15) determined the rate constants of aggregation and deaggregation of monodisperse latexes in water. Parenthetically, there is also an extensive literature (13) on both the theoretical and experimental aspects of the associated phenomena of aggregation.

The present study was carried out to study quantitatively the deaggregation rates of a relatively insoluble benzoic acid derivative and its sodium salt from different dosage forms. Equations were developed based on the postulate that the deaggregation rate followed a first-order process. The effects of a wetting agent and a dispersant on the rate were evaluated. A correlation between the rates of solution and deaggregation was established.

THEORETICAL CONSIDERATIONS

Smoluchowski (16) showed that deaggregation follows a first-order process. If, therefore, for a particular system there are a number of large aggregates Na, broken down to a number b, of small aggregates N, the following mechanism can be written to describe this occurrence:[1]

$$Na \xrightarrow{k} bN$$

k is described as an apparent rate constant for the deaggregation process. The rate of disappearance of Na with time (t) is then:

$$\frac{dNa}{dt} = -kNa \qquad \text{(Eq. 1)}$$

The following conditions can also be set: at $t = 0$, $Na = Na^0$, $N = 0$; at $t = t$, $Na = Na$, $N = N$; at $t = \infty$, $Na = 0$, $N = Ns$; where Na^0 is the concentration of large aggregates at zero time and Ns is the concentration of small aggregates at the final state.

[1] The reaction is considered irreversible since under the experimental conditions employed in this study, the reverse reaction is negligible.

Received April 14, 1967, from the Product Development Department, Parke, Davis & Co., Detroit, MI 48232
Accepted for publication July 5, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors thank Dr. J. C. Samyn for the Coulter counter measurements, Mr. H. R. Sotherland for preparing the tablets, and Dr. L. M. Wheeler for his interest and encouragement.

Integrating Eq. 1 using the limits $t = 0$ and $t = t$, one obtains:

$$\ln \frac{Na}{Na^0} = -kt \qquad \text{(Eq. 2)}$$

Since the number of small aggregates Ns at the final state is proportional to Na^0, one can set $Ns = bNa^0$. Then:

$$bNa = Ns - N \text{ or } Na = \frac{Ns - N}{b}$$

Equation 2 can now be written in terms of Ns and N:

$$\ln \frac{Na^0}{Na} = \ln \frac{Ns}{Ns - N} = kt \qquad \text{(Eq. 3)}$$

or

$$2.303 \log \frac{Ns}{Ns - N} = kt \qquad \text{(Eq. 4)}$$

The applicability of Eq. 4 in this study is based on the hypothesis that the only species present in the system at any time are the large aggregates Na and/or small aggregates N. This implies that the large aggregates Na are broken down into the small aggregates without going through intermediate stages, or if these are formed, they are extremely short-lived. Experimental verification of the hypothesis was obtained by microscopic examination of samples removed at various times during the runs. To prevent shearing the aggregates during examination, the samples were removed with an inverted pipet and a drop was placed gently on a slide and viewed microscopically without a cover slip.

The number of aggregates N present in the system at any time can be evaluated from the time dependence of any property which is directly proportional to N. In this study, absorbance was used since it could be conveniently determined without disturbing the system during the measurements.

Writing Eq. 4 in terms of absorbance A and rearranging, one has:

$$\log \frac{As}{As - At} = \frac{k}{2.303} t \qquad \text{(Eq. 5)}$$

where As is the absorbance at the final state and At is the absorbance at time t.

If the deaggregation process follows Eq. 5, a plot of $\log As/As - At$ *versus* t should be linear and the rate constant k can be calculated by multiplying the slope of the resulting line by 2.303.

Vold (13), who used absorbance measurements in his studies of deaggregation rates of carbon black, has shown that although aggregates in many suspensions are of irregular shape, they are still likely to be spherically symmetrical if they are built up at random.

It is also apparent that absorbance can be used only if A^0 (absorbance at time zero) is much smaller than As (absorbance at the final condition). Furthermore, the absorbance should be a linear function of the concentration of aggregates present in the system. These two conditions were met in this study. The absorbance at time zero was negligible and the linear relationship between the absorbance and concentration was checked by measuring the absorbance of the aggregates (at the final state) over a hundredfold dilution. Measurements were carried out with a Coleman junior spectrophotometer and repeated with a Beckman DU spectrophotometer. Beer's law was obeyed in each case.

From the absorbance measurements, neither the absolute size of the aggregates nor their concentration in the system can be determined. These measurements are simply an indirect estimate of the size and number of aggregates present, *i.e.*, the total surface area presented by the aggregates to the light beam. However, one may reasonably assume that if the volume of the dispersing liquid, concentration of the drug used, and primary particle size are fixed, the higher the turbidity (*i.e.*, higher the absorbance), the greater is the number of aggregates present in the system. Material balance would then require that the greater the number of aggregates present, the smaller would be their size.

The surface area of the aggregates present at the final condition was obtained from Coulter counter measurements. A correlation between the surface area of the aggregates at the final state and absorbance was established. This was done by taking samples of the aggregates having different absorbance values at the final state and measuring their surface area, using a Coulter counter. A working curve was then prepared by plotting the absorbance *versus* the surface area in cm.^{2}/Gm. The surface area of the aggregates at the final state for other runs was estimated from this curve. The Coulter counter measurements were carried out by diluting a volume of the "slurry" in normal saline solution.

Admittedly, some change could occur during the Coulter counter measurements. Nevertheless, it was felt that the data would be of sufficient value to estimate the relative aggregate sizes.

EXPERIMENTAL

Materials—The benzoic acid derivative used in the studies was prepared by a special procedure to obtain the drug in a small but uniform particle size powder. The drug was further classified by passing through a No. 100 mesh screen (U.S. Standard) and collecting on a No. 120 mesh screen.

Capsules, tablets, and a suspension were prepared from the screened powder. The screened powder was hand filled into No. 1 and No. 00 hard gelatin capsules at a concentration of 250 mg. per capsule. The powder was also preblended with 0.5% of a nonionic polyol surfactant[2] and with 0.5% of the sodium salt of polymerized alkyl naphthalene sulfonic acid,[3] respectively, and made into capsules in a similar manner.

Compressed tablets were made containing 250 mg. of the acid, a disintegrant, a binder, and a filler. Another lot of tablets was prepared containing in addition 1.25 mg. of the polyol surfactant.

The suspension contained 250 mg. of the acid in 5 ml., a water-soluble gum, a preservative, a buffer, and a protective colloid.

The sodium salt was also sieved in the same manner as the acid and hand filled in a No. 1 capsule. A second lot of capsules was prepared containing in addition 0.5% of the polyol surfactant. Each capsule contained 275 mg. of the sodium salt, equivalent to 250 mg. of the acid.

[2] Marketed as Pluronic F-68 by the Wyandotte Chemical Corp., Wyandotte, Mich.

[3] Marketed as Daxad No. 11, Dewey and Almy Chemical Division, W. R. Grace Co., Cambridge, Mass.

The deaggregation studies were carried out in simulated gastric and intestinal fluid T.S. (U.S.P. XVI) without the enzymes. The enzymes were excluded as their turbidity interfered with the absorbance measurements. To compensate for the loss of wetting which the enzymes normally provide, 500 mg. of the polyol surfactant was added to 500 ml. of the test solution.

In carrying out the deaggregation studies with the tablets, it was necessary to add an amylolytic and a cellulolytic[4] enzyme to hydrolyze and solubilize the disintegrant and filler present in the tablet. Ten milligrams of each enzyme was dissolved in the 500 ml. of simulated intestinal fluid T.S., and the solution was filtered prior to use. In this way the turbidity caused by the filler and disintegrant was considerably minimized. It was not possible to determine the deaggregation rates of the tablets in the simulated gastric fluid T.S. since the amylolytic and cellulolytic enzymes have little activity at the low pH of the fluid.

Methodology—Five hundred milliliters of simulated intestinal or gastric fluid T.S. was added to an 800-ml. beaker placed in a constant-temperature bath set at 37° ± 0.5°. The solution was stirred at 70 r.p.m. and allowed to come to temperature. The design of the all glass stirrer used was such that it provided random agitation throughout the depth of the liquid, without creating a vortex. For all the studies, except those with the capsules, an empty capsule was dissolved in the test medium to provide a uniform background for the spectrophotometric measurements.

To study the deaggregation rate of the (loose) powders, 250 mg. of the acid (or 275 mg. of sodium salt) was sprinkled lightly on the surface of the agitated liquid. A stopwatch was started and readings were taken at appropriate intervals of time. Similarly, 5 ml. of the suspension containing 250 mg. of the acid was pipeted directly into 495 ml. of the agitated liquid. The capsules containing the drugs were added directly to the 500 ml. of simulated gastric fluid T.S. The stopwatch was started as soon as the gelatin dissolved and first traces of the drug appeared in the liquid, generally in about 2 min.

The gelatin shell composing the capsule was found to dissolve very slowly in the simulated intestinal fluid T.S. To minimize the difference in the time necessary to dissolve the capsule shell in the two fluids, a slightly different approach was used when studying the deaggregation rates of the capsules in the intestinal fluid. In this case, the capsule was first treated with 50 ml. of simulated gastric fluid T.S. (maintained at 37°) in a 100-ml. graduate. The graduate was gently rocked manually every 10 sec. At the end of 2 min., the contents of the graduate were emptied into 450 ml. of stirred simulated intestinal fluid T.S. also maintained at 37° ± 0.5°. This treatment of the capsule with the simulated gastric fluid T.S. effectively dissolved most of the gelatin leaving a very thin film. The remaining gelatin dissolved within a few minutes after the addition to the simulated intestinal fluid T.S. To neutralize the excess acid and maintain the pH of the simulated intestinal fluid T.S. at pH 7.5, 1.55 ml. of 2 *N* sodium hydroxide was added to it prior to adding the contents of the graduate. The treatment effectively cancelled any difference in the time necessary to dissolve the gelatin shell in the simulated gastric or intestinal fluid T.S.; the lag time in the two media could then be equated more easily.

Spectrophotometric Measurements—The per cent transmission (from which absorbance was calculated) was measured using a Coleman junior spectrophotometer. The wavelength used was 650 mμ. A Manostat Varistaltic pump[5] circulated the fluid *via* a 3 mm. diameter Tygon tubing from the beaker through a flow-through cell located in the spectrophotometer. The diameter of the tubing and the pumping rate provided a continuous flow of the liquid from the beaker through the cell and back, without shear. Thus, measurements could be carried out directly without disturbing the system or necessitating pipeting samples, a procedure which was found to introduce considerable errors.

The force necessary to deaggregate the drugs was provided by the shearing action of the stirred liquid. In each instance, the stirring was continued until the absorbance measured was constant, indicating that the final state had been reached.

Dissolution Studies—Part of the same lot of the acid used in the deaggregation studies was used in the dissolution studies. A 250-mg. quantity of the drug as a loose powder was added rapidly to 500 ml. of simulated intestinal fluid T.S. containing 500 mg. of the polyol surfactant instead of pancreatin. The solution was maintained at 37° ± 0.5° and stirred at 70 r.p.m. with the same glass stirrer described previously. After the addition of the powder, 4-ml. samples were withdrawn from the system at measured time intervals. The sample was filtered through Millipore filters (pore sizes 0.45 μ), using a Swinney syringe adapter. The concentration of the drug in solution was measured spectrophotometrically in the ultraviolet region at 335 mμ after appropriate dilution with the solvent. All spectrophotometric measurements were made with a Beckman DU spectrophotometer. When the solubility and dissolution rates were measured with the drug in the capsules, these were pretreated as described with 50 ml. of simulated gastric fluid T.S. to dissolve the gelatin prior to its addition to the simulated intestinal fluid T.S. The polyol surfactant at the concentration added facilitated the wetting of the powders, without affecting appreciably the solubility of the drug in the medium.

RESULTS AND DISCUSSION

The deaggregation profiles of the acid added to the simulated gastric fluid T.S. as a powder, suspension, and in two capsule sizes, are shown in Fig. 1. The profiles of these dosage forms and also of the tablet in the simulated intestinal fluid T.S. are shown in Fig. 2. These plots of logarithm of per cent transmission *versus* time provide a convenient comparison of the rates. They also compare the relative surface area of the aggregates present at the steady-state condition. The data presented in Figs. 1 and 2 indicate that there is no significant measurable difference in the deaggregation rates in the simulated gastric or intestinal fluid T.S. under the experimental condition used in these studies.

[4] Marketed as Mylase 100 and Cellase 1000, respectively, by the Wallerstein Co., Staten Island, N. Y.

[5] Manostat Corp., New York, N.Y.

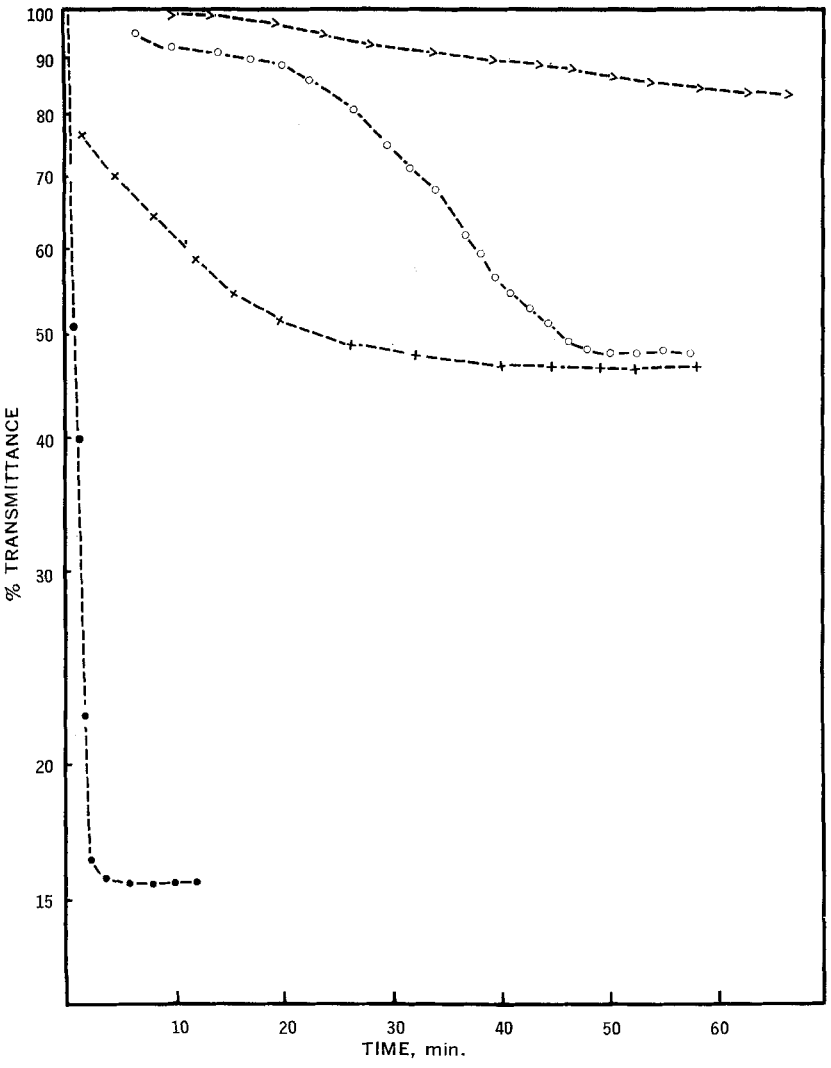

Fig. 1—Plot of per cent transmittance vs. time for the dosage forms studied showing relative rates of deaggregation in simulated gastric fluid T.S. Key: ●, suspension; ×, loose powder; ○, No. 00 capsule; >, No. 1 capsule.

The similarity of the plots also stresses the reproducibility of the experimental method used.

It is readily apparent from these plots that the suspension has the fastest rate in both media, and the aggregates formed have the largest surface area, and therefore the smallest aggregate size at the steady state. This is not surprising since the drug is deaggregated intentionally by homogenization during the manufacture of the suspension. The aggregates which form in the suspension on storage are generally readily redispersed in the test media.

The deaggregation rate of the acid in a No. 1 capsule is the slowest in both the simulated gastric and intestinal fluid T.S. and requires approximately 3 hr. to reach the steady-state condition. The surface area of the aggregates at this condition was similar to that shown for the loose powder. When the acid was packed in a No. 00 capsule, the rate was much faster. Although the steady state was reached sooner, the surface area of the aggregates was again the same as that of the loose powder.

The tablet showed a considerably faster initial rate than the capsules tested in the simulated intestinal fluid T.S.; however, the surface area of the aggregates at the steady state was much smaller. The presence of the disintegrants appeared to aid in breaking up the tablet readily, apparently exposing the granules; however, further deaggregation seemed to be difficult, probably due to the strong cohesive bonds formed between the primary particles during the granulation and compression processes.

The data shown in Figs. 1 and 2 are replotted in accordance with Eq. 5 in Figs. 3 and 4. The deaggregation rates of the acid added to the simulated gastric and intestinal fluid T.S. as a loose powder and as a suspension follow closely a first-order process. The apparent rate constant k, for the deaggregation in simulated gastric fluid T.S., calculated from the slope of each of these lines is given in Table I. The apparent rate constants for the deaggregation in simulated intestinal fluid T.S. are given in Table II.

The deaggregation process for the drug in the capsules, in both the simulated gastric and intestinal fluid T.S., shown in Fig. 4, seems to be more complicated. The curves for the capsules emerge from the abscissa with a very small slope which continues

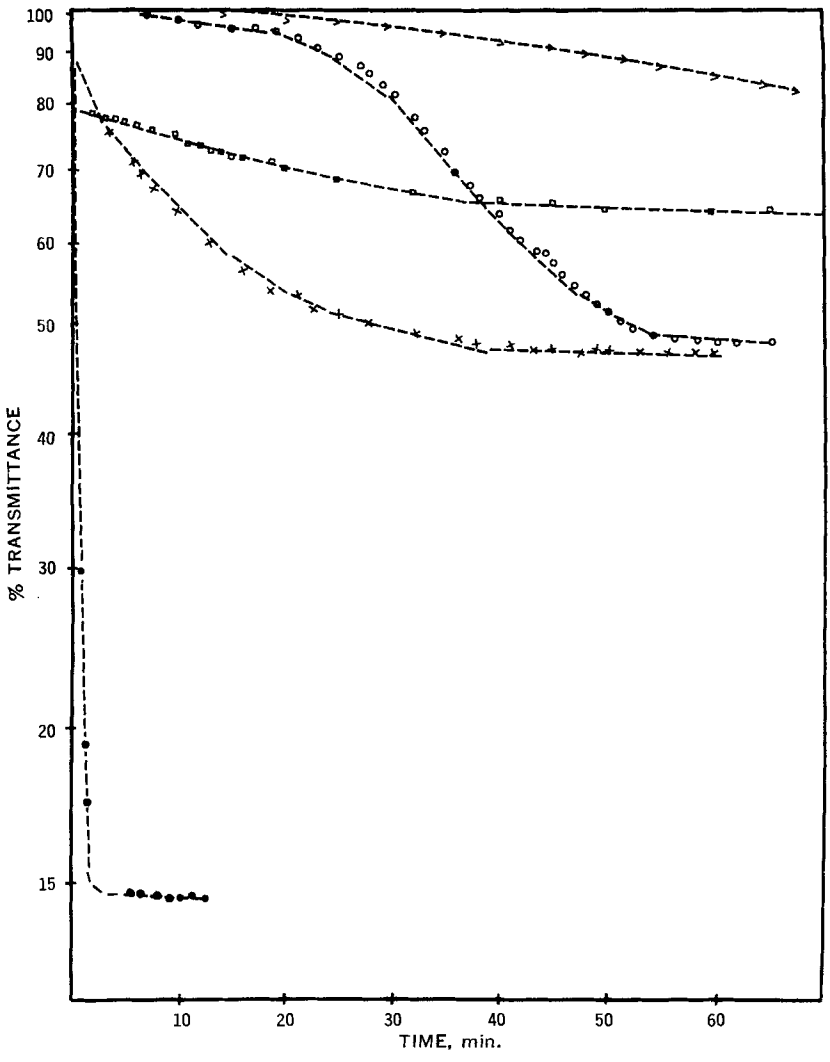

Fig. 2—Plot of per cent transmittance vs. *time for the dosage forms studied, showing relative rates of deaggregation in simulated intestinal fluid T.S. Key:* ●, *suspension;* ×, *loose powder;* □, *tablet;* >, *No. 1 capsule;* O, *No. 00 capsule.*

over a period of time; the curve then gradually bends upward and continues as a long straight line. There is, therefore, a lag time before the rate follows a first-order process. This lag time was apparently a function of the degree of compactness of the powder since it was longer for the smaller capsule. The rate was also approximately three times faster for the No. 00 than for the No. 1 capsule.

The observed deaggregation rate of the tablet in the simulated intestinal fluid T.S., shown in Fig. 4, was also complicated and indeterminate initially apparently due to the turbidity caused by the disintegrant and filler as they were released in the medium. However, after they were broken down and solubilized by the enzymes, the rate followed a first-order process fairly closely.

The apparent rate constant k, time necessary to reach the final state, and the estimated surface area at this state determined from Coulter counter measurements in the simulated gastric fluid T.S. are given in Table I and in the simulated intestinfluid T.S. are given in Table II.

The effects of compacting the powder in the capsules are evident from Table I. For example, the estimated surface areas for the aggregates of the acid at the final state are very close for the loose powder and two capsule sizes. However, the rate of deaggregation of the drug in the larger capsule was approximately two times faster than that in the smaller one. Similarly, the time to reach the final state was also faster for the drug in the No. 00 capsule.

The data in Table II for the deaggregation measurements in simulated intestinal fluid T.S. also emphasize the same aspects. They also show that while the deaggregation rate for the tablet was relatively fast compared to the smaller capsule, and the final state was reached in only 50 min. *versus* 180 min. for the capsule, the estimated surface area of the aggregates at the final state was considerably smaller than that obtained with the other dosage forms in the same medium.

It is difficult to assess *a priori* which of these would govern the rate of absorption. However, one can assume that in the initial phases the deaggregation rate, lag time, and time to reach the final state would be more important than the final surface area of the aggregates, for the simple reason that

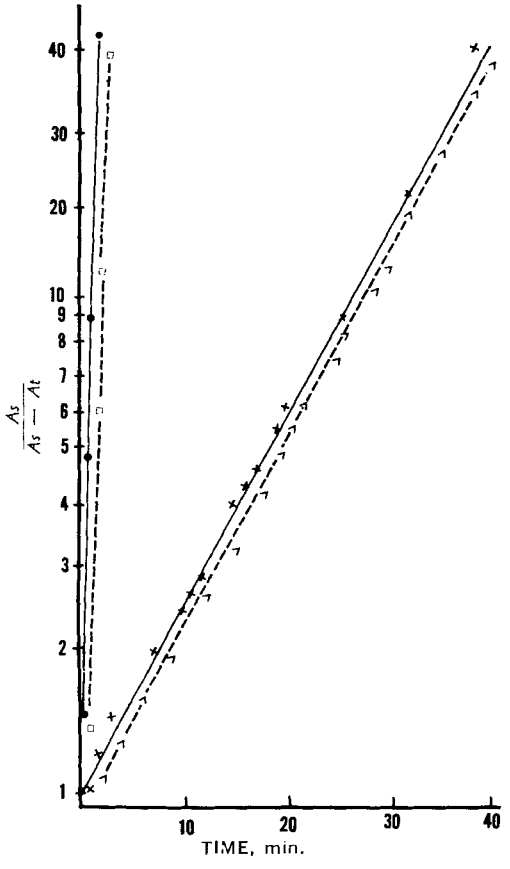

Fig. 3—Plot showing deaggregation rates of the acid as a loose powder and in suspension in simulated gastric fluid T.S. and intestinal fluid T.S. Key: ●, suspension in simulated intestinal fluid T.S.; □, suspension in simulated gastric fluid T.S.; ×, loose powder in simulated intestinal fluid T.S.; >, loose powder in simulated gastric fluid T.S.

they make the drug available earlier for dissolution in the gastrointestinal fluids at the absorption sites. The surface area would become more important after the initial exposed drug is dissolved and the rate of solution becomes dependent only on the area. Ideally, the fastest dissolution and absorption would occur in a preparation which is deaggregated rapidly, and in which the surface area of the aggregates at the final state is large. The drug in a suspension has these characteristics and would

TABLE I—COMPARISON OF THE DATA FOR DEAGGREGATION OF THE ACID IN SIMULATED GASTRIC FLUID T.S.

Dosage Form	Lag Time, min.	k, min.$^{-1}$	Time to Reach Final State, min.	Estimated Surface Area of the Aggregates at the Final State, cm.²/Gm.
Powder	0	1.94	38	5,500
Suspension	0	41.0	3.5	44,500
No. 1 capsule	51	0.28	170	5,400
No. 00 capsule	34	0.74	48	5,400

TABLE II—COMPARISON OF THE DATA FOR DEAGGREGATION OF THE ACID IN SIMULATED INTESTINAL FLUID T.S.

Dosage Form	Lag Time, min.	k, min.$^{-1}$	Time to Reach Final State, min.	Estimated Surface Area of the Aggregates at the Final State, cm.²/Gm.
Powder	0	1.94	42	6,000
Suspension	0	41.3	3	45,000
No. 1 capsule	53	0.25	180	6,000
No. 00 capsule	39	0.65	54	6,000
Tablet	5	0.41	50	3,000

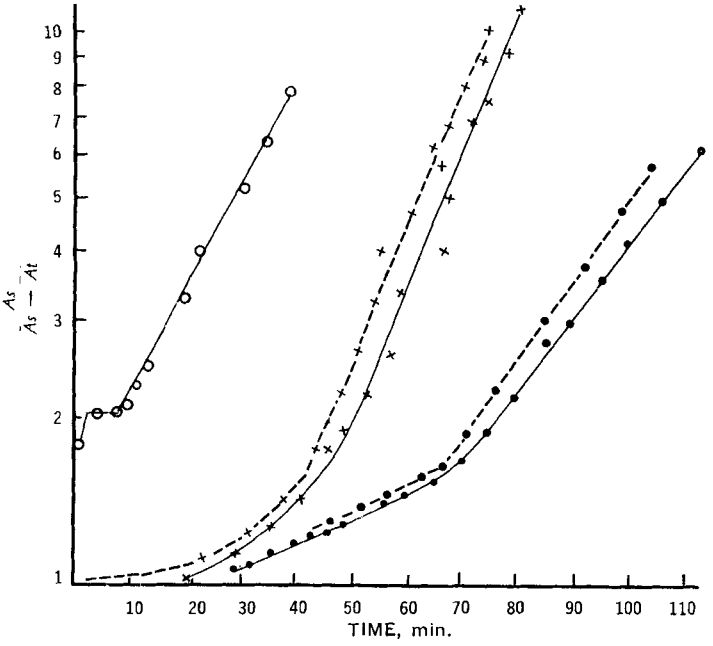

Fig. 4—Plot showing the deaggregation rates of the acid dosage forms in simulated gastric fluid T.S. and intestinal fluid T.S. Key: ○, tablet; ●, No. 1 capsule; ×, No. 00 capsule; ——, simulated intestinal fluid T.S.; – – –, simulated gastric fluid T.S.

therefore be absorbed more rapidly, since the drug is exposed to the dissolving medium at the site of absorption rapidly and the dissolution rate would be fast because of the large surface area present.

The effect on the deaggregation rate of adding 0.5% of the polyol surfactant to the acid powder is shown in Fig. 5. The presence of the surfactant slowed the initial rate, possibly due to the tackiness it induced in the powder; however, the rate was increased perceptibly in the later stages. The exact mechanism by which the surfactant increased the rate was not clear and is under investigation at the present time. It could be due simply to the enhanced wetting of the powder by the liquid or coating of the surface of the particles, preventing their adhesion.

It is also seen from Fig. 5 that the sulfonate salt at the same concentration also increased the deaggregation rate of the powder in the simulated intestinal fluid T.S. The sodium salt of polymerized alkyl naphthalene sulfonic acid cannot be classified as a true wetting agent (17) since it does not reduce the interfacial tension between the powder and the

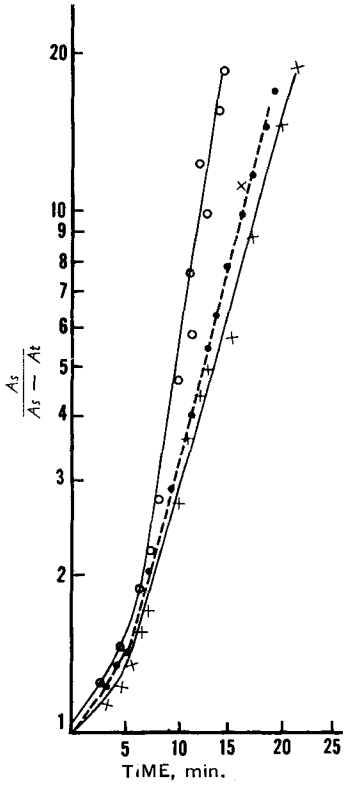

Fig. 5—Plot showing the effect of 0.5% nonionic polyol surfactant on the deaggregation rates of the acid as a loose powder in simulated gastric fluid T.S. and intestinal fluid T.S., and the effect of 0.5% sodium salt of polymerized alkyl naphthalene sulfonic acid on the rate of powder in simulated intestinal fluid T.S. Key: ●, loose powder with 0.5% polyol surfactant in simulated gastric fluid T.S.; ×, loose powder with 0.5% polyol surfactant in simulated intestinal fluid T.S.; ○, loose powder with 0.5% sulfonic acid salt in simulated intestinal fluid T.S.

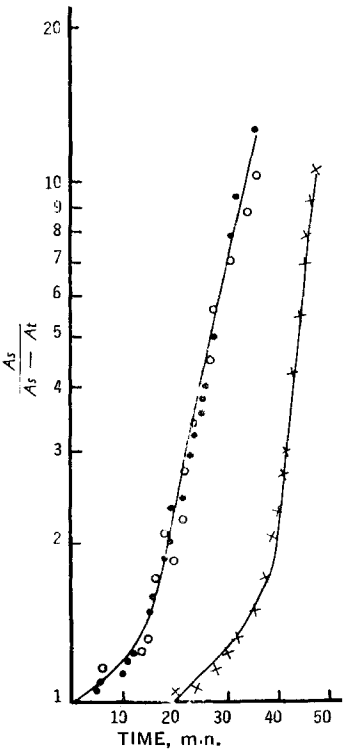

Fig. 6—Plot showing the effect of 0.5% sulfonic acid salt and polyol surfactant on the deaggregation rate of the acid in No. 1 capsule. Key: ×, No. 1 capsule with 0.5% sulfonic acid salt in simulated intestinal fluid T.S.; ●, No. 1 capsule with 0.5% polyol surfactant in simulated intestinal fluid T.S.; ○, No. 1 capsule with 0.5% polyol surfactant in simulated gastric fluid T.S.

TABLE III—EFFECT OF 0.5% OF THE POLYOL SURFACTANT AND OF THE SULFONATE SALT ON THE DEAGGREGATION OF THE ACID IN SIMULATED GASTRIC FLUID T.S. AND INTESTINAL FLUID T.S.

Dosage Form	Lag Time, min	k, min.$^{-1}$	Time to Reach Final State, min.	Estimated Surface Area of the Aggregates at the Final State, cm.^{2}/Gm.
Powder + 0.5% polyol surfactant (gastric and intestinal T.S.)	4	2.76	23	15,000
Powder + 0.5% sulfonate salt (intestinal T.S.)	4	2.92	20	26,000
No. 1 capsule + 0.5% polyol surfactant (gastric and intestinal T.S.)	12	1.01	40	13,000
No. 1 capsule + 0.5% sulfonate salt (intestinal T.S.)	37	2.3	52	24,000

liquid. Its action is not completely understood, but it seems (17) that it induces like charges on the individual particles so that they repel each other, thus preventing strong interparticulate bonds being formed.

The addition of 0.5% of the polyol surfactant and of the sulfonate salt to the acid in the capsule also has a significant effect on the deaggregation rate. The data are shown in Fig. 6. It is evident that the polyol surfactant effectively decreases the lag time, probably due to its wetting properties and also increases the deaggregation rate. The sulfonate salt also increases the deaggregation rate; however, its effect on the lag time is not so great as that of the surfactant. The surface area of the aggregates for the drug containing 0.5% sulfonate salt in No. 1 capsule was also larger than that for the polyol surfactant at the same concentration. The data are summarized in Table III.

Deaggregation Rates of the Sodium Salt—Only simulated gastric fluid T.S. was used to evaluate the deaggregation characteristics of the sodium salt since it is soluble in the simulated intestinal fluid T.S. It should be pointed out that the deaggregation rates measured are actually those of the nascent acid, since the sodium salt is rapidly converted to the acid when it is exposed to the simulated gastric fluid T.S.

The results of determinations with the sodium salt in the simulated gastric fluid T.S. are plotted in accordance with Eq. 5 in Fig. 7. It is seen that the loose powder again showed the fastest rate. By encapsulating the drug in a No. 1 capsule, the rate was considerably slowed. It was observed during the determinations that after the removal of the gelatin shell from the capsule, the salt exposed to the simulated gastric fluid T.S. was converted to the acid. The action of simulated gastric fluid T.S. on the exposed salt formed an acid mantle around the unconverted sodium salt, maintaining the shape of the capsule and preventing the liquid from reaching the inner core. It is also obvious from Fig. 7 that the addition of the polyol surfactant had a limited effect on the rate when the salt was encapsulated. It should be pointed out that of all the determinations, the deaggregation rate of the sodium salt with the polyol surfactant in the No. 1 capsule was the least reproducible. The reason is probably that a nonuniform acid mantle forms on repeated runs when the gelatin is removed, exposing uneven pockets of the surfactant at the surface.

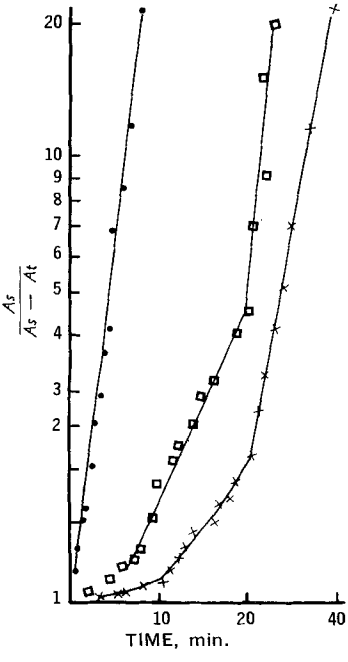

Fig. 7—Plot showing the deaggregation rate of the sodium salt in simulated gastric fluid T.S. as a loose powder, in No. 1 capsule, and in No. 1 capsule with 0.5% polyol surfactant. Key: ●, loose powder; □, No. 1 capsule with 0.5% polyol surfactant; ×, No. 1 capsule.

TABLE IV—COMPARISON OF THE RATE CONSTANT, LAG TIME, AND TIME TO REACH STEADY STATE FOR THE SODIUM SALT IN SIMULATED GASTRIC FLUID T.S.

Dosage Form	Lag Time, min.	k, min.$^{-1}$	Time to Reach Final State, min.
Powder	...	9.21	9
No. 1 capsule	18	6.12	30
No. 1 capsule + 0.5% polyol surfactant	≈15	8.0	24

The rate constant, calculated from the slope of these lines, lag time, and time to reach the steady state are summarized in Table IV. The data presented in Table IV stress the influence which the surfactant had on the deaggregation rate, time to reach the final state, and lag time when the salt was encapsulated.

DISSOLUTION STUDIES

The results of rate of solution studies carried out with the acid as a loose powder and in different dosage forms are shown in Fig. 8. It is obvious that the drug in suspension has the fastest dissolution rate and reaches the plateau level in approximately 8 min. On the other hand, the acid in a No. 1 capsule shows the slowest rate. These results appear to correlate with the deaggregation rates, which is to be expected, as it is well established that the dissolution rate is dependent on the surface area exposed to the dissolving media. Since the suspension deaggregates very rapidly, the surface area presented is much greater than that of the capsules or other dosage forms studied.

The dissolution behavior of the acid in a tablet also reflects its deaggregation pattern. The tablets broke up readily in the medium exposing some of the drug to the dissolving medium. This gave a comparatively fast dissolution rate in the initial phases. However, as the exposed drug dissolved, the rate of solution at the later time became solely dependent on the surface area of the large aggregates present, and the dissolution rate was considerably slowed.

The effect on the dissolution rate of adding 0.5% polyol surfactant to the acid powder, tablet, and No. 1 capsule is demonstrated in Fig. 9. It is apparent that the presence of polyol surfactant increased significantly the dissolution rate of the acid as a loose powder and in the No. 1 capsule. The increase in the dissolution rate of the powder and capsule can again be attributed to the faster

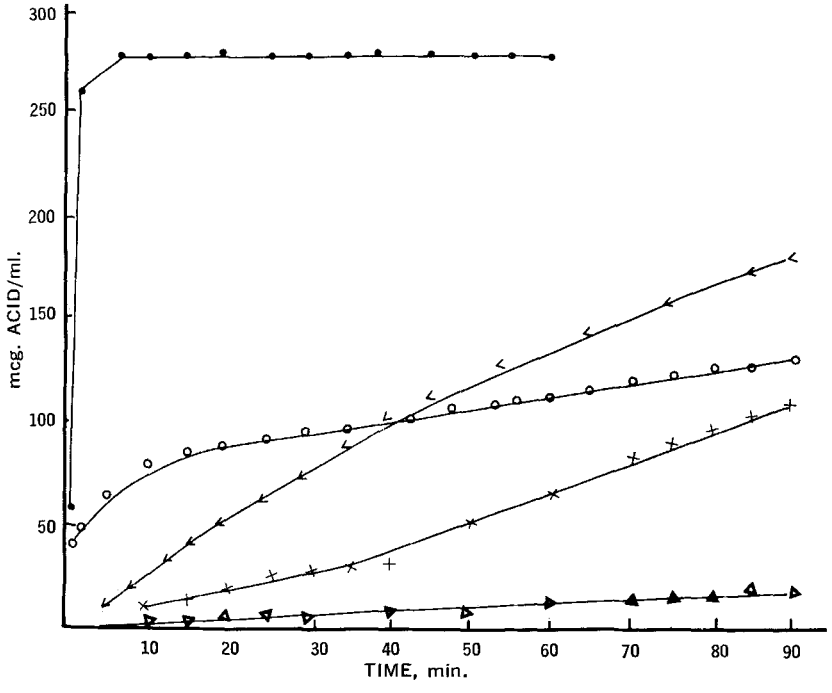

Fig. 8—Plot showing the dissolution rates of various dosage forms of the acid. Dissolution rate measured in simulated intestinal fluid T.S. Key: ●, suspension; O, tablet; <, loose powder; ×, No. 00 capsule; ▶, No. 1 capsule.

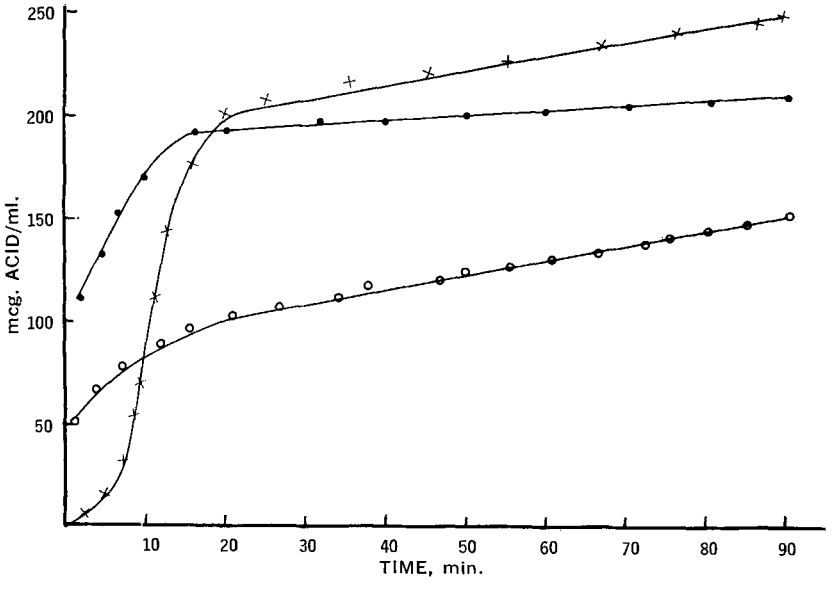

Fig. 9—Plot showing the effect of 0.5% polyol surfactant on the dissolution rates of the acid as a tablet, in a No. 1 capsule, and as a loose powder. Dissolution rates measured in simulated intestinal fluid T.S. Key: O, tablet with 0.5% polyol surfactant; ×, loose powder with 0.5% polyol surfactant; ●, No. 1 capsule with 0.5% polyol surfactant.

deaggregation rates induced by the surfactant in these dosage forms. The effect of tackiness, due to the surfactant, in the initial phases of the dissolution of the powder was also evident.

The effect of the surfactant on the deaggregation rate of the tablet was small, and this was reflected in the small increase in the rate of solution. Possibly higher concentrations of the surfactant are necessary to break up and disperse the aggregates.

BIOPHARMACEUTICAL IMPLICATIONS

The study emphasizes the importance of deaggregation rates. If the absorption is dependent on the dissolution rates, which in turn are dependent on the rates of deaggregation, the study would predict that the highest and earliest blood levels would be obtained with the suspension followed in order by the sodium salt, the acid as a loose powder, in the No. 00 capsule, in the tablet, and last the No. 1 capsule. It is probable that the addition of a surfactant to the acid to increase the deaggregation rate would also increase the absorption rate.

The absorption profiles in man of the acid and sodium salt are currently being investigated in these laboratories. Preliminary results of these investigations support the findings of these studies, and will be published elsewhere.

Recently Wagner and his co-workers (10) studied the effect of dosage form on serum levels of indoxole. They found that the drug administered as a suspension produced a greater serum level response than did the hard gelatin capsules, although both were prepared from the same lot of drug with identical small particle size. They state that the most likely explanation is that the indoxole particles were less agglomerated when administered in suspension form than when given in capsules, and the surface area of indoxole presented for dissolution in the gastrointestinal fluids at the absorption sites must have been greater with the suspension.

In a recent publication, Higuchi *et al.* (6) cited various other examples where the deaggregation rates were the controlling factors in absorption.

It would appear that in dealing with insoluble drugs which are difficult to wet and disperse, the rate of deaggregation from different dosage forms should be routinely determined, just as the rate of solution, particle size, and polymorphic forms are investigated.

REFERENCES

(1) Atkinson, R. M., Bedford, C., and Child, K. J., *Antibiot. Chemotherapy*, **12**, 232(1962).
(2) Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961).
(3) Lees, K. A., *J. Pharm. Pharmacol.*, **15**, 43T(1963).
(4) Nelson, E., *Clin. Pharmacol. Therap.*, **3**, 673(1962).
(5) Wheeler, L. M., private communication.
(6) Higuchi, T., Finger, K. F., and Higuchi, W. I., in "Annual Reports in Medicinal Chemistry, 1965," Cain, C. K., ed., Academic Press Inc., New York, N.Y., 1966, p. 331.
(7) Calesnick, B., Katchen, B., and Black, J., *J. Pharm. Sci.*, **54**, 1277(1965).
(8) Wood, J. H., *ibid.*, **54**, 1207(1965).
(9) Hollister, L. E., and Kanter, S. L., *Clin. Pharmacol. Therap.*, **6**, 5(1965).
(10) Wagner, J. G., Gerard, E. S., and Kaiser, D. G., *ibid.*, **7**, 610(1966).
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(12) *Ibid.*, **54**, 233(1965).
(13) Hiemenz, P. C., and Vold, R. D., *J. Colloid. Sci.*, **20**, 635(1965).
(14) Reich, I., and Vold, R. D., *J. Phys. Chem.*, **63**, 1497 (1959).
(15) Gillespie, T., *J. Colloid. Sci.*, **15**, 313(1960).
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(17) Technical Bulletin, Dewey and Almy Chemical Division, W. R. Grace & Co., 1954.

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Mouse fibroblast cells, strain L-929, in static tube culture were exposed to 6, 9, and 12 mmole doses of triethyl citrate (TEC) for periods up to 78 hr. At 1.5, 48, and 78 hr. after TEC exposure, the cells were pulsed with ^{14}C-labeled purine and pyrimidine metabolic precursors in an attempt to differentiate the site of purine–pyrimidine metabolism interference. U.V. spectrophotometric analyses were run concurrently with liquid scintillation analyses. The mechanism of inhibition appeared to be initial mitochondrial involvement, with secondary DNA repression.

SINCE ALL pharmacology and toxicology begins ultimately at the cellular level, it seems only logical to elucidate mechanisms involving basic biochemical pathways by way of cellular techniques, and project or apply these findings to the whole organ or body. The advantages of cell culture in a variety of applications are many. Highly specialized cells, differentiating cells, or abnormal cells may be used so that drug effects may be seen under widely varying circumstances. Another convenience is that quantitative evaluation of activity is possible, whereas pharmacodynamic quantitization in the intact animal is often impossible due to the nature of the compound or of the system. Using cell culture techniques, one can test solids, liquids, and gases, and evaluate changes both to the cells and to the growth medium (1–3).

Recently in this laboratory, Rosenbluth *et al.* (2), found that triethyl citrate (TEC), a commonly used plasticizer for polyvinyl chloride resin, was a growth inhibitor to mouse fibroblast cells, strain L-929, in static tube culture at 35° at several dose levels. Through a series of simple experiments, he was able to eliminate chelation of calcium ions as the mechanism of triethyl citrate toxicity. By correlating parachor and other physiochemical values assigned to triethyl citrate, it was postulated that TEC is probably a physically toxic compound. Triethyl citrate is relatively lipid soluble. Being

Received April 14, 1967, from the Drug-Plastic and Toxicology Laboratory, College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication June 19, 1967.
Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This research was supported in part by grant CA-06120 from the National Institutes of Health, and contract PH43-64-557 from the Division of Biologic Standards, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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REFERENCES

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soluble in water only to the extent of 6.25% w/v, it would be classified according to Ferguson (4) on the basis of its thermodynamic activity as a physical toxicant as opposed to a chemically specific toxicant. Thermodynamic activity is an expression of threshold activity concentration relative to its solubility in water. Because of its lipid solubility and molecular volume, triethyl citrate should have a certain affinity for proteins, nucleic acids, lipoproteins, enzymes, and other macromolecules normally found within the cell and in the cell membranes.

From the previous studies, it was known that triethyl citrate in some way interfered with purine and pyrimidine metabolism to the extent that cell growth was inhibited relative to the dose. The interference need not be a direct effect on mitotic cells, since purines and pyrimidines are indispensable in energy metabolism and cytoplasmic synthetic activities as well as chromosomal replication.

In the above-mentioned study, the method of analysis did not differentiate the site of interference, but merely represented total activity. Since it is widely accepted that thymidine and uridine are precursors of the nucleic acids, DNA and RNA (5), respectively, their uptake could easily be adapted to the original method of McIntire and Smith (6). If adenine, a constituent of the mitochondrial enzymes, adenosine triphosphate (ATP), and the nucleic acids were assayed simultaneously within the same cell population, a more complete profile of purine and pyrimidine metabolism could be easily obtained.

Consequently, it was decided to follow purine and pyrimidine utilization with ^{14}C-labeled precursors and liquid scintillation techniques in an attempt to explain the mechanism of triethyl citrate toxicity. The liquid scintillation study was designed to analyze the parameters of exposure time to TEC, concentration effects, and precursor utilization within each time period and concentration.

MATERIALS AND METHODS

Cell Culture—Mouse fibroblast cells, strain L-929[1] were maintained in static culture in 120 ml. prescription bottles or in Blake culture bottles at 35° in a 5% carbon dioxide atmosphere. Eagle's basal medium 1X (1) supplemented with 1% L-glutamine,[1] 4% calf serum, and 0.5% streptomycin sulfate (Pfizer) per milliliter was used routinely. At regular intervals, the cells were subcultured with the aid of a 0.05% trypsin solution.

Static Tube Culture—The mouse fibroblast cells used in the study were placed in Eagle's basal medium (BME 1X) with 10% calf serum and 1% L-glutamine. Cell population was determined by hemocytometer count and volume adjustments made to provide 3.0×10^5 cells per 1.5 ml. The cells were delivered into sterile disposable culture tubes by a Cornwall automatic pipet. An additional 1.0 ml. of medium (BME 1X) was added to control tubes for assay at 48 and 78 hr. To the test sample tubes was added 1.0 ml. of triethyl citrate[2] at the desired concentrations dissolved in BME 1X. The tubes were covered with sterile aluminum or plastic closures, placed upright in racks in the incubator, and withdrawn at the specified time intervals for pulse-labeling and assay procedures. All tubes were left in the incubator for 1.5 hr. prior to initial assay to allow for resumption of normal cellular activities which are disturbed during the subculture and inoculation techniques.

The tubes to be used in liquid scintillation studies were pulsed by the addition of 1.0 ml. of ^{14}C-purine and pyrimidine precursors[3] dissolved in sterile deionized water to give an activity of 0.15 μc./ml. One milliliter of Eagle's basal medium 2X (7) was added to ensure tonicity of the radioisotope solution. The tubes were then incubated for 1 hr., at the end of which time they were assayed for total purine–pyrimidine content (2).

Assay Procedure for Absorbance Readings—Tubes for initial assay were centrifuged at 2000 r.p.m. for 15 min. No others were centrifuged unless the cells appeared to be loose or had the tendency to be washed from the test tube walls. The supernatant medium was decanted, and the cells were washed twice with 2.5-ml. portions of phosphate buffered saline solution (PBS 1X) (2), to remove color interference. Cells were then covered with 5.0 ml. of 0.3 N H_2SO_4 in 20% NaCl. The closures were replaced and the tubes placed in a sterilizer, covered, and maintained at 100° for 30 min. The tubes were quickly removed to a 4° refrigerator for 20 min. At the end of this period, all tubes were centrifuged at 2000 r.p.m. for 15 min. The resulting supernatant solution was drawn off with a bulb-tipped capillary pipet and read in a quartz cell against the acid-salt blank in a Beckman DB spectrophotometer at 268 mμ (3).

Assay Procedure for Liquid Scintillation—The supernatant medium was decanted after the 1-hr. incubation period, and the cells were washed twice with 2.5-ml. portions of PBS 1X to remove all unbound, extracellular, radioactive material. Centrifugation was carried out only when cells appeared loose from the test tube walls. The cells were then covered with 0.5 ml. of 0.25% trypsin[4] to remove the monolayer from the glass. After trypsinization, the cells were covered with 1.0 ml. of 0.3 N H_2SO_4. Tubes were recapped and boiled at 100° for 30 min., then quick-cooled in a 4° refrigerator for 20 min. After centrifuging at 2000 r.p.m. for 15 min., the supernatant solution was decanted into 15 ml. of the liquid scintillation solvent mixture. A 1.0-ml. quantity of 0.3 N sulfuric

[1] All media supplements obtained from Microbiological Associates, Baltimore, Md.

[2] Citroflex 2, Chas. Pfizer & Co.

[3] Adenine was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Thymidine and uridine were obtained from International Chemical and Nuclear Corp., City of Industry, Calif.

[4] Trypsin, 2.5%, 10%; PBS 1X, 15%; sterile deionized water, 75%.

acid wash was added to the scintillation cocktail to make a total of 17.5 ml. The scintillation cocktail, a modified Bray's solution (8), was composed of dioxane–ethylene glycol monomethyl ether–xylene[5] (12:2:1) with an additional 4.2% of a premix solution (50 Gm. PPO and 0.625 Gm. POPOP/L. of toluene).[6] Each Wheaton snapcap potassium-free counting vial was appropriately labeled, placed in the refrigerated detection chamber, and allowed to cool to −7.0° before counting. Control vials contained 15.0 ml. of scintillation solvent, 0.5 ml. of 0.25% trypsin solution, 1.0 ml. of 0.6 *N* H_2SO_4, and 1.0 ml. of radioisotope with an activity of 0.15 μc./ml.

Counting Technique—Counting was performed with a Nuclear-Chicago, series 720, liquid scintillation spectrometer. The channels ratio method (9) was used to correct for quenching with an efficiency of 49%.

RESULTS

All data were compiled by summing the means of four replicate experiments and obtaining the ratios of treated to control cells. Variance analysis was used to determine statistical significance within the parameters of time, concentration, and precursor utilization. Unless otherwise mentioned, all changes referred to as significant were at the 95% confidence level or greater.

Liquid scintillation data showed that within all concentrations of TEC used, adenine utilization was decreased significantly (C.L. >90%) at the initial time period, with respect to controls, and to the other precursors. At a TEC dose level of 6 mmoles, however, adenine uptake was apparently resumed after 48 hr. (Fig. 1.) At a dose level of 9 mmoles, adenine uptake was decreased at 1.5 hr. and at 48 hr., but had resumed normal utilization at 78 hr. (Fig. 2). At a dose level of 12 mmoles, adenine uptake was more significantly reduced than at other dose levels and continued to decrease throughout the 78-hr. period. (Fig. 3.)

Thymidine incorporation was not significantly affected by a dose of 6 mmoles over a time period of 78 hr. However, at 9 mmoles, it decreased significantly at 48 hr. and began an upward trend again at 78 hr. The same pattern was true of the 12-mmoles TEC dose, except that decreased uptake was more pronounced at all time periods.

At a TEC dose level of 6 mmoles, uridine incorporation was decreased only with respect to controls, and by 78 hr. had exceeded control uptake. It was more significantly decreased at the 9-mmole dose level at 1.5 hr., but again resumed uptake by 48 hr. and exceeded that of controls at 78 hr. There was little difference between 9 and 12 mmole TEC dose effects on uridine uptake at all time periods.

Adenine uptake levels remained significantly lower than thymidine and uridine in all TEC dose levels. Uridine uptake increased significantly over adenine and thymidine at 48 to 78 hr., but only at the 9 and 12 mmoles TEC dose levels.

From the standpoint of time of exposure of the cells to TEC, the initial assay revealed a more significant decrease in uridine and adenine utilization than in thymidine, regardless of dose. Adenine

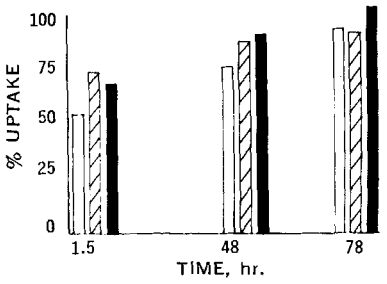

Fig. 1—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 6-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

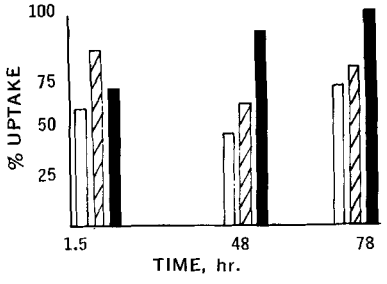

Fig. 2—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 9-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

uptake was most depressed at the 12 mmole TEC level at 48 hr., while thymidine did not vary appreciably among 9 and 12 mmole TEC doses during this time period. At all time periods, uridine uptake was significantly higher at 9 and 12 mmole dose levels than at a 6-mmole dose with respect to other precursors. Of all three precursors, adenine incorporation into the cell was lowered to the greatest extent at all dose levels. The decreased adenine uptake continued with time only at 12 mmoles, while at the other dose levels, it recovered and approached normal values by 78 hr. Thymidine remained relatively unchanged with respect to TEC dose level except at 48 hr. where a significant decrease in uptake was observed. Uridine uptake increased with both time and dose, and was therefore not inhibited in its incorporation into the cell by TEC.

Spectrophotometric data yielded results similar to those obtained in previous studies (Fig. 4). Liquid scintillation data were compared to spectrophotometric data to ascertain what fractions each of the labeled purines and pyrimidines occupied in the total cell extract. It should be realized at this point that the cell extracts for both spectrophotometry and liquid scintillation contained total purines and pyrimidines, but that the extract for liquid scintillation "read out" only the ^{14}C-labeled purines and pyrimidines. The differences between readings on the two instruments can be accounted for by guanine and cytosine, the base pairs found in both RNA and DNA, as well as other areas of intermediary metabolism.

Ratios of uptake percentages taken from counts per minute and absorbance (CPM/*A*) showed the rel-

[5] All solvents were Fisher, reagent quality.
[6] Liquifluor, Nuclear-Chicago Corp.

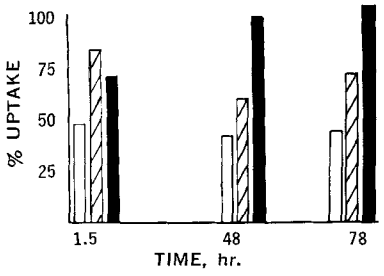

Fig. 3—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 12-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

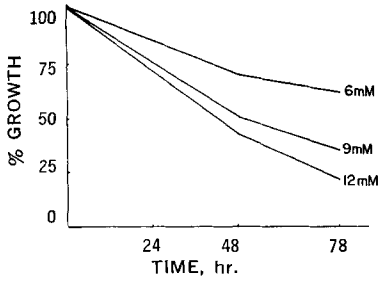

Fig. 4—Percentage growth of L cells after exposure to TEC, assayed for total purine–pyrimidine content by U.V. spectrophotometry at 268 mμ.

ative quantities of each precursor in the total purine and pyrimidine content. These ratios are higher than true values because labeled guanine and cytosine were not introduced into the system in this study.

Assuming there is no significant change between initial absorbance values and those at 1.5 hr., the zero-time value was derived by taking the ratio of the individual precursor in question to total precursors as expressed by counts per minute under each of the three initial assay conditions and obtaining an average value. Since there is no real number, *per se*, for initial assay by absorbance, the absorbance values were representative of 100% growth.

At all dose levels, adenine had an approximate CPM/*A* ratio of 0.24 initially, 0.63 at 48 hr., and 1.45 at 78 hr. Thymidine had a CPM/*A* ratio of about 0.66 initially, 0.51 at 48 hr., and 0.91 at 78 hr. Uridine gave a ratio of about 0.13 initially, 0.82 at 48 hr., and 1.25 at 78 hr. There occurs, then, the general tendency for uridine/adenine and adenine/thymidine ratios to increase significantly with time. These data essentially substantiated the liquid scintillation data, indicating a major, over-all increase in uridine content when compared to the other purines and pyrimidines. (Table I.)

In general then, only the 9 and 12-mmole dose levels of TEC showed significant effects on cellular utilization of the test precursors except at initial assay. At both the 9 and 12-mmole dose levels, however, adenine was apparently inhibited preferentially and did not recover to normal values.

TABLE I—FRACTION OF EACH ^{14}C-LABELED PRECURSOR IN TOTAL PURINE–PYRIMIDINE EXTRACT AS EXPRESSED BY ABSORBANCE AT 268 mμ

^{14}C-Labeled Precursor	CPM/*A* $_{268\ m\mu}$ Time of Assay After Exposure to TEC, hr. 1.5	48	78
Adenine	0.24	0.63	1.45
Thymidine	0.66	0.51	0.91
Uridine	0.13	0.82	1.25

DISCUSSION

The immediate and sustained adenine decrease was one of the most noticeable effects of treatment of mouse fibroblasts by triethyl citrate. With this in mind, one should first look into the possibility of mitochondrial involvement since generation of adenosine triphosphate (ATP) occurs within this particular organelle. The energy processes involving mitochondria are electron transport, oxidative phosphorylation, and the citric acid cycle. The substrates oxygen, adenosine diphosphate, and inorganic phosphate enter the mitochondria and leave as carbon dioxide, water, and ATP. ATP is a high energy phosphate-ester moiety which mediates reactions such as cleavage of molecules, synthesis and coupling of molecules, transfer of materials across cell membranes, protein synthesis, enzyme synthesis, lipid metabolism, and nucleic acid metabolism. Without ATP, there would occur an increase in entropy and an alteration in balance of endergonic biological processes (10).

Adenine is also involved in the coenzymes participating in the electron transport system within the mitochondria, and in acetyl coenzyme A, which mediates glucose, and fatty acid metabolism. Blockage of either the electron transport system or proper metabolism of coenzyme A would favor inhibition of ATP production, accumulation of polysaccharides and lipids, and decreases in synthetic processes such as DNA and RNA polymerization.

It is of interest that uridine uptake increased while there was a concurrent decrease in adenine utilization and a relative decrease in thymidine uptake. The fact that thymidine decreased proportional to the increase in uridine, but tended to remain fairly constant within a 78-hr. period indicated that there was little or no chromosomal replication and cell duplication (11, 12, 15, 17). This could in part account for the observed inhibitory effect on cellular growth by TEC. It is, however, still possible for RNA to be synthesized as evidenced by the now common knowledge that RNA synthesis continues in the G_2 phase after DNA synthesis has stopped (14) and that "heterochromatin" of a nongenetic nature is apparently the source of a large amount of RNA which functions in the metabolism of the cell (12, 13). As long as there is DNA template available, RNA, and particularly messenger RNA, will be synthesized. It is quite possible, then, that as long as sufficient precursors, primers, and ATP are available, RNA synthesis will continue (14, 16). However, it seemed that in this study, the amount of uridine accounted for by liquid scintillation far exceeded that of adenine, and was so disproportionate that

synthesis of RNA after 48 hr. would be impossible. This is only reasonable since adenine not only is a major constituent of RNA, but is necessary in the form of ATP for the polymerization of RNA. By referring to Figs. 2 and 3, it can be seen that uridine appeared to be involved in a cyclic relationship with thymidine. Initially, at a TEC dose level of 12 mmoles, thymidine was not depressed significantly, indicating that some DNA synthesis was still occurring. At 48 hr., the decreased thymidine uptake could be representative of the RNA depression which resulted from 1.5-hr. exposure to TEC. If there is an insufficient quantity of RNA for synthesis of proteins and enzymes, a secondary depression in DNA will ensue, and it appears quite likely that this is happening at 48 and 78 hr. with triethyl citrate (18). The uridine increase at 48 and 78 hr. could then be explained by the presence of pre-existing DNA which functions only as a template.

It is also possible, but highly unlikely, that uridine was being aminated *via* a small proportion of available ATP and glutamine to form cytidine; in this way, the same level of radioactivity would be maintained and the amount of adenine consumed would probably be less than half of that required to phosphorylate and polymerize new ribonucleic acid.

There is yet another possibility that triethyl citrate might serve as a substrate to enzymes mediating citrate transformations into and out of the citric acid cycle (19). This possibility is feasible only if the ethyl groups of TEC do not sterically hinder "active sites" of the enzymes. If such were the case, however, inhibition of ATP would occur.

After reviewing individual possibilities, there still remains the question of TEC exerting its effects by multiphasic involvement. This would include accumulation of TEC in membranes of a lipoprotein nature, including those of the mitochondria, or clathrate formation with macromolecules in a nonspecific manner (22). Such a mechanism would make nucleic acids, enzymes, and other proteins virtually unavailable for cellular metabolism (23). Since there is a rapid labeling, degeneration, and turnover of soluble RNA normally occurring with the cell (20), it is more plausible that the increased uridine count is actually the result of pooled degradation products caught in the cytoplasmic gel which forms at the higher dose levels studied in this paper (21).

CONCLUSION

At the TEC dose levels reported in this paper, it might be concluded that the action of triethyl citrate is fairly specific. Its apparent initial site of action is in the mitochondria which generate energy necessary for other cellular processes to occur. Secondary cyclic functions are then noticeably altered at 48 and 78 hr., especially at 9 and 12 mmole dose levels. The DNA content of the cell does not increase sufficiently to support duplication, but does serve as a template for some further RNA synthesis at the expense of adenine before the 48-hr. assay. Finally, that RNA which was built up between initial assay and 48 hr. is probably degraded, pooled, and kept within the cell membranes by gelation (21), accounting for the resulting uridine levels as revealed by the liquid scintillation techniques. Other sites of action are possible but were not investigated in this study.

REFERENCES

(1) Guess, W. L., Rosenbluth, S. A., Schmidt, B., and Autian, J., *J. Pharm. Sci.*, **54,** 1545(1965).

(2) Rosenbluth, S. A., "Inhibitory Effects of Citric Acid Ester Plasticizers on the Growth of Mammalian Cell Culture," Ph.D. Dissertation, University of Texas, Austin, Tex., August 1966.

(3) Ferguson, J., Hawkins, S. W., and Doxey, D., *Nature*, **165,** 1021(1950).

(4) Ferguson, J., *Proc. Royal Soc. London, Ser. B*, **127,** 387(1939).

(5) Zimmerman, E. F., and Greenberg, S. A., *Mol. Pharmacol.*, **1,** 113(1965).

(6) McIntire, F. C., and Smith, M. F., *Proc. Exptl. Biol. Med.*, **98,** 76(1958).

(7) Eagle, H., *Science*, **122,** 501(1955).

(8) Bray, G. A., *Anal. Biochem.*, **1,** 279(1960).

(9) Bruno, G. E., and Christian, J. E., *Anal. Chem.*, **33,** 650(1961).

(10) "General Pathology," 3rd ed., Florey, H., ed., W. B. Saunders Co., Philadelphia, Pa., 1962, p. 389.

(11) Davidson, J. N., *Biochem. Pharmacol.*, **4,** 164(1960).

(12) Gahan, P. B., and Chayen, J., in "International Review of Cytology," vol. 18, Bourne, G. H., and Danielli, J. F., eds., Academic Press Inc., New York, N. Y., 1963, p. 223.

(13) Roels, H., *ibid.*, vol. 19, 1966, p. 1.

(14) Prescott, D. M., in "Use of Radioisotopes in Animal Biology and the Medical Sciences," vol. 1, Academic Press Inc., New York, N. Y., 1961.

(15) Stanners, C. P., and Till, J. E., *Biochim. Biophys. Acta*, **37,** 406(1960).

(16) Gale, G. R., *Biochem. Pharmacol.*, **14,** 1705(1965).

(17) Taylor, J. H., in "Cell Growth and Cell Division," Symposium of International Society for Cell Biology, Harris, R. J. C., ed., vol. 2, Academic Press Inc., New York, N. Y., 1963.

(18) Terasima, T., and Yasukawa, M., *Exptl. Cell Res.*, **44,** 669(1966).

(19) White, A., Handler, P., and Smith, E., "Principles of Biochemistry," 3rd ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1965, pp. 245, 317.

(20) Rake, A. V., and Graham, A. F., *Biochem. Biophys. Acta Commun.*, **55,** 267(1962).

(21) Jones, A. B., Guess, W. L., and Autian, J., unpublished data.

(22) Pauling, L., *Science*, **134,** 15(1961).

(23) Danielli, J. F., "Cell Physiology and Pharmacology," Elsevier Publishing Co., New York, N. Y., 1950.

Polymeric Pharmaceutical Coating Materials II

In Vivo Evaluation as Enteric Coatings

By LEWIS C. LAPPAS and WAYNE MCKEEHAN

A series of polymeric films exhibited a gradient in dissolution pH within the pH range of the gastrointestinal tract. The *in vivo* release properties of these films were evaluated clinically as enteric coated tablets using acetylsalicylic acid as the marker. The absorption and excretion of these tablets were studied through plasma salicylate and urinary salicylurate measurements in normal, fasting subjects. A direct relationship was observed between the polymer dissolution pH and the onset and induction times of these enteric tablets in the plasma and urine, respectively. No significant difference in absorption rates existed between the stomach for the control and the upper intestine for two of the enteric products. A further increase in the dissolution pH of the polymers released the drug in the lower intestine effecting a progressive decrease in the absorption rate and total absorption. Variation in the upper intestine was significantly less than in the lower intestine.

THE INCREASING emphasis on the clinical efficacy of drug formulations has greatly stimulated the study of those physical and chemical properties which contribute directly to the release and subsequent absorption of orally administered pharmaceuticals. Many drugs are absorbed at various rates throughout the gastrointestinal tract due to their degree of dissociation and solubility (1). The pH gradient of the gastrointestinal tract may then be utilized to control and study the release and ultimate absorption of a drug in specific areas of the intestine. This factor is significant for those medicinals which are absorbed primarily in the intestine from enteric and sustained-release dosage forms. A series of films, half esters of poly(vinyl methyl ether maleate), was prepared to dissolve at various pH values within the pH range of the intestine (2). Earlier reports described the *in vivo* utility of some of these materials as enteric and sustained-release coatings (3–5).

The drug, acetylsalicylic acid, has been studied extensively relative to the effect of the gastrointestinal pH gradient on its rate of absorption. Proven analytical procedures are available for its detection as free salicylate in the blood plasma and as conjugated metabolites in the urine. In view of these considerations, this drug is suitable for use as a marker in this study.

Rapidly disintegrating tablet cores of acetylsalicylic acid were coated with the various films to produce a gradient in their disintegration pH. The effect of these products on the intestinal absorption was measured clinically in normal, fasting subjects through plasma salicylate and urinary salicylurate measurements.

Received April 14, 1967, from the Pharmaceutical Research Department, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206

Accepted for publication June 19, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Previous paper: Lappas, L. C., and McKeehan, W., *J. Pharm. Sci.*, **54**, 176(1965).

The authors express their appreciation to Dr. M. E. Amundson, Mr. W. M. Seidler, and Miss M. M. Webster, Eli Lilly and Co., for their assistance in this work.

EXPERIMENTAL

Physiological Aspects—The essentially unionized form of acetylsalicylic acid and its hydrolysis product, salicylic acid, are passively and preferentially absorbed throughout the entire gastrointestinal tract (6). The pKa of acetylsalicylic acid is 3.49, and the pH of the gastrointestinal tract will determine its degree of dissociation at various absorption sites. Its rate of absorption is highest in the stomach (6, 7), decreases progressively in the intestine (8), and becomes minimal in the colon (9). At the fasting gastric pH range of 1.2–3.5, the ionic state of acetylsalicylic acid is highly favorable for absorption (10); the extent of its absorption is limited by the low solubility in this pH range and the emptying time of the stomach. The pH of the intestine varies from 4 to 7.5, increasing from the proximal to the distal end (11); therefore, the degree of unionized molecule is reduced to approximately 1%. The increased dissolution rate and greater mucosal surface in the upper intestine could effect an equivalent or more rapid absorption from this area than from the stomach (12–14). It is apparent that salicylic acid (pKa 3.0) might produce a similar absorption pattern in the gastrointestinal tract. Eriksen *et al.* demonstrated a similarity of absorption for salicylate in the stomach and 2–3 ft. past the pylorus (15). These investigators noted the rate and efficiency of absorption in the cecum were less than in the stomach and small intestine.

The absorption of acetylsalicylic acid from solid dosage forms is limited and controlled by the dissolution rate of the drug in the gastrointestinal fluids (16). Enteric coatings are designed to transfer the dosage form intact through the gastric fluid and render the drug available for dissolution in the intestinal fluid. Assuming the dissolution properties of a pharmaceutical dosage form are reproducible, it is evident that gastric emptying

time can become a source of significant variation in the intestinal absorption for enteric products. Those factors contributing greatly to this variation are the presence or absence of food (17) and position of the body during sleep (18). Another source of variation in intestinal absorption is the transit or resident time of the dosage form as it traverses the intestine. In general, transit time is minimal proximally and gradually increases distally. These potential variables make it essential to assess the dissolution rates of both the enteric coating and the drug from the solid dosage form.

Plasma and urinary salicylate measurements have been used frequently to study the relative absorption rates of various orally administered dosage forms. The majority of the plasma salicylate is present as protein bound salicylate or free salicylate ion in solution (19). Metabolism of acetylsalicylic acid occurs in the tissues, and elimination as conjugated salicylates is effected readily at 90% yield. Salicyluric acid is the chief metabolite excreted in a normal acid urine, and its rate of formation may become the limiting step in the excretion process. This metabolic lag is seen in the differences between peak times of the plasma and urinary salicylate levels indicating the metabolic rate is slower than the absorption rate from the gastrointestinal tract.

Cummings and Martin studied the kinetics of acetylsalicylic acid metabolism relative to dosage and noted the urinary excretion rates between doses of 325 mg. and 650 mg. were decreased with the higher dose (20). This change in half-life was attributed to the attainment of a saturation excretion level which was related to plasma concentration. Other investigators have studied the half-life of salicylate in the blood and observed a range from 4.7 to 19 hr. depending on the plasma concentration (21–23).

Preparation of Enteric Polymers—A series of half esters of the copolymer poly(vinyl methyl ether/maleic anhydride) (PVM/MA) was prepared, and the dissolution pH of these films was determined according to the procedures described by Lappas and McKeehan (2).[1] The dissolution pH of each enteric film, determined after 24 hr. at 25°, is listed in Table I.

Preparation of Tablet Core—A circular, biconvex tablet was formulated from a starch base and contained 0.325 Gm. of acetylsalicylic acid by the U.S.P. method of analysis (24). The disintegration property of this tablet was designed to rupture and disperse completely in 1–2 min. at pH 1.2, 6.0, and 7.5 using the U.S.P. disintegration apparatus, without disks, at 37° (25). The test fluids of designated pH were prepared by mixing solutions of enzymeless, U.S.P. simulated gastric and intestinal fluids (26). In order to promote the adhesion of these enteric polymers to this tablet, two types of subcoatings were used. In the film coating process, methylcellulose 10 cps. (Dow Chemical Co.) was applied at 3 mg./tablet, with a DeVilbiss sprayer, from a solution of equal volumes of methanol and methylene chloride. In the alternate process, approximately 15 mg./tablet of a powder subcoat was applied by ladling syrup on the tablets to produce tackiness and then dusting to dryness with talc–acacia (88:12). The disintegration times of these subcoated cores were the same as that of the uncoated tablet core.

TABLE I—DISSOLUTION pH OF ENTERIC FILM COATINGS

Half Ester of PVM/MA	Dissolution pH[a]
Ethyl	4.25
Isopropyl	5.25
n-Butyl	5.75
Cyclopentyl	6.25

[a] pH of complete solubility; the film is intact at 0.25 pH unit below this pH.

Preparation of Enteric Coated Tablets—The polymeric film coatings were applied to the subcoated tablets in batch sizes of 3000 in a conventional 16-in. diameter tablet coating pan. Solutions of 4% w/v polymer in a mixture of acetone–isopropanol (85:15) were applied from a DeVilbiss sprayer. The final weight of coating per tablet was determined as the amount required to withstand the disintegration test for 1 hr. in enzymeless gastric fluid without physical change. The maximum amount of acetylsalicylic acid extracted from any tablet under these conditions was 4.8 mg. The disintegration pH of the tablet was defined as a function of pH and time required to dissolve the enteric film coat and disperse the tablet contents. The apparatus and method of preparation for the pH-adjusted test solutions were described previously for the uncoated tablet core. The pH at which the tablet remained intact for a minimum period of 1 hr. demonstrated the small range in pH between solubility and insolubility. The presence of enzymes in the test solutions did not alter the disintegration times of the enteric tablets. The disintegration properties of the various enteric coated tablets are summarized in Table II. The rate of disintegration at pH 7.5 increased in direct relationship from a mean of 6 min. for the 1/2 ethyl PVM/MA coated tablet to 12 min. for the 1/2 cyclopentyl PVM/MA coated tablet. At the lower pH's, the differences in disintegration time between the various enteric tablets are more clearly defined than at pH 7.5.

Clinical Procedure—A total of 12 healthy male subjects, who were known to be absorbers of acetylsalicylic acid, were used throughout the study; however, not all members of this panel participated in all of the experiments. The subjects were selected at random for testing the various enteric coated tablets as follows: five subjects in a cross-over study with the uncoated control, the 1/2 ethyl PVM/MA and the 1/2 isopropyl PVM/MA; eight subjects were used to study the 1/2 *n*-butyl PVM/MA; and six subjects constituted the group for the 1/2 cyclopentyl PVM/MA. A total of 0.650 Gm. of acetylsalicylic acid, two tablets, was administered to overnight fasting subjects with 200 ml. of water. A light breakfast was permitted 1 hr. after the administration of this dose. Although liquids were taken freely after 1 hr., 100 ml. of water was administered after each urine void. Preliminary evaluation of these materials determined the optimal time for the withdrawal of blood and the collection of urine samples. Urinalysis was continued for 48 hr., the time of complete excretion for all products tested.

[1] Polymer-grade used was Gantrez AN 139 for PVM/MA which is available from General Aniline and Film Corp.

TABLE II—DISINTEGRATION PROPERTIES OF ENTERIC COATED TABLETS OF ACETYLSALICYLIC ACID 0.325 Gm.

Enteric Coating	Subcoat	Enteric Coat, mg./Tablet	Disintegration pH[a]	Disintegration min.	Insolubility pH[b]
1/2 Ethyl PVM/MA	Methyl-cellulose	28	5.75	25	5.0
1/2 Isopropyl PVM/MA	Methyl-cellulose	20	6.0	38	5.5
1/2 *n*-Butyl PVM/MA	Powder	16	6.2	38	6.0
1/2 Cyclopentyl PVM/MA	Powder	10	6.75	15	6.5

[a] pH of solutions was monitored constantly. [b] Tablet was physically unaltered for at least 1 hr.

Analytical Procedures—A modification of the procedure reported by Schachter and Manis was used to determine the plasma salicylate fluorimetrically (19). Freshly drawn blood samples were mixed with heparin, and the plasma was separated by centrifugation. An aliquot of this sample was acidified and extracted with chloroform to remove the free salicylate. This solution was alkalinized with sodium hydroxide and activated at a wavelength of 314 mμ. Its fluorescence was measured at an emission wavelength of 410 mμ using an Aminco-Bowman spectrophotofluorometer.

Generally, about 75% of the dose of acetylsalicylic acid is excreted as the metabolite, salicyluric acid; 10% is found as the free salicylate. In order to economize time and effort, the metabolite salicyluric acid was measured to evaluate the comparative absorption and total excretion of acetylsalicylic acid from the various preparations. Individual urine samples were pooled and collected at 4, 8, 12, 18, 24, 32, and 48 hr. These samples were refrigerated prior to analysis for pH and salicyluric acid content. Although the pH of the urine samples varied for the individuals, the range of pH 5.0 to 6.8 was considered normal for acid urine. The method of analysis for salicyluric acid was essentially that described by Schachter and Manis (14). An aliquot of the urine sample was acidified with hydrochloric acid and extracted with ether. This layer was subsequently extracted with sodium hydroxide solution, and its fluorescence was measured at an emission wavelength of 410 mμ after activation at 335 mμ.

RESULTS AND DISCUSSION

This investigation was undertaken to demonstrate the *in vivo* release properties of a series of polymeric

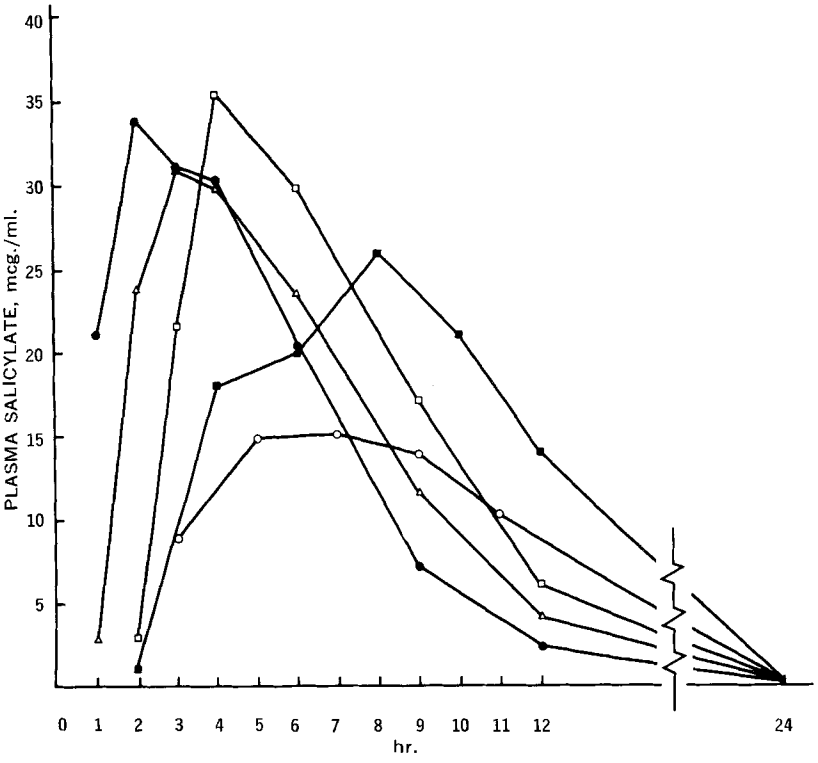

Fig. 1—Plasma salicylate concentration vs. *time: effect of polymer dissolution pH gradient. ASA blood levels; single dose, 650 mg. (two tablets). Key:* ●, *control uncoated;* △, *1/2 ethyl PVM/MA;* □, *1/2 isopropyl PVM/MA;* ■, *1/2* n-*butyl PVM/MA;* ○, *1/2 cyclopentyl PVM/MA.*

films having a dissolution pH gradient within the pH range of the intestinal tract. These films, applied over a rapidly disintegrating core of acetylsalicylic acid, exert a direct control over the release, dissolution, and subsequent absorption of this active ingredient. The significant physiological variables affecting its absorption are the pH of the intestine, the gastric emptying time of the stomach, and the transit time of the drug in the intestine. In consideration of these factors, the absorption aspects are revealed for areas of the intestine rather than exact locations.

The absorption-excretion data are expressed as average plasma salicylate and urinary salicylate levels as a function of time. These measurements illustrate the relative differences in the absorption of acetylsalicylic acid as it is presented to various pH values along the gastrointestinal tract. The frequency of the urine collection is a factor which limits the use of these data for quantitative kinetic study of the absorption-distribution-excretion properties of this drug.

The plasma salicylate levels plotted in Fig. 1 reveal similar peak levels for the control, the 1/2 ethyl PVM/MA and 1/2 isopropyl PVM/MA coated tablets at 2, 3, and 4 hr., respectively. The absorption slopes are steep and similar for the three products indicating equivalent absorption rates in the stomach for the control and two locations in the upper part of the small intestine for the enteric tablets. This observation is in accord with that of other investigators (6–14) and may be explained by the increased solubility of the drug in an area of vast intestinal mucosal surface which equalizes the optimal ionic conditions for absorption of this drug in the stomach. The 1/2 *n*-butyl PVM/MA coated tablet effected a decrease in the absorption slope and peak level; a salicylic acid concentration of 26 mcg./ml. was attained in 8 hr. Similarly, the 1/2 cyclopentyl PVM/MA coated product plateaued at 5–9 hr. reaching its peak salicylate value of 15.1 mcg./ml. after 7 hr. This gradient in the absorption rates of acetylsalicylic acid is attributed to the decreased efficiency in absorption from the lower parts of the intestine.

In Fig. 2, the average cumulative urinary excretion is expressed as mg. of salicylic acid and as the per cent released relative to the uncoated control. These data indicate the onset of absorption was progressively delayed with an increase in the disintegration pH of the enteric coated tablets. The cumulative excretions of the control, the 1/2 ethyl PVM/MA, and the 1/2 isopropyl PVM/MA coated tablets were equivalent after 12 hr. This level of

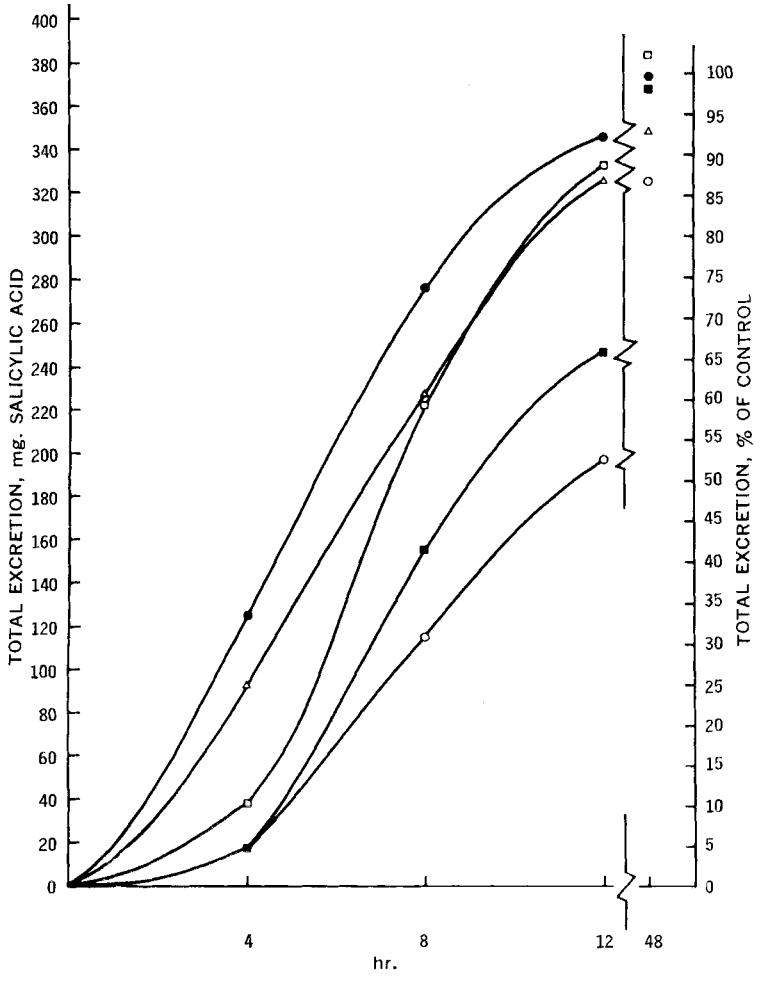

Fig. 2—Total urinary salicylate excretion vs. *time: effect of polymer dissolution pH gradient. ASA urinary excretion; single dose, 650 mg. (two tablets). Key:* ●, *control uncoated;* △, *1/2 ethyl PVM/MA;* □, *1/2 isopropyl PVM/MA;* ■, *1/2* n-*butyl PVM/MA;* ○, *1/2 cyclopentyl PVM/MA.*

excretion was approached by the 1/2 *n*-butyl PVM/MA and the 1/2 cyclopentyl PVM/MA coated tablets in 18 and 24 hr., respectively. The excretion study was conducted through 48 hr. indicating complete availability for all enteric tablets except for the 1/2 cyclopentyl PVM/MA coated tablet which was 87% available. The deposition of an enteric tablet at a lower absorption site than the latter tablet would be expected to result in slower and less complete absorption with a lower plasma peak and lower degree of availability.

The variation of the tested products was considered by examination of the distribution of peak times for individuals. This parameter is a reflection of the combined variables of gastric emptying time, pH, and intestinal transit time. The distribution patterns for the control, the 1/2 ethyl PVM/MA, and the 1/2 isopropyl PVM/MA coated tablets were equivalent. The 1/2 *n*-butyl PVM/MA coated tablet indicated increased variation, and the 1/2 cyclopentyl PVM/MA coated tablet exhibited the greatest variation. In these fasting ambulatory subjects the absorption reproducibility of the enteric tablets was greatly dependent upon the absorption site of the intestine, *i.e.*, the upper part of the intestine exhibited significantly less variation in absorption than the lower part of the intestine.

These data reveal that the gradation in coating dissolution pH is significant in permitting some control over the absorption of acetylsalicylic acid. Many other drugs are absorbed at various rates throughout the intestinal tract. Therefore, each drug should be considered an individual problem in formulating enteric-release products to effect optimal absorption.

REFERENCES

(1) Schanker, L. S., *Ann. Rev. Pharmacol.*, **1**, 29(1961).
(2) Lappas, L. C., and McKeehan, W., *J. Pharm. Sci.*, **54**, 176(1965).
(3) *Ibid.*, **51**, 808(1962).
(4) Kleber, J. W., Nash, J. F., and Lee, C.-C., *ibid.*, **53**, 1519(1964).
(5) Nessel, R. J., DeKay, H. G., and Banker, G. S., *ibid.*, **53**, 882(1964).
(6) Hogben, C. A. M., Schanker, L. S., Tocco, D. J., and Brodie, B. B., *J. Pharmacol. Expil. Therap.*, **120**, 540 (1957).
(7) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **120**, 528(1957).
(8) Schanker, L. S., Tocco, D. J., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **123**, 81(1958).
(9) Schanker, L. S., *ibid.*, **126**, 283(1959).
(10) James, A. N., and Pickering, G. W., *Clin. Sci.*, **49**, 181(1949).
(11) Rovelstad, R. A., *Gastroenterology*, **31**, 530(1956).
(12) Garrett, E. R., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 584(1957).
(13) Edwards, L. J., *Trans. Faraday Soc.*, **47**, 1191(1951).
(14) Weikel, J. H., Jr., and Lish, P. M., *Arch. Intern. Pharmacodyn.*, **119**, 398(1959).
(15) Eriksen, S. P., Swintosky, J. V., Serfass, E. J., Lin, T. H., Abrams, J., and Sturtevant, F. M., *J. Pharm. Sci.*, **50**, 151(1961).
(16) Levy, G., Gumtow, R. H., and Rutowski, J. M., *Can. Med. Assoc. J.*, **85**, 414(1961).
(17) Blythe, R. H., Grass, G. M., and MacDonnell, D. R., *Am. J. Pharm.*, **131**, 206(1959).
(18) Lappas, L. C., unpublished data.
(19) Schachter, D., and Manis, J. G., *J. Clin. Invest.*, **37**, 800(1958).
(20) Cummings, A. J., and Martin, B. K., *Biochem. Pharmacol.*, **13**, 767(1964).
(21) Brodie, B. B., Burns, J. J., and Weiner, M., *Med. Exptl.*, **1**, 290(1959); through *Chem. Abstr.*, **54**, 25254 (1960).
(22) Swintosky, J. V., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 395(1956).
(23) Levy, G., *J. Pharm. Sci.*, **54**, 959(1965).
(24) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 20.
(25) *Ibid.*, p. 934.
(26) *Ibid.*, pp. 1072, 1073.

Hydrolysis of Solubilized Aspirin

By A. G. MITCHELL* and J. F. BROADHEAD

The hydrolysis of aspirin in buffered solutions of the nonionic surfactant, cetomacrogol, has been studied at 37° over the pH range 1 through 7. Cetomacrogol reduces the hydrolysis rate of unionized aspirin but not that of ionized aspirin. The reaction occurs mainly in the aqueous phase but at low pH the contribution of a hydrogen ion catalyzed reaction within the micelles becomes significant. A kinetic expression has been derived to account for reaction both in the aqueous phase and in the micelles.

THE HYDROLYSIS of aspirin in aqueous solution has been investigated extensively by Edwards (1, 2) and Garrett (3, 4). They determined overall first-order rate constants as a function of pH and proposed various reaction mechanisms.

The effect of anionic, cationic, and nonionic surfactants on the stability of solubilized aspirin has been studied by Nogami *et al.* (5). It was shown that each surfactant suppressed the hydrolysis of unionized aspirin, while the hydrolysis of the anionic form of aspirin was suppressed only by cationic surfactants.

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EXPERIMENTAL

Reagents and Solutions—Aspirin was recrystallized from acetone, m.p. 134–136°. Salicylic acid was recrystallized from alcohol and then water,

excretion was approached by the 1/2 *n*-butyl PVM/MA and the 1/2 cyclopentyl PVM/MA coated tablets in 18 and 24 hr., respectively. The excretion study was conducted through 48 hr. indicating complete availability for all enteric tablets except for the 1/2 cyclopentyl PVM/MA coated tablet which was 87% available. The deposition of an enteric tablet at a lower absorption site than the latter tablet would be expected to result in slower and less complete absorption with a lower plasma peak and lower degree of availability.

The variation of the tested products was considered by examination of the distribution of peak times for individuals. This parameter is a reflection of the combined variables of gastric emptying time, pH, and intestinal transit time. The distribution patterns for the control, the 1/2 ethyl PVM/MA, and the 1/2 isopropyl PVM/MA coated tablets were equivalent. The 1/2 *n*-butyl PVM/MA coated tablet indicated increased variation, and the 1/2 cyclopentyl PVM/MA coated tablet exhibited the greatest variation. In these fasting ambulatory subjects the absorption reproducibility of the enteric tablets was greatly dependent upon the absorption site of the intestine, *i.e.*, the upper part of the intestine exhibited significantly less variation in absorption than the lower part of the intestine.

These data reveal that the gradation in coating dissolution pH is significant in permitting some control over the absorption of acetylsalicylic acid. Many other drugs are absorbed at various rates throughout the intestinal tract. Therefore, each drug should be considered an individual problem in formulating enteric-release products to effect optimal absorption.

REFERENCES

(1) Schanker, L. S., *Ann. Rev. Pharmacol.*, **1**, 29(1961).
(2) Lappas, L. C., and McKeehan, W., *J. Pharm. Sci.*, **54**, 176(1965).
(3) *Ibid.*, **51**, 808(1962).
(4) Kleber, J. W., Nash, J. F., and Lee, C.-C., *ibid.*, **53**, 1519(1964).
(5) Nessel, R. J., DeKay, H. G., and Banker, G. S., *ibid.*, **53**, 882(1964).
(6) Hogben, C. A. M., Schanker, L. S., Tocco, D. J., and Brodie, B. B., *J. Pharmacol. Expil. Therap.*, **120**, 540 (1957).
(7) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **120**, 528(1957).
(8) Schanker, L. S., Tocco, D. J., Brodie, B. B., and Hogben, C. A. M., *ibid.*, **123**, 81(1958).
(9) Schanker, L. S., *ibid.*, **126**, 283(1959).
(10) James, A. N., and Pickering, G. W., *Clin. Sci.*, **49**, 181(1949).
(11) Rovelstad, R. A., *Gastroenterology*, **31**, 530(1956).
(12) Garrett, E. R., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 584(1957).
(13) Edwards, L. J., *Trans. Faraday Soc.*, **47**, 1191(1951).
(14) Weikel, J. H., Jr., and Lish, P. M., *Arch. Intern. Pharmacodyn.*, **119**, 398(1959).
(15) Eriksen, S. P., Swintosky, J. V., Serfass, E. J., Lin, T. H., Abrams, J., and Sturtevant, F. M., *J. Pharm. Sci.*, **50**, 151(1961).
(16) Levy, G., Gumtow, R. H., and Rutowski, J. M., *Can. Med. Assoc. J.*, **85**, 414(1961).
(17) Blythe, R. H., Grass, G. M., and MacDonnell, D. R., *Am. J. Pharm.*, **131**, 206(1959).
(18) Lappas, L. C., unpublished data.
(19) Schachter, D., and Manis, J. G., *J. Clin. Invest.*, **37**, 800(1958).
(20) Cummings, A. J., and Martin, B. K., *Biochem. Pharmacol.*, **13**, 767(1964).
(21) Brodie, B. B., Burns, J. J., and Weiner, M., *Med. Exptl.*, **1**, 290(1959); through *Chem. Abstr.*, **54**, 25254 (1960).
(22) Swintosky, J. V., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 395(1956).
(23) Levy, G., *J. Pharm. Sci.*, **54**, 959(1965).
(24) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 20.
(25) *Ibid.*, p. 934.
(26) *Ibid.*, pp. 1072, 1073.

Hydrolysis of Solubilized Aspirin

By A. G. MITCHELL* and J. F. BROADHEAD

The hydrolysis of aspirin in buffered solutions of the nonionic surfactant, cetomacrogol, has been studied at 37° over the pH range 1 through 7. Cetomacrogol reduces the hydrolysis rate of unionized aspirin but not that of ionized aspirin. The reaction occurs mainly in the aqueous phase but at low pH the contribution of a hydrogen ion catalyzed reaction within the micelles becomes significant. A kinetic expression has been derived to account for reaction both in the aqueous phase and in the micelles.

THE HYDROLYSIS of aspirin in aqueous solution has been investigated extensively by Edwards (1, 2) and Garrett (3, 4). They determined overall first-order rate constants as a function of pH and proposed various reaction mechanisms.

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EXPERIMENTAL

Reagents and Solutions—Aspirin was recrystallized from acetone, m.p. 134–136°. Salicylic acid was recrystallized from alcohol and then water,

m.p. 159–161°. Cetomacrogol 1000 B.P.C.[1] has the general formula $CH_3 \cdot (CH_2)_m [O \cdot CH_2 \cdot CH_2]_n \cdot OH$, where m may be 15 or 17 and n may be 19 to 23. The molecular weight was taken as 1300. Solutions of cetomacrogol were passed through a column of mixed bed ion-exchange resins to remove alkaline impurities (6). Using a Zeiss laboratory interferometer the refractive index of the effluent was measured as an instrument scale reading and the concentration of cetomacrogol determined from a standard curve of instrument scale reading *versus* molar concentration.

Cetomacrogol solutions were buffered as follows: pH 1.0–2.2 HCl–KCl buffer (7), pH 2.2–2.9 glycine–HCl buffer (7), pH 2.9–4.6 acetate buffer (8), and pH 6.8–7.1 phthalate buffer (8). Ferric chloride reagent (1% $FeCl_3 \cdot 6H_2O$ in 0.05 N HCl). All solutions were made using freshly boiled and cooled glass-distilled water.

Apparatus—Beckman research pH meter (relative precision ± 0.001 under optimum conditions). The glass-saturated calomel electrode system was standardized, using 0.05 M potassium hydrogen phthalate, and the electrode response was checked on this buffer after each reading. The pH/E.M.F. relationship of the electrode was checked periodically using 0.05 M sodium borate. pH measurements were made at 37 ± 0.1° in a constant-temperature jacketed beaker.

Colorimetric analyses were made using a Unicam SP 1300 colorimeter and an Ilford No. 624 bright spectrum green filter (495–575 mμ).

Analytical Method—Solutions were analyzed for salicylic acid, using the blue color developed on reaction with ferric chloride (9).

Solubility of Aspirin—Excess aspirin was equilibrated with aqueous buffer or buffered cetomacrogol solutions by rotation in sealed cylinders in a water bath thermostatically controlled at 37 ± 0.1°. After filtration through a jacketed filter-funnel, aliquots of filtrate were assayed for salicylic acid present due to hydrolysis. Further samples were made alkaline with 0.5 N KOH, heated on a steam bath for 60 min., reacidified with 0.5 N HCl, and assayed for total salicylic acid. The solubility of aspirin was calculated from the difference between the amount of salicylic acid present initially and the amount found after complete hydrolysis.

Kinetic Procedure—An accurately weighed amount of aspirin was dissolved in aqueous buffer or buffered cetomacrogol solution maintained at 37 ± 0.1° in a thermostatically controlled water bath. Duplicate samples were withdrawn at zero time and then at suitable time intervals. One sample was assayed immediately for salicylic acid and the other for salicylic acid after complete hydrolysis. The concentration of aspirin remaining at each sampling time was calculated.

Density Measurements—A pycnometer of approximately 10-ml. capacity was used to measure the density of the cetomacrogol solutions at 37° (10). Each density was the mean of at least two determinations.

RESULTS AND DISCUSSION

Hydrolysis—The hydrolysis of aspirin proceeds as a first-order reaction both in aqueous buffer and in buffered cetomacrogol solutions (Fig. 1). Reaction rate constants were determined over the pH range 1 through 7 and the pH profiles in various surfactant concentrations are shown in Fig. 2. (Table I.) At the pH of maximum stability, pH 2.27, where aspirin exists largely in the unionized form, the half-life increases with cetomacrogol concentration and in 0.07 M cetomacrogol is approximately twice that in aqueous buffer. In the plateau region where aspirin is largely ionized, the rate of hyrolysis is independent of cetomacrogol concentration.

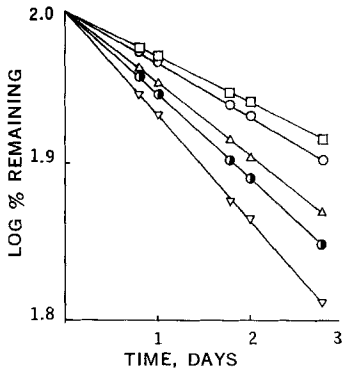

Fig. 1—First-order plots for the hydrolysis of aspirin solubilized in cetomacrogol solutions at 37°, pH 2.27. Key: cetomacrogol, moles L.$^{-1}$—▽, 0.00; ◑, 0.01; △, 0.02; ○, 0.05; □, 0.07.

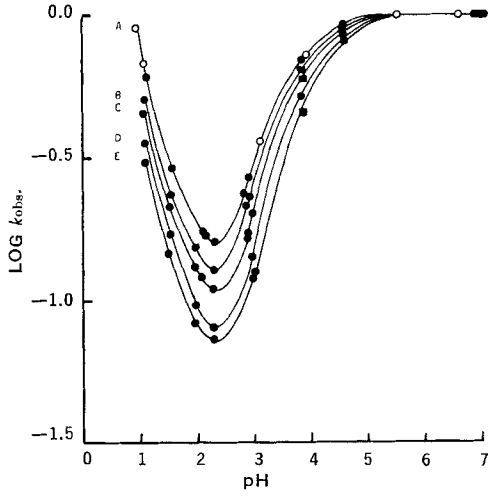

Fig. 2—Variation of observed first-order rate constant with pH for hydrolysis of aspirin solubilized in cetomacrogol solutions at 37°. Key: cetomacrogol, moles L.$^{-1}$—A, 0.00; B, 0.01; C, 0.02; D, 0.05; E, 0.07; ●, present study; ■, calculated; ○, Nogami (5).

On the two-phase model of micelle formation, solute will partition between the aqueous phase and the micellar phase. Using the two-phase model for a first-order process in surfactant solution, Yamada and Yamamoto (11) derived Eq. 1:[2]

$$k_{obs.} = \frac{1 - v}{Kv + (1 - v)} k_w \qquad \text{(Eq. 1)}$$

[1] Texofor A24, Glovers Chemicals Ltd., Leeds, England.

[2] See the *Appendix*.

TABLE I—RATE CONSTANTS FOR DECOMPOSITION OF ASPIRIN IN AQUEOUS BUFFER AND BUFFERED CETOMACROGOL SOLUTIONS

pH	Cetomacrogol, moles L.$^{-1}$	$k_{obs.}$, Day^{-1}
1.11	0	0.600
1.56	0	0.290
1.96	0	0.190
2.08	0	0.173
2.16	0	0.165
2.27	0	0.160
2.83	0	0.234
2.87	0	0.271
2.95	0	0.300
3.84	0	0.691
4.60	0	0.918
6.88	0	0.981
1.11	0.01	0.507
1.54	0.01	0.232
1.95	0.01	0.155
2.27	0.01	0.123
2.86	0.01	0.213
2.94	0.01	0.231
4.60	0.01	0.894
1.11	0.02	0.460
1.54	0.02	0.213
1.95	0.02	0.130
2.05	0.02	0.120
2.27	0.02	0.109
2.85	0.02	0.165
2.90	0.02	0.171
2.96	0.02	0.203
4.61	0.02	0.868
7.05	0.02	0.984
1.11	0.05	0.356
1.55	0.05	0.165
1.96	0.05	0.0953
2.28	0.05	0.0804
2.95	0.05	0.140
3.86	0.05	0.486
4.61	0.05	0.830
7.10	0.05	1.000
1.11	0.07	0.308
1.54	0.07	0.143
1.96	0.07	0.0828
2.28	0.07	0.0726
2.96	0.07	0.119
3.02	0.07	0.126
4.62	0.07	0.780
6.98	0.07	0.981

where $k_{obs.}$ is the observed rate constant in surfactant solutions at a given pH, k_w is the rate constant in aqueous solution at the same pH, K is the apparent partition coefficient for the distribution of solute between the micelles and aqueous phase, v is the volume fraction of micellar phase, and $1-v$ is the volume fraction of aqueous phase.

To test the applicability of Eq. 1 to the hydrolysis of aspirin in surfactant solutions, it was first necessary to estimate v and K.

Partial Molar Volume—The partial molar volume of cetomacrogol at 37° calculated from the density measurements shown in Fig. 3 was 1170 ml. mole^{-1}. Florence (12) has reported a value of 1099 ml. mole^{-1} at 30°. The partial molar volume was used to calculate v at each cetomacrogol concentration. The CMC of cetomacrogol is sufficiently low (13) for the concentration of monomeric surfactant to be neglected at the cetomacrogol concentrations under consideration.

Apparent Distribution Coefficient—The pH-dependent apparent distribution coefficient, K, is given by:

$$K = \frac{D_m}{v} \cdot \frac{(1 - v)}{D_w} \qquad \text{(Eq. 2)}$$

where D_m is the amount of solute in the micellar phase and D_w is the amount of solute in the aqueous phase. The total amount of solute, D, is given by:

$$D = D_w + D_m \qquad \text{(Eq. 3)}$$

In a given volume of a saturated solution the total amount of solute is equal to the solubility, C_s, and Eq. 3 becomes:

$$C_s = D_w + D_m \qquad \text{(Eq. 4)}$$

D_w was calculated from:

$$D_w = C_{sw}(1 - v) \qquad \text{(Eq. 5)}$$

where C_{sw}, the total solubility of aspirin in aqueous solution, is given by Eq. 6 (14):

$$C_{sw} = C'_{sw}\,[1 + \text{antilog (pH–pKa)}] \qquad \text{(Eq. 6)}$$

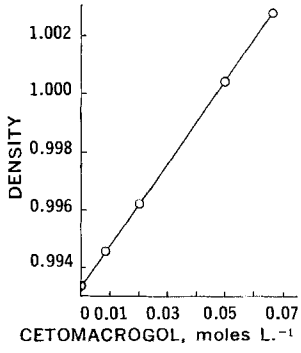

Fig. 3—Densities of cetomacrogol solutions at 37°.

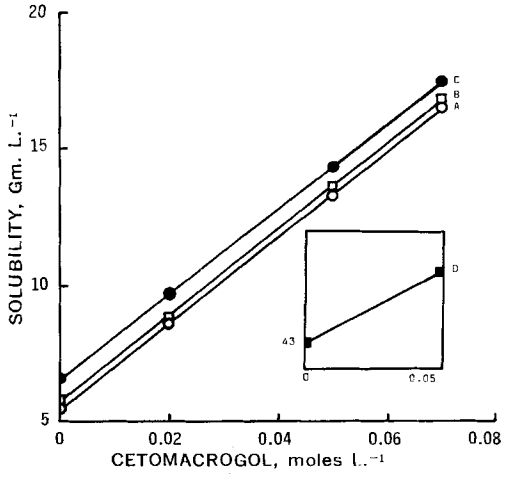

Fig. 4—Solubility of aspirin in aqueous buffer and buffered cetomacrogol solutions at 37°. Key: ○, pH 1.11; □, pH 2.04; ●, pH 2.75; ■, pH 4.36. Curves B–D *calculated from solubilities at pH 1.11, and Eqs. 4–7; all points determined by experiment.*

TABLE II—DISTRIBUTION OF ASPIRIN BETWEEN MICELLES AND AQUEOUS PHASE OF CETOMACROGOL SOLUTIONS AT VARIOUS pH VALUES

	Aspirin, mg. ml.$^{-1}$								
	Cetomacrogol, 0.02 *M*			Cetomacrogol, 0.05 *M*			Cetomacrogol, 0.07 *M*		
pH	*Dw*	*Dm*	*K*[a]	*Dw*	*Dm*	*K*[a]	*Dw*	*Dm*	*K*[a]
1.11	5.40	3.30	25.5	5.14	8.15	25.7	5.00	11.50	25.9
2.04	5.63	3.29	24.4	5.40	8.30	24.5	5.20	11.65	24.9
2.76	6.35	3.37	21.7	6.12	8.23	21.6	5.97	11.53	21.7
4.37				42.20	8.20	3.1			

[a] *K* calculated from the mean value of *Dm* at each cetomacrogol concentration.

C'_{sw}, the aqueous solubility of unionized aspirin at 37°, is 5.46 Gm. L.$^{-1}$, and the pKa for aspirin calculated by the solubility method (14) is 3.5 ± 0.03.

The solubility of aspirin at various pH values is shown as a function of cetomacrogol concentration in Fig. 4. The amount of aspirin in the micelles, D_m, at each cetomacrogol concentration is constant and from Eqs. 4 and 5 is given by:

$$D_m = C_s - C_{sw}(1 - v) \qquad \text{(Eq. 7)}$$

Table II shows some of the values for D_w, D_m, and K.

Agreement between the theoretical solubility curves shown in Fig. 4 (calculated from solubilities measured at pH 1.11 and Eqs. 4–7) and solubility values determined experimentally over the pH range where direct estimations were possible confirms the validity of this approach. Apparent distribution coefficients at any required pH can be determined from the plot of *K versus* $1/1 + k_a/[H^+]$, shown in Fig. 5.

Kinetics—One of the assumptions implicit in Eq. 1 is that only drug in the aqueous phase undergoes hydrolysis. It has been shown recently (15) that this assumption is valid for the hydrolysis of chlorobutol in nonionic surfactants at pH 8.7 and pH 9.2. If Eq. 1 is applicable to the hydrolysis of aspirin in cetomacrogol solutions, then a plot of the observed rate constant, $k_{obs.}$, for each pH against $(1-v)/Kv+(1-v)$ should be a line of slope k_w passing through the origin. Figure 6 shows that the equation holds at pH 2.95 and above, but at lower pH values the lines intercept the ordinate above the origin. The occurrence of an intercept indicates that at pH < 2.95 a reaction additional to that in the aqueous phase is making a significant contribution toward the over-all reaction.

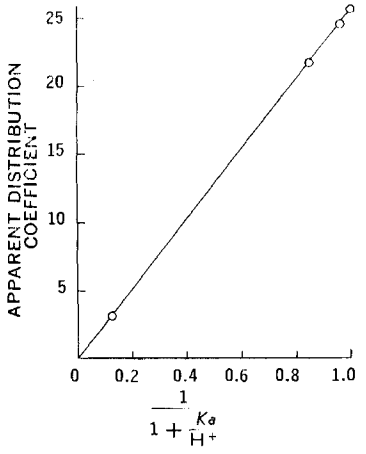

Fig. 5—Apparent distribution coefficients for the partition of aspirin between the micelles and aqueous phase of cetomacrogol solutions at 37°, plotted as a function of the ionization constant and hydrogen ion concentration.

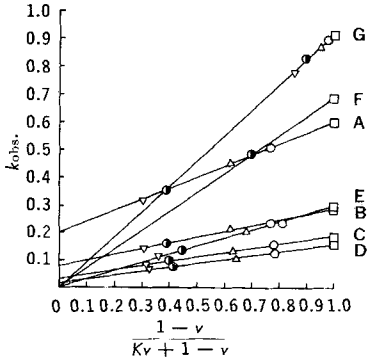

Fig. 6—Plot of observed rate constant against $(1 - v)/Kv + (1 - v)$ *in accordance with Eq. 1. Key: cetomacrogol, moles L.*$^{-1}$*— □, 0.00; ○, 0.01; Δ, 0.02; ◑, 0.05; ▽, 0.07; A, pH 1.11; B, pH 1.55; C, pH 1.95; D, pH 2.27; E, pH 2.95; F, pH 3.84; G, pH 4.60*

When the term $(1-v)/Kv + (1-v) = 0$, the volume of the micellar phase, v, is equal to 1. Hence the additional reaction must be taking place in the micellar phase and has a rate constant, k_m, equal to the intercept. It is apparent from Fig. 7 that k_m is directly proportional to the hydrogen-ion concentration which suggests that the reaction is due to hydrogen-ion attack on unionized aspirin within the micelles. To account for the reaction in the micelles, Eq. 8[3] was derived in a manner similar to the derivation of Eq. 1.

$$k'_m = \frac{Kv}{Kv + (1 - v)} \cdot k_m \qquad \text{(Eq. 8)}$$

where k'_m is the contribution of the micellar rate constant to the observed rate constant in the solubilized system. If k'_w is the corresponding contribution of the aqueous rate constant then the over-all rate constant is given by:

$$k_{obs.} = k'_m + k'_w \qquad \text{(Eq. 9)}$$

Equation 1 can now be rewritten to account for the micellar reaction:

$$k'_w = \frac{1 - v}{Kv + (1 - v)} \cdot k_w \qquad \text{(Eq. 10)}$$

where from Eqs. 9 and 8:

[3] See the *Appendix*.

$$k'_w = k_{obs.} - k'_m = k_{obs.} - \frac{Kv}{Kv + (1 - v)} \cdot k_m$$

At pH > 2.95, the reaction within the micelles is negligible and $k'_w = k_{obs.}$. Hence at any pH, a plot of k'_w, the contribution of the aqueous rate constant to the observed rate constant, *versus* the term $(1 - v)/Kv + (1 - v)$ should be a straight line

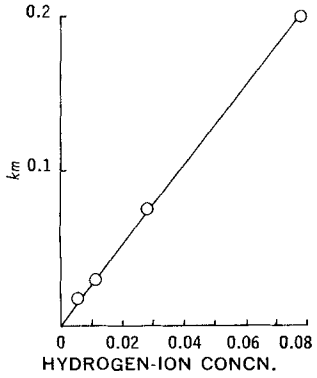

Fig. 7—Influence of hydrogen-ion concentration on the micellar rate constant.

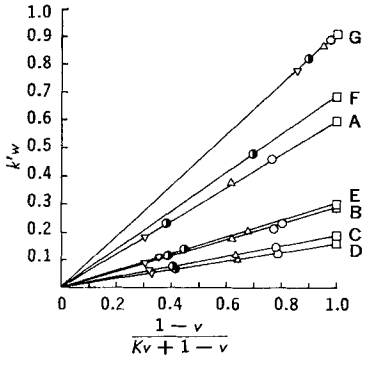

Fig. 8—Plot of contribution of the aqueous rate constant to the observed rate constant against (1 − v)/Kv + (1 − v) *in accordance with Eq. 10. Key: cetomacrogol, moles L.$^{-1}$— □, 0.00; ○, 0.01; △, 0.02; ◑, 0.05; ▽, 0.07; A, pH 1.11; B, pH 1.55; C, pH 1.95; D, pH 2.27; E, pH 2.95; F, pH 3.84; G, pH 4.60*

TABLE III—INFLUENCE OF ASPIRIN CONCENTRATION AND IONIC STRENGTH ON THE OBSERVED RATE CONSTANT

pH	Ionic Strength	Cetomacrogol, moles L.$^{-1}$	K	Aspirin— mg. ml.$^{-1}$	$k_{obs.}$ Day^{-1}
1.11	0.147	0.00		2.48	0.600
1.11	0.147	0.00		3.00	0.605
1.11	0.400	0.00		2.12	0.600
1.11	0.147	0.02	25.7	2.52	0.460
1.11	0.147	0.02	25.7	6.06	0.460
1.11	0.147	0.05	25.7	2.26	0.354
1.11	0.147	0.05	25.7	6.06	0.360
1.96	0.060	0.05	24.9	4.00	0.0950
1.95	0.060	0.05	24.9	8.48	0.0956
1.95	0.200	0.05	24.9	4.88	0.0953
1.96	0.400	0.05	24.9	4.40	0.0956
2.78	0.012[a]	0.00		2.78	0.239
2.78	0.100[a]	0.00		2.50	0.242

[a] Different strength glycine–HCl buffer, all other ionic strengths adjusted with KCl.

of slope k_w, which passes through the origin. Such a plot is shown in Fig. 8. Equation 10 therefore separates the contributions of the aqueous and micellar rate constants toward the observed rate constant and is essentially the same as an equation derived by Winters and Grunwald (16) for the reaction of methyl bromide with cyanide ions in anionic and cationic surfactants.

Both Eqs. 1 and 10 are derived on the assumption that the distribution of aspirin between the micelles and aqueous phase obeys the simple partition law. This appears to be true for some solutes, but for others Mitchell and Brown (17) have shown that the distribution coefficient depends on the degree of saturation of the system with solute. In the present work direct estimation of the apparent distribution coefficient was difficult because of the unstable nature of aspirin. The apparent distribution coefficients therefore were calculated using solubility data and Eqs. 2–7. This procedure is open to criticism since the method is limited to measurements at saturation below which it cannot be assumed that the calculated distribution coefficients will be valid. If, however, the apparent distribution coefficient varies with the degree of saturation of the system with aspirin, then $k_{obs.}$ should also vary. Results in Table III show that, at each pH for a given cetomacrogol concentration, values of $k_{obs.}$ are independent of aspirin concentration. It is reasonable to conclude therefore that the apparent distribution coefficients are independent of aspirin and cetomacrogol concentrations. Table III also includes values of $k_{obs.}$ determined at the same pH but different ionic strengths to show that the reaction is independent of ionic strength.

APPENDIX

Derivation of Eq. 1—Let C = initial reactant concentration (moles L.$^{-1}$ total system), C_w = initial reactant concentration in the aqueous phase (moles L.$^{-1}$ aqueous phase), and x = concentration reacting in time t (moles L.$^{-1}$ total system). Then, on the assumption that hydrolysis in a solubilized system takes place only in the aqueous phase, the concentration of reactant remaining after time t, is $C_w(C - x)/C$ moles L.$^{-1}$ aqueous phase or $C_w(1 - v)(C - x)/C$ moles L.$^{-1}$ total system. The differential rate equation for the concentration change with respect to the total system is:

$$\frac{dx}{dt} = k_w C_w (1 - v) \frac{(C - x)}{C} \qquad \text{(Eq. 11)}$$

which on integration and rearrangement gives:

$$k_w = \frac{C}{C_w(1 - v)} \frac{1}{t} \ln \frac{C}{C - x} \qquad \text{(Eq. 12)}$$

From Eq. 2, the distribution coefficient:

$$K = \frac{C - C_w(1 - v)}{C_w v} \qquad \text{(Eq. 13)}$$

which on rearrangement becomes:

$$\frac{C}{C_w} = Kv + (1 - v) \qquad \text{(Eq. 14)}$$

Substitution into Eq. 12 gives:

$$k_w = \frac{Kv + (1 - v)}{1 - v} \cdot \frac{1}{t} \ln \frac{C}{C - x} \quad \text{(Eq. 15)}$$

where $1/t \ln C/(C - x)$ is the observed rate constant $k_{obs.}$, assuming that hydrolysis takes place only in the aqueous phase. Substitution of $k_{obs.}$ into Eq. 15 and rearrangement leads to Eq. 1:

$$k_{obs.} = \frac{1 - v}{Kv + (1 - v)} k_w \quad \text{(Eq. 1)}$$

Derivation of Eq. 8—On the assumption that decomposition in solubilized systems takes place only in the micelles, the differential rate equation is given by:

$$\frac{dx}{dt} = k_m C_m v \frac{(C - x)}{C} \quad \text{(Eq. 16)}$$

where C_m is the intiial reactant concentration in the micelles (moles L.$^{-1}$ micellar phase). Integration and rearrangement gives:

$$k_m = \frac{C}{C_m v} \frac{1}{t} \ln \frac{C}{C - x} \quad \text{(Eq. 17)}$$

From Eq. 2, the distribution coefficient:

$$K = \frac{C_m (1 - v)}{C - C_m v} \quad \text{(Eq. 18)}$$

which on rearrangement becomes:

$$\frac{C}{C_m} = \frac{K_v + (1 - v)}{K} \quad \text{(Eq. 19)}$$

Substitution into Eq. 17 gives:

$$k_m = \frac{Kv + (1 - v)}{Kv} \frac{1}{t} \ln \frac{C}{C - x} \quad \text{(Eq. 20)}$$

where the term $1/t \ln C/(C - x)$ is the observed rate constant, assuming that hydrolysis takes place only in the micelles. In a system where reaction takes place in both micelles and aqueous phase this term is equal to k'_m, the contribution of the micellar rate constant to $k_{obs.}$, where $k_{obs.}$ is now the over-all observed rate constant defined in Eq. 9. Substitution of k'_m into Eq. 20 and rearrangement leads to Eq. 8:

$$k'_m = \frac{Kv}{Kv + (1 - v)} \cdot k_m \quad \text{(Eq. 8)}$$

REFERENCES

(1) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723(1950).
(2) *Ibid.*, **48**, 696(1952).
(3) Garrett, E. R., *J. Am. Chem. Soc.*, **79**, 3401(1957).
(4) Garrett, E. R., *J. Org. Chem.*, **26**, 3660(1961).
(5) Nogami, H., Awazu, S., and Nakajima, N., *Chem. Pharm. Bull. Tokyo*, **10**, 503(1962).
(6) Ginn, M. E., and Church, C. L., *Anal. Chem.*, **31**, 551 (1959).
(7) Colowick, S. P., and Kaplan, N. O., "Methods in Enzymology," vol. I, Academic Press Inc., New York, N. Y., 1955, pp. 138–140.
(8) Long, C., "Biochemists Handbook," E. and F. N. Spon, Ltd., London, England, 1961, p. 30.
(9) De Marco, J. D., and Marcus, A. D., *J. Pharm. Sci.*, **51**, 1010(1962).
(10) Bauer, N., and Lewin, S. Z., "Physical Methods of Organic Chemistry," vol. I, 3rd ed., Interscience Publishers, Inc., New York, N. Y., 1960, pp. 142–162.
(11) Yamada, H., and Yamamoto, R., *Chem. Pharm. Bull. Tokyo*, **13**, 1279(1965).
(12) Florence, A. T., *J. Pharm. Pharmacol.*, **18**, 384(1966).
(13) Mitchell, A. G., and Wan, L. S. C., *J. Pharm. Sci.*, **53**, 1467(1964).
(14) Albert, A., and Serjeant, E. P., "Ionization Constants of Acids and Bases," Methuen & Co., Ltd., London, England, 1962, pp. 106–112.
(15) Anderson, R. A., and Slade, A. H., *J. Pharm. Pharmacol.*, **18**, 640(1967).
(16) Winters, L. J., and Grunwald, E., *J. Am. Chem. Soc.*, **87**, 4608(1965).
(17) Mitchell, A. G., and Brown, K. F., *J. Pharm. Pharmacol.*, **18**, 115(1966).

Ultrastructural Hepatotoxicity Induced by an Organotin Ester

By DAVID CALLEY, W. L. GUESS, and J. AUTIAN*

Adult mice and rats were intubated with daily one-fourth LD_{50} amounts of the organotin dibutyltin diacetate. Histopathology by light microscopy, and cellular ultrastructural changes by electron microscopy to hepatic tissue was determined at 24-hr. intervals to elucidate the mode of hepatotoxic action of the compound. It was found that early mitochondrial damage with cristae swelling and subsequent hydropic degeneration occurred. Swelling of granular and agranular endoplasmic reticulum followed soon after mitochondrial changes were seen, and progressed to hydropic vesicular formation. There was a progressive congestion of bile canaliculi due to cellular and microvilli swelling. The proposed mechanism of action of the organotin was dithiol inhibition of ATP production in the mitochondria, with consequent interference of ATP-dependent membrane activity in other parts of the cell. Rats maintained greater cellular integrity possibly due to early inhibition of phagocytosis or phagolysosomal activity.

A SERIES OF organotin esters has previously been studied in this laboratory for their toxic effects to biological systems *in vivo* and *in vitro*. It was found that certain of these compounds even in low concentrations, and particularly those possessing the acetate ester moiety, were highly toxic. In investigations by Guess *et al.* (1) and Calley *et al.* (2), these compounds have been shown to rapidly cause death of cell cultures *in vitro;* have an initial CNS depressant effect when administered orally or parenterally to rabbits, rats, or mice; are highly irritating to dermal, muscle, and ocular issue; and bring about hepatic disturbances resulting in biliary obstruction, jaundice, and liver necrosis in mice, and altered liver function in rats and rabbits.

No study reported to date has discussed the mechanism of toxicity to liver tissue by organotin at the subcellular level. According to Rees (3), exogenous toxic agents may cause biochemical changes in liver tissue which lead either to fat accumulation or to necrosis with resultant autolysis. One of these mechanisms of toxic action may be evidenced without the other, or both may occur simultaneously. A reduction or blockage of protein synthesis is the key event in fat accumulation (4–8). The changes in protein synthesis are detectable at a time when intracellular fat accumulations become visible by light microscopy. Harris, Robinson, and Seakins (4–6) have proposed that lack of protein, normally constituting part of the lipoprotein molecule, causes the formation and deposition of triglycerides intracellularly. According to Rees and Shotlander (7, 8), this process is coincidental with a fall in serum lipoprotein levels.

It is generally agreed that intracellular changes which lead to tissue necrosis involve at least two events. These are cell permeability changes and mitochondrial injury. In addition, lysosomal damage may sometimes occur. The latter is thought by de Duve and co-workers (9) to be a terminal stage of liver cell injury and may be the event precipitating necrosis. Disagreement on this point is based upon the late occurrence of above normal amounts of unbound serum lysosomal enzymes, signifying lysosomal damage. Investigations by Slater, Greenbaum, and Wang (10) at this level of liver cell injury have shown a preponderance of unbound enzymes only after necrosis has occurred. Mitochondrial injury is also thought to be a relatively late event in hepatic poisoning, although preceding lysosomal damage. The manner in which mitochondrial change occurs is uncertain, but according to Rees *et al.* (11) may be secondary to other changes. Rees (3) suggests that the initial point of attack of all liver poisons is the endoplasmic reticulum and that this sets into motion a series of events leading to necrosis or fatty degeneration, depending upon whether smooth or rough endoplasmic reticulum is affected, *i.e.*, whether cell permeability and ionic changes occur, or alternatively, protein synthesis is inhibited.

Status of knowledge concerning organotin toxicity through 1958 has been well documented in a monograph by Barnes and Stoner (12). Inhibition of oxidative phosphorylation has been demonstrated by Sone (13) in triethyltin poisoning. Tetraethyltin was found by Cremer (14) to be converted in small amounts to triethyltin, primarily by liver cells, when administered intraven-

Received April 10, 1967, from the College of Pharmacy, University of Texas, Austin, TX 78712

Accepted for publication July 5, 1967.

Presented to the Pharmacology and Biochemistry Section, A. PH. A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This research was supported in part by grant CA-06120 from the National Institutes of Health and contract PH43-64-557 from the Division of Biologics Standards, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and by grant SRF-665 from the University of Texas Research Institute.

The authors are indebted to Mr. James F. Smith for technical assistance in the electron microscopic work.

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ously in rats. Barnes and McGee (15) found that dibutyltin salts produced lesions of the bile duct in rats and mice, and was found in highest concentration in liver and kidney tissue and was excreted unchanged in bile. They also found that species difference was noted as to bile duct damage, with rabbits, guinea pigs, and cats showing no effect, rats and mice showing lesions.

A serious lack of knowledge exists as to the mechanisms involved in toxicity induced to cellular material by the organotin esters. Changes which may be brought about to the cytoplasmic and nuclear membranes, to cell organelles such as mitochondria, lysosomes, Golgi apparatus, chromosomes, endoplasmic reticulum, *etc.*, and how these changes are related to enzyme levels and normal body function is virtually unknown. The electron microscope is, of course, the most useful tool in determining structural changes at the subcellular level. It was decided that a study combining the capabilities of this instrument with customary light microscope pathological determinations of liver tissue from dosed animals would be a rational approach to delineate some of the more subtle aspects of toxicity.

MATERIALS AND METHODS

Young adult female Holtzman rats, weighing between 275 and 300 Gm., were intubated daily with one-fourth the LD_{50} concentration of dibutyltin diacetate (LD_{50} 109 mg./Kg.). Cottonseed oil was employed as the vehicle and control. One rat was sacrificed at each 24-hr. interval, with the dosage and sacrifice schedule covering a 10-day period. A similar schedule was followed in which young adult female, Swiss-Webster white mice weighing between 17 and 20 Gm. were dosed and sacrificed daily. Liver tissue from both rats and mice was taken immediately upon sacrifice and prepared for histological examination by light microscopy and for ultrastructural interpretation by electron microscopy.

Liver tissue selected for light microscopy was fixed in 10% phosphate buffered formalin, paraffin embedded, and the mounted sections were hematoxylin-eosin stained for examination.

A cross-section of tissue from the center portion of the left lobule of the liver was prepared for electron microscopy. Liver slices approximately 2 mm. in thickness were placed in 2.5% gluteraldehyde buffered with 0.2 *M* collidine. All tissue was placed in preservative within 1 min. after sacrifice to prevent autolysis. After 10 days, the tissue was removed from the buffered gluteraldehyde, cut into 2 mm. by 2 mm. cubes, washed twice for 30 min. in collidine buffer, and post-fixed for 1.5 hr. in cold (4°) 1% osmium tetroxide buffered with collidine. After removal from the osmium fixative, the tissues were dehydrated in a graded series of ethanol solutions, with final dehydration of the tissue in acetone. The material was then embedded in a resin[1] and sectioned on a Porter-Blum Servall MT 1 microtome fitted with a diamond knife. Sections were post-stained with 0.5% uranyl acetate and lead citrate, examined, and photographed on an RCA model 3G electron microscope. Negative prints were taken at ×8,330 magnification and developed to obtain ×21,000 photomicrographs. In the liver tissue, parenchymal, Kupffer, and endothelial lining cells, as well as bile ductules were observed for ultrastructural changes from normal condition represented by the control tissues. Comparisons were made of the size, configuration, spatial relationship, and relative numbers of all cell organelles, including mitochondria, rough and smooth endoplasmic reticulum, lysosomes, phagosomes, Golgi bodies, lipid droplets, or other storage particles. In addition, changes in nuclear size, shape, content, and nuclear and cytoplasmic membranes were also observed.

Data from all procedures were analyzed and compared to determine whether changes had been induced in rats or mice as the result of oral administration of the organotin.

[1] An Epon resin, Shell Chemical Co., New York, N. Y.

RESULTS

Histopathological Determination by Light Microscopy

After about a 2 to 3-day lag period in the development of visible liver damage, both mice and rats began to exhibit progressive liver involvement that reached a peak of pathological change on the sixth and seventh days of the 24-hr. repeating dosage schedule (Fig. 1). In the rat the severity lessened after this critical point, whereas in the mouse the degree of histopathology continued to be manifest.

At the peak of hepatocellular response in the mouse on the seventh through ninth doses, loss of hepatic architecture was severe, often involving the histologic organization of the entire lobule. Cells showed a spectrum of responses including cloudy swelling, opacification, cytoplasmic granulation, some fatty degeneration and infiltration, hyaline change, rupture of cell wall, cellular debris, changes in nuclear size, nuclear dust, and chromatin condensation. Vascular channels lost their continuity, protein was precipitated, and necrosis was common. No repair processes were evident, and the injury to these liver cells was deemed irreversible.

In the rat all changes which were seen in mice were visible except those which might be considered irreversible and lethal to the hepatic cell. There was

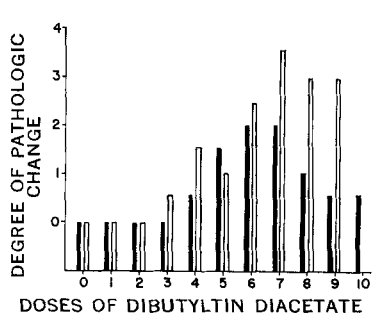


Fig. 1—Histopathological response to daily intubation with one-fourth LD_{50} concentration of dibutyltin diacetate in mice and rats. Degree of pathologic change was rated as: 0, normal; 1, minimal; 2, moderate; 3, moderately severe; 4, severe. Key: ■, rat; □, mouse.

minimal necrosis, rupture of cell walls, profound nuclear change, or vascular discontinuity even at the height of liver damage on the sixth and seventh day of dosing. In other words, the injury to the rat cells was not so extensive, and rats exhibited a terminal recovery pattern.

Ultrastructural Histopathology

Mitochondria—Normal mouse liver mitochondrial profiles (Fig. 2) exhibit a dense, nonvacuolated matrix with a few electron dense narrow lines representing the cristae, the structure bounded by a double membrane. Both mouse and rat hepatic parenchyma cells underwent vacuolar degeneration of the mitochondria as a direct consequence of poisoning with dibutyltin diacetate. These changes were visible 24 hr. after the first dose and were progressive in nature. Mice were more drastically affected than rats.

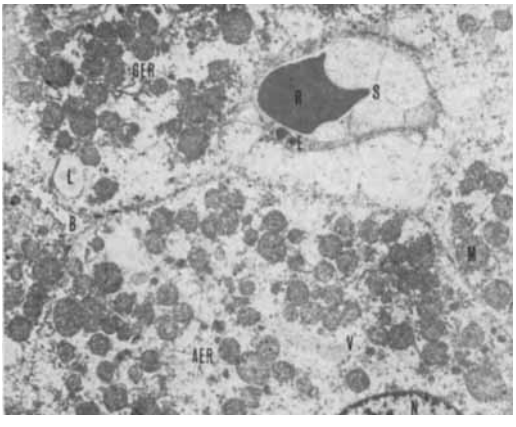


Fig. 2—Micrograph of control mouse hepatic cells showing normal parenchyma cell and organelle architecture. Key: AER, *agranular endoplasmic reticulum;* B, *bile canaliculus;* E, *endothelial lining cell;* GER, *granular endoplasmic reticulum;* L, *lysosome;* M, *mitochondria;* N, *nucleus;* R, *red blood cell;* S, *sinusoid;* V, *Golgi vesicle.*

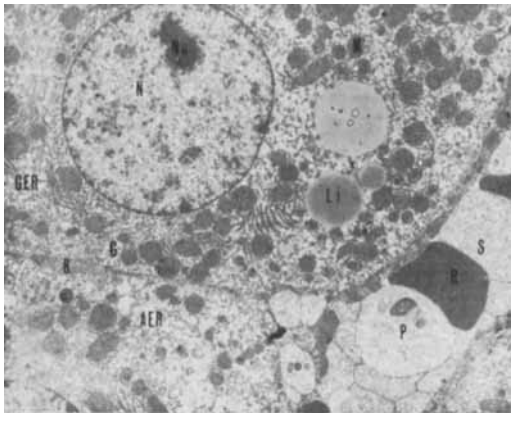


Fig. 3—Micrograph of mouse hepatic cells following the third successive dose of dibutyltin diacetate showing accumulation of lipid (Li), *condensation of chromatin in nucleolus* (Nu) *inside the nucleus, extrusion of phagolysosomes* (P) *into sinusoid* (S). *Several mitochondria* (M) *contain swollen cristae membranes. Lumen of bile canaliculi* (B) *is reduced and contains swollen microvilli. Also appearing unchanged are granular* (GER) *and agranular* (AER) *endoplasmic reticulum, a Golgi complex* (G), *red blood cell* (R), *and sinusoid* (S).

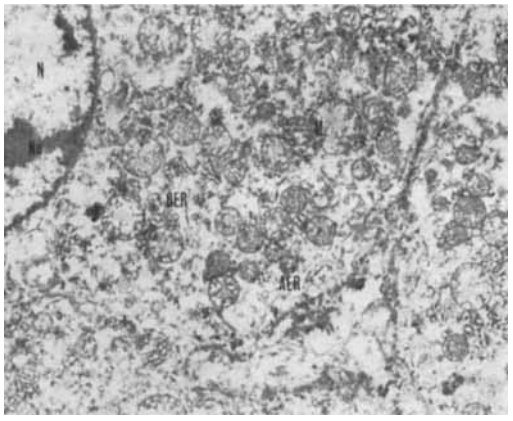


Fig. 4—Micrograph at higher magnification of mouse hepatic parenchyma cell following the sixth successive dose of dibutyltin diacetate showing hydropic degeneration and the formation of myelin figures in mitochondria (M), *swelling and vacuolization of granular* (GER), *and agranular* (AER) *endoplasmic reticulum.*

Initially, the cristae in a few mitochondria of both species became slightly distended (Fig. 3). After six successive doses, the mouse showed a pattern as illustrated in Fig. 4, in which the cristae were swollen and disrupted, the matrix of the mitochondria became less dense, and membranous whorls developed inside. It is obvious at this point that the mitochondrial damage was extensive and probably irreversible. With this degree of organelle injury, it is doubtful that a functional unit existed. Rat mitochondria did not degenerate to the extent described for mice, although they did develop numerous swollen cristae. A recovery pattern in rats was observed to take place beginning with dose seven. Here the cristae began to return to a more normal condition, although swelling of cristae, sometimes developing small vacuoles, could still be seen in some cells. Many cristae appeared normal, indicating a good recovery pattern or lack of original injury by the organotin.

Granular Endoplasmic Reticulum (GER)—In normal parenchyma cells, the double layered membranes of this organelle are often found in close proximity to mitochondria (Fig. 2). Their planar surfaces are covered with small electron dense particles called ribosomes. The cisternae between the membranes have a narrow, undulating appearance. Very little if any change in this pattern was seen after initial organotin dosing (Fig. 3). After repeated doses of organotin, both mice and rat GER showed a progressive swelling but with no noticeable loss of ribosomes. It can be seen in Fig. 4 that after six successive doses in mice, no normal GER can be found. Most cisternae have undergone gross swelling or enlargement and much GER appears to have dispersed as small vesicles, ribosomes intact, into the cytoplasm. Rats showed a similar pattern but to a lesser degree. Considerably greater amounts of GER either remained intact or were more rapidly regenerated in the rat cells (Fig. 5).

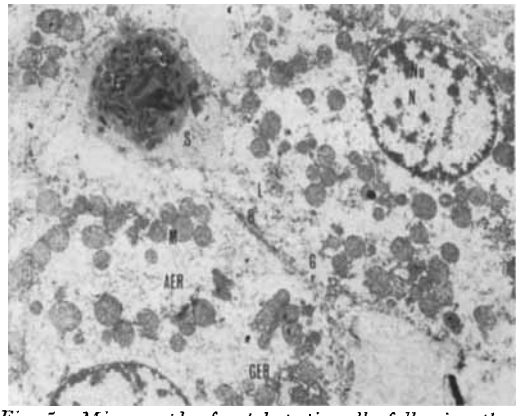


Fig. 5—Micrograph of rat hepatic cells following the tenth successive dose of dibutyltin diacetate in which organelles have been preserved or regenerated, in contrast to the condition found in the mouse at this dose interval. Agranular (AER) *and granular* (GER) *endoplasmic reticulum have near normal structure, bile canaliculus* (B) *remains obstructed, eosinophils* (Eo) *are present in sinusoid* (S), *Golgi* (G) *and lysosome* (L) *activity is present, mitochondria* (M) *appear normal except for a few vacuolated forms, chromatin in nucleolus* (Nu) *remains condensed.*

Agranular Endoplasmic Reticulum (AER)—In normal hepatic cells the AER were visualized as minute channels, often appearing in cross section as tiny vesicles (Fig. 2). These were most often irregularly dispersed in areas within the cell which were lacking in other structures, and having a somewhat transparent cytoplasmic matrix. The complexity of these structures greatly increased after the first three doses in both species, depicted in the mouse in Fig. 3. Further dosing led to irregular dispersion of these tubules, with some disruption into discrete vesicles appearing. Random invasion by cellular components of the areas formerly occupied only by the AER was evident (Fig. 4) creating a disorganized assemblage of vesicles from both AER and GER plus abnormal mitochondria and other vacuolar material. This pattern was seen in certain hepatic cells of both species, although much more prevalent in the mouse. Many of the rat cells appeared to regain or retain much of the original internal architecture (Fig. 5) and the AER was again found in normal amounts and sites inside the cells.

Bile Canaliculi—The normal condition of this structure in both rats and mice, as shown in Fig. 2, was an uncongested lumen into which projected a few small microvilli. Membranous whorls or myelin figures were commonly found unattached in the lumen, which was otherwise free of particles. In both study species, beginning with the second dose of organotin, these bile capillaries were rapidly closed (Fig. 3) so that after the third dose, no lumen was visible. The closing was attributed to two primary factors, one of these being the swelling of the parenchyma cell. The microvilli also appeared to have become swollen, further contributing to the congestion. No relief from this pattern was noted through the dose series in either mice or rats.

Lysosomes—Normal lysosomal activity involves discharge of contents from this aging organelle into the bile canaliculi as depicted in Fig. 2. After dosing with organotin, the bile capillaries became swollen and obstructed and lysosomes were seen to empty into the sinusoid instead, as shown in Fig. 3. As dosing frequency increased beyond the sixth in the series, an inhibitory effect was seen on parenchyma cell lysosomal activity, especially in mice. This lack of lysosomal activity was associated in both species with extreme disorganization and random dispersion of numerous vesicles and particles inside many cells (Fig. 4). In mouse cells, lysosomal activity was never reactivated to any appreciable extent. In rat cells, however, there was an apparent regeneration of lysosomal activity as shown in Fig. 5, even though the structures seen were much smaller and less numerous than in controls. Phagocytic activity, often associated with lysosome activity, was initially stimulated by dosing in mice but appeared to be inhibited in rats. This could have accounted in part for the damage seen in mouse cells as compared to rats.

Golgi Complex—The Golgi complex in normal cells is seen to arise from normal AER and is easily recognized by its structure (Fig. 2). Since the Golgi complex may be one of the sources for lysosomal production, it would be expected that damage to the AER would damage the Golgi and hence the lysosomal enzyme production. It was apparent that lysosomal activity showed a decrease at about the same time as the disappearance of the Golgi was noted. After the sixth dose of organotin in mice, the Golgi were not seen (Fig. 4) but are readily distinguishable in rat cells even after dose ten (Fig. 5).

Nucleus—The primary effect of organotin on the nucleus was a condensation of the nucleolus in both species to create a more dense often somewhat reticulated pattern shown in Fig. 3. This effect was noted after dose three and remained unchanged throughout the remainder of the series in mice and rats. Another effect was the accumulation of lipid and other small droplets in the nucleus seen during the later phases of dosing, primarily in mice.

Lipid Droplets—A noticeable increase in the number of lipid droplets began to be apparent from the first dose of organotin and were evident through the third dose (Fig. 3) in mice. These droplets were seen only rarely thereafter, indicating little or no fat accumulation in the cell or, alternatively, an increased mobilization from the cell, which was doubtful. In rats, small amounts of lipid were found after dose four and little or none at successive doses. This observation confirms the general observation that rat cells are still functioning.

Kupffer and Endothelial Lining Cells—Endothelial lining cells of both species showed increased thickness and granularity after dosing. Excessive swelling in some cells developed later, with sparse matrix containing small amounts of tiny vesicles. Kupffer cells increased in matrix density and showed greater numbers of dark staining granules in the cytoplasm.

DISCUSSION

The earliest noted ultrastructural change attributable to the dibutyltin diacetate dosing schedule was in the parenchymal cell mitochondria of both rats and mice. In this organelle, the cristae membranes responded initially by swelling and this became more

pronounced through time as doses of the organotin were repeated daily. This mitochondrial reaction was much more advanced in mice at any given dose than in the corresponding rat hepatic cells. As dosing continued in the mouse, the swollen cristae developed into the formation of large vacuoles and finally into a complete disorganization of the mitochondrial structures. At the latter stages of dosing, myelin figures were noted in grossly distorted mitochondria.

The observed fact that the mitochondria were the earlier organelles to be visually injured by the organotin appears to be in some disagreement with the views proposed by Rees (3). According to the scheme which he proposed, the granular endoplasmic reticulum is the first subcellular organelle to be injured by a liver poison. This injury to the GER would result in cellular permeability changes and ionic imbalance, resulting in a later mitochondrial injury.

Based on the over-all morphological changes in the mitochondria as observed in this study, one could propose that the first alteration of consequence would be the failure of the mitochondria to produce adequate ATP for the cell to maintain normal energy requiring functions. It is known that the cristae of the mitochondria are involved with electron transport enzymes and are more susceptible to swelling than the outer limiting membrane (16). Christie and Judah (17) concluded from their studies on vacuoles of mitochondrial origin obtained from poisoned hepatic cells that such poisons could induce hydropic changes to mitochondria and this in turn interferred with the tricarboxylic acid cycle at the citrate and maleate level, thus preventing final metabolism of pyruvate and acetate necessary for eventual ATP formation. The possible mechanism of interference with the tricarboxylic acid cycle has been proposed by Peters (18) and Williams (19) as interfering with the activity of α-keto acid oxidases. A molecule of the divalent toxic substance, in this case, dibutyltin diacetate, will combine with two SH groups in the enzyme, which is thereby inhibited. The enzymes inhibited by this mechanism would be pyruvic and α-keto glutaric dehydrogenases, enzymes which are essential for the formation of ATP *via* these cycles. Robinson (20) found that 2,4-dinitrophenol, which makes energy released by oxidation unavailable for ATP formation, causes tissues to take up water, implying that energy is normally expended by cells to prevent hydropic degeneration. Price *et al.* (21) found that isolated liver mitochondria maintained normal water content when incubated under conditions favorable to oxidative phosphorylation. Other investigators (22–24) have also found that failure of ATP synthesis appears to be a critical factor in determining whether cell degeneration will proceed to autolysis. Mouse liver mitochondria showed a progressive degeneration and this was reflected in areas of eventual liver necrosis in these animals, but was not as extensively shown in rats. However, as pointed out previously, the rat mitochondria were never injured to the same extent as the mouse tissue, thereby explaining the apparent partial cellular recovery in rats.

It can be postulated, then, that early mitochondrial injury in mouse and rat hepatic parenchyma cell poisoning is probably one of the underlying causes for toxic manifestations to other cellular organelles having a dependency on ATP for normal function.

Under the conditions of this study the granular or rough endoplasmic reticulum (GER) was the second cell organelle to manifest degenerative change. A progressive hydropic change with gross swelling and vacuolization of the cisternae of this structure is considered to be a contributing factor to the cloudy swelling as seen through the light microscope. Confirming studies by Molbert (25) and Oberling (26) also have shown that vacuolization of the endoplasmic reticulum and dilation of its cisternae were contributing factors to hydropic degeneration and cloudy swelling. It was apparent from the series of micrographs of normal cells that a very close spatial relationship existed in these cells between mitochondria and the GER. There is, no doubt, a direct functional dependency. The GER, for example, depends upon material elaborated by the mitochondria for membrane transport. In pathological cells, particularly those showing greatest degenerative change, the close relationship between these two organelles was lost. In the rat, the ability to maintain to a greater degree the integrity of both structures in certain areas of the cell was evident. It is probable that in such cases the GER maintains its membrane activity by ATP supply from mitochondria, and in the absence of this supply, cannot adjust to ionic and/or water imbalance. When the latter exists, swelling of the cisternae develops.

Porter and Bruni (27) postulate that the agranular or smooth endoplasmic reticulum (AER) portion of the vacuolar system is formed from the terminals of the GER and has a function in carbohydrate metabolism. In addition Essner and Novikoff (28) proposed that secretory and hydrolytic Golgi complexes were formed from the AER. Shnikta (29) indicated that microbodies may also arise from the AER, but in a different manner than do the secretory or hydrolytic bodies. As a result of the administration of the organotin compound, there was an obvious disruption of the AER as well as the GER, followed by a disappearance of the above-mentioned elaborated structures. If these structures are no longer elaborated by the AER, there would follow a cessation of various enzyme formation and activity, an accumulation of precursors and raw materials usually subject to metabolism by enzyme action.

Of particular importance, perhaps, was the loss of ysosomal action which maintains intracellular digestion and either recycles or extrudes from the cell the breakdown products. According to de Duve and Wattiaux (30), lysosomes are almost always involved in the response of the cell to challenging agents and by failing to perform their lytic actions adequately or performing them in injurious fashion may contribute to the cell pathology. Their malfunction may be due to uptake of inhibitors by a derangement of enzyme biosynthesis, or as Triederici and Pirani (31) suggest, by the production of abnormal proteins of low molecular weight which contribute to edema of the cell. It is not thought, however, that compressive or congestive lysosomal damage was a major factor in poisoning by dibutylin diacetate in rats, although in mice there was an obvious initial lysosomal or phagolysosome stimulation which declined with successive dosing.

One of the prime responses of the hepatic cells of both mice and rats was the rapid congestion of the bile capillaries upon dosing with the test compound.

This feature was not observed after the first dose, but some congestion was observed after the second dose, and almost complete reduction of the lumen of the canal was observed after the third repeated dose. This injury was of great significance to the welfare of the hepatic cells, since it obstructs a primary route of discharge of unwanted waste products, causing them to pile up inside the cell or, when possible, to be discharged into the sinusoid or extracellular spaces. In rats, lysosomal enzymes are all present in the bile, according to de Duve and Wattiaux (30), at a concentration indicating a daily unloading of about 5% of the total hepatic lysosomes. Confer and Stenger (32) and Bruni and Porter (33) suggest that the peribiliary location of many liver lysosomes and the presence of amembranous dense bodies and myelin figures in the lumen of bile capillaries suggest elimination by exocytosis into this structure. This suggests that the bile may be the major pathway for unloading lysosomes or their contents by hepatic parenchyma cells. The release of lysosomal enzymes into body fluids or into extracellular fluids has been observed by several researchers (34–37) to be associated with pathological phenomena. This congestion then left the parenchyma cell without a normal route of elimination of these cellular waste products, possibly resulting in an intracellular accumulation and subsequent necrosis.

SUMMARY AND CONCLUSIONS

Dibutyltin diacetate was administered daily in one-fourth LD_{50} doses for a period of 10 days. Ultrastructural damage to liver parenchyma cells after each 24 hr. of dosing was evaluated by the electron microscope and by the light microscope. Chronologically, the order of injury to the subcellular organelles and structures were as follows.

1. Mitochondrial damage with early cristae swelling followed by vacuolization and hydropic swelling.

2. Swelling and vacuolization of the granular endoplasmic reticulum.

3. Progressive congestion of the bile canaliculi due to cellular and microvilli swelling.

4. Formation of a dense population of small vacuoles resulting from the disruption of the rough and smooth endoplasmic reticulum.

It is felt then that the initial and progressive mitochondrial lesions interfered with normal biochemical and synthetic activity through a reduction and/or blockage of ATP production by dithiol inhibition. As a consequence, other cell functions involving active transport across membranes as well as certain synthetic and metabolic functions were inhibited. The net result of these inhibitory processes was cell necrosis in both mice and rats, although moderated in the latter. The ability of the rat hepatic cells to maintain better internal organization throughout the dosage schedule seemed to be related in part to an early cessation of phagocytosis or phagolysosomal activity, thus preventing intake of lethal (cellular) doses of the organotin by these routes.

REFERENCES

(1) Guess, W. L., O'Leary, R., Calley, D., and Autian, J., *Soc. Plastic Engrs.*, **XXV**, 4(1966).

(2) Calley, D., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **55**, 158(1966).

(3) Rees, K. R., in "Ciba Foundation Symposium on Cellular Injury," Little, Brown and Co., Boston, Mass., 1964, p. 53.

(4) Harris, P. M., and Robinson, D. S., *Biochem. J.*, **80**, 253(1961).

(5) Robinson, D. S., and Harris, P. M., *ibid.*, **80**, 361 (1961).

(6) Robinson, D. S., and Seakins, A., *ibid.*, **82**, 1(1962).

(7) Rees, K. R., and Shotlander, V. L., *Ann. N. Y. Acad. Sci.*, **704**, 905(1963*a*).

(8) Rees, K. R., and Shotlander, V. L., *Proc. Roy. Soc. London, Ser. B*, **157**, 517(1963*b*).

(9) Beaufay, H., Van Campenhaut, E., and de Duve, C., *Biochem. J.*, **73**, 617(1959).

(10) Slater, T. F., Greenbaum, A. L., and Wang, D. Y., in "Ciba Foundation Symposium on Lysosomes," Little, Brown and Co., Boston, Mass., 1963, p. 311.

(11) Rees, K. R., Sinha, R. P., and Spector, W. B., *J. Pathol. Bacteriol.*, **81**, 107(1961).

(12) Barnes, J. M., and Stoner, H. B., *Brit. J. Ind. Med.*, **15**, 15(1958).

(13) Sone, N., *J. Biochem. (Japan)*, **56**, 151(1964).

(14) Cremer, J. E., *Biochem. J.*, **68** (4), 685(1958).

(15) Barnes, J. M., and McGee, P. N., *J. Pathol. Bacteriol.*, **75**, 267(1956).

(16) André, J., *J. Ultrastruct. Res.*, **3**, 1(1962).

(17) Christie, G. S., and Judah, J. D., *Proc. Roy. Soc. London, Ser. B*, **142**, 241(1954).

(18) Peters, R. A., *Brit. Med. Bull.*, **5**, 313(1948).

(19) Williams, R. T., "Detoxication Mechanisms," Chapman and Hall, Ltd., London, England, 1959.

(20) Robinson, J. R., *Proc. Roy. Soc. London, Ser. B*, **140**, 135(1952).

(21) Price, C. A., Fonnesu, A., and Davis, R. E., *Biochem. J.*, **64**, 754(1956).

(22) Dawkins, M. J. R., and Rees, K. R., "A Biochemical Approach to Pathology," Edwd. Arnold, London, England, 1959, chap. 3.

(23) Fonnesu, A., in "The Biochemical Response to Injury," Blackwell Scientific Publications, Oxford, England, 1960, p. 85.

(24) Dawkins, M. J. R., Judah, J. D., and Rees, K. R., *J. Pathol. Bacteriol.*, **77**, 257(1959).

(25) Molbert, E., *Verhandl. Deut. Pathol. Ges.*, **41**, 303 (1958).

(26) Oberling, C., *Intern. Rev. Cytol.*, **8**, 1(1954).

(27) Porter, K. R., and Bruni, C., *Cancer Res.*, **19**, 997 (1959).

(28) Essner, E., and Novikoff, A. B., *J. Biophys. Biochem. Cytol.*, **9**, 773(1961).

(29) Shnitka, T. K., *J. Ultrastruct. Res.*, **16**, 598(1966).

(30) de Duve, C., and Wattiaux, R., *Ann. Rev. Physiol.*, **28**, 435(1966).

(31) Triederici, H. H. R., and Pirani, C. L., *Lab. Invest.*, **13**, 250(1964).

(32) Confer, D. E., and Stenger, R. J., *Am. J. Pathol.*, **45**, 533(1964).

(33) Bruni, C., and Porter, K. R., *ibid.*, **46**, 691(1965).

(34) Thomas, L., in "Injury, Inflammation and Immunity," Thomas, L., Uhr, J. W., and Grant, L., eds., Williams and Wilkins, Baltimore, Md., 1964, p. 312.

(35) Weissman, G., *Federation Proc.*, **23**, 1038(1964).

(36) Weissman, G., and Thomas, L., *Recent Prog. Hormone Res.*, **20**, 215(1964).

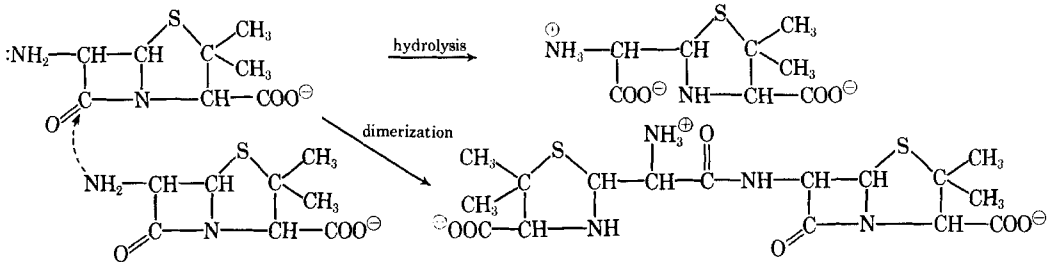
(37) de Duve, C., in "Injury, Inflammation and Immunity," Thomas, L., Uhr, J. W., and Grant, L., eds., Williams and Wilkins, Baltimore, Md., 1964, p. 283.

Degradation Kinetics of 6-Aminopenicillanic Acid

By DAVID W. DENNEN

The degradation of 6-aminopenicillanic acid (6APA), as measured by the loss of the β-lactam ring, followed pseudo first-order kinetics with initial concentrations of 0.05–0.92 *M* and in the pH and temperature range of 5.8–6.6 and 35–90°, respectively. Higher orders of degradation rates were approached with increasing pH while the maximum stability of the molecule at all temperatures examined was at approximately pH 8.0. Polymeric materials, measured by ultraviolet absorbance, were formed under all conditions examined.

THE FINDING that solutions of 6APA yielded increasing antibiotic activity to *Staphylococcus aureus* with time was observed in these laboratories (1), and it was suggested that a 6APA dimer was being formed in solution, presumably through nucleophilic attack of the amino group of one molecule to the β-lactam of another to form a penicillin (2). (Scheme I.)



Scheme I

Confirmatory studies remained unpublished when this mechanism was postulated by Grant *et al.*, who isolated a number of antimicrobially inactive polyamides with 7–8 residues from concentrated solutions of 6APA (3). In addition, a material termed factor 1 has subsequently been obtained with antimicrobial activity against *Bacillus subtilis* from 6APA preparations (4). The factor was apparently not an impurity carried with 6APA during purification and appeared to be an antimicrobial condensation product of this molecule containing at least one intact β-lactam ring.

Studies concerned with isolation and subsequent acylation of 6APA for the synthesis of semisynthetic penicillins required an examination of the stability of this molecule. This paper describes the stability of 6APA in terms of its rate of degradation as a function of temperature and pH.

Received May 24, 1967, from the Antibiotic Development Department, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication July 5, 1967.
The author acknowledges helpful discussions with Dr. W. W. Davis, who introduced the author to this interesting reaction. The assistance of Mr. D. O. Woolf, Jr., in infrared evaluation and Dr. E. H. Flynn and Dr. J. L. Spencer for criticizing the manuscript is appreciated.

EXPERIMENTAL

Preparation of 6APA—Penicillin nucleus was prepared by the cleavage of the side chain of phenoxymethylpenicillin by amidase (5). The 6APA was then precipitated quickly as the zwitterion at pH 4.7. Repeated solution in base and titration with acid to the isoelectric point produced the product which crystallized as hexagonal plates. The percentage composition of the product was measured as theoretical, m.p. 208–210°, and the pK's 5.5, 4.05 in DMF.

Preparation of Penicic Acid (Open Lactam 6APA)—Penicillinase A (Riker Laboratories) at a concentration of 200,000 units in 5 ml. was suspended within a dialysis tubing in a solution of 6APA (1 mg./ml.) for 24 hr. at 25°. After the 6APA solution gave a negative hydroxamate test, the material was lyophilized. The percentage composition of penicic acid was measured as theoretical and the molecule had pK's 4.7 and 7.8 in water. In 66% acetonitrile, the compound had an R_f of 0.54 and formed a blue color with ninhydrin.

Measurement of β-Lactam Content—Measurement of β-lactam content was performed according to the procedure of Ford (6) by reacting intact β-lactam with neutral hydroxylamine to give the hydroxamate, followed by color development with ferric chloride.

Preparation of 6APA Dimer—6APA solutions (20%) were allowed to stand at room temperature for 1 week, after which they were passed through a G-10 Sephadex column as described in Fig. 6. Hydroxamate positive material eluting between 22–27 ml. was collected and lyophilized. Infrared measurements (Beckman IR-7) revealed the appearance of mono-substituted amide at 6.0 and 6.6 μ and the loss of β-lactam at 5.6–5.7 μ. Titration of the material gave a molecular weight of 430. The

bulk of the sample moved 5.85 cm. toward the anode during paper strip electrophoresis (pyridine–acetate buffer; pH 4.3, 17 v./cm., 4 hr., 25°) and coincided with the value reported elsewhere for antimicrobially active material derived from 6APA (4). Degradation of the sample at pH 5.8, 75°, yielded an ultraviolet absorbing chromophore with a maximum at 304 mμ.

Degradation of 6APA—6APA was weighed and diluted to volume with preheated buffer of the desired pH. The buffers used were acetate, phosphate, and tris(hydroxymethyl)aminomethane in their respective ranges. The concentration of buffer was systematically twice the molar concentration of the initial concentration (C_0) of 6APA used. The constancy of pH was checked periodically throughout the time studies. No attempt was made otherwise to control the ionic strength to constancy. The buffered 6APA solution was then incubated at constant temperature under nitrogen in order to eliminate access of CO_2 and the possible formation of 8-hydroxypenillic acid (7). Aliquots were taken periodically for various assays described above. Cooling or diluting the aliquots were shown substantially to stop the reaction.

RESULTS

The loss of β-lactam content in 6APA solutions (0.05–0.92 *M*) followed first-order kinetics in the pH range 5.8–6.6 and shifted to second-order rates at pH 7.5–8.0 and above. In Fig. 1, the degradation of β-lactam is plotted in a fashion that designates a second-order reaction as a linear curve, while those data deviating from linearity obey the first-order rate law. Since below pH 7.5, the degradation of 6APA could be described by first-order kinetics at an initial concentration of 0.184 *M*, suggesting simple β-lactam hydrolysis, it was desirable to see if increased initial concentrations of 6APA would lead to second-order rates concomitant with bimolecular polymerization. However, as illustrated in Fig. 2, the first-order rate law was obeyed in increasing initial 6APA concentrations to 0.92 *M*.

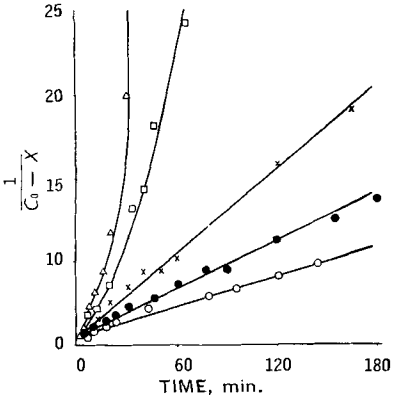


Fig. 1—Rate of β-lactam disappearance at constant initial concentration of 6APA, as a function of pH. First-order curves: pH 5.80 (△), pH 6.60 (□), second-order curves: pH 8.50 (×), pH 8.0 (●), and pH 7.50 (○). $C_0 - x$ is the amount of intact β-lactam 6APA. Initial concentrations of 6APA were 0.184 M and the degradation was measured at 90°.

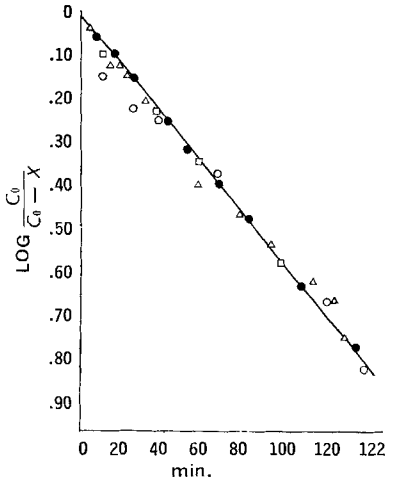


Fig. 2—Rate of β-lactam disappearance with increasing initial concentrations of 6APA. 6APA solutions incubated at pH 6.30, 80°. Initial concentrations: 0.13 M (○), 0.365 M (□), 0.42 M (●), 0.92 M (△). C_0 is the initial concentration of 6APA, and $C_0 - x$ is the amount of intact β-lactam.

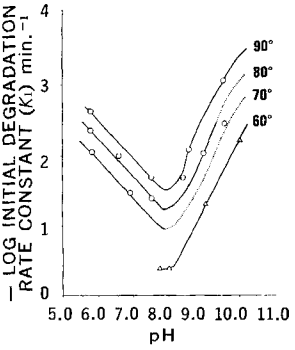


Fig. 3—Initial rate of β-lactam loss in 6APA solutions. Initial rate constants determined in 6-APA solutions by the rate of hydroxamate disappearance (○) and by titration in a pH-stat (△).

An examination of 6APA degradation at various pH's and temperatures revealed maximum stability of this molecule at approximately pH 8.0, well above the pKa (5.5) of its free amino group. In Fig. 3, the stability of 6APA β-lactam is depicted in relation to the initial rates of degradation. The data presented were obtained by two methods: disappearance of the 6APA reactivity to hydroxamate, and electrometric titration of the proton liberated by the opened β-lactam. It is important to note that in the degradation reaction below pH 8.0, the free amino group shifts from pKa 5.5 to that characteristic of an α-amino group and no base is consumed in the pH-stat titration.

The initial rates of degradation of β-lactam at an initial concentration of 0.184 *M* are plotted according to the method of Arrhenius in Fig. 4 as a function of temperature. The activation energy (E_a) for the degradation was calculated to be 17 Kcal./mole, comparing with the inactivation of phenethicillin (8), for example, at 17.6 Kcal./mole and benzylpenicillin (9) at 20 Kcal./mole. The activation energy associated with these latter penicillins can be essentially attributed to the formation of penicilloates (10) (β-lactam hydrolysis) at those pH's examined. The energy associated with 6APA

degradation, however, must take into account that activation energy pre-empting any polymerization that occurred simultaneously with β-lactam hydrolysis. Since second-order kinetics were visualized only in alkaline solutions, the distinct possibility was presented that penicic acid, formed initially from 6APA, could in turn prime the polymerization of 6APA. Below pH 7.5 it could not participate as a nucleophile, and 6APA would degrade simply through β-lactam hydrolysis as observed kinetically. The consequence of such a polymerization mechanism, however, would preclude the retention of an intact β-lactam in the dimer or polymer formed to account for the observed increase in antimicrobial activity.

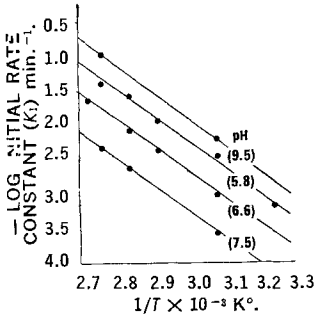


Fig. 4—Effect of temperature on the initial rate loss of β-lactam as a function of pH. Rate constants determined with 0.184 M solutions of 6-APA.

Since solutions of 6APA are not routinely allowed to remain at pH's much above neutrality, it was of importance to examine the character of the degradation product at pH's below 7.0 rather than in alkaline solution in order to determine whether polymerization was occurring at a rate proportional to the first rather than second order of the remaining 6APA concentration.

To measure polymerization, advantage was taken of an ultraviolet absorbing material that appeared during the progress of 6APA degradation. The ultraviolet spectra of this material shown in Fig. 5, had a maxima at 304 mμ while penicic acid, when similarly degraded from nearly the same initial concentration, had very little absorbance at this wavelength.

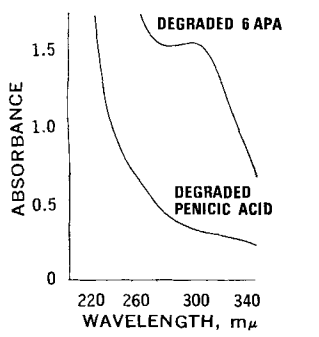


Fig. 5—Ultra violet spectra of degraded 6APA and penicic acid. Initial 6APA concentration, 2.5%; initial penicic acid concentration, 2.0%. Degradation for 2.5 hr., 75°. Degradation mixtures diluted 1:-12.5 for assay.

A degradation mixture of 6APA containing this chromophoric material was passed through a G-10 Sephadex column, and as shown in Fig. 6, it is clear that this material represented a molecular size greater than 6APA. Species were present in the effluent which coincided in position to phenoxymethylpenicillin and benzylpenicillin while the leading edge of the effluent yielded materials of greater molecular size than the penicillins. Any condensation that could occur with 6APA or penicic acid to form diketopiperazines did not seem to involve an increase in the absorbance at 304 mμ. The 304 mμ chromophore thus appeared to represent subsequent rearrangement of 6APA to penaldehyde and/or azlactone-type compounds following the acylation of one molecule by another.

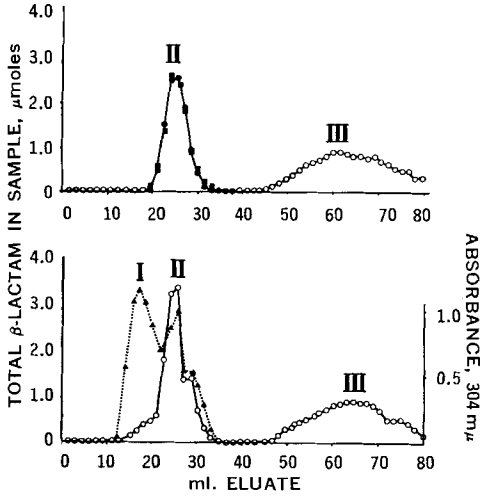


Fig. 6—Separation of penicillins, 6APA, and 6APA degradation mixture by gel-filtration. Top: 14.9 μmoles penicillin and 11.50 μmoles 6APA applied to column, phenoxymethylpenicillin (■), benzylpenicillin (●), 6APA (○). Recovery of total β-lactam, 88%. Bottom: the starting material was 6APA which was degraded and then applied to the column. Intact β-lactam of 6APA and polymeric material (○), 304 mμ absorbing material (▲). Sephadex G-10 column 1 × 39 cm. with 39 cm. head of buffer for elution. Peaks I, II, and III represent polymeric material, penicillin, or 6APA dimer and 6APA, respectively.

In Fig. 7, the formation of the 304 mμ absorbing material is shown in degradation mixtures of 6APA at an initial concentration of 0.184 *M* as a function of temperature at pH 6.30. It is apparent that a constant amount of polymeric material, represented by ultraviolet absorbance, was formed at this initial concentration. The addition of varying amounts of penicic acid to 6APA failed to increase the 304 mμ absorbance yield, and thus it appeared that the former was not necessary to prime the polymerization. It is also interesting to note that the total polymeric material produced from increasing initial concentrations of 6APA did not increase as the square of the concentration, but rather was directly proportional to the initial concentration of 6APA as shown in Fig. 8. Furthermore, the formation of polymeric materials adjudged from the 304 mμ absorbance in 6APA degradation reactions at pH 6.3 was confirmed at pH 5.8 and 6.0 as well, indicating that polymerization occurred as a parallel reaction to 6APA hydrolysis.

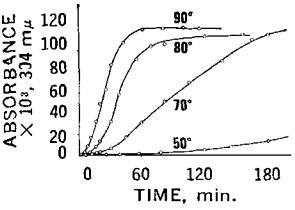


Fig. 7—Formation of 304 mμ absorbing material from degraded 6APA solutions. Initial 6APA concentration, 0.184 M, pH 6.30.

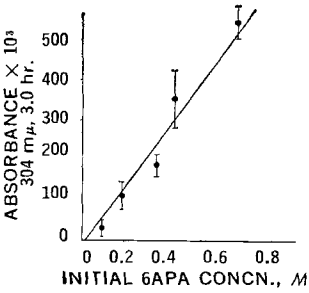


Fig. 8—Maximum level of 304 mμ absorbance as a function of the initial 6APA concentration. 6-APA degraded at pH 6.30, 80°.

DISCUSSION

The rates of β-lactam hydrolysis and dimerization of 6APA are both proportional to the first order of the intact β-lactam content in solutions below pH 7.5. This is not a surprising result since no direct measure of 6APA monomeric concentration is obtained by the assay of intact β-lactam in a system undergoing hydrolysis and polymerization. The dimerization or further polymerization of 6APA involving a β-lactam acylation by another 6APA molecule always leaves one β-lactam intact per two molecules of 6APA. The rate of dimerization would thus be proportional to the square of the 6APA concentration, whereas it would be directly proportional to the first-order loss of β-lactam.

Since the activation energy (E_a) for the hydrolysis of other penicillins is similar to that measured in the degradation of 6APA, it is reasonable to conclude that both β-lactam hydrolysis and intermolecular acylation occur with equal ease. This is one suggested explanation for the proportionately direct increase in the amount of polymer formed as a function of the initial 6APA concentration. Otherwise, one might expect that the rate of dimerization, which is proportional to the square of the initial concentration of 6APA, would be reflected by a proportionate increase in the polymer yield.

In alkaline solution, second-order kinetics of β-lactam loss are apparent from the data, and in these instances the β-lactam content can be visualized to closely approach the actual remaining concentration of 6APA. The increased lability of an acylated β-lactam over that of 6APA affects the apparent loss of two β-lactams per dimer rather than one.

REFERENCES

(1) Wright, W. E., Eli Lilly and Co., Indianapolis, Ind., personal communication.
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(4) Batchelder, F. R., Cole, M., Gazzard, D., and Rolinson, G. N., *Nature*, **195**, 954(1962).
(5) Rolinson, G. M., Batchelder, F. R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G. C., Hart, M., Richards, M., and Chain, E. B., *ibid.*, **187**, 236(1960).
(6) Ford, J. H., *Anal. Chem.*, **19**, 1004(1947).
(7) Johnson, D. A., and Hardcastle, G. A., *J. Am. Chem. Soc.*, **83**, 3534(1961).
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Cytotoxicity of Rubber Closures in Tissue Culture Systems

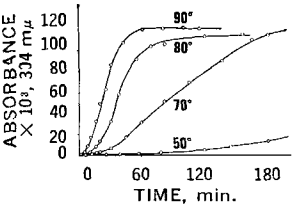
By P. J. VASINGTON, H. D. PIERSMA, J. J. CORBETT, and J. L. BITTLE

Rubber closures used in the pharmaceutical industry were tested for cytotoxicity in primary cultures of chick embryo cells and in cultures of cells passaged from human malignant tissue. Assay was by three methods: direct contact, growth-extract, and maintenance-extract. In the first, rubber samples were placed in direct contact with cell monolayers. In the growth-extract system, a sample was incubated in growth medium, and the resultant extract was placed in a cell suspension. In the maintenance-extract method, following incubation of a sample in maintenance medium, the extract was overlayed on a cell monolayer. Tissue culture provided a more sensitive test of toxicity in comparison with the generally accepted implantation of rubber in the paravertebral muscles of rabbits.

TESTING THE toxicity of rubber closures for pharmaceutical applications has been primarily physicochemical. To date, there is no acceptable biological assay system, although the rabbit implantation method of Brewer and Bryant (1) for screening plastics has gained considerable favor.

The need for a rapid, economical, and reproducible *in vitro* test has led to the investigation of

Received January 11, 1967, from Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965
Accepted for publication July 5, 1967.



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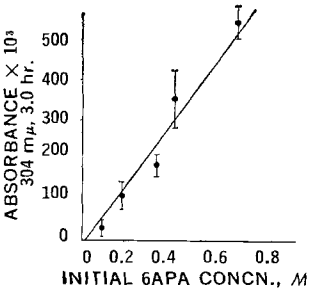

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TESTING THE toxicity of rubber closures for pharmaceutical applications has been primarily physicochemical. To date, there is no acceptable biological assay system, although the rabbit implantation method of Brewer and Bryant (1) for screening plastics has gained considerable favor.

The need for a rapid, economical, and reproducible *in vitro* test has led to the investigation of

Received January 11, 1967, from Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965
Accepted for publication July 5, 1967.

the use of tissue culture systems. The cytotoxicity of some rubber closures used to stopper cell culture tubes and bottles of synthetic medium became quite obvious to early investigators. Parker *et al.* (2) found a number of rubber closures to be toxic to cell cultures. Cruickshank *et al.* (3) employed tissue explants of chick embryo and guinea pig skin to test the toxicity of rubbers and plastics used in transfusion-giving sets. They concluded that tissue culture explants might prove valuable as a screening method to select suitable materials for clinical trials. The method involved peripheral fibroblastic outgrowths away from the area of toxic samples.

Rosenbluth *et al.* (4) reported the similar use of fibroblastic-like "L" cells for testing the toxicity of plastic materials employed in medical practice. They compared their results with those obtained in rabbit implantation and found the cell culture system to be more sensitive. They placed a sample in direct contact with the cell monolayer. Zones of degeneration developed when samples were toxic.

This report compares the use of primary and continuous cell cultures for evaluating the toxicity of rubber closures using three methods of assay in tissue culture. These are defined as: (*a*) direct contact with the cell monolayer, (*b*) maintenance medium-extract overlay on the cell monolayer, and (*c*) growth medium-extract in a cell suspension. The rubber closures tested were received in the laboratory for routine use. They were composed of natural rubber or synthetic butyl rubber and ranged in size from 13 mm. to 30 mm. in diameter.

EXPERIMENTAL

Direct Contact Method—Primary cell monolayers were prepared as follows. Nine-day-old chicken embryos were decapitated, minced, washed with phosphate buffered saline (PBS, pH 7.4), and the cells dissociated with 0.25% trypsin. Approximately 2×10^6 cells were inoculated into 60-mm. tissue culture plates[1] containing 5 ml. of Eagle's basal medium (BME) supplemented with 10% bovine serum. The plates were incubated at 37° in a humidified CO_2 incubator which maintained a pH of 7.0–7.4. When the cell monolayer was confluent, usually 2–3 days, the medium was evacuated and a rubber sample placed carefully at the center of the monolayer. Growth medium (BME + 10% fetal bovine serum) was added and the plate incubated as above. The rubber samples were tested as received unless the geometry of the closure necessitated cutting them. They were washed in pyrogen-free distilled water prior to testing. Samples were cut to approximately 5 mm.[3] and the cut surface exposed to the cell monolayer. Plates were observed daily for 3 days for evidence of cellular degeneration. When the sample was toxic, macroscopic observation revealed clear zones surrounding the sample, because of degradation of the cell monolayer (Figs. 1 and 2). Microscopic observation was made with a low power objective (10×). A sample was considered to be toxic when cells appeared granular, rounded, and detached from the surface of the culture plate.

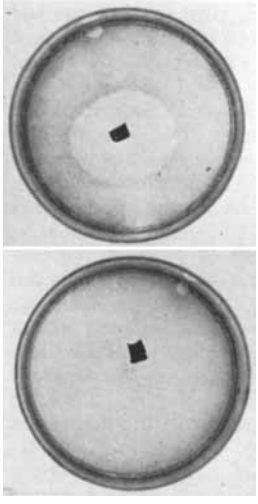

Fig. 1—Direct contact method of assay on cell monolayer. Key: top, toxic rubber sample showing an area of degeneration; bottom, nontoxic rubber sample. Cells stained with neutral red (1:20,000).

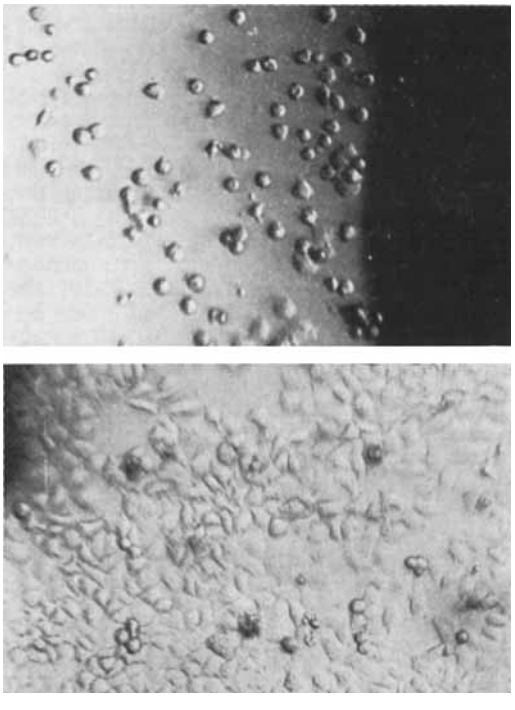

Fig. 2—Microscopic view of areas adjacent to rubber sample shown in Fig. 1. Key: top, toxic sample (shadow) showing degenerative cells; bottom, nontoxic sample (shadow) showing a healthy confluent epithelial cell monolayer. Cells are HEp-2 cultures. 100× magnification.

[1] Falcon Plastics, B-D Laboratories.

Human epidermoid carcinoma cell cultures (HEp-2), originally derived from a carcinoma of the larynx by Toolan (5) in 1955, were grown in 60 cm.^{2} milk dilution bottles (MDB). The cells had undergone an unknown number of passages before receipt. The cells were grown as monolayers by transferring them twice weekly using 0.25% trypsin in phosphate buffered saline (PBS, pH 7.4) to detach the cells from the glass. This was accomplished by removing the maintenance medium from MDB and adding 20 ml. of the trypsin solution to bathe the cell sheet. Following incubation at room temperature for 1 min., the trypsin was removed and the cultures incubated at 37° for 15 min. Sufficient growth medium (BME plus 10% fetal bovine serum) was added to give a cell suspension which was subdivided into MDB or 25 cm.^{2} Falcon plastic flasks at a split ratio of 1 to 6. The concentration of fetal bovine serum was dropped to 2% for maintenance. When the cell sheet became confluent, the tests were performed in the same manner as described for primary cultures except as noted.

Maintenance-Extract Method—Primary and continuous cell cultures were prepared as described above. When the cell monolayer was confluent, the growth medium was removed and replaced with a maintenance medium to which a sample extract had been added. The extract was prepared by suspending a rubber sample, as received, into 2 ml. of maintenance medium and incubating the suspension at room temperature for 24 hr. The medium was aseptically drawn off and added to sufficient maintenance medium diluent to give a final extract concentration of 25%. The cell cultures were incubated at 37° and examined daily for 3 days under low power magnification (10×). Cellular granulation, rounding, and detachment from the surface of the container were indicative of a toxic response.

Growth Medium-Extract Method—The sample extract was prepared as described above except that growth medium was used in place of maintenance medium. Cell suspensions of chick embryo and HEp-2 cultures were prepared by detaching the cells from MDB or plastic flasks with 0.25% trypsin as previously described. The cells were suspended in the growth medium-extract mixture to give a planting concentration of 1.6×10^5 cells per ml. for chick embryos and 75×10^3 cells per ml. for HEp-2 cultures. The resulting cell suspensions were inoculated into plastic plates, incubated at 37°, and observed daily for 3 days. Absence of cell growth and attachment indicated a toxic response.

Rabbit Implantation—Intramuscular implantation of rubber samples was performed and evaluated by the method described in the USP XVII or in the NF XII for plastics.

Samples used for rabbit implantation were prepared by cutting strips from the top of the rubber closure with a razor blade to a size approximating $1 \times 1 \times 10$ mm. This method resulted in smooth cut surfaces on all sides of the sample.

RESULTS AND DISCUSSION

A comparison between HEp-2 and primary chicken cell cultures was made using the direct contact method to determine the effect of subcultivation on the sensitivity of the cell cultures to toxic samples. Differences were noted by measuring the approximate diameter of the zones of degeneration surrounding the samples as shown in Figs. 1 and 2. Variation in cell sensitivity occurred in both types of cell culture. However, it was interesting to note (Figs. 3 and 4) that the degree of sensitivity of the sample and positive control varied in the same direction with HEp-2 cells as the substrate, whereas there was random variation in primary cell cultures. This may be attributed to the uniformity of epithelial-like cell populations from continuous culture, even though sensitivity changes with different passage levels. In contrast, primary cell cultures are generally disorganized and contain cells of many types. The degree of cytotoxicity in primary cultures may be dependent on the differing distribution and concentration of cell types with each culture preparation.

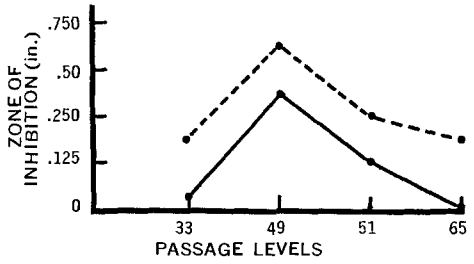

Fig. 3—Effect of passage levels of continuous (HEp-2) cell cultures on rubber closures. Key: - - - -, positive control; ——, sample 2342-2.

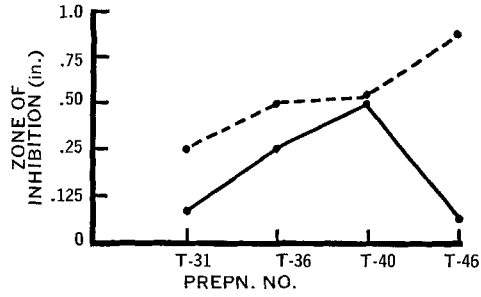

Fig. 4—Effect of different preparations of primary chick embryo (ChE) cell cultures on rubber closures. Key: - - - -, positive control; ——, sample 2342-2.

This study suggested that HEp-2 cells provided more reproducible results than did primary chicken cell cultures, although primary cells appeared more sensitive than HEp-2 cells as judged by the generally larger zones of degeneration.

Table I summarizes the results of an experiment designed to compare the three tissue culture assay systems in the more sensitive primary chicken cells. Sample 7 was completely nontoxic in all three systems, and sample 5 was toxic, and only slightly, by the direct contact method. The growth-extract system was the least sensitive, with little inhibition of cell proliferation by samples that were toxic in the direct contact and maintenance-extract tests. It may be significant that in the last two methods samples are introduced into established cell monolayers. Since the degree of sensitivity

between the direct contact and maintenance-extract methods did not vary markedly, the former was chosen because of its simplicity for a comparison of toxicity determinations in tissue culture and by rabbit implantation.

TABLE I—COMPARISON OF THREE TISSUE CULTURE METHODS IN EVALUATING TOXICITY OF RUBBER SAMPLES

Sample	Contact (Zone in.)	Extract: Growth (Confluency %)	Extract: Maintenance (Confluency %)
1	.25	95	10
2	.25	80	10
3	.125	80	25
4	.25	70	40
5	.0625	100	100
6	.25	95	75
7	Nontoxic	100	100
8	.50	95	10
9	.50	90	5
10	.25	90	40
Normal control	...	100	100
Neg. control	Nontoxic	100	100
Pos. control	.25	10	25

Table II shows results of implants in rabbits and direct contact in both HEp-2 and chick embryo cell cultures. The findings in the two types of cell cultures correlate well, although the primary cells were significantly more sensitive. The first five samples varied in toxicity between the two preparations, while samples *6*, *7*, and *8* were unquestionably toxic in both. The tissue culture system compared favorably with intramuscular implantation and appeared to be more sensitive, since *2A* and *4*, negative by implantation, were positive in both culture systems. It is also interesting to note that the nonchlorinated segment of sample *2* (*2A*) and the chlorinated (*2B*) were both toxic for tissue culture, but only the chlorinated sample was positive in rabbits.

TABLE II—COMPARISON OF RABBIT IMPLANT AND TISSUE CULTURE TESTING OF RUBBER CLOSURES

Sample	Rabbit Implant	Direct Contact: HEp-2	Direct Contact: ChE
1	+	3/3[a](1-ST)[b]	3/3
2A	−	3/3(1-ST)	4/4(1-ST)
2B (chlorinated)	+	3/3(1-ST)	3/3
3	Questionable	1/3(ST)	3/3
4	−	3/4(ST)	4/4(1-ST)
5	+	4/4(3-ST)	4/4(1-ST)
6	+	4/4	4/4
7	+	4/4	3/3
8	+	3/3	3/3
9	Questionable	0/1	0/1
Neg. control	− (Sometimes reactive)	0/7	0/4
Pos. control	+	5/5	4/4

[a] Denotes number of times the sample was toxic over the number of tests performed. [b] Slightly toxic.

Another study was designed to determine the effect of autoclave sterilization on rubber closures. Eight samples shown to be toxic by the direct contact method were compared in the growth-extract system. The results (Table III) showed two samples, *N* and *O*, to be negative when autoclaved and allowed to stand 1 hr. prior to assay. There was good correlation between the direct contact method and the growth-extract method for samples which were not autoclaved, except for sample *K*.

TABLE III—EFFECT OF AUTOCLAVE STERILIZATION ON CYTOTOXICITY IN HEp-2 CELL CULTURES

Sample	Direct Contact	Autoclave Sterilized Growth-Extract	Growth-Extract
H	Very toxic	No cell attachment	No cell attachment
I	Nontoxic	75% confluent	65% confluent
J	Very toxic	No cell attachment	No cell attachment
K	Sl. toxic	30–35% confluency	40% confluent
L	Nontoxic	75% confluent	65% confluent
M	Sl. toxic	60% confluent	70% confluent
N	Toxic	75% confluent	30–35% confluency
O	Sl. toxic	75% confluent	55% confluent
Neg. control	Nontoxic	75% confluent	75% confluent

The results of this investigation suggest that tissue culture may be useful as a biological assay to screen rubber closures prior to use in the manufacture of parenteral drug products. The two methods of assay involving established cell monolayers were in general agreement with each other but not with the growth-extract method. The direct contact method appears to be the most useful because of the ease of performance and evaluation. Although it would be difficult to equate cytotoxicity with reactions in man, such a screening mechanism could reasonably be assumed to reduce the risk of potential danger in man.

Pilot studies on a number of plastics using the same methods described for rubber closures compare favorably with the results of Rosenbluth *et al.* cited earlier in this report (4).

REFERENCES

(1) Brewer, J. H., and Bryant, H. H., *J. Am. Pharm Assoc., Sci. Ed.*, **49**, 652(1960).
(2) Parker, R. C., Morgam, J. F., and Morton, H. J., *Proc. Soc. Exptl. Biol. (N.Y.)*, **76**, 444(1951).
(3) Cruickshank, C. N. D., Hooper, C., Lewis, H. B. M., and MacDougall, J. D. B., *J. Clin. Pathol.*, **13**, 42(1960).
(4) Rosenbluth, S. A., Weddington, G. R., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **54**, 156(1965).
(5) Moore, A. E., Sabachewsky, L., and Toolan, H. W., *Cancer Res.*, **15**, 598(1955).

Methods for the Qualitative and Quantitative Analysis of Some Hydroxystilbenes

By JOSEPH E. SINSHEIMER and ROBERT VICTOR SMITH*

Paper, thin-layer, and gas chromatographic systems for the separation of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene have been developed. Quantitative analysis of 4-hydroxy- and 4,4′-dihydroxystilbene *via* spectrofluorimetry on thin-layer plates is described along with an assay of these compounds as their trimethylsilyl ethers using vapor phase chromatography (VPC) with flame ionization detection. The use of electron capture detection in the quantitative VPC analysis of three derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene has been investigated. A colorimetric assay, based on the reduction of blue tetrazolium, has been devised for 4,4′-dihydroxystilbene.

INVESTIGATIONS in these laboratories of the metabolism of certain stilbenes require assay procedures for 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene. Qualitative and quantitative literature analyses of these compounds are lacking and thus the purpose of this investigation was to devise such methods.

Paper chromatography of hydroxystilbenes indigenous to plants is well described in the literature (1) and served as a reference point for the development of paper and thin-layer systems in the present investigation. The recent commercial availability of fluorescent thin-layer scanners made a study of the quantitative evaluation of fluorescent hydroxystilbenes directly from thin-layer plates of interest.

Langer *et al.* (2) have successfully gas chromatographed simple monobenzenoid phenols as their trimethylsilyl (TMS) ethers. Formation of these derivatives from hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine has been reported to take place rapidly and nearly quantitatively at room temperature (3). An application of these techniques to the hydroxystilbenes was proposed. Of further interest was an evaluation of the sensitivity and utility of electron capture detection of these compounds for quantitative analysis. Chloromethyldimethylsilyl (CMDMS) ethers (4), with their added aliphatic chlorine, were expected to be of particular usefulness in the electron capture procedure.

The reduction of blue tetrazolium by compounds embodying the hydroquinone or quinone nucleus has been shown to be analytically useful (5). However, the analysis of hydroquinone vinylogs by this reaction has not been reported. Since 4,4′-dihydroxystilbene represents a hydroquinone vinylog, its reactivity with blue tetrazolium was also of interest.

EXPERIMENTAL

Reference Compounds

Commercially obtained *trans*-stilbene (Eastman Chemicals), 4-hydroxy-, and 4,4′-dihydroxystilbene (K & K Laboratories) were recrystallized prior to use. 2-Hydroxystilbene was prepared according to Singh, Chand, and Chojer (6), m.p. 143–145°.[1] [Lit. 147° (7).]

4-Hydroxy-3-methoxystilbene—A modified procedure of Dey and Row (8) was used for the preparation of this compound. A mixture of 1.52 Gm. of 4-hydroxy-3-methoxybenzaldehyde, 1.36 Gm. of phenylacetic acid, and 0.5 ml. of piperidine was refluxed for 4 hr., then poured over ice. The precipitate formed was recrystallized from ethanol–water to give 1.45 Gm. of a brown solid which was chromatographed over silica gel (75 Gm., 100 mesh) (Mallinckrodt) with benzene–methanol (96:4) to give 773 mg. of a pink crystalline solid. This solid was recrystallized from cyclohexane to give 707 mg. (31.2%) of white leaflets, m.p. 132.5–133.5°. [Lit. m.p. 134° (8).]

4-Acetoxystilbene—A mixture of 1.96 Gm. of 4-hydroxystilbene, 7 ml. of acetic anhydride, and 27 mg. of *p*-toluenesulfonic acid was refluxed for 2 hr., then poured over ice. Recovery of this precipitate by ether extraction and recrystallization from ethanol–water gave 1.91 Gm. (80.2%) of white needles, m.p. 151–152°. [Lit. m.p. 152° (9).]

4,4′-Diacetoxystilbene—A mixture of 531 mg. of 4,4′-dihydroxystilbene, 4 ml. of acetic anhydride, and 16 mg. of *p*-toluenesulfonic acid monohydrate was refluxed for 1.5 hr., then poured over ice. The precipitate formed, was recrystallized from chloroform–methanol to give 636 mg. (85.8%) of white plates, m.p. 218–219°. [Lit. m.p. 213° (10).]

4-Methoxystilbene—The procedure of Hewitt, Lewcock, and Pope (11) was used as the basis for the synthesis of this compound. To a stirred solution of 500 mg. of 4-hydroxystilbene in 30 ml. of methanol and 50 ml. of 30% potassium hydroxide, 20 ml. of dimethyl sulfate was added dropwise

Received April 4, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication July 5, 1967.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

* American Foundation for Pharmaceutical Education Fellow. Holder of the Sir Henry S. Wellcome Memorial Fellowship, 1966–1967.

[1] Melting points were determined with a Koller hot stage and are corrected.

TABLE I—PAPER CHROMATOGRAPHY

Hydroxystilbene	Rf in Solvent Systems[a] 1	2	3	Detection: Diazotized Sulfanilic Acid[b]	Gibb's Reagent[c]	Fluorescence[d]
2-Hydroxy-	0.90	0.91	0.88	Orange	Blue	Bright blue
4-Hydroxy-	0.85	0.89	0.84	Yellow	Yellow-gray	Pale blue
4,4′-Dihydroxy-	0.65	0.64	0.69	Yellow	Grey	Bright blue
4-Hydroxy-3-methoxy-	0.80	0.92	0.79	Red-orange	Yellow-gray	Blue

[a] Solvent systems used were: *1*, *n*-butanol–14% ammonium hydroxide (1:1) (top layer); *2*, *n*-butanol–benzene–acetic acid–water (0.5:2:2:1) (top layer); *3*, isopropanol–28% ammonium hydroxide–water (8:1:1). [b] Chromatogram sprayed with 0.5% sulfanilic acid–0.5% sodium nitrite (1:1), then oversprayed with 10% sodium carbonate. [c] Spraying with 0.1% ethanolic *N*-2,6-trichloro-*p*-benzoquinoneimine, followed by saturated sodium bicarbonate solution. [d] Excitation at 320–400 mμ with chromatographic viewer.

TABLE II—THIN-LAYER CHROMATOGRAPHY

Hydroxystilbene	Rf in Solvent Systems[a] 1	2	3	4	5	6
2-Hydroxy-	0.27	0.59	0.14	0.52	0.76	0.90
4-Hydroxy	0.13	0.52	0.05	0.47	0.71	0.85
4,4′-Dihydroxy-	0.00	0.18	0.00	0.21	0.58	0.76
4-Hydroxy-3-methoxy-	0.34	0.67	0.27	0.49	0.76	0.82

[a] Solvent systems employed were: *1*, benzene; *2*, benzene–methanol (92:8); *3*, cyclohexane–chloroform (1:1); *4*, cyclohexane–*p*-dioxane (2:1); *5*, benzene–*p*-dioxane–acetic acid (90:25:4); *6*, benzene–isopropanol–28% ammonium hydroxide (5:4:1). Detection reagents, methods, and color reactions are the same as indicated in Table I.

during a period of 50 min. Following an additional 30 min. of stirring, the reaction mixture was exhaustively extracted with methylene chloride. The methylene chloride layer was washed with water and saturated NaCl, dried over anhydrous sodium sulfate, and passed over 20 Gm. of alumina (Woelm, grade 1, neutral). Reduction to dryness *in vacuo* and recrystallization from ethanol–water gave 348 mg. (65.0%) of white leaflets, m.p. 137–138.5°. [Lit. m.p. 135–136° (9).]

Analytical Procedures

Paper Chromatography—Whatman No. 1 was used throughout for ascending development of 15–16 cm. Table I lists the solvent systems used.

Thin-Layer Chromatography—Silica Gel G plates (20 × 20 cm.) were prepared from a slurry containing 3 Gm. of Silica Gel G[2] and 7 ml. of water per plate. After air drying, plates were activated at 105° for 1 hr. Developing distance employed was 10 cm. using the solvent systems listed in Table II.

TLC Fluorimetry—Activated Silica Gel G plates were scored into 0.5-cm. channels, spotted (each concentration in triplicate) with 2.0–12.0 mcg. of 4-hydroxystilbene or 0.25–1.50 mcg. of 4,4′-dihydroxystilbene and developed (10 cm.) in benzene–isopropyl alcohol–28% ammonium hydroxide (5:4:1). The developed plates were overlayed with a clean glass plate after air drying and scanned with an Aminco-Bowman thin film scanner (No. 4-8221A) connected to an Aminco-Bowman spectrofluorometer (SPF 4-8202) and a 100-mv. Beckman recorder (93500). Measurement of 4-hydroxystilbene was performed under the following conditions: λ excitation, 370 mμ; λ emission, 425 mμ; sensitivity, 23; meter multiplication, 0.01; scanner motor, 1 r.p.m.; recorder speed, 60 in./hr. 4,4′-Dihydroxystilbene was measured under the same conditions used for 4-hydroxystilbene except that the λ emission was set at 435 mμ.

Gas Chromatography—Columns were packed according to Maunder *et al.* (12), with 3% SE-52 on Chromosorb W (60/80 mesh-HMDS treated). Qualitative VPC (Figs. 3 and 4) was performed with an F & M model 700 gas chromatograph equipped with dual flame ionization detectors, using the following conditions: columns, 4 ft. × 1/8 in. copper; injection port temperature, 270° ± 5°; column temperature, 174° (TMS ethers, Fig. 3), 220° (CMDMS ethers, Fig. 4); detector temperature, 261° ± 1°; carrier gas flow, 60 ml./min., helium (40 p.s.i.); hydrogen flow, 50 ml./min. (16 p.s.i.); air flow, 380 ml./min. (16 p.s.i.); attenuation, 5; range, 10^2. Quantitative VPC of 4-hydroxy- and 4,4′-dihydroxystilbene as their TMS ethers was conducted with an F & M model 300 gas chromatograph equipped with a model 1609 flame ionization detector, under the following conditions: column, 2 ft. × 1/4 in. copper; injection port temperature, 261° ± 2°; column temperature, 174°; detector temperature, 271° ± 2°; helium rotometer setting 9.0 (flow about 60 ml./min.) (30 p.s.i.); hydrogen rotometer setting, 7.0 (24 p.s.i.); air rotometer setting, 9.0 (20 p.s.i.); attenuation, 8; range, 10^2.

Trimethylsilyl (TMS) Ethers—Hydroxystilbenes (1–20 mg. each), dissolved in 0.7 ml. of dry pyridine (over potassium hydroxide pellets), were reacted with 0.2 ml. of hexamethyldisilazane (HMDS) and 0.1 ml. of trimethylchlorosilane (TMCS) for 10–15 min. at room temperature. With flame ionization detection, reaction mixtures were injected directly, while with electron capture detection, they were first diluted twentyfold with high purity *n*-hexane[3] prior to injection.[4]

Chloromethyldimethylsilyl (CMDMS) Ethers—CMDMS ethers were prepared as the TMS ethers only using di(chloromethyl)tetramethyldisilazane (CMTMDS) (0.2 ml.) and chloromethyldimethylchlorosilane (CMDMS)[3] (0.1 ml.) as silating reagents. Injections of these derivatives were made as indicated for the TMS ethers.

[2] Warner-Chilcott Laboratories, Richmond, Calif.

[3] Burdick and Jackson Laboratories, Inc., Muskegon, Mich.

[4] Hamilton 1 and 10-μl. syringes used throughout.

Blue Tetrazolium Reduction—Reagents were prepared as described previously (5). To 0.1612 mg. of 4,4′-dihydroxystilbene in 10 ml. of absolute alcohol was added 2 ml. of tetramethylammonium hydroxide and 2 ml. of blue tetrazolium reagent. Absorbance readings at 530 mμ were obtained at room temperature (23° ± 1°) against an identically prepared blank with a Zeiss PMQ II spectrophotometer. A solution prepared with 0.2728 mg. of 4-hydroxystilbene was reacted and measured similarly.

RESULTS AND DISCUSSION

Paper and Thin-Layer Chromatography—Tables I and II summarize conditions and results of the paper and thin-layer chromatographic separation and detection of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene.

The mobilities of the hydroxystilbenes in the paper chromatographic systems, where partition phenomena prevail, are readily explicable on the basis of the number of polar functions present. However, in thin-layer systems *1* through *4* (Table II), where adsorption effects predominate, the mobilities of 2-hydroxy- and 4-hydroxy-3-methoxystilbene are greater than might be predicted. In the former compound, this could result from steric hindrance of the approach of the phenolic group to the silica gel surface (13). While for 4-hydroxy-3-methoxystilbene, intramolecular hydrogen bonding would explain its high R_f values relative to that of 4-hydroxystilbene (14).

TLC Fluorimetry—The use of fluorescent scanning of thin-layer plates with system *6* for the analysis of 4-hydroxy- and 4,4′-dihydroxystilbene was investigated. In the apparatus employed, plates are scanned perpendicular to the solvent front with an activation wavelength slit limited to 0.5 cm. in length. Thus, while diffusion of spots toward the solvent front is related to the width of the recorded peaks, lateral diffusion beyond 0.5 cm. is not recorded. It was found necessary to limit such lateral diffusion by scoring the thin-layer plate into 0.5-cm. columns.

Figures 1 and 2 show the relationship of peak area to concentration obtained for 4-hydroxy- and 4,4′-dihydroxystilbene. Both curves reveal a restricted but usable linear range, limited at high concentrations by quenching effects (15).

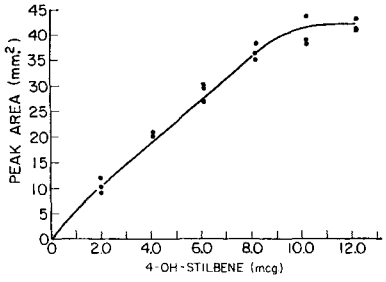

Fig. 1—TLC fluorimetry of 4-hydroxystilbene.

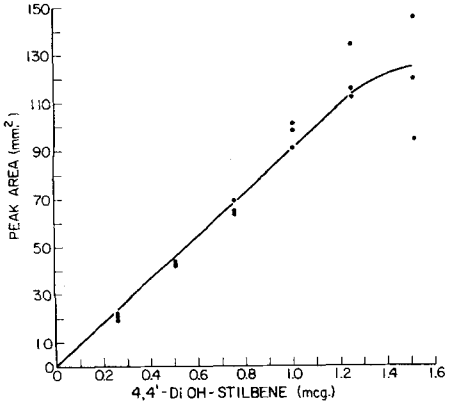

Fig. 2—TLC fluorimetry of 4,4′-dihydroxystilbene.

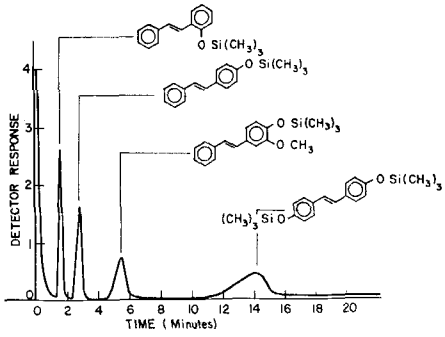

Fig. 3—Gas chromatographic separation of 2-hydroxy-, 4-hydroxy-, 4-hydroxy-3-methoxy-, and 4,4′-dihydroxystilbene as their TMS ethers.

Gas Chromatography—The TMS ethers of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene were prepared and separated by VPC as shown in Fig. 3. The chloromethyldimethylsilyl (CMDMS) ethers of these compounds also showed suitable development and resolution when subjected to gas chromatographic analysis (Fig. 4).

The utility of the TMS ethers in the quantitative analysis of 4-hydroxy- and 4,4′-dihydroxystilbene was studied. Based on the characteristics of their peaks (*e.g.*, Fig. 3) assessment to three significant places was achieved by using peak height and peak area measurements for 4-hydroxy- and 4,4′-dihydroxystilbene, respectively.[5] Linearity was exhibited in the range of 1 to 8 mcg. for 4-hydroxystilbene and 2 to 14 mcg. for 4,4′-dihydroxystilbene.[6]

Though the use of flame ionization detection was found suitable for the quantitative analysis of the TMS ethers of 4-hydroxy- and 4,4′-dihydroxystilbene, it was felt that an increase in sensitivity might be realized by employing electron capture detection for these or other volatile derivatives.

Studies concerning electron capture detection in the vapor phase chromatography of certain aromatic

[5] Peak height calculations were made according to the base line technique of Gaul (16) while peak area approximations (b 1/2 × h) were performed as indicated by Kaiser (17).

[6] Plots of peak height or peak area *versus* amount of hydroxystilbene did not pass through the origin. Wotiz and Clark (18) note that this may be due to loss on the column or irreversible adsorption of a constant amount of derivative during development.

hydrocarbons, has shown *trans*-stilbene to possess four times the electron affinity of chlorobenzene (19). The effect of substitution, specifically of oxygenated functions, on this affinity, however, has not been reported.

Table III lists the detection sensitivity relative to *trans*-stilbene and the limit of detection of three derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene. The detection limit is calculated as that amount of compound giving a peak height of two times the noise level, the latter taken as 2 mm.

Relative sensitivities were calculated from a comparison of peak areas derived from 10^{-9} moles of *trans*-stilbene and each stilbene derivative. For

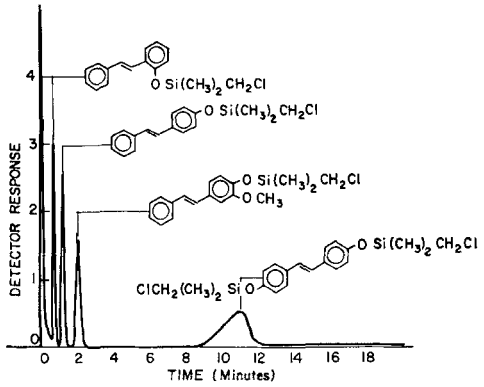

Fig. 4—Gas chromatographic separation of 2-hydroxy-, 4-hydroxy-, 4-hydroxy-3-methoxy-, and 4,4′-dihydroxystilbene as their CMDMS ethers.

this comparison, both compounds were chromatographed under identical conditions. When column temperatures above 200° were required for the development of a given compound (*Footnotes d, e*, Table III), direct comparison to *trans*-stilbene was not possible. At these temperatures, *trans*-stilbene elicits a peak which is not readily measurable due to its proximity to the solvent peak. Thus, in these cases, the compound was compared to a derivative which had previously been related to *trans*-stilbene.

Since *trans*-stilbene captures electrons by a process involving the propagation of a molecular ion (20, 21), a derivative of it, possessing an electron donating substituent might be expected to show a decrease in electron affinity. This effect is noted as a sensitivity decrease for 4-methoxystilbene (Table III).

The increase in sensitivity of the acetates of *trans*-stilbene is interesting since simple esters usually show little affinity for electrons (22). However, the increase in sensitivity of the TMS ethers of 4-hydroxy and 4,4′-dihydroxystilbene is explicable on the basis of increased electron affinity due to the considerable π character of the silicon-oxygen bond (21).

The significant increase in sensitivity of the CMDMS ethers, due to the ready dissociation of aliphatic halides in the electron capture detector (23), provides an extremely sensitive mode of analysis for these compounds and should have general applicability with analogous compounds.

Blue Tetrazolium Reduction—The oxidation of 4,4′-dihydroxystilbene by blue tetrazolium was established and the analytical applications of this reaction were studied. Since tetrazolium salts have been employed as detection reagents on paper

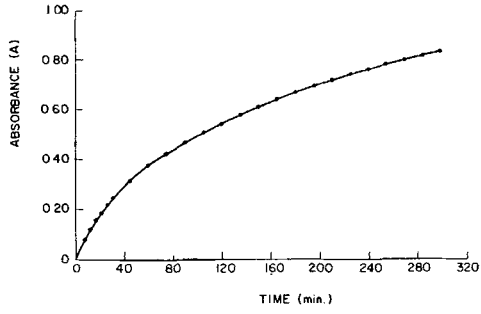

Fig. 5—Reduction of blue tetrazolium by 4,4′-dihydroxystilbene (5.4×10^{-5} M in reaction solution).

TABLE III—RELATIVE SENSITIVITY AND DETECTION LIMIT OF DERIVATIVES OF 4-HYDROXY- AND 4,4′-DIHYDROXYSTILBENE USING VPC AND ELECTRON CAPTURE DETECTION

Compd.	Conditions[a]	*Rt* min.[b]	Sensitivity[c] mm.²/10^{-9} mole	Relative Sensitivity	Detection Limit, ng.
trans-Stilbene	*A*	0.43	14.7	1	...
4-Methoxystilbene	*A*	1.32	11.8	0.8	403
4-Acetoxystilbene	*A*	2.42	48.7	3.2	95
4,4′-Diacetoxystilbene[d]	*B*	2.73	32.1	7.0	177
trans-Stilbene	*C*	0.40	19.6	1	...
4-Trimethylsiloxystilbene	*C*	2.06	26.7	1.4	106
4,4′-Di(trimethylsiloxy)stilbene	*C*	8.52	157	8.0	92
4-Chloromethyldimethylsiloxystilbene	*C*	5.48	775	40	8
4,4′-Di(chloromethyldimethylsiloxy)-stilbene[e]	*D*	9.77	867	71	11

[a] An F & M model 700 gas chromatograph, fitted with a 4 ft. × 1/8 in. column (copper) packed as indicated under *Experimental* and equipped with a model 700A-3 electron capture detector (tritium), was used throughout with the following constant conditions: injection port temperature, 225° ± 5°; detector temperature, 215° ± 3°; carrier gas, argon–methane (95:5) (50 p.s.i.); purge, 10 ml./min.; pulse, 15. Varied conditions: *A*, carrier gas flow, 60 ml./min.; column temperature, 183° ± 1°; range 10^2; attenuation, 2; *B*, carrier gas flow, 60 ml./min.; column temperature, 217° ± 1°; range, 10^2; attenuation, 2; *C*, carrier gas flow, 60 ml./min.; column temperature, 183° ± 1°; range, 1; attenuation, 5; *D*, carrier gas flow, 90 ml./min.; column temperature, 216° ± 1°; range, 1; attenuation, 2. [b] Relative to solvent front. [c] Calculated from the average area of three runs. The magnitude of response (peak area) of all compounds studied was found to be inversely proportional to the column temperature emphasizing the need for strict adherence to conditions during quantitative studies. [d] Compared to 4-acetoxystilbene under conditions *B*. [e] Compared to 4-chloromethyldimethylsiloxystilbene under conditions *D*.

chromatograms (24), similar procedures were applied for the detection of 4,4′-dihydroxystilbene. However, the slow rate of the tetrazolium–dihydroxystilbene reaction caused this procedure to be unsuccessful.

A measure of the formazan produced with time, when blue tetrazolium was reacted with 4,4′-dihydroxystilbene (5.4×10^{-5} *M*), is shown in Fig. 5. Similar measurement of a solution containing 4-hydroxystilbene (1×10^{-4} *M*) and blue tetrazolium showed no absorbance attributable to formazan production, over a period of 3 hr. Furthermore, spectrophotometric measurement of solutions containing varying amounts of 4,4′-dihydroxystilbene after 60 min. reaction with blue tetrazolium, showed a good correlation between absorbance and concentration. Thus, the feasibility of assaying 4,4′-dihydroxystilbene in the presence of 4-hydroxystilbene was established.

SUMMARY AND CONCLUSIONS

Paper, thin-layer, and gas chromatographic methods have been presented for the qualitative analysis of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene.

A sensitive and rapid method for the analysis of 4-hydroxy- and 4,4′-dihydroxystilbene has been devised, based on fluorescent scanning of thin-layer plates. Gas chromatographic analysis of these compounds as their trimethylsilyl ethers has also been shown to be a reliable quantitative procedure.

Vapor phase chromatography, coupled with electron capture detection has been shown to be a highly sensitive tool for the analysis of certain derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene.

The analysis of 4,4′-dihydroxystilbene, even in the presence of 4-hydroxystilbene, is possible through the use of blue tetrazolium.

REFERENCES

(1) Smith, I., "Chromatographic and Electrophoretic Techniques," vol. I, 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1960, pp. 320–321.

(2) Langer, S. H., Pantages, P., and Wender, I., *Chem. Ind. (London)*, **1958**, 1664.

(3) Horii, Z., Makita, M., Takeda, I., Tamura, Y., and Ohnishi, Y., *Chem. Pharm. Bull. (Japan)*, **13**, 636(1965).

(4) "Gas-Chrom Newsletter," vol. 7, No. 4, Applied Science Laboratories, Inc., State College, Pa., 1966.

(5) Sinsheimer, J. E., Salim, E. F., and Manni, P. E., *J. Pharm. Sci.*, **53**, 391(1964).

(6) Singh, R. N., Chand, V., and Chojer, T. N., *Agra Univ. J. Res. Sci.*, **1**, 153(1952); through *Chem. Abstr.*, **48**, 2654*b*(1954).

(7) Kostanecki, S. V., and Tambor, J., *Chem. Ber.*, **42**, 826(1909).

(8) Dey, B. B., and Row, K. K., *Quart. J. Indian Chem. Soc.*, **1**, 277(1925).

(9) "Dictionary of Organic Compounds," vol. 3, 4th ed., Oxford University Press, New York, N. Y., 1965, p. 1810.

(10) Auwers, K., *Chem. Ber.*, **36**, 1887(1903).

(11) Hewitt, J. T., Lewcock, W., and Pope, F. G., *J. Chem. Soc.*, **101**, 606(1912).

(12) Maunder, M. J. deF., Egan, H., and Roburn, J., *Analyst*, **89**, 157(1964).

(13) Fike, W. W., *Anal. Chem.*, **38**, 1697(1966).

(14) Lederer, E., and Lederer, M., "Chromatography—A Review of Principles and Applications," Elsevier Publishing Corp., New York, N. Y., 1957, p. 47.

(15) Fischer, L. J., and Riegelman, S., *J. Chromatog.*, **21**, 268(1966).

(16) Gaul, J. A., *J. Assoc. Offic. Anal. Chemists*, **49**, 389 (1966).

(17) Kaiser, R., "Gas Phase Chromatography," vol. I, Butterworth, Inc., Washington, D. C., 1963, pp. 179–186.

(18) Wotiz, H. H., and Clark, S. J., "Gas Chromatography in the Analysis of Steroid Hormones," Plenum Press, New York, N. Y., 1966, p. 55.

(19) Lovelock, J. E., Zlatkis, A., and Becker, R. S., *Nature*, **193**, 540(1962).

(20) Lovelock, J. E., *ibid.*, **189**, 729(1961).

(21) Gaston, L. K., *Residue Rev.*, **5**, 21(1964).

(22) Lovelock, J. E., and Lipsky, S. R., *J. Am. Chem. Soc.*, **82**, 431(1960).

(23) Lovelock, J. E., *Anal. Chem.*, **33**, 162(1961).

(24) Bush, I. E., and Willoughby, M., *Biochem. J.*, **67**, 689(1957).

Comparative Study in Man of the Absorption and Excretion of Amobarbital-^{14}C from Sustained-Release and Nonsustained-Release Dosage Forms

By EARL ROSEN*, ANDREW POLK*, SPENCER M. FREE*, PHILIP J. TANNENBAUM†, and ARCHER P. CROSLEY, JR.†

Amobarbital was tagged with a radioactive carbon atom and incorporated into sustained-release and nonsustained-release dosage forms to obtain comparative absorption and excretion data. Radioactivity levels in plasma and urine showed that the sustained-release formulation released about one-third of its amobarbital promptly and the remaining portion gradually, whereas the nonsustained-release dosage form showed a steady decrease in plasma concentration after 1 hr. and a higher urinary excretion rate during the first 12-hr. period. These performance characteristics indicate that a moderately short-acting drug, such as amobarbital, can be pharmaceutically modified so as to be a candidate for use with longer-acting drugs, such as trifluoperazine, in a single dose unit.

TESTS IN ANIMALS showed that a combination of amobarbital and trifluoperazine has a broader spectrum of activity than the individual drugs, but combining them in a single dose unit posed a pharmaceutical problem because amobarbital is a moderately short-acting drug (1) while trifluoperazine is long acting. Incorporating the amobarbital component into a sustained-release formulation would solve the problem if a formulation could be prepared that would release amobarbital promptly and gradually over an extended period. This report describes the specific sustained-release dosage form and how it was evaluated in man.

EXPERIMENTAL

Preparation of Dosage Forms—Based on the results of preliminary clinical studies, a combination of 30 mg. of amobarbital and 2 mg. of trifluoperazine was chosen for testing. However, conventional bioanalytic techniques to measure levels of amobarbital and its metabolites in blood and urine are not sufficiently sensitive to accurately measure drug concentration from a 30-mg. dose, so it was necessary to label the amobarbital with a radioactive carbon atom. The utility of this technique for evaluating pelleted sustained-release preparations has been reported previously (2–4).

Based on human safety considerations, an upper limit of 50 μc. of ^{14}C was selected for the 30-mg. amobarbital dose. To obviate the problems involved in uniformly mixing labeled and unlabeled drug, the 5-ethyl-5-isoamyl barbituric acid-2^{14}C was synthesized with a specific activity of 1.57 μc./mg.

The dosage forms for this study were prepared by a method similar to that reported previously (2, 4). One kilogram of nonsustained-release amobarbital pellets was prepared in a 12-in. stainless steel coating pan located in a disposable glove-box inside a walk-in fume hood fitted with special filters. A portion (150 Gm.) of these nonsustained-release pellets was set aside and the remaining pellets were coated with a wax-fat coating.

Six separate groups of sustained-release pellets with approximately 6, 7, 8, 9, 10, and 11% wax-fat (WF) coating were prepared to insure a sufficient spread in patterns to facilitate blending for the *in vitro* release pattern selected on the basis of controlled clinical studies. This desired *in vitro* pattern was 35, 60, 75, and not less than 80% released at the 0.5, 2, 4.5, and 7-hr. intervals, respectively.

The *in vitro* release patterns of the six experimental groups are shown in Table I; they were determined by the method of Souder and Ellenbogen (5). In these groups, it was found that the blend consisting of 35% nonsustained-release pellets, 10% WF-1, 20% WF-2, 15% WF-3, 10% WF-4, and 10% WF-5 gave a calculated pattern of 35, 56, 77, and 86% released at the desired time intervals.

The *in vitro* patterns of this blend assayed before and after it was encapsulated with 2 mg. of nonsustained-release trifluoperazine are shown in Table II. These data indicated that the *in vitro*

TABLE I—*In Vitro* RELEASE PATTERNS OF AMOBARBITAL-^{14}C WAX-FAT COATED GROUPS

Group	% *In Vitro* Release at Time Interval 0.5 hr.	2 hr.	4.5 hr.	7 hr.
WF-1	0	72	88	91
WF-2	0	51	75	84
WF-3	0	23	62	77
WF-4	0	9	51	63
WF-5	0	4	41	57
WF-6	0	3	20	51

Received April 14, 1967, from the * Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101, and the † Section of Clinical Pharmacology, Research Institute and Department of Medicine, Presbyterian-University of Pennsylvania Medical Center, Philadelphia, PA 19104

Accepted for publication June 22, 1967.

Presented to the Industrial Pharmaceutical Technology Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

TABLE II—BLEND AND CAPSULE *In Vitro* RELEASE PATTERNS (AMOBARBITAL-^{14}C)

Material	% *In Vitro* Release at Time Interval 0.5 hr.	2 hr.	4.5 hr.	7 hr.
Blend	34	54	80	86
Sustained-release amobarbital-^{14}C, trifluoperazine capsule	34	53	77	83

release pattern of the experimental capsule closely approximated the desired pattern.

The nonsustained-release pellets of labeled amobarbital, previously set aside, were encapsulated with 2 mg. of nonsustained-release trifluoperazine.

In Vivo Protocol—Two dosage regimens, *A* and *B*, were compared in 10 normal subjects, using a cross-over design: (*A*) sustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluoperazine, 2 mg., given at 0 hr.; (*B*) nonsustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluoperazine, 2 mg., given at 0 hr.

These regimens were tested to determine whether the sustained-release amobarbital formulation provided a prompt initial dose, whether the remaining portion of amobarbital was delivered gradually, and whether there was any significant reduction in the total amount of amobarbital made available from the sustained-release formulation.

Prior to the tests, the subjects were examined and found to be in good health. All underwent studies to rule out impairment of renal, hepatic, and hematopoietic function. The subjects were then divided into two groups of five and each group was studied on alternate weeks over a 4-week period. The order in which the test formulations were given was alternated within each group.

Each morning of the study, the subjects reported in a fasting state and were given a test dose with fruit juice. They received no food or drink for 90 min.; then they resumed their normal diets. For the first 12 hr., this consisted of a free selection of hospital cafeteria food.

Plasma and Urine Sampling—A blood specimen was taken from each subject before drug administration and at hourly intervals after it for a total of 12 hr., by means of an indwelling anticoagulated venous needle system (4). The plasma was separated and immediately frozen until assayed.

Complete urine specimens were collected for 96 hr. after drug administration. The collection intervals were 0–3, 3–6, 6–9, 9–12, 12–24, 24–48, 48–72, and 72-96 hr. Urine specimens were also frozen until assayed.

It was also decided to determine the pH of all urine specimens passed in the first 12 hr. of each segment of this cross-over study. The authors did not intend to try to establish a relationship between urinary pH and drug excretion (6, 7). An increase in the excretion of a weak acid like amobarbital (pKa 7.96), or its principal metabolite hydroxyamobarbital, could be expected only in an alkaline urine and Elliot *et al.* (8) have found that only 1% of urine specimens have a pH of 7.2 or more. Rather, the authors wanted to determine whether pH profiles would be similar on two separate occasions. Information of this sort is not presently available and would be useful in the design of dosage form performance studies involving drugs which *are* influenced by urinary pH fluctuations.

Analysis of the Specimens—Plasma and urine samples were assayed for radioactivity using a method previously used to assay dextroamphetamine-^{14}C (4). The pH of the urine specimens was determined immediately after voiding, using a Beckman model G glass electrode pH meter.

RESULTS AND DISCUSSION

Plasma Data—These data, as shown in Fig. 1, are reported as micrograms of amobarbital per ml. of plasma, but include radioactivity that originated from the metabolites as well as from the parent compound. A comparison of the two regimens at the first hour after administration shows that the plasma level of radioactivity resulting from the sustained-release formulation was 36% of the amount resulting from the nonsustained-release formulation. This value for the sustained-release formulation agrees closely with the *in vitro* release value of 34% at 0.5 hr.

The plasma levels of radioactivity following the administration of the nonsustained-release regimen were consistently and significantly higher ($P < 0.01$) than the levels of the sustained-release regimen. And there was a statistically significant difference ($P < 0.01$) in the patterns: the nonsustained-release regimen showed a steady decrease in plasma

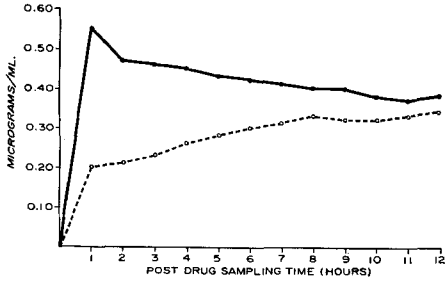

Fig. 1—Plasma levels of radioactivity reported as micrograms of amobarbital/ml. of plasma. Each line represents the averages of 10 volunteers. Key: – – – –, (A) sustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluorperazine, 2 mg.; ————, (B) nonsustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluoperazine, 2 mg.

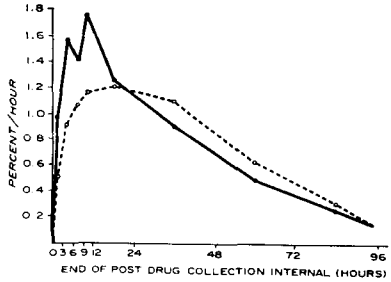

Fig. 2—Urinary excretion rate of radioactivity reported as per cent of dose excreted per hour. Each line represents the averages of 10 volunteers. Key: – – – –, (A) sustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluoperazine, 2 mg.; ————, (B) nonsustained-release amobarbital-^{14}C, 30 mg., nonsustained-release trifluoperazine, 2 mg.

TABLE III—AVERAGE CUMULATIVE URINARY RECOVERY OF RADIOACTIVITY REPORTED AS PER CENT OF ADMINISTERED DOSE

Regimen, %	Cumulative Period, hr. 0–3	0–6	0–9	0–12	0–24	0–48	0–72	0–96
A	1.6	4.4	7.7	11.3	26.2	53.4	69.0	77.4
B	3.0	7.8	12.2	17.7	32.1	57.1	69.8	76.2

TABLE IV—URINARY pH PROFILES FOR TWO 0–12-hr. PERIODS

	Regimen							
	A				*B*			
	Intervals, hr.				Intervals, hr.			
Subject	0–3	3–6	6–9	9–12	0–3	3–6	6–9	9–12
1	5.42	7.45	7.14	6.70	5.37	6.31	6.32	6.75
2	5.45	5.42	5.57	5.46	6.18	5.95	6.23	5.85
3	7.21	6.95	6.36	6.07	5.68	6.02	6.90	6.80
4	7.21	7.70	7.80	6.95	6.83	7.17	7.42	6.54
5	6.34	5.80	6.29	6.62	5.50	7.20	7.52	6.35
6	5.52	6.45	6.70	6.20	5.55	6.76	6.22	5.35
7	5.50	5.28	5.28	5.46	5.96	6.35	6.20	4.93
8	5.30	6.50	5.75	5.70	5.50	6.65	5.48	6.85
9	5.51	6.51	7.50	6.55	6.06	6.38	6.30	5.60
10	5.71	7.50	7.05	7.08	5.35	6.38	5.42	6.17
Av.	5.92	6.56	6.54	6.28	5.80	6.51	6.40	6.12
S.D.	0.738	0.860	0.843	0.595	0.465	0.428	0.705	0.665

concentration after 1 hr.; in contrast, the sustained-release regimen showed a slowly rising pattern after 1 hr. All these data indicated that the sustained-release formulation made approximately one-third of its amobarbital content available for absorption promptly and that it delivered the remaining portion gradually.

Urine Data—The radioactivity excretion rates for the two regimens were computed by dividing the per cent of radioactivity excreted per collection-interval by the number of hours in the interval. Each value was then plotted at the midpoint of the collection interval, as shown in Fig. 2.

An analysis of the plots for the two regimens showed a statistically significant difference ($P < 0.01$). Furthermore, the data showed that less amobarbital was excreted from the sustained-release regimen than from the nonsustained-release regimen during the first 24-hr. period. But the reverse was true thereafter; more amobarbital was excreted from the sustained-release regimen. These findings are consistent with the plasma findings and give further proof that the sustained-release amobarbital formulation makes its drug gradually available for absorption.

The cumulative urinary recovery data, reported in Table III as the per cent of administered dose in each collection interval, are essentially identical for the two regimens over a 96-hr. period and indicate that amobarbital in the sustained-release formulation was as available for absorption as amobarbital in the nonsustained-release dose.

Urinary pH Data—The 0–12 hr. urinary pH profiles for each of the 10 subjects during the two test periods are reported in Table IV. The profiles were very similar and, as could be expected, showed a peak in the 3–6-hr. interval consistent with the afternoon postprandial tide and range reported by Elliot *et al.* (8). But the most interesting finding was the similarity in the averages of the profile values: the figures for the two test periods varied by less than 0.2 pH units.

SUMMARY

This study in man was undertaken to determine the performance characteristics of a sustained-release amobarbital formulation. To obtain comparative absorption and excretion data, amobarbital was tagged with a radioactive carbon atom and incorporated into sustained-release and nonsustained-release dosage forms. Data from plasma and urine studies showed that the sustained-release formulation made about one-third of its amobarbital content promptly available for absorption and that the remaining portion was absorbed gradually. This was in contrast to the nonsustained-release dosage form, which showed a steady decrease in plasma concentration after 1 hr. and had a higher urinary excretion rate during the first 12-hr. period. Furthermore, there was no reduction in drug availability from the sustained-release formulation: the cumulative urinary recovery of radioactivity from both dosage forms after 96 hr. was about 75%. These data indicate that it is possible to combine this sustained-release amobarbital formulation in a single dose unit with a long-acting drug like trifluoperazine.

During this study, urinary pH data were also recorded which showed that 0–12-hr. urinary profiles taken on two separate occasions were very similar and that profile averages varied by less than 0.2 pH units.

REFERENCES

(1) Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics," The Macmillan Co., New York, N. Y., 1965.

(2) Rosen, E., and Swintosky, J. V., *J. Pharm. Pharmacol.*, **12**, 237(1960).

(3) Rosen, E., *J. Pharm. Sci.*, **52**, 98(1963).

(4) Rosen, E., Ellison, T., Tannenbaum, P., Free, S. M., and Crosley, A., *ibid.*, **56**, 365(1967).

(5) Souder, J. C., and Ellenbogen, W. C., *Drug Std.*, **26**, 77(1958).

(6) Milne, M. D., Schribner, B. H., and Crawford, M. A., *Am. J. Med.*, **24**, 709(1958).

(7) Beckett, A. H., and Rowland, M., *J. Pharm. Pharmacol.*, **17**, 628(1965).

(8) Elliot, J. S., Sharp, R. F., and Lewis, L., *J. Urol.*, **81**, 339(1959).

Diffusion, Permeation, and Solubility of Selected Agents In and Through Polyethylene

By M. A. GONZALES, J. NEMATOLLAHI, W. L. GUESS, and J. AUTIAN*

Even though polyethylene containers have and are being used with more frequency to package drug products, diagnostic agents, biological products, and cosmetic preparations, little emphasis apparently has been placed on the possible interactions between solute molecules in the product and the polyethylene. This report presents a study on the diffusion, permeation, and solubility of six compounds (acetophenone, benzaldehyde, benzoic acid, benzyl alcohol, 4-methylacetophenone, and 4-methylbenzaldehyde) in and through polyethylene. Results reveal that benzyl alcohol and benzoic acid had the lowest permeation (P) and diffusion (D) values as compared to the other compounds in the series. This was ascribed to the intermolecular hydrogen bonding potential of benzyl alcohol and benzoic acid; thus the formation of dimer or trimer whose permeation through the polymer is assumed to be restricted. In the case of 4-methylbenzaldehyde and 4-methylacetophenone, the D values obtained were similar to those of the parent compounds (benzaldehyde and acetophenone). Higher P values for the 4-methyl derivatives of benzaldehyde and acetophenone were related to the higher solubility of the molecules in the plastic.

THE PACKAGING of drugs, biological products, diagnostic aids, and cosmetic products in polymeric materials has increased greatly in the past decade. A number of advantages of plastics over the conventional glass, paper, and metals have helped to accelerate the adoption of plastic packaging systems to a host of old and new products entering the medical and para-medical fields. As might be expected with the introduction of new materials for a specific application, certain problems have arisen or may arise which justify research into drug–plastic interactions (1–4).

Presently the most commonly used polymeric material for containers is polyethylene. It has been found to be a good moisture-vapor barrier for aqueous products as well as a good barrier for a number of organic solvents. Pinsky (5) has studied the permeation rates of several classes of organic liquids through polyethylene and has found the rate was lowest with the highly polar substances, increasing in the following order of chemical structures: alcohol, acids, nitro derivatives, aldehydes and ketones, esters, ethers, hydrocarbons, and halogenated hydrocarbons. Salame and Pinsky have been able to develop an arithmetical method for predicting the permeability of liquids through polyethylene (6). They refer to their method as the "Permachor" method. It is interesting to note, however, that only a few reports are available on the barrier properties of polyethylene in contact with aqueous solutions of specific compounds. It should be apparent that any loss of an ingredient in a drug or similar type of product stored in a polyethylene container would decrease the effectiveness of the drug product leading to possible serious consequences to the patient and the manufacturer of the product.

To develop a body of knowledge on the barrier properties of polyethylene which may be of value to the pharmaceutical or cosmetic scientist, a study was undertaken to determine the diffusion, permeation, and solubility of some commonly found pharmaceutical compounds, namely, benzyl alcohol, benzoic acid, and benzaldehyde. Also included in the study were acetophenone, 4-methylbenzaldehyde, and 4-methylacetophenone which are structurally related to benzoic acid and benzaldehyde. Study of the latter compounds was designed to provide contrasting information on the effect of certain substituents on the rate of diffusion and permeation of the parent compounds.

EXPERIMENTAL

Materials and Supplies—Polyethylene film, 3 mil thickness (Gulf Oil Corp., Chemicals Department, Orange, Tex.); acetophenone, certified reagent grade (Fisher Scientific Co., Fairlawn, N. J.); benzaldehyde, Eastman grade (J. T. Baker Chemical Co., Phillipsburg, N. J.); benzyl alcohol, Eastman grade (Eastman Organic Chemicals, Rochester, N. Y.); 4-methylacetophenone, Eastman grade (Eastman Organic Chemicals, Rochester, N. Y.); 4-methylbenzaldehyde, Eastman grade (Eastman Organic Chemicals, Rochester, N. Y.).

Properties of Polyethylene Film—The density of the film as determined by the use of a density gradient column at 25° was found to be 0.9246 Gm./ml. This value indicates that the film falls in the category of a low density polyethylene. Weight average

Received March 20, 1967, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78756

Accepted for publication July 5, 1967.

This investigation was supported in part by contract PH43-64-557 from the Division of Biologic Standards, National Institutes of Health, Bethesda, Md., and from a graduate fellowship from Abbott Laboratories, North Chicago, Ill.

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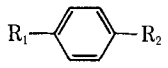
TABLE I—MOLECULAR STRUCTURES OF COMPOUNDS INVESTIGATED OF THE GENERAL FORMULA



Compd.	R_1	R_2	Mol. Wt.	Dipole[a] Moment	Solubilities[b] in Water, % w/v
Benzyl alcohol	$-CH_2OH$	$-H$	108.13	1.68	4.0
Benzoic acid	$-\overset{O}{\overset{\|}{C}}-OH$	$-H$	122.12	1.00	0.34
Benzaldehyde	$-\overset{O}{\overset{\|}{C}}-H$	$-H$	106.12	2.77	0.33
Acetophenone	$-\overset{O}{\overset{\|}{C}}-CH_3$	$-H$	120.14	2.77	0.30
4-Methylbenzaldehyde	$-\overset{O}{\overset{\|}{C}}-H$	$-CH_3$	120.14	3.30	0.10
4-Methylacetophenone	$-\overset{O}{\overset{\|}{C}}-CH_3$	$-CH_3$	134.17	3.20	0.10

[a] Values are in Debye units. These values were determined in benzene at 25° as reported in Wesson, L. G., "Tables of Electric Dipole Moments," The Technology Press, Massachusetts Institute of Technology, Cambridge, Mass., 1948. [b] The solubilities in water at approximately 25° are taken from "The Merck Index of Chemicals and Drugs," Merck and Co., Inc., Rahway, N. J., 1960, for benzyl alcohol, benzoic acid, and benzaldehyde. The solubilities for the other compounds were determined experimentally by the authors.

molecular weight was equal to 83,430 while the number average molecular weight was 15,820.[1] The polyethylene had an average of 19.3 methyl groups per 1,000 monomeric units (methylene groups) indicating a high degree of branching and a terminal vinylidene content equal to 0.37 per 1,000 methylene units.[1]

Permeation Experiments—The compounds studied in this report with some of their physical constants are listed in Table I. Aqueous solutions of each of these compounds were prepared at a specific original concentration as shown in Table II. Each agent was studied at four different temperatures (40°, 50°, 60°, and 70° ± 0.02°). The permeation experiment was conducted in a cell consisting of two chambers separated by the polyethylene film[2] secured in place by a clamp (Fig. 1) following the general procedure of Rodell (7). In the left chamber was placed 500 ml. of the solution under study and in the right, 500 ml. of distilled water. The solution and water were heated to a temperature slightly higher than the temperature of the water bath prior to adding them to the permeation cell. The cell was then placed into a constant-temperature water bath adjusted to the desired temperature. At various time periods aliquots from the right chamber were withdrawn and the quantity of the compound was determined spectrophotometrically at the wavelength of maximum absorbance. (See Table II.) From the data a figure was prepared plotting the quantity of the compound passing through the film against time. Each of the compounds was studied in this manner at various temperatures.

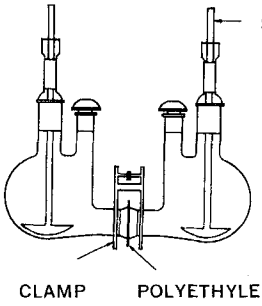


Fig. 1—Diagram of permeation cell.

TABLE II—ORIGINAL CONCENTRATIONS OF SOLUTIONS AND WAVELENGTHS OF MAXIMUM ABSORBANCE

Compd.	% Concn., Wt./Vol.	Wavelength Max. Abs., mμ	Diluting Solvent
Benzyl alcohol	1.0	258	Distilled water
Benzoic acid	0.3	230	0.1 *N* HCl
Benzaldehyde	0.3	250	Distilled water
Acetophenone	0.3	245	Distilled water
4-Methylbenzaldehyde	0.1	260	Distilled water
4-Methylacetophenone	0.1	257	Distilled water

Representative plots are shown for only two of the compounds (benzyl alcohol and benzaldehyde) in Figs. 2 and 3.

Extrapolation of lines in the various plots to the time axis permitted the calculation of the apparent diffusion coefficient (D) using the Barrer relationship (8):

[1] Statistics on molecular weights, branching, and olefinic bonds were generously furnished by Mr. T. O. J. Kresser, Plastics Laboratory, Gulf Oil Corp., Chemicals Department, Orange, Tex.

[2] Surface area exposed to solution was 22.07 cm.².

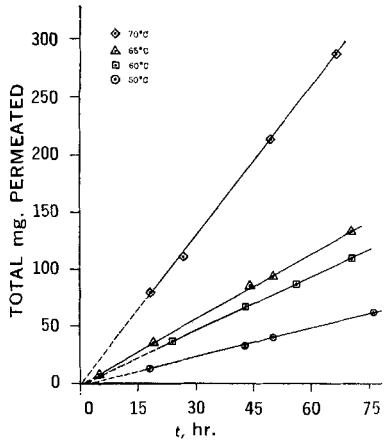


Fig. 2—Permeation plot of benzyl alcohol (1.0%) through polyethylene at several temperatures.

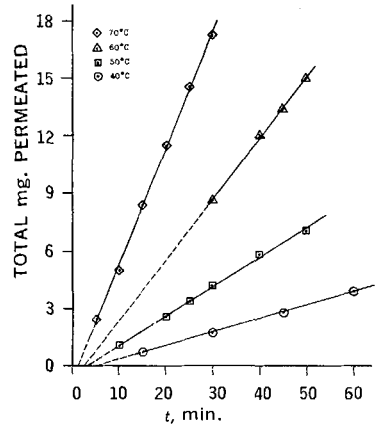


Fig. 3—Permeation plot of benzaldehyde (0.3%) through polyethylene at several temperatures.

$$D = L^2/6\tau \qquad \text{(Eq. 1)}$$

where L is the thickness of the film and τ is the time lag obtained from the extrapolated line.

The D values for each of the agents at the specific temperature of the experiment are listed in Table III. The same table also includes the activation energy (ΔE_D) of diffusion calculated by the use of the Arrhenius expression:

$$\log D = \log D_0 - \Delta E_D/2.303\ RT \qquad \text{(Eq. 2)}$$

The plots of log D *versus* $1/T$ for the solutes under study gave linear relationship, thus permitting the evaluation of ΔE_D from the slopes.

Permeability constants (P) were evaluated for each of the compounds for a given temperature using the expression:

$$P = c/t \cdot LV/CA \qquad \text{(Eq. 3)}$$

where c/t is the slope of the linear portion of the permeation plot (Figs. 2, 3), L is the thickness of the film, V the total volume of solution, C the original concentration, and A the surface area of the film exposed to the solution. The P values are listed in Table IV, which also includes the activation energies of permeation, evaluated in a manner similar to that described for the activation energy of diffusion.

Solubility coefficients (S) for each of the compounds were calculated by the relation of $P = SD$, and these values are listed in Table V. Heats of solution (ΔH), calculated by the use of the Arrhenius relationship are also included in Table V.

RESULTS AND DISCUSSION

Initial permeation studies were directed to compare the barrier properties of polyethylene toward benzyl alcohol, benzoic acid, and benzaldehyde. As are depicted in Tables III and IV, considerable difference is noted between the aldehyde and either the alcohol or the acid. At 50° benzaldehyde has an apparent diffusion coefficient of 542.0×10^{-10} cm.^{2}/sec., while for benzoic acid and benzyl alcohol the D values are 14.6×10^{-10} cm.^{2}/sec. and 11.6×10^{-10} cm.^{2}/sec., respectively. Comparison of the permeability constants (Table IV) for the three compounds indicates that benzaldehyde possesses a higher permeation rate than benzoic acid or benzyl alcohol and although the difference is not so large as with the diffusion coefficients, it is still significant.

Since the benzaldehyde gave much higher values for D and P than demonstrated for either the benzyl alcohol or benzoic acid, similar studies on three structurally related compounds (acetophenone, 4-methylbenzaldehyde, and 4-methylacetophenone) were undertaken.

Diffusion Coefficients and Activation Energies of Diffusion—As has been indicated above, the diffusion coefficient for benzaldehyde at any temperature was found to be greater than either the benzyl alcohol or the benzoic acid. These latter two compounds have nearly similar D values as may be noted in Table III. The similar apparent diffusion coefficients suggest a similar molecular volume. All other factors being equal, the diffusion coefficient is strongly dependent on the molecular volume of a

Table III—Diffusion Coefficients and Activation Energies of Selected Compounds in Polyethylene

Compd.	$D \times 10^{10}$ cm.^{2}/sec. 40°C.	50°C.	60°C.	70°C.	ΔE_D, Kcal./mole
Benzyl alcohol[a]		11.6	21.3	71.0	12.6
Benzoic acid[b]	5.29	14.6	22.5		15.0
Benzaldehyde	339.0	542.0	737.0	1400.0	8.16
Acetophenone	355.0	592.0	769.0	1210.0	8.05
4-Methylbenzaldehyde	137.0	403.0	557.0	1100.0	6.90
4-Methylacetophenone	179.0	502.0	611.0	1260.0	10.2

[a] Experiment at 40° replaced by one at 65°. D at 65° was 27.3. [b] Experiment at 70° discontinued.

TABLE IV—PERMEABILITY CONSTANTS AND ACTIVATION ENERGIES OF SELECTED COMPOUNDS THROUGH POLYETHYLENE

Compd.	$P \times 10^8$[a] 40°C.	50°C.	60°C.	70°C.	ΔE_p, Kcal./mole
Benzyl alcohol[b]		0.771	1.49	4.00	11.5
Benzoic acid	0.330	1.00	1.74		17.2
Benzaldehyde	12.7	27.7	56.8	109.0	15.5
Acetophenone	11.2	30.4	60.0	95.9	17.4
4-Methylbenzaldehyde	35.0	55.3	124.0	203.0	17.2
4-Methylacetophenone	22.3	62.3	124.0	203.0	16.5

[a] P or the permeability constant is the quantity (in mg.) of agent passing through a film of 1 cm.^{2} surface area and 1 cm. thickness per second when the concentration gradient is 1 mg./ml. P expressed in following units: (mg.)(cm.)/(sec.)(cm.^{2})(mg./ml.). [b] The permeability constant at 65° was 1.78×10^{-8}.

TABLE V—SOLUBILITY COEFFICIENTS[a] AND HEATS OF SOLUTION OF SELECTED COMPOUNDS IN POLYETHYLENE

Compd.	S 40°C.	50°C.	60°C.	70°C.	ΔH_s, Kcal./mole
Benzyl alcohol[b]		7.03	6.97	5.63	−1.01
Benzoic acid	6.25	6.86	7.72		2.18
Benzaldehyde	3.74	5.11	7.70	7.87	7.52
Acetophenone	3.16	5.13	7.80	8.50	9.39
4-Methylbenzaldehyde	25.6	13.7	22.2	18.5	10.32
4-Methylacetophenone	12.5	12.4	20.2	16.1	6.25

[a] The solubility coefficients are unitless, since they express the ratio of the amount of solute in a volume of plastic over the amount of solute in a volume of solution at equilibrium (mg./ml. per mg./ml.). [b] S at 65° was 6.48.

diffusate molecule, D decreasing with an increase in molecular volume. One may cite as an example the diffusion coefficient of helium at 30° in a low density polyethylene reported in the literature as 81.5×10^{-7} cm.^{2}/sec., while that for oxygen under similar conditions is 6.05×10^{-7} cm.^{2}/sec. (9).

Since the diffusion coefficients for benzyl alcohol and benzoic acid were similar, there is a possibility that the diffusing species of these two compounds had a similar molecular volume. It is known that both benzoic acid and benzyl alcohol are capable of forming dimers by hydrogen and hydrophobic bonding, and it could be that the dimerization gives rise to large diffusate molecules (10–13). The larger diffusion coefficients for benzaldehyde were probably due to smaller diffusing species than those for benzyl alcohol and benzoic acid, since benzaldehyde does not possess a hydrogen capable of hydrogen bonding.

Further support for the existence of larger diffusate molecules with benzyl alcohol and benzoic acid is found in the activation energies for diffusion (Table III). The relatively high activation energies for benzyl alcohol, 12.6 Kcal./mole, and benzoic acid, 15.0 Kcal./mole, as compared to that of benzaldehyde, 8.16 Kcal./mole, may be interpreted as resulting from the high energy required to make a hole in the polymer matrix large enough to allow diffusion of the large dimerized molecules. According to Barrer and other investigators (14, 15) the activation energy for diffusion is strongly influenced by the size of the diffusate molecule and increases with an increase in molecular size. Since benzyl alcohol and benzoic acid require more energy to diffuse in polyethylene than does benzaldehyde, it should follow that benzyl alcohol and benzoic acid possess larger diffusing species than benzaldehyde.

The ability of carboxylic acids to form dimers in aqueous solutions by intermolecular hydrogen bonding and by hydrophobic interactions has been documented in the literature (10–12). Indirect proof for dimerization of benzyl alcohol has come through several sources. Scheraga *et al.* has shown that alcohols interact with each other through hydrophobic bond formation in model systems (13). Infrared spectra of various concentrations of benzyl alcohol in carbon tetrachloride reveal that the O–H stretching frequency band shifts to lower frequency as a function of concentration. The high boiling point of benzyl alcohol (205.2°), as compared to the lower boiling point of ethylbenzene (136.2°), a molecule having the same molecular weight as benzyl alcohol, is ascribed to the hydrogen bonding capacity of the former.

In the case of acetophenone, 4-methylbenzaldehyde, and 4-methylacetophenone the diffusion coefficients observed were very similar to those of benzaldehyde at 50°. Acetophenone had a D value of 592.0×10^{-10} cm.^{2}/sec., while 4-methylbenzaldehyde and 4-methylacetophenone had values of 403.0×10^{-10} cm.^{2}/sec. and 502.0×10^{-10} cm.^{2}/sec., respectively. Lack of intermolecular hydrogen bonding in this series of compounds would seem to be responsible for the almost identical D values. As with benzaldehyde, these compounds do not form dimers and are very slightly soluble in water (solubilities listed in Table I). The similarity of the diffusion coefficients and activation energies of diffusion of benzaldehyde and its methyl analogs, as listed in Table III, also indicates that the addition of a methyl group does not increase the molecular size enough to alter the diffusivity of these compounds significantly.

Permeability Constants and Activation Energies of Permeability—The permeability constants calculated for benzyl alcohol, benzoic acid, benzaldehyde, and acetophenone were found to have a similar relationship to that existing between the values for the diffusion coefficients of these compounds. The permeability constants for benzyl alcohol and benzoic acid at 50° were 0.771×10^{-8} and $1.00 \times$

10^{-8}, respectively. The values for benzaldehyde and acetophenone were 27.7×10^{-8} and 30.4×10^{-8}. Thus, the trend was generally an increase in the permeation of the compounds in the same order as the diffusion. The permeability constants, however, for the methyl substituted derivative of the latter two compounds were found to be twice as large as those for the parent compounds. The P value at 50° for 4-methylbenzaldehyde was 55.3×10^{-8} while the same constant for 4-methylacetophenone was 62.3×10^{-8}. No such relationship was observed with the diffusion coefficients. The D values for the *p*-methyl analogs were essentially the same as those of the parent compounds.

Except for benzyl alcohol, the values obtained for the activation energies of permeability for all the compounds were in the range of 17 Kcal./mole. Benzyl alcohol had the lowest activation energy, 11.5 Kcal./mole (Table IV). To explore the differences in the permeability, it was necessary to consider the solubility coefficients and the heats of solution of the permeating agents in polyethylene.

Solubility Coefficients and Heats of Solution—In this study the solubility coefficients (S) were found to be approximately the same for benzyl alcohol, benzoic acid, benzaldehyde, and acetophenone (Table V). With benzyl alcohol, however, the S values decreased with an increase in the temperature of the experiment, while the opposite occurred with the other compounds. By definition, the solubility coefficient is a ratio of the concentration of solute in a unit weight or volume of plastic to the concentration of the solute in a unit weight or volume of solution. The solubility coefficient, thus, can be visualized as a distribution ratio, which makes it possible to interpret the solubility data with facility.

Upon increasing the temperature of benzyl alcohol solution, dimerization decreases due to a disruption of intermolecular hydrogen bonding. The relatively high capacity of benzyl alcohol to form hydrogen bonds with water molecules renders it less soluble in polyethylene, thus resulting in a decrease of the S values. An Arrhenius plot of the values of the log of S *versus* $1/T$ gives a positive slope which yields a negative heat of solution. This may be interpreted as meaning that the exothermic heat resulting from the binding of benzyl alcohol molecules to the polymer chain segments predominates over the endothermic heat of mixing, and the resulting heat of solution is negative (-1.01 Kcal./mole).

All the other compounds studied had positive heats of solution, which would imply that the endothermic heat of mixing was predominant. It could follow that the increase in the solubility coefficients noted for benzoic acid, benzaldehyde, and acetophenone was due primarily to an increase in the mobility of the polymer chains which allowed a faster increase in the solubility of the solute in the polymer than in the solution. In the case of benzoic acid, however, the increase in temperature will result in the cleavage of intermolecular hydrogen bonds, as is true in the case of benzyl alcohol—differing in that benzoic acid molecule possesses two sites for the formation of intermolecular hydrogen bonds. The increase in monomer fraction will result in an increase in the solubility of benzoic acid in water. However, due to the relatively high polar character of benzoic acid molecule the increase in its solubility coefficient is not so rapid as that for benzaldehyde or acetophenone.

In the case of 4-methylbenzaldehyde and 4-methylacetophenone, the solubility coefficients were found to be twice as high as those of the parent compounds. At 50° 4-methylbenzaldehyde had an S value of 13.72 and 4-methylacetophenone had a similar value of 12.41. The same constants for benzaldehyde and acetophenone were 5.11 and 5.13, respectively. These high solubility coefficients could be ascribed to a decrease in the dielectric constant due to the presence of the methyl substituent.

After reviewing the solubility data, it is possible to explain the apparent anomalous results obtained for the permeability constants and activation energies of permeability. The high solubility coefficients for 4-methylacetophenone accounted for the high permeability constants obtained for these compounds. Since their diffusion coefficients were generally the same as those of their parent compounds, it follows from the relationship $P = DS$ that the permeability constants will vary according to the variation in the solubility coefficients. The solubility coefficients of 4-methylbenzaldehyde and 4-methylacetophenone are twice as large as those of benzaldehyde and acetophenone; thus, the permeability values as well would be expected to rise by twofold.

To propose a rationale for the resultant activation energies of permeability, it is necessary to recall that the activation energy for permeability is a measure of the energy required to allow the net transfer of a mole of solute through the amorphous regions of a plastic. This energy will then be the sum of the activation energy for diffusion and the heat of solution. Since benzyl alcohol had a negative heat of solution, the energy to accomplish the permeation of benzyl alcohol molecules through the polyethylene chains was less than the energy for diffusion. Benzyl alcohol also had the lowest activation energy of permeability.

Although a reasonable hypothesis has been presented in an attempt to explain the data, there are still several complications that should be emphasized. One of these is that the concentrations used in these studies were not always the same and although the constants derived mathematically are concentration independent, the possibility that the differences in concentration may contribute to some unexpected experimental results should not be overlooked. Finally, it should be remembered that with the type of polyethylene used in this experiment the degree of crystallization will decrease with an increase in temperature. In fact, studies above temperatures higher than 70° gave anomalous results which may have been due to the changes in crystallinity. This facet must, however, be investigated more thoroughly before the exact effects of changes of crystallinity on sorption can be presented.

SUMMARY

A series of structurally related aromatic compounds was employed for the evaluation of their permeation through a polyethylene film. Six compounds were used in the study: benzyl alcohol, benzoic acid, benzaldehyde, acetophenone, 4-methylbenzaldehyde, and 4-methylacetophenone.

Results of the experiments revealed that benzyl

alcohol and benzoic acid had the lowest P and D values as compared with the other compounds in the series. This was ascribed to intermolecular hydrogen bonding potential of benzyl alcohol and benzoic acid which produce dimers or trimers whose penetration into the polymer is not favorable.

In the case of 4-methylbenzaldehyde and 4-methylacetophenone, the D values obtained were similar to those of the parent compounds, benzaldehyde and acetophenone. The P and S values, however, were found to be larger. The difference in the P and S values was attributed to the effect of the methyl substituents which gave rise to higher solubility in the plastic.

Quantitative studies as reported here and similar studies with other compounds should be of value to those who may desire to package drugs, cosmetics, or other products in polyethylene.

REFERENCES

(1) Autian, J., *J. Pharm. Sci.*, **52**, 1(1963).
(2) *Ibid.*, **52**, 105(1963).
(3) Neuwald, F., *Specialities*, **1**, 24(1965).
(4) Autian, J., "Interaction between Medicaments and Plastics," presented to the Scientific Section of the FIP meeting, Madrid, Spain, September 1966.
(5) Pinsky, J., *Mod. Plastics*, **34**, 145(April 1957).
(6) Salame, M., and Pinsky, J., *Mod. Packaging*, **November**, 86(1962).
(7) Rodell, M. B., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **55**, 1429(1966).
(8) Barrer, R. M., *Trans. Faraday Soc.*, **35**, 628(1939).
(9) Bixler, H. J., Ph.D. Thesis, M.I.T., Cambridge, Mass., 1959.
(10) Katchalsky, A., Eisenberg, H., and Lifson, S., *J. Am. Chem. Soc.*, **73**, 5880(1951).
(11) Cartwright, D. R., and Monk, C. B., *J. Chem. Soc.*, **1955**, 2500.
(12) Schrier, E. E., Pottle, M., and Scheraga, H. A., *J. Am. Chem. Soc.*, **86**, 3444(1964).
(13) Schneider, H., Kresheck, G. C., and Scheraga, H. A., *J. Phys. Chem.*, **69**, 1310(1965).
(14) van Amerongen, G. J., *J. Polymer Sci.*, **5**, 307(1950).
(15) Barrer, R. M., *J. Phys. Chem.*, **61**, 178(1957).

Crystal and Molecular Structure of 5-[1-(2′-Deoxy-α-D-ribofuranosyl)uracilyl] Disulfide

By ELI SHEFTER, MICHAEL P. KOTICK, and THOMAS J. BARDOS

The molecular structure of 5-[1-(2′-deoxy-α-D-ribofuranosyl)uracilyl] disulfide (I) has been determined by X-ray crystallographic analysis. The molecule crystallizes in the orthorhombic space group $P2_12_12_1$, with the following cell dimensions: a = 9.492 Å., b = 14.061 Å., c = 16.328 Å. The valence state about the S-S linkage appears to be markedly different from that observed in other disulfide structures. The torsional angle about this bond is 49° and the S-S bond length was found to be 2.108 Å. The configurations of both the uracil and the deoxyribosyl residues are different in the two nucleoside units of the molecule. The crystal is bound together by hydrogen bonds.

A STRUCTURAL ANALOG of thymidine, 5-mercapto-2′-deoxyuridine (MUDR), has been recently synthesized (1), and found to be a potent inhibitor in several bacterial systems. This compound and some of its congeners are of potential chemotherapeutic interest because of their ability to inhibit the biosynthesis of DNA (2, 3). Both the α and β anomers of MUDR exhibited rapid autoxidation in aqueous solutions to the corresponding disulfides (4). These disulfides have interesting biochemical and physicochemical properties. They are capable of being reduced nonenzymatically at a virtually instantaneous rate by stoichiometric amounts of thiols (2, 5), and their high optical activities showed temperature dependence (6).

In an effort to gain further insight into the various properties that could be pertinent as to the mode of action on a molecular level of MUDR and its congeners, X-ray structural studies have been initiated on several of these compounds. This report concerns itself with the crystallographic structure of the first of these compounds to be completed, the disulfide of the α-anomer, 5-[1-(2′-deoxy-α-D-ribofuranosyl)uracilyl] disulfide (I). This compound has additional interest as it appears to be the first α-nucleoside whose crystal structure has been elucidated.

EXPERIMENTAL

The compound crystallizes from water as slightly yellow prisms belonging to space group $P2_12_12_1$, with the following crystallographic data:

$a = 9.492 \pm 0.003$ Å.
$b = 14.061 \pm 0.002$ Å.
$c = 16.328 \pm 0.003$ Å.
ρ(meas.) = 1.577 Gm./cm.^{3}
ρ(calcd.) = 1.579 Gm./cm.^{3}
$Z = 4$

Received April 21, 1967 from the Departments of Pharmaceutics and Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication June 30, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This research was supported by grants CA-10104-01 and CA-06695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
The authors thank Professor David Harker, Center for Crystallographic Research, Roswell Park Memorial Institute, for permitting the use of his facilities, and the computing center of this university for a generous gift of computing time.

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Quantitative studies as reported here and similar studies with other compounds should be of value to those who may desire to package drugs, cosmetics, or other products in polyethylene.

REFERENCES

(1) Autian, J., *J. Pharm. Sci.*, **52**, 1(1963).
(2) *Ibid.*, **52**, 105(1963).
(3) Neuwald, F., *Specialities*, **1**, 24(1965).
(4) Autian, J., "Interaction between Medicaments and Plastics," presented to the Scientific Section of the FIP meeting, Madrid, Spain, September 1966.
(5) Pinsky, J., *Mod. Plastics*, **34**, 145(April 1957).
(6) Salame, M., and Pinsky, J., *Mod. Packaging*, **November**, 86(1962).
(7) Rodell, M. B., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **55**, 1429(1966).
(8) Barrer, R. M., *Trans. Faraday Soc.*, **35**, 628(1939).
(9) Bixler, H. J., Ph.D. Thesis, M.I.T., Cambridge, Mass., 1959.
(10) Katchalsky, A., Eisenberg, H., and Lifson, S., *J. Am. Chem. Soc.*, **73**, 5880(1951).
(11) Cartwright, D. R., and Monk, C. B., *J. Chem. Soc.*, **1955**, 2500.
(12) Schrier, E. E., Pottle, M., and Scheraga, H. A., *J. Am. Chem. Soc.*, **86**, 3444(1964).
(13) Schneider, H., Kresheck, G. C., and Scheraga, H. A., *J. Phys. Chem.*, **69**, 1310(1965).
(14) van Amerongen, G. J., *J. Polymer Sci.*, **5**, 307(1950).
(15) Barrer, R. M., *J. Phys. Chem.*, **61**, 178(1957).

Crystal and Molecular Structure of 5-[1-(2′-Deoxy-α-D-ribofuranosyl)uracilyl] Disulfide

By ELI SHEFTER, MICHAEL P. KOTICK, and THOMAS J. BARDOS

The molecular structure of 5-[1-(2′-deoxy-α-D-ribofuranosyl)uracilyl] disulfide (I) has been determined by X-ray crystallographic analysis. The molecule crystallizes in the orthorhombic space group $P2_12_12_1$, with the following cell dimensions: a = 9.492 Å., b = 14.061 Å., c = 16.328 Å. The valence state about the S-S linkage appears to be markedly different from that observed in other disulfide structures. The torsional angle about this bond is 49° and the S-S bond length was found to be 2.108 Å. The configurations of both the uracil and the deoxyribosyl residues are different in the two nucleoside units of the molecule. The crystal is bound together by hydrogen bonds.

A STRUCTURAL ANALOG of thymidine, 5-mercapto-2′-deoxyuridine (MUDR), has been recently synthesized (1), and found to be a potent inhibitor in several bacterial systems. This compound and some of its congeners are of potential chemotherapeutic interest because of their ability to inhibit the biosynthesis of DNA (2, 3). Both the α and β anomers of MUDR exhibited rapid autoxidation in aqueous solutions to the corresponding disulfides (4). These disulfides have interesting biochemical and physicochemical properties. They are capable of being reduced nonenzymatically at a virtually instantaneous rate by stoichiometric amounts of thiols (2, 5), and their high optical activities showed temperature dependence (6).

In an effort to gain further insight into the various properties that could be pertinent as to the mode of action on a molecular level of MUDR and its congeners, X-ray structural studies have been initiated on several of these compounds. This report concerns itself with the crystallographic structure of the first of these compounds to be completed, the disulfide of the α-anomer, 5-[1-(2′-deoxy-α-D-ribofuranosyl)uracilyl] disulfide (I). This compound has additional interest as it appears to be the first α-nucleoside whose crystal structure has been elucidated.

EXPERIMENTAL

The compound crystallizes from water as slightly yellow prisms belonging to space group $P2_12_12_1$, with the following crystallographic data:

$a = 9.492 \pm 0.003$ Å.
$b = 14.061 \pm 0.002$ Å.
$c = 16.328 \pm 0.003$ Å.
ρ(meas.) = 1.577 Gm./cm.^{3}
ρ(calcd.) = 1.579 Gm./cm.^{3}
$Z = 4$

Received April 21, 1967 from the Departments of Pharmaceutics and Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication June 30, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This research was supported by grants CA-10104-01 and CA-06695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
The authors thank Professor David Harker, Center for Crystallographic Research, Roswell Park Memorial Institute, for permitting the use of his facilities, and the computing center of this university for a generous gift of computing time.

The density was measured by means of flotation in a mixture of chloroform and dibromoethane. The unit cell dimensions were obtained by least squares refinement of 30 general reflections measured on a General Electric XRD-6 diffractometer, using Cu Kα radiation.

The intensity data were collected by the stationary counter-stationary crystal technique using balanced filters and Cu Kα radiation. The crystal utilized for the data collection was mounted with a parallel to the ϕ axis of the goniostat and its dimensions were 0.66 mm. along a, 0.16 mm. along b, and 0.38 mm. along c. In the 2 θ range between 0° and 142°, 2328 reflections of a total possible number of 2408, had intensities significantly greater than their background counts. The intensities of the various reflections were corrected for Lorentz and polarization factors in the usual manner. A correction for absorption as a function of the angle ϕ was measured for various h.0.0. reflections. The average transmission factor obtained was then used to correct the intensities for anisotropy of absorption.

Structure Determination—The positions of the two sulfurs were easily obtained from a Patterson synthesis. A Fourier synthesis calculated using the phases of the sulfur atoms uncovered all but 8 of the nonhydrogen atoms. From successive difference syntheses the positions of these eight atoms were found. The refinement was then continued by block diagonal least squares, using a modified version of the Trueblood, Sparks, and Gantzel program (ACA No. 317), which minimizes the function $\Sigma w||Fo| - |Fc||^2$. The initial isotropic refinement was carried out with the weight equal to $1/f_{(n)}$, where $f_{(n)}$ is the scattering curve for nitrogen. After anisotropic temperature factors were introduced, a difference Fourier showed positive electron density (ranged between 0.2 to 0.4 e/Å.^{3}) in the regions where the hydrogen atoms were expected to be found. In the final least squares refinement the hydrogen atoms were included with isotropic temperature factors, and the weighting scheme utilized was $1/\sigma(F^2)$, where the standard deviations of the structure factors were calculated in the manner described by Evans (7). The final R value (usual discrepancy index) was 0.054 for the observed reflections, and 0.062 for all the reflections, where the unobserved data were given an intensity equal to half the minimum observable value. Throughout the refinement the unobserved data were given zero weight.

The atomic form factors used in all the above computations were those given by Cromer and Waber (8), except for the hydrogens. McWeeny's (9) scattering curve for the hydrogen atom was utilized. The sulfur atoms were corrected for anomalous scattering using both the real and complex terms (10).

Results and Discussion—The final atomic positions and thermal parameters for the molecule together with their respective estimated standard deviations (ESD) are shown in Tables I, II, and III. The ESD's were calculated from the inverted matrix of the normal least squares equations. The intramolecular bond lengths and angles are shown in Figs. 1 and 2. The atomic names shown in these figures will be used throughout the text. The average standard deviation in bond length is 0.008 Å. for the nonhydrogen atoms, with the exception of the disulfide bond which is 0.003 Å. The intramolecular angles between the various nonhydrogen bonds have an average error of 0.4°. The ESD's for the bonds and angles involving the hydrogens average around 0.1 Å. and 3°, respectively. These angles are not shown in Fig. 2, and only those relevant to the discussion of the molecule will be given.

TABLE I—ATOMIC COORDINATES FOR NONHYDROGEN ATOMS AND (IN PARENTHESES) THEIR STANDARD DEVIATIONS × 10^4

Name	x/a	y/b	z/c
S(1)	0.06212(2)	0.11176(1)	0.04133(1)
S(2)	0.22564(2)	0.21267(1)	0.05023(1)
1C5	0.13620(7)	0.01399(4)	0.09336(4)
1C4	0.12121(7)	−0.00103(5)	0.18075(4)
1O4	0.05717(5)	0.05052(3)	0.22987(3)
1N3	0.19054(6)	−0.08150(4)	0.20969(3)
1C6	0.21492(7)	−0.04713(4)	0.04872(4)
1C2	0.27194(7)	−0.14425(4)	0.16434(3)
1O2	0.32836(5)	−0.21203(3)	0.19594(3)
1N1	0.28048(5)	−0.12315(3)	0.08241(3)
1C1′	0.37220(7)	−0.18663(4)	0.03081(3)
1O1′	0.42662(4)	−0.13351(3)	−0.03338(2)
1C4′	0.36997(7)	−0.16612(4)	−0.11185(4)
1C3′	0.24462(7)	−0.23028(4)	−0.08848(4)
1C2′	0.29047(7)	−0.26992(4)	−0.00598(4)
1C5′	0.48862(7)	−0.21631(5)	−0.15740(4)
1O5′	0.44439(5)	−0.24139(3)	−0.23777(3)
1O3′	0.12131(5)	−0.17595(3)	−0.07703(3)
2C5	0.37218(7)	0.14789(4)	0.01707(4)
2C6	0.45885(7)	0.11237(4)	0.07660(4)
2N1	0.57756(6)	0.06194(4)	0.05893(3)
2C2	0.61891(7)	0.04136(5)	−0.02101(4)
2O2	0.72334(5)	−0.00253(3)	−0.03887(3)
2N3	0.52770(6)	0.07775(4)	−0.07989(3)
2C4	0.40222(7)	0.12551(4)	−0.06700(3)
2O4	0.32689(5)	0.14612(4)	−0.12560(3)
2C1′	0.65684(7)	0.01613(5)	0.12807(4)
2O1′	0.55915(5)	−0.04542(3)	0.16858(2)
2C2′	0.71567(7)	0.08665(5)	0.19145(4)
2C3′	0.62658(7)	0.06851(4)	0.26775(4)
2O3′	0.50564(5)	0.13261(3)	0.27191(3)
2C4′	0.56642(7)	−0.03245(4)	0.25573(4)
2C5′	0.65621(8)	−0.10901(5)	0.29494(4)
2O5′	0.80245(5)	−0.09730(3)	0.27145(3)

In discussing the crystal structure, the various portions of the molecule shall be considered individually and then the packing of the molecule will be discussed.

Disulfide Linkage—The various structural features of some of the more accurately determined disulfides are compared to the present structure in Table IV. There is a striking difference between the various values listed for I and the other compounds.

The torsional angle, ϕss, defined as the projected angle of the two C—S bonds down the S—S bond (see Fig. 3), is much smaller in I than in any of the open chain disulfides. It is also much larger than the angle found in the cyclic disulfide structures. The angle in the open-linked disulfides is believed to be determined primarily by the repulsion of the electrons in the p orbitals of the sulfur atoms. This results in an energy minimum at 90°. Abrahams, in a review (18) of disulfide bonding, found that open disulfide structures have values distributed within the vicinity of 90°. This is seen to be true for the open-chain disulfides listed with the exception of I. The values for the constrained disulfides are

TABLE II—THERMAL PARAMETERS FOR NONHYDROGEN ATOMS AND (IN PARENTHESES) THEIR STANDARD DEVIATIONS[a]

Atom	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
S(1)	624(19)	338(9)	213(6)	282(25)	−155(21)	37(14)
S(2)	754(20)	246(8)	177(6)	262(24)	− 39(21)	− 56(13)
1C5	653(82)	229(33)	105(23)	111(95)	− 10(75)	− 38(47)
1C4	581(79)	298(37)	131(23)	−161(100)	− 80(77)	− 78(50)
1O4	785(64)	412(29)	172(18)	233(78)	122(62)	−152(40)
1N3	694(71)	301(30)	91(18)	21(82)	65(63)	− 6(41)
1C6	527(74)	241(31)	106(22)	21(87)	− 62(77)	4(48)
1C2	505(73)	317(35)	101(22)	−237(98)	14(71)	− 26(49)
1O2	889(65)	298(26)	180(18)	334(75)	95(60)	168(39)
1N1	568(61)	218(26)	76(17)	176(79)	59(59)	− 40(38)
1C1′	523(75)	298(36)	100(22)	194(92)	34(71)	− 50(48)
1O1′	552(52)	316(25)	103(16)	−334(65)	99(50)	− 41(35)
1C4′	568(79)	259(34)	110(23)	43(91)	111(75)	− 11(48)
1C3′	560(81)	292(36)	159(25)	−117(96)	− 38(77)	−120(51)
1C2′	830(92)	192(35)	142(24)	−126(97)	148(84)	28(48)
1C5′	697(85)	311(36)	123(23)	149(105)	158(77)	− 10(55)
1O5′	1214(78)	392(29)	154(18)	−112(88)	220(70)	− 89(40)
1O3′	455(53)	455(30)	227(19)	−136(72)	42(57)	−135(42)
2C5	546(77)	281(35)	121(23)	34(94)	21(74)	47(49)
2C6	632(79)	263(34)	113(21)	87(97)	− 37(74)	− 34(50)
2N1	584(65)	317(30)	91(19)	272(78)	− 34(61)	51(40)
2C2	691(86)	296(36)	128(23)	78(99)	261(79)	60(50)
2O2	811(67)	469(29)	236(21)	432(84)	394(68)	77(45)
2N3	794(75)	298(30)	73(17)	78(87)	35(64)	31(42)
2C4	700(81)	279(36)	100(22)	42(97)	1(72)	− 3(49)
2O4	1080(71)	599(35)	119(17)	342(91)	−173(63)	− 11(43)
2C1′	663(84)	308(37)	129(24)	152(102)	−209(79)	− 10(52)
2O1′	1058(71)	296(25)	127(17)	−330(78)	−352(62)	64(36)
2C2′	478(76)	334(38)	164(25)	− 91(102)	− 74(79)	− 75(54)
2C3′	558(78)	212(33)	188(26)	17(93)	−221(81)	− 70(50)
2O3′	762(58)	295(26)	255(21)	119(73)	42(64)	−159(42)
2C4′	733(85)	284(37)	116(23)	−111(103)	−132(80)	2(50)
2C5′	1022(102)	278(38)	173(26)	−139(115)	−113(90)	85(56)
2O5′	887(68)	372(29)	259(79)	282(79)	−274(68)	2(44)

[a] Values multiplied by 10^5. The temperature factor is of the form: $\exp(-b_{11}h^2 - b_{22}k^2 - b_{33}\rho^2 - b_{12}hk - b_{13}h\rho - b_{23}k\rho)$.

TABLE III—POSITIONAL AND ISOTROPIC THERMAL PARAMETERS FOR THE HYDROGEN ATOMS[a]

Name	x/a	y/b	z/c	B (Å.^{2})
H1	0.181	−0.095	0.271	0.1
H2	0.239	−0.043	−0.013	−1.7
H3	0.449	−0.917	0.068	1.8
H4	0.337	−0.317	−0.016	−0.4
H5	0.203	−0.303	0.024	−0.4
H6	0.227	−0.274	−0.135	2.6
H7[b]	0.145	−0.152	−0.050	0.9
H8	0.345	−0.113	−0.145	1.1
H9	0.516	−0.276	−0.132	−0.7
H10	0.584	−0.167	−0.145	1.9
H11	0.445	−0.214	−0.250	−0.1
H12	0.568	0.070	−0.133	0.7
H13	0.431	0.127	0.134	−0.5
H14	0.748	−0.026	0.093	0.5
H15	0.790	0.069	0.201	−2.5
H16	0.701	0.155	0.172	−0.5
H17	0.692	0.072	0.327	2.7
H18	0.534	0.169	0.275	1.3
H19	0.449	−0 040	0.275	1.3
H20	0.654	−0.101	0.357	0.0
H21	0.601	−0.165	0.273	2.7
H22	0.860	−0.132	0.256	4.3

[a] Average standard deviations in the positional parameters for the hydrogens are 0.06 Å. Standard deviations for B's average 1.7 Å^2. [b] See text for discussion of this atom.

very much lower. Recently an analysis of these cyclic compounds has been presented by Hordvik (19), and as it is felt to be nonrelevant to the bonding in I, these structures will not be discussed.

The electronic structure about the disulfide linkage of I also appears to be quite different from the other compounds listed in this table. A large degree of double bond character appears to exist in the C—S bond of I, as evidenced by the significantly shorter lengths for these bonds. This could increase the electron density in the d orbitals of the sulfurs and thereby cause an overlap of the

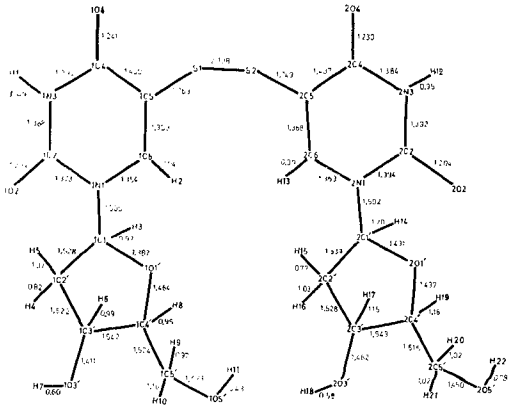

Fig. 1—Schematic drawing of I showing intramolecular bond distances. (Reduced to about 1/4.)

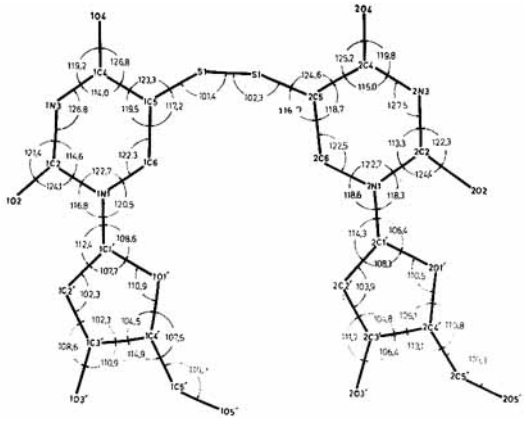

Fig. 2—Schematic drawing of I with intramolecular bond angles for nonhydrogen atoms. (Reduced to about $^1/_4$.)

TABLE IV—COMPARISON OF THE STRUCTURAL FEATURES OF SOME DISULFIDES WITH I

Compd.	φss (deg.)	S-S (Å.)	C-S (Av.) Å.	C-Ŝ-S (Av.) (deg.)	Ref.
I	49.1	2.108	1.763	101.4 (0.2)	
		(0.003)	1.749 (0.007)	102.3 (0.2)	
L-Cystine	74	2.032	1.82	114.5	(11)
Dimethyldisulfide	84.7	2.038	1.81	102.8	(12)
Perfluorodimethyldisulfide	90[a]	2.05	1.83	105	(13)
N,N^1-Diglycyl-L-cystine dihydrate	89	2.04	1.87	103	(14)
1,2-Dithiolane-4-carboxylic acid	27	2.096	1.84	95	(15)
Sporidesmin	9	2.08	1.90	98	(16)
Gliotoxin	12	2.076	1.875	98	(17)

[a] φss assumed to be 90°.

3 *d* electrons, which in turn may result in repulsion between the sulfurs, and subsequent lowering of the torsional angle. The greater S—S bond length in this structure, as compared to other disulfides, is in agreement with this postulation. The valence state of the C—S bonds appears to be influenced by the uracil moieties. The C5—C6 bonds in the two pyrimidines are substantially longer than the average value observed for this bond in a number of pyrimidine nucleosides and nucleotides (average 1.33 ± 0.01 Å.).

Uracil Moieties—Within experimental error, the bond lengths and angles of the two rings are quite similar. The most striking difference between the two residues exists between the planarity of the pyrimidine rings. The least squares planes calculated through the six ring atoms of the two bases are shown in Table V. The atoms comprising ring 1 are coplanar within their standard deviations, whereas those comprising ring 2 are significantly nonplanar. Pyrimidine ring 2 is puckered substantially about N3-C4-C5, with N3 and C5 being displaced on the opposite side of the plane to C4. Puckering has been observed in a number of pyrimidine nucleosides (20), and is possibly the result of the valence state of the nitrogen involved being intermediate between sp^2 and sp^3 hybridization. The puckering in this ring may also reflect the close proximity of portions of the two pyrimidine rings to each other, as a result of the disulfide conformation. The distance between 2C5 and 1C5 is 3.2 Å., less than the sum of the van der Waals radii of the two atoms, 3.4 Å. In the puckered uracil residue, both C1′ and O4 are displaced from the pyrimidine plane to a much greater extent than in ring 1.

The C4—O4 carbonyl groups have a significantly longer bond length than the C2—O2 groups. The greater degree of polarization in the 4 position of uracil derivatives has been discussed with respect to their hydrogen bonding (21). It can be pointed out that both O4 atoms are participating in hydrogen bonds, while only one O2 is involved in such a bond.

With the exception of the C4—C5 bonds of the two uracil moieties, the bond distances and angles are similar to those found in other pyrimidine containing nucleosides and nucleotides.

Deoxyribose Moieties—It is well established from structural data that the furanose ring in nucleosides

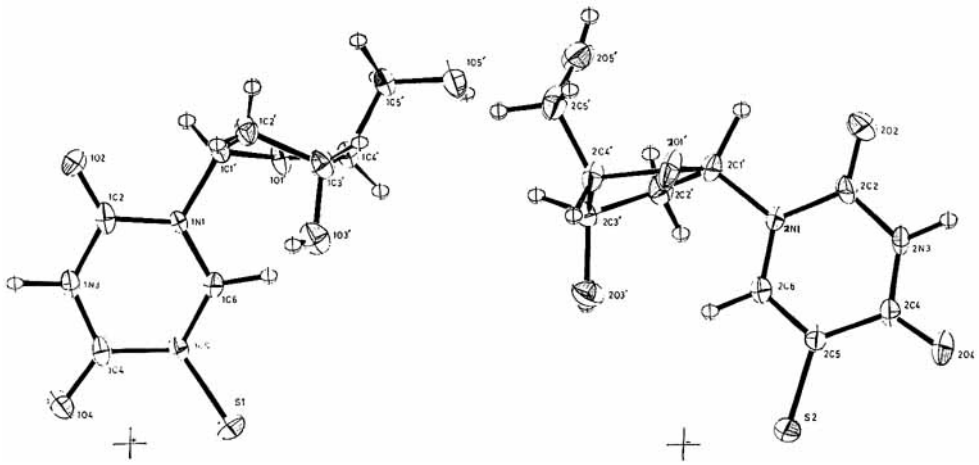

Fig. 3—The two nucleosidic portions of the molecule as viewed down the S1 → S2 bond. In order to visualize the complete molecule the + sign should be overlayed on the − sign. The thermal ellipsoids of the nonhydrogen atoms are shown. (Reduced to about $^1/_3$.)

TABLE V—LEAST SQUARES PLANES CALCULATED IN THE MANNER OF SCHOMAKER *et al.*[a]

Atoms Comprising the Plane	Displacements, Å.	Displacements of Other Atoms, Å.	
Uracil 1			
1N1	0.003	$1O_4$	0.056
1C2	0.001	$1O_2$	−0.006
1N3	−0.007	S(1)	0.045
1C4	0.008	$1C_1'$	0.068
1C5	−0.004	H1	−0.015
1C6	−0.001	H2	0.045
Uracil 2			
2N1	0.010	$2O_4$	0.124
2C2	−0.001	$2O_2$	−0.005
2N3	−0.023	$2C_1$	0.210
2C4	0.036	S(2)	−0.056
2C5	−0.028	H_{12}	−0.163
2C6	0.006	H_{13}	0.001

[a] Schomaker, V., Waser, J., Marsh, R. E., and Bergman, G., *Acta Cryst.*, **12**, 600(1959).

is puckered and not planar. The common puckering found in β-nucleosides usually involves the C2′ and C3′ atoms (22). In sugar 1, one of the modes of puckering observed in β-nucleosides is found, with C2′ and C3′ displaced on opposite sides of the plane through C1′, O1′, and C4′. The puckering in sugar 2 is quite different from that found in other furanose structures (23). Its distortion involves the displacement of C3′ and C4′ from the plane of the other 3 atoms. This is evident from Figs. 3 and 4.

One of the best ways to describe the puckering of furanose structures is in terms of a twist angle about each ring bond (23, 24). This conformational angle for a given bond, *e.g.*, φ C2′ → C3′, is the projected angle between the C1′—C2′ bond and the C3′—C4′, in that order, measured clockwise. The twist angles for the sugars are given in Table VI. The smaller the absolute value of the twist angle, the greater is the displacement of the atom opposite the bond concerned.

The conformation about the exocyclic C4′—C5′ is of interest, as it may be important in considering the hindered rotation about the disulfide bond. This can be seen in Fig. 4, which indicates that these exocyclic atoms may come in close contact upon rotation about the S—S bond. The conformation about this bond can be designated by the projected angles made by C5′—O5′ with C4—O1′ and C4′—C3′ (25). These angles are listed in Table VI, as φOO and φOC, the former of these refers to the angle between C5′—O5′ and C4′—O1′. A value near 60° is considered to be *gauche* and a value near 180°, *trans*. Sugar 1 has *tg* conformation about this bond, while sugar 2 has the *gg* conformation (the first letter refers to φOO and the second to φOC). These conformations would possibly afford a minimum amount of contact between these exocyclic atoms if the nucleoside residues are rotated about the disulfide bond.

The C—C bonds average 1.530 Å. and 1.536 Å.

TABLE VI—CONFORMATION ANGLES

	Residue 1	Residue 2
φ C1′ → C2′	−27° 36′	−7° 58′
φ C2′ → C3′	−34° 19′	21° 0′
φ C3′ → C4′	30° 2′	−27° 39′
φ C4′ → O1′	−13° 39′	23° 32′
φ O1′ → C1′	−8° 44′	9° 51′
φ OC	70° 2′	49° 27′
φ OO	174° 9′	68° 16′
φ CN	−30°	−72°

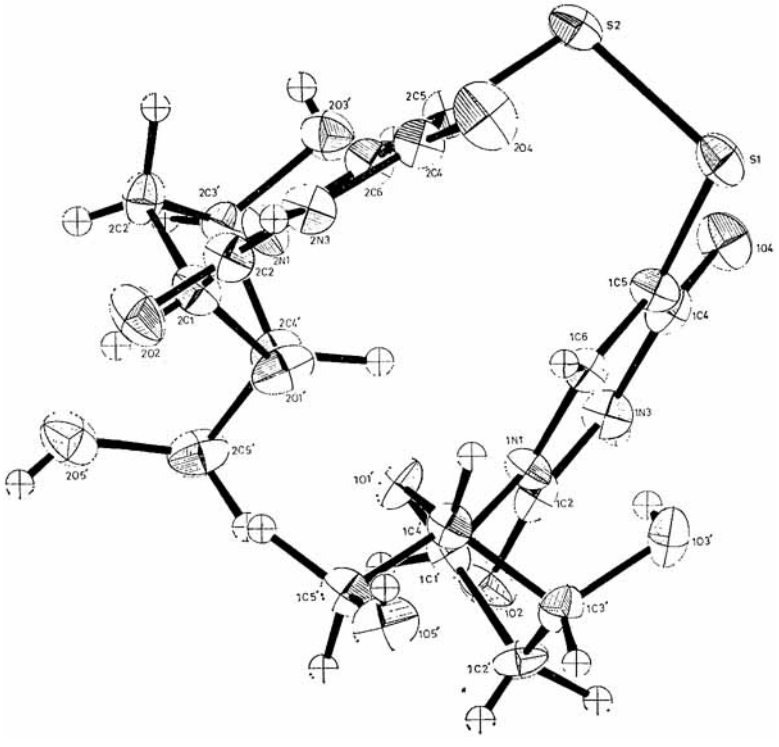

Fig. 4—The structure viewed along the c *axis. (Reduced to about* 1/2.)

TABLE VII—SHORT MOLECULAR CONTACTS

Hydrogen Bonds Acceptor	Donor[a]	Pertinent Distances and Angles	
104	H11-105′ (1)	104 .....105′	2.736
		104 .....H11	2.32
		104 .....H11-105′	161°
102	H18-203′ (3)	102 .....203′	2.745
		102 .....H18	2.17
		102 .....H18-203′	168°
105′	H22-205′ (3)	105′.....205′	2.695
		105′.....H22	1.97
		105′.....H22-205′	154°
204	H1-1N3 (4)	204 .....1N3	2.84
		204 .....H1	1.82
		204 .....H1-1N3	167°
205′	H12-2N3 (5)	205′.....2N3	2.927
		205′.....H12	2.02
		205′.....H12-2N3	157°
203′	103′ (1)	203′.....103′	2.813
		203′.....103′-1C3′	110.9°
	Other Short Hydrogen Contacts		
203′	H13-2C6	203′.....2C6	3.233
		203′.....H13	2.37
		203′.....H13-2C6	145°
103′	H2-1C6	103′.....1C6	2.879
		103 .....H2	2.42
		103′.....H2-1C6	106°

[a] The value in parentheses following the donor atom name denotes its equivalent position in fractional coordinates, as follows: (1) $0.5 - x, -y, 0.5 + z$, (2) $1 - x, y - 0.5, 0.5 - z$, (3) $x - 0.5, -(y + 0.5), -z$, (4) $0.5 - x, -y, z - 0.5$, (5) $1.5 - x, -y, 0.5 + z$.

for sugars 1 and 2, respectively. These values do not deviate significantly from the bond length between two tetrahedral carbon atoms, 1.533 ± 0.003 Å. (26). The exocyclic C4′—C5′ in both sugars is substantially shorter than this distance, and as suggested by Sundaralingham and Jensen (27) for other nucleoside structures, that this effect may be due to the distortion of the C4′ atom from a tetrahedral conformation (see angles in Fig. 2). In other nucleoside structures, it has been observed that the C—O bond lengths can deviate substantially from the commonly accepted value for a C—O single bond, 1.425 ± 0.007 Å. (28). There are a number of significantly longer and shorter C—O bonds in the two sugars. The C—O bonds in furanose ring 1 show a commonly occurring deviation that is found in a number of nucleosides (22), that is the C1′—O1′ is significantly shorter than O1′—C4′. In sugar 2 this effect does not manifest itself to as great an extent, which may be a direct indication that this phenomenon is dependent on the conformation of the sugars.

Hydrogen Atoms and Bonding—The intramolecular bond lengths and angles (latter not shown) involving the hydrogen atoms agree fairly well with those found in other crystal structures. The O—H bond lengths are the only ones that deviate considerably from the commonly found values of 1.06 Å. for this bond. From various considerations (*i.e.*, C—O—H angles and hydrogen bond angles), it appears that only H7 has been positioned erroneously.

Table VII contains a list of all the hydrogen bonding contacts found in this structure. From a consideration of the distances and angles of various short intermolecular contacts, it appears that 103′ is forming a hydrogen bond with a 203′ atom. This would require that the 103′ hydrogen (H7) be placed at the cross in Fig. 5, rather than the position given for this atom. Though difference syntheses did not show any electron density at the likely position, this may be a reflection of the difficulty in obtaining accurate hydrogen positions in such a complex molecule. With H7 relocated, the hydrogen bonding scheme as shown in Fig. 5 is a very plausible one, *i.e.*, all angles and distances are reasonable. The molecules are held together in the lattice framework by these intermolecular hydrogen bonds.

Aside from the usual hydrogen bonding interactions, there also appears to be short C6—H . . . O3′ intramolecular contacts. The H13 . . . 203′ and H2 . . . 103′ distances are 2.37 Å. and 2.42 Å., respectively. These are shorter than the normal van der Waals contacts between an oxygen and hydrogen. The sum of the van der Waals radii of hydrogen and oxygen is 2.60 Å. The existence of such interactions has been presented by Sutor (29), and the C6 hydrogen in other pyrimidine nucleosides has been found to form such interactions (25). These C—H . . . O close contacts may be a determining factor for the conformations about the glycosidic C1′—N1′ bonds. The torsional angles (30), ϕCN, *i.e.*, the dihedral angle between the trace of the plane of the base with the C1′—O1′ bond, are given in Table VII for each of these bonds. The short C—H . . . O contacts are represented in Fig. 5 by dotted lines.

Thermal Parameters—In Figs. 3 and 4 the magnitudes and directions of the thermal motion for the various atoms are shown. The thermal ellipsoids were drawn using a Cal. Comp. plotter with a program written by Johnson (31).

The isotropic temperature factors for the hydrogens are quite low. This behavior has been found in a large number of other organic structures, where the scattering factor curve used for hydrogen was that of the free atom (32).

CONCLUSION

In conclusion it is worthwhile to compare the

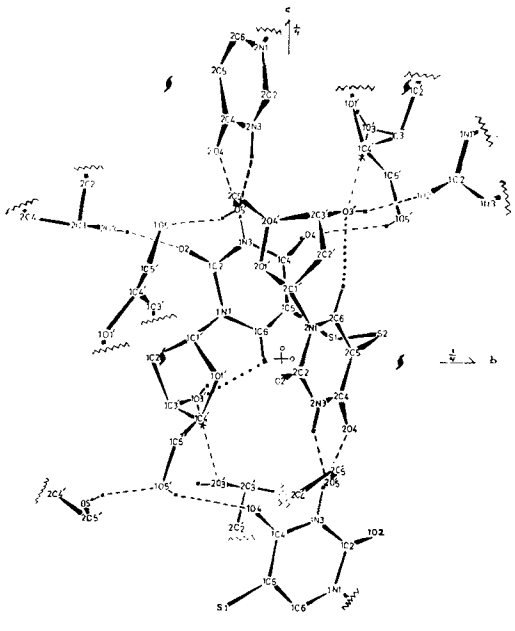

Fig. 5—Projection of molecule along the a *axis. Key: – – –, postulated hydrogen bonding scheme; . . . , short CH . . . O intramolecular contacts. (Reduced to about* 1/3.)

above structural observations with some of the properties found for this compound.

(*a*) The high optical rotation and its temperature dependence might find its explanation in that the electronic state about the disulfide linkage and the conformation of the sugar moieties may hinder free rotation of the two nucleoside residues about the S—S bond.

(*b*) The facile reducibility of the disulfide linkage for this compound appears to be attributable to the valence state of the disulfide bond.

REFERENCES

(1) Bardos, T. J., Kotick, M. P., and Szantay, C., *Tetrahedron Letters*, **1966**, 1759.
(2) Kalman, T. I., Baranski, K., and Bardos, T. J., "Abstracts of Papers Presented to the American Chemical Society," p.M., 1967.
(3) Bardos, T. J., Ambrus, J. L., Baranski, K., Kalman, T. I., and Ambrus, C. M., *Proc. Am. Assoc. Cancer Res.*, **8**, 4(1967).
(4) Kalman, T. I., and Bardos, T. J., *J. Am. Chem. Soc.*, **89**, 1171(1967).
(5) Bardos, T. J., and Kalman, T. I., *J. Pharm. Sci.*, **55**, 606(1966).
(6) Szantay, C., Kotick, M. P., Shefter, E., and Bardos, T. J., *J. Am. Chem. Soc.*, **89**, 713(1967).
(7) Evans, H. T., Jr., *Acta Cryst.*, **14**, 689(1961).
(8) Cromer, D. T., and Waber, J. T., *ibid.*, **18**, 104(1951).
(9) McWeeny, R., *ibid.*, **4**, 513(1951).
(10) Cromer, D. T., *ibid.*, **18**, 17(1965).
(11) Oughton, B. M., and Harrison, P. M., *ibid.*, **12**, 396 (1959).
(12) Sutter, D., Dreizler, H., and Rudolph, H. D., *Z. Naturforsch.*, **20**, 1676(1965).
(13) Bowen, H. J. M., *Trans. Faraday Soc.*, **50**, 452(1954).
(14) Yakel, H. L., and Hughes, E. W., *Acta Cryst.*, **7**, 291(1954).
(15) Foss, O., Hordvik, A., and Sletten, J., *J. Acta Chem. Scand.*, **20**, 1169(1966).
(16) Fredrichson, J., and Mathieson, A. M., *Acta Cryst.*, **18**, 1043(1965).
(17) Kartha, G., personal communication.
(18) Abrahams, S. J., *Quart. Rev. (London)*, **10**, 407(1956).
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(21) Shefter, E., and Mautner, H. G., *J. Am. Chem. Soc.*, **89**, 1249(1967).
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(24) Brown, G. M., and Levy, H. A., *Science*, **141**, 478 (1963).
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(30) Donohue, J., and Trueblood, K. N., *J. Mol. Biol.*, **2**, 363(1960).
(31) Johnson, C. K., publication ORNL-3794, rev., Oak Ridge National Laboratory, Oak Ridge, Tenn., 1966.
(32) Jensen, L. H., and Sundaralingham, M., *Science*, **145**, 1185(1964).

Essential Oils and Their Constituents XXXIV

Isolation of Khusenic Acid and Isokhusenic Acid from Oil of Vetiver and Some Observations Concerning Their Structures

By ISHWAR C. NIGAM and HISASHI KOMAE

Khusenic acid and isokhusenic acid, two new tricyclic sesquiterpene acids, were isolated from oil of vetiver (*Vetiveria zizanioides*, Linn). The physicochemical properties of the acids and their methyl esters were determined. Infrared, NMR, and mass spectral data as well as the results of dehydrogenation suggest that the two acids differ in the location of the double bond. Khusenic acid and methyl khusenate possess a terminal methylene group which rearranges in the presence of mineral acid to a tetrasubstituted double bond leading to the formation of isokhusenic acid and methyl isokhusenate, respectively. Dehydrogenation of the acids as well as their esters failed to yield any aromatic products, the main reaction being decarboxylation of the compounds to a C_{14} hydrocarbon.

OIL OF VETIVER, an important essential oil used by the perfume and cosmetic industries, is obtained by steam distillation of the roots of *Vetiveria zizanioides* (*Gramineae*). Chemical examination of the oil has been carried out by many investigators for almost a century. Most of this work concerned its ketone, alcohol, and hydrocarbon fractions. The acids present in the oil have so far received only limited attention (1–4). The present report describes the isolation and characterization of the major acidic constituent as well as of a closely related isomer.

It is suggested that the major acidic constituent be named khusenic acid (3) rather than vetivenic acid (5) as the latter nomenclature may erroneously imply structural relationship with vetivane. The isomeric compound will accordingly be named isokhusenic acid.

Received May 2, 1967, from the Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication July 5, 1967.
Previous paper: *Perfumery Essent. Oil Rec.*, **56**, 783(1965).
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REFERENCES

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Angola oil (acid value: 29.1) gave a higher recovery of acids than reunion oil (acid value: 6.3), and was, therefore, used in the present investigations. Since gas chromatographic analysis of carboxylic acids is best carried out on the esters, the acids were isolated as methyl esters using Amberlite IRA-400 ion-exchange resin (6). This method was superior to that involving extraction with $NaHCO_3$, hydrolysis of the salt with HCl, and subsequent methylation, as it yielded the esters directly in a higher yield and the isolated fraction was lighter in color.

The gas chromatograms of the ester fractions obtained by either method exhibited essentially the same peaks but the relative proportions of the constituents appearing at 16.2 min. and 13.1 min. were reversed. It appeared that isolation of the acids by the $NaHCO_3$ method, particularly regeneration of the acids from the sodium salts by treatment with hydrochloric acid, brought about isomerization of the main component, khusenic acid to isokhusenic acid. This was confirmed by treating methyl khusenate with aqueous methanolic HCl, which yielded methyl isokhusenate as the major product of reaction (see Fig. 1 and Table I).

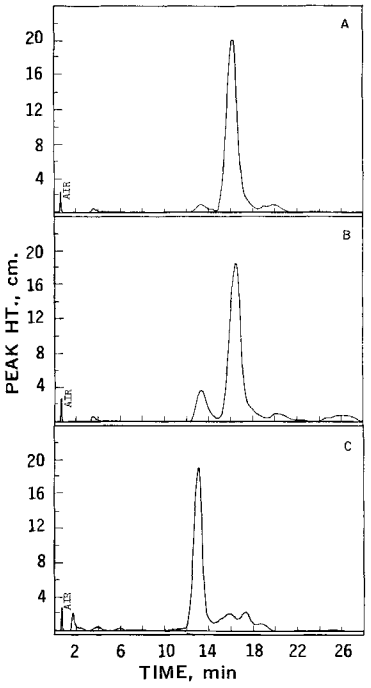


Fig. 1—Gas chromatograms of methyl esters of free acids present in oil of vetiver: A, resin treatment and methylation at room temperature; B, resin treatment and methylation under reflux; C, isomerization of A with HCl. Column: Reoplex 400, operated at 220°. Carrier gas: helium, 75 ml./min.

Analytical samples of methyl khusenate and methyl isokhusenate were obtained by preparative gas chromatography of the crude ester fraction and its isomerization product, respectively. Elemental analysis of methyl isokhusenate and molecular weights of both esters determined by mass spectrometry agreed with the molecular formula $C_{16}H_{24}O_2$. Hence, the corresponding acids are $C_{15}H_{22}O_2$.

Saponification of methyl isokhusenate yielded isokhusenic acid, which on purification *via* its cyclohexylamine salt (m.p. 131–133°) was obtained as a crystalline solid (m.p. 80–81°). For the preparation of pure khusenic acid, the crude acid obtained *via* $NaHCO_3$ extraction was converted into the cyclohexylamine salt (m.p. 146–148°), and the derivative decomposed with dilute hydrochloric acid. The acid so obtained was a liquid. Gas chromatographic analysis (Reoplex 400 column) showed it to be a mixture of two substances, which were isolated by preparative gas chromatography. The faster eluting one was a solid (m.p. 80–81°), the other a liquid. On the basis of infrared and NMR spectral data, the compounds were identified as isokhusenic acid and khusenic acid, respectively.

Hydrogenation of methyl khusenate and methyl isokhusenate confirmed the presence of one double bond in each molecule and hence the tricyclic character of the esters as well as the corresponding acids (2–4). The nature of unsaturation in both compounds was revealed by infrared and NMR spectral analysis as well as ozonolysis.

TABLE I—GAS CHROMATOGRAPHIC ANALYSIS OF METHYL ESTERS OF FREE ACIDS ISOLATED BY DIFFERENT METHODS

No.	Origin of Oil	Method of Obtaining Esters	—% of Main Components[a]— Methyl Khusenate	Methyl Isokhusenate
1	Reunion	$NaHCO_3$ treatment and methylation of the regenerated acid	13	60
2	Reunion	Resin treatment and methylation at room temperature	66	11
3	Angola	Same as in 2	88	5
4	Angola	Resin treatment and methylation under reflux	69	14
5	Angola	Sample from 3 isomerized with HCl	14	73

[a] The balance of 100% represents unidentified minor constituents.

The infrared spectra of the two acids and their esters are shown in Fig. 2. Khusenic acid exhibited infrared absorption bands at 3080, 1635, and 888 cm.$^{-1}$, characteristic of an unsymmetrical disubstituted double bond (>C=CH_2) (7). The corresponding peaks in the spectrum of methyl khusenate appeared at 3085, 1637, and 890 cm.$^{-1}$. The infrared spectra of isokhusenic acid and its methyl ester lacked characteristic olefinic absorption bands, thereby indicating the tetrasubstituted nature of the double bond in both these compounds.

The NMR spectra of the two acids and their methyl esters supported the infrared assignments and in addition suggested the presence of two more methyl groups in these compounds. Thus khusenic acid and methyl khusenate showed peaks for the >C=CH_2 group at 4.75 and 4.61 δ and at 4.77 and 4.63 δ, respectively. Isokhusenic acid and methyl isokhusenate did not give any signal attributable to an olefinic proton. These compounds, on the other hand, exhibited peaks at 1.47 and 1.46 δ, respectively, which could be assigned to a CH_3 group attached to an olefinic carbon atom. In addition, all four compounds gave peaks indicative of the presence of two paraffinic methyl groups. For isokhusenic acid and its ester, these groups possessed identical chemical shifts and generated a single peak. The positions and assignments of characteristic peaks observed in the NMR spectra of these compounds are summarized in Table II.

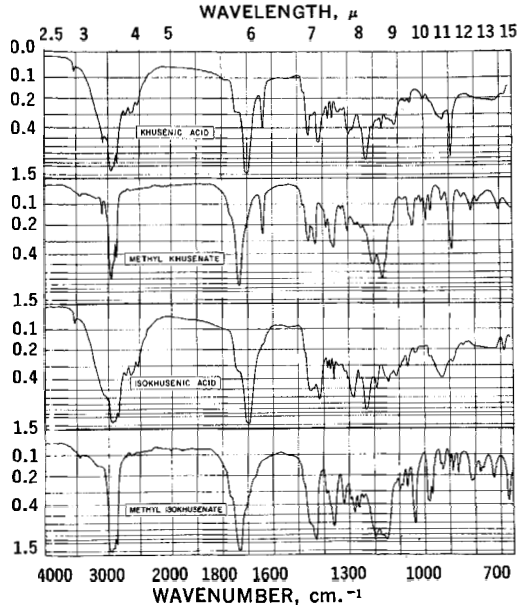
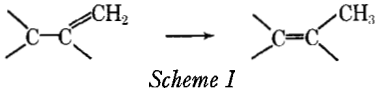


Fig. 2—Infrared spectra of khusenic acid, isokhusenic acid, and their methyl esters. The ordinate is labeled absorbance.

On ozonolysis methyl khusenate lost one carbon atom and yielded a monoketo ester ($C_{15}H_{22}O_3$). The infrared spectrum of the product exhibited carbonyl bands at 1708 and 1728 cm.$^{-1}$ for the keto group and the ester group, respectively. The observation confirmed the presence of an exocyclic double bond in methyl khusenate and hence, in the corresponding acid.

On the basis of the above data it would appear that the acid-catalyzed isomerization of khusenic acid and its methyl ester involves the rearrangement of the unsaturated linkage shown in Scheme I. Close resemblance between the



Scheme I

infrared spectra of the two acids and their methyl esters in the fingerprint region and the generation of peaks at the same positions in their mass spectra (Fig. 3) suggests that the isomerization involves only the double bond and does not affect any other part of the molecule. This deduction was further supported by the results of dehydrogenation experiments, in which the acid as well as their esters yielded the same major reaction product.

In none of these experiments was any aromatic or azulenic compound obtained. A hydrocarbon ($C_{14}H_{22}$) was always the main reaction product. The infrared spectrum of this hydrocarbon did not exhibit any absorption characteristic of aromatic or olefinic groups. The NMR spectrum indicated the presence of three methyl groups, one of them attached to an olefinic carbon atom

TABLE II—NMR SPECTRAL DATA FOR KHUSENIC ACID, ISOKHUSENIC ACID, AND THEIR DERIVATIVES

Compd.	Positions (δ) of Characteristic Peaks and Assignments			
	=CH_2	$COOCH_3$	=C—CH_3	C—CH_3
Khusenic acid	4.75, 4.61 (2H)	...	...	1.09 (3H)
				1.03 (3H)
Isokhusenic acid	...	...	1.47 (3H)	1.00 (6H)
Methyl khusenate	4.77, 4.63 (2H)	3.67 (3H)	...	1.08 (3H)
				1.05 (3H)
Methyl isokhusenate	...	3.64 (3H)	1.46 (3H)	1.00 (6H)
C_{14} Hydrocarbon obtained by	...	...	1.47 (3H)	1.02 (3H)
dehydrogenation				1.98 (3H)

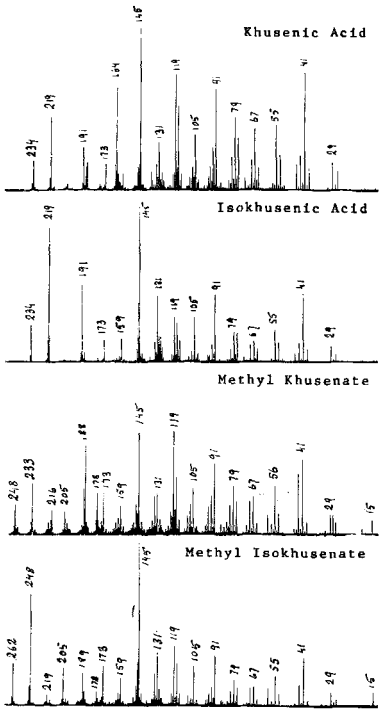


Fig. 3—Mass spectra of khusenic acid, isokhusenic acid, and their methyl esters.

(see Table II). These data suggested that in the case of isokhusenic acid and its methyl ester the reaction involved primarily loss of the carboxyl or the ester group, respectively. In the case of khusenic acid and its methyl ester it involved, in addition, rearrangement of the olefinic methylene group to a tetrasubstituted double bond, a reaction analogous to the isomerization of these compounds observed under the influence of acid.

The experimental data presented describe, for the first time, standardized procedures for the preparation of khusenic acid, isokhusenic acid, and their methyl esters, and yield some preliminary information regarding the structures of these substances. Isokhusenic acid and its methyl ester are crystalline solids and can, therefore, be readily characterized. They may also serve for the identification of khusenic acid and its methyl ester from which the former may be easily prepared *via* isomerization. The spectral and gas chromatographic data should prove of value as additional criteria of identity. Although both acids yield solid cyclohexylamine salts, these compounds were not found to be sufficiently stable for use as characteristic derivatives.

EXPERIMENTAL

All melting points and boiling points are uncorrected. Microanalyses were carried out gas chromatographically employing an F&M, C, H, and N analyzer model 185. Infrared spectra were recorded on a Perkin-Elmer model 221 spectrophotometer and NMR spectra measured in $CDCl_3$ solution employing TMS as internal reference using a Varian A-60A spectrometer equipped with a Varian C-1024 time averaging computer (CAT). Mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6D spectrometer. Measurements of optical rotations were carried out in $CHCl_3$ solution using a Rudolph model 200 photoelectric polarimeter. Gas chromatographic analyses were carried out on two instruments: (*a*) Burrell Kromo-Tog K-2 equipped with a 20% Reoplex 400 column prepared as previously described, the carrier gas being helium (75 ml./min.) (8); (*b*) aerograph Hi-Fi 600-C equipped with a stainless steel column (outside diameter $^1/_8$ in., length 5 ft.) containing 3% silicone rubber SE-30 on silanized 60 to 80 mesh Chromosorb W prepared according to the method of Horning *et al.* (9, 10), the carrier gas being nitrogen (16 ml./min.).

Isolation of Acids from Reunion Vetiver Oil by Extraction with $NaHCO_3$—The oil (175 Gm.) was dissolved in hexane (350 ml.) and shaken with five 175-ml. portions of 10% $NaHCO_3$ solution. The aqueous extract was washed with water (100 ml.), acidified with 1 *N* HCl solution, and the liberated acid was extracted with five 200-ml. portions of ether. The ether extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated. Yield of crude acids: 1.86 Gm. (1.1%).

Preparation of Methyl Esters—A portion of the crude acids (840 mg.) was dissolved in the methylating reagent (20 ml.) prepared by adding acetyl chloride dropwise to ten times its volume of cold absolute methanol. The mixture was left overnight, then diluted with water (80 ml.), and extracted five times with 20-ml. portions of ether. The ether extract was freed from HCl by repeated washings with water, dried over anhydrous Na_2SO_4, and evaporated. Yield of methyl esters: 572 mg. (68.1%).

Isolation of Free Acids as Methyl Esters from Reunion Vetiver Oil by Treatment with Ion-Exchange Resin—Oil of vetiver (2 Gm.) was dissolved in hexane (25 ml.), and the mixture was stirred for 5 min. with Amberlite IRA-400 (10 Gm.) converted into the OH form by pretreatment with alkali. Following decantation the resin was washed with 25-ml. portions of hexane until evaporation of the washings did not yield an oily residue. The oil-free resin was allowed to react with the above described methylating reagent (25 ml.) for 30 min. with frequent shakings. The supernatant liquid was decanted and the resin washed twice with 10 ml. of the methylating reagent. The supernatant liquid and the washings were combined, and the methyl esters were isolated from the reaction mixture in accordance with the procedure described. Yield of esters: 118 mg. (5.9%).

Isolation of Free Acids as Methyl Esters from Angola Vetiver Oil by Treatment with Ion-Exchange Resin—A sample of the oil (5.05 Gm.), was allowed to react with Amberlite IRA-400 resin (40 Gm.), as described above. The acid-bearing resin was divided in two equal parts. One part was methylated (50 ml. reagent) for 30 min. as previously described and the other part was refluxed with the same volume of reagent for the same length of time. The

methyl esters were recovered from each reaction mixture as described before. Yields of the ester fractions: obtained at room temperature, 661 mg. (26.2%); under reflux, 581 mg. (23.0%).

Acid-Catalyzed Isomerization of Esters Obtained by Resin Treatment—A sample of the ester (104 mg.) obtained from Angola oil by methylation at room temperature was dissolved in a mixture of methanol (9 ml.) and concentrated HCl (1 ml.), and refluxed for 2 hr. Following conventional processing the reaction mixture gave the isomerized product, yield: 101 mg. (97.1%).

Gas Chromatography of Ester Fractions—The esters obtained in accordance with the procedures described above were gas chromatographed on Reoplex 400 and SE-30 columns, operated at 220° and 180°, respectively. All samples exhibited two main peaks corresponding to methyl khusenate and methyl isokhusenate. The relative retention times of the two constituents on the Reoplex 400 column (reference standard: 1,2-dimethylnaphthalene) were 1.59 and 1.31, and on the SE-30 column (reference standard: phenanthrene) 0.85 and 0.77, respectively. The relative proportions of methyl khusenate and methyl isokhusenate in the different samples are recorded in Table I.

Preparation and Properties of Methyl Khusenate and Methyl Isokhusenate—The crude ester fractions were subjected to fractional distillation under reduced pressure—methyl khusenate fraction, yield 87%, purity by GLC 92%; methyl isokhusenate fraction, yield 90%, purity by GLC 95%. Methyl isokhusenate was further purified by recrystallization from ethanol, and an analytical sample of methyl khusenate was obtained by preparative gas chromatography on the Reoplex 400 column. The properties of the two esters are described below.

Methyl Khusenate—$b_{0.7}$ 130°; $[\alpha]_D$ +43.5° (c 3.54); n_D^{26} 1.5058; mol. wt. (mass spectrum) 248.

Anal.—Calcd. for $C_{16}H_{24}O_2$: C, 77.37; H, 9.74. Found: C, 77.60; H, 9.79.

Infrared absorption bands (liquid film) at 3085, 1730, 1637, 1457, 1430, 1385, 1365, 1351, 1303, 1200, 1165, 1045, 991, 973, 930, 890, 850, 815, 790, and 707 cm.$^{-1}$; NMR peaks at 4.63, 4.77, 3.67, 1.08, and 1.05 δ.

Methyl Isokhusenate—Melting point 51°; $b_{0.6}$ 125°; $[\alpha]_D$ +46.8° (c 1.64); mol. wt. (mass spectrum) 248.

Anal.—Calcd. for $C_{16}H_{24}O_2$: C, 77.37; H, 9.74. Found: C, 77.60; H, 9.66.

Infrared absorption bands (melt film) at 1732, 1452, 1432, 1387, 1360, 1320, 1293, 1278, 1261, 1243, 1199, 1190, 1168, 1153, 1098, 1087, 1069, 1040, 981, 968, 937, 929, 902, 888, 867, 815, 780, 770, and 728 cm.$^{-1}$; NMR peaks at 3.64, 1.46, and 1.00 δ.

Preparation of Isokhusenic Acid from Methyl Isokhusenate—Methyl isokhusenate (203.9 mg.) was refluxed with 1 *N* methanolic KOH (5 ml.) for 3 hr. The reaction mixture was acidified (pH 3) with 1 *N* HCl and extracted with three 20-ml. portions of ether. The ether extract on conventional processing yielded crude isokhusenic acid (160.4 mg.). Reaction of the crude acid with cyclohexylamine (0.2 ml.) in acetone (2 ml.) yielded the cyclohexylamine salt recrystallized from the same solvent (m.p. 131–133°). Isokhusenic acid was regenerated from the salt by treatment with 1 *N* HCl (2 ml.). The liberated acid was extracted with ether (3 × 2 ml.); the extract was washed with water, dried over Na_2SO_4, and evaporated. The residue, on recrystallization from aqueous ethanol, yielded pure isokhusenic acid (26.4 mg.), m.p. 80–81°; $[\alpha]_D$ +27.9° (c 2.78).

Anal.—Calcd. for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46. Found: C, 77.32; H, 9.61.

Infrared absorption bands (CCl_4 solution) at 3520, 3000–2400 (broad band), 1700, 1453, 1418, 1384, 1372, 1359, 1281, 1231, 1191, 1144, 1068, and 938–928 cm.$^{-1}$; NMR peaks at 1.47 and 1.00 δ.

Isolation of Khusenic and Isokhusenic Acids from Crude Acid Fraction—The dark viscous acid fraction (1.0 Gm.) obtained from Reunion vetiver oil following treatment with $NaHCO_3$ was allowed to react with cyclohexylamine (1.0 ml.) in acetone solution (5 ml.). The cyclohexylamine salt separated as a solid which was recrystallized from acetone (m.p. 146–148°). On treatment with 1 *N* HCl (3 ml.) it yielded an oil (650 mg.), which by gas chromatographic analysis was found to be composed of khusenic acid (52%) and isokhusenic acid (48%) (retention times: 55.0 min. and 44.5 min., respectively; column: Reoplex 400; temperature 220°). Each of the two acids were collected separately by gas chromatography. The I.R. spectrum and melting point of isokhusenic acid were identical with those of the sample prepared from methyl isokhusenate. Khusenic acid had the following properties: $b_{0.5}$ 158°; $[\alpha]_D$ +17.2 (c 4.39); n_D^{26} 1.5198.

Anal.—Calcd. for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46. Found: C, 77.34; H, 9.55.

Infrared absorption bands (CCl_4 solution) at 3520, 3080, 1700, 1635, 1457, 1415, 1378, 1362, 1335, 1297, 1225, 1165, 1114, 943–928, and 888 cm.$^{-1}$; NMR peaks at 4.75, 4.61, 1.09, and 1.03 δ.

Hydrogenation of the Methyl Esters—Methyl khusenate and methyl isokhusenate were hydrogenated in glacial acetic acid solutions employing Adam's platinum oxide catalyst. The volume of hydrogen absorbed by 105 mg. of methyl khusenate at 27° and 754 mm. pressure was 13 ml. (1.2 moles of H_2 per mole of ester). Hydrogen uptake by 100 mg. of methyl isokhusenate under the same conditions was 10 ml. (1.0 mole).

Attempted Dehydrogenation of Methyl Khusenate and Methyl Isokhusenate—Each of the esters was mixed with twice its weight of selenium and refluxed at 300 ± 10° in an atmosphere of nitrogen. The resultant products were extracted with hexane and chromatographed as described below.

Products from Methyl Khusenate—The reaction mixture (reaction time: 4 hr.) from methyl khusenate (100 mg.) was chromatographed on grade I alumina (5.5 Gm.). The following fractions were obtained: hexane (8 ml.), 13.1 mg.; hexane (24 ml.), 3.8 mg.; hexane (96 ml.), 1.9 mg.; benzene (32 ml.), 13.4 mg.

Products from Methyl Isokhusenate—Reaction of this ester (70 mg.) was allowed to proceed for 9 hr. The products were chromatographed on grade I alumina (5 Gm.) and three fractions were collected: hexane (8 ml.), 2.7 mg.; hexane (64 mg.), 0.3 mg.; hexane–benzene (4:1, 72 ml.), 4.4 mg.

Attempted Dehydrogenation of Crude Acid Mixture—*Employing Selenium*—The crude acid (356 mg.) was allowed to react with selenium for 14 hr. under experimental conditions described above for esters. The acidic constituents present in the reac-

tion mixture were separated as methyl esters employing Amberlite IRA-400. Yields of the neutral and acidic fractions (isolated as methyl esters) were 197 mg. and 22.1 mg., respectively. The neutral fraction was purified by chromatography on grade I alumina (5 Gm.) when a colorless hydrocarbon fraction (72 mg.) was obtained.

Employing Metal Catalysts—The experiment was repeated with the crude acid (250 mg.), employing 5% Pt on alumina (50 mg.) as the dehydrogenating agent. Analogous processing of the reaction mixture yielded a neutral oil (148 mg.) and methyl esters (41 mg.). Chromatography of the neutral fraction on alumina gave hydrocarbons (58 mg.). I.R. and U.V. analyses showed the hydrocarbon fraction did not undergo any chemical change on further refluxing with 5% Pd-on-carbon (31 mg.) for 2 hr. at 320–350°.

Characterization of the Hydrocarbon Fractions Obtained by Dehydrogenation Experiments—Similar hydrocarbon fractions (I.R. spectroscopy and gas chromatography) were obtained in each of the dehydrogenation experiments. The main constituent (79%) of the hydrocarbon fraction obtained by Se-dehydrogenation of the acids was isolated by gas chromatography on a Reoplex 400 column. It possessed the following characteristics: relative retention times on Reoplex 400 (column temperature, 200°) and SE-30 (column temperature, 185°): 0.49 and 1.76, respectively (reference standard: naphthalene; retention times: 6.70 min. and 1.65 min., respectively). NMR peaks at 1.47, 1.02, and 0.98 δ. Mol. wt. (mass spectrum) 190.

Ozonolysis of Methyl Khusenate—Methyl khusenate (1.00 Gm.) was dissolved in ethyl acetate (5 ml.), cooled to 0°, and ozonized using a Gallenkamp ozone apparatus GE-150. The ozonide was decomposed with hydrogen in the presence of Pd-C and the product treated with Amberlite IRA-400 (5 Gm.) in accordance with the procedure described above. The neutral fraction (918 mg.) obtained was purified by chromatography on grade III alumina (50 Gm.) using hexane–benzene mixture (1:1) as solvent and recrystallized from ethanol. Yield: 52% (by GLC), m.p. 102–103°.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.95; H, 8.86. Found: C, 71.97; H, 8.87.

Infrared absorption bands (CCl_4 solution) at 1728, 1708, 1458, 1430, 1388, 1356, 1300, 1195, 1164, and 1042 cm.$^{-1}$.

REFERENCES

(1) Anh, N. T., and Fetizon, M., *Am. Perfumer*, **80**, 40 (1965).
(2) Guenther, E., "The Essential Oils," vol. IV, D. Van Nostrand Co., Inc., New York, N.Y., 1950, p. 171.
(3) Zutshi, N. L., and Sadgopal, *Perfumery Essent. Oil Rec.*, **48**, 333(1957).
(4) Novotel'nova, N. F., and Rafanova, R. Y., *Khim. Nauka i Promy.*, **3**, 539(1958).
(5) Ruzicka, L., Capato, E., and Huyser, H., *Rec. Trav. Chim.*, **47**, 370(1928).
(6) Hornstein, I., Alford, J. A., Elliott, L. E., and Crowe, P. F., *Anal. Chem.*, **32**, 540(1960).
(7) Bellamy, L. J., "The Infra-Red Spectra of Complex Molecules," John Wiley & Sons, Inc., New York, N.Y., 1958, p. 34.
(8) Nigam, I. C., and Levi, L., *Can. J. Chem.*, **40**, 2083 (1962).
(9) Horning, E. C., Moscatelli, E., and Sweeley, C. C., *Chem. Ind.*, **1959**, 751.
(10) Horning, E. C., Vanden Heuvel, W. J. A., and Creech, B. G., in "Methods of Biochemical Analysis," Glick, D., ed., vol. XI, Interscience Publishers, Inc., New York, N.Y., 1963, p. 82.

Drug Standards

New Electromechanical Method for the Assay of Heparin *In Vitro*

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A rapid, simple, and accurate *in vitro* assay for heparin employing a diatomaceous silicate modified plasma and a precision coagulation timer is reported. The method reveals standard deviations of ±2.5 sec. from the average clotting times with a coefficient of variation of about 2 per cent. Sodium heparin U.S.P., heparin reference standard lot H, and the second international standard for heparin have been successfully assayed by this method. The assays have also revealed the stability of sodium heparin to autoclaving and high energy electron irradiation.

WALTON *et al.* (1) have recently reviewed current methods of heparin assay. One of the most commonly employed methods is that of Reinert and Winterstein (2) which is the basis for the U.S.P. heparin assay procedure. A second method is that of Adams (3) which is prescribed by the British Pharmacopoeia. There exist numerous other methods which are essentially modi-

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(2) Guenther, E., "The Essential Oils," vol. IV, D. Van Nostrand Co., Inc., New York, N.Y., 1950, p. 171.
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(6) Hornstein, I., Alford, J. A., Elliott, L. E., and Crowe, P. F., *Anal. Chem.*, **32**, 540(1960).
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(8) Nigam, I. C., and Levi, L., *Can. J. Chem.*, **40**, 2083 (1962).
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(10) Horning, E. C., Vanden Heuvel, W. J. A., and Creech, B. G., in "Methods of Biochemical Analysis," Glick, D., ed., vol. XI, Interscience Publishers, Inc., New York, N.Y., 1963, p. 82.

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fications of the original Howell procedure (4) upon which the various current heparin activity units are based.

Elliott (5) reported the use of a coagelometer in the determination of heparin concentration. A linear relationship was observed at heparin concentrations of 0 to 2.0 mcg./ml. of whole blood with coagulation times varying from 12 to 30 min. The coagelometer employed, based on a photoelectric readout of the coagulation, was complex and no details were given as to the reproducibility and reliability of the method employed. No data were presented on the assay of heparin employing plasma.

The accepted procedures employed in the assay of heparin are generally time-consuming (a few hours to several days) and subjective to a large extent. It was considered desirable to simplify the assay procedure and to obtain a more precise end point determination by detecting clotting times by the use of the Fibrometer[1] precision coagulation timer. Recently Barnett and Pinto (6) and Fletcher *et al.* (7) presented data describing the performance of the instrument in the determination of coagulation times.

Attempts to employ the standard U.S.P. heparin assay procedure using the precision coagulation timer were unsuccessful in our hands.

The use of a diatomaceous silicate[2] in the partial thromboplastin time assay by Struver and Bittner (8) prompted an investigation of the effect of this "activating" agent on plasma clotting times. As a result, the necessary conditions for a simple and reliable assay for heparin activity were obtained by modification of the U.S.P. assay procedure by the addition of the diatomaceous silicate. This report summarizes the new *in vitro* assay procedure, its assets, and limitations.

EXPERIMENTAL

Apparatus—The precision coagulation timer and standard accessory equipment were employed. The automatic pipet was set to deliver 0.1 ml. The thermal Prep-Block precision heating unit was used to provide a constant supply of disposable plastic Fibrotube[1] cups preheated to 37 ± 0.5°.

Freezing point depression readings were obtained on 0.9% sodium chloride solution using a Beckman molecular weight apparatus to assure isotonicity.

Chemicals and Reagents—Dry sodium heparin preparations were obtained from three sources; these preparations were stored at −20° in their original containers over desiccant; the units/mg. are their assigned potencies.

(*a*) Second international standard for heparin, described by Bangham and Mussett (9), from the National Institute for Medical Research, Mill Hill, England: 130 units/mg.

(*b*) U.S.P. sodium heparin reference standard; lot H, U.S.P., New York, N.Y.: 2.8 units/mg.

(*c*) Sodium heparin; Pharmaceutical Division, Wilson Laboratories, Chicago, Ill: 143 units/mg.

A stock diatomaceous silicate reagent was made by suspending 1.5% of the diatomaceous silicate (w/v) in 0.025 *M* calcium chloride in distilled water and stored at room temperature.

Citrated plasma was obtained by bleeding healthy sheep as directed under *assay* in the U.S.P. monograph entitled "Sodium Heparin" (10). Various plasma lots, from individual sheep, were obtained in approximately 100-ml. volumes and stored in the frozen state at −20°. The 100-ml. volumes of plasma were subsequently thawed, thoroughly mixed, subdivided into 10-ml. portions in screw-capped borosilicate vials, and stored at −20°.

Assay Solution Preparations—A fresh heparin sample (tablet or powder) was used for each assay. Dry heparin was weighed and appropriate dilutions made in 0.9% sodium chloride for stock concentrations as follows: 0.33, 0.36, 0.39, 0.42, 0.45, and 0.48 units/ml., according to labeled potency. The heparin assay solutions were maintained at room temperature during the assay period.

A 10-ml. portion of frozen plasma was thawed, mixed, brought to 37°, and decanted into 10-ml. borosilicate glass-stoppered volumetric flasks, then placed for use in an ice bath at 0°. A freshly thawed 10-ml. portion of plasma was prepared daily for each assay. One 10-ml. portion is more than sufficient to complete an assay as presented in Table III.

The diatomaceous silicate reagent was maintained at 37° ± 0.5° in a water bath.

Clotting Time Assay—The control clotting time (CCT) of citrated sheep plasma was determined by delivering 0.1 ml. of 0.9% sodium chloride into each of three or four disposable plastic cups which were then equilibrated at 37° for 3 min. or longer. One-tenth milliliter of plasma was then added to the first plastic cup placed in the center well of the coagulation timer. Precisely at the end of 30 sec., 0.1 ml. of the diatomaceous silicate reagent, preheated to 37° ± 0.5° and well mixed by a magnetic stirring bar, was delivered, with moderate force, into the 0.9% sodium chloride–plasma mixture and the timer started automatically.

When the coagulation timer digital read-out mechanism stopped, the probe assembly was immediately removed, the probes rinsed with fresh 0.9% sodium chloride, brushed gently with a soft-bristle toothbrush, rinsed once again, and blotted dry. The clotting time, in seconds, was recorded. The digital timer was reset to zero and the probe assembly reinserted into the instrument and the next determination immediately made using the second plastic cup containing 0.1 ml. of 0.9% sodium chloride. The procedure was repeated again until three or four determinations were obtained. Each determination required approximately 4 min. to complete.

For the assay of heparin standards and unknowns 0.1-ml. volumes of the various heparin concentrations were substituted for the 0.9% sodium chloride solution.

[1] Fibrometer and Fibrotube are registered trademarks of the Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.

[2] Celite 315 diatomite mineral filler. Celite is a registered trademark of Johns-Manville, New York, N. Y.

Plasma control clotting times (CCT) were determined before and after heparin assay. Times were averaged and subtracted from the clotting times (HCT) obtained from the heparin determinations.

RESULTS

At a constant calcium chloride concentration (0.025 *M*), as seen in Fig. 1, a concentration of 1.5% (w/v) of the diatomaceous silicate was found suitable for the determinations since the least variation of plasma clotting times was observed at this value.

It was apparent that the average CCT was dependent upon the plasma lot employed, as shown in Table I. In practice it was found that the CCT was constant to within ± 5 sec. of the average during the assay period. Qualitative observation on plasma stored in the frozen state for a period of 1 year did not reveal apparent changes in clotting time. For example, lot No. 1 plasma assayed initially gave an average CCT value of 123.6 ± 8 sec. for 15 assays; 11 months later, the average CCT value of 125.4 ± 8 sec., for 7 assays, was obtained.

A study was made to determine the optimum plasma concentration for use in the assay in order to establish the plasma control clotting time (CCT). Various dilutions of plasma were assayed and the results are shown in Fig. 2. A concentration of 0.33 ml. plasma per ml., in the final assay mixture, was chosen for experimental convenience and reliability. The clotting times obtained on the same lot of plasma on assays performed on *different days* are summarized in Table II. The change observed in Table II (9/7/66 to 12/15/66) is attributed to the introduction of a new preparation of the diatomaceous silicate reagent as indicated. The average value of 7 CCT determinations on lot No. 3 of sheep plasma, performed on the *same day* (12/19/66), during a regular assay was 92.8 sec., with a standard deviation of ± 2.02 sec., and a coefficient of variation of 2.19% (Table III). It was observed that thawed plasma, maintained at 0° (ice bath), displayed a continuous increase in clotting time as a function of the duration of storage as shown in Fig. 3. Attempts to correct for this effect of plasma inactivation, on standing during the assay procedure, provided no significant improvement in linearity of response.

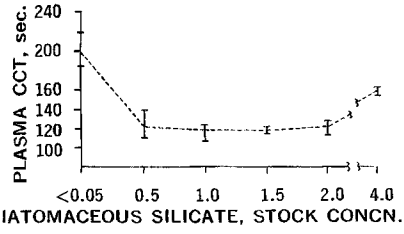

Fig. 1—Effect on the control clotting time (CCT) of citrated sheep plasma, in seconds, due to varying the concentration of the diatomaceous silicate in 0.025 M *calcium chloride. The concentration of citrated sheep plasma and 0.9% sodium chloride diluent were held constant.*

TABLE I—VARIATION OF CCT BETWEEN SHEEP PLASMA LOTS

Plasma Lot	Av. CCT
1	125.4 ± 8 sec. (7 assays)
2	94.4 ± 8 sec. (7 assays)
3	100.2 ± 8 sec. (6 assays)

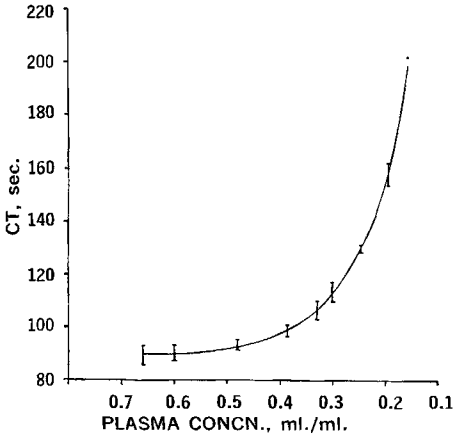

Fig. 2—Effect on the clotting time, in seconds, due to varying the final concentration of citrated sheep plasma. The diluent (0.9% sodium chloride) and the 1.5% diatomaceous silicate–calcium chloride reagent were held at constant concentration.

TABLE II—VARIATION OF CLOTTING TIMES WITHIN THE SAME SHEEP PLASMA LOT NO. 3

Day	Av. CCT, sec.
8/25/66	107.6
9/2/66	105.8
9/7/66	106.0
12/15/66	96.0[a]
12/16/66	93.3
12/19/66	92.8

[a] New preparation of diatomaceous silicate–calcium chloride reagent introduced.

For the assay of heparin potency, concentrations of the various heparin preparations were chosen which would result in a range of clotting times from 110 to 250 sec. corresponding to approximately 0.11 to 0.16 heparin units/ml. in the final assay mixture. A comparative assay was performed between U.S.P. heparin reference standard lot H (curve U), and the second international standard for heparin (curve I), Fig. 4. From the curves (Fig. 4) there were obtained slope values corresponding to about 0.001 Δ heparin units of activity/second. Additional slope values of calibration curves plotted for eight heparin assays employing sheep plasma lot No. 3, ranged from 0.0007 to 0.0016 units/second and together averaged 0.001 Δ units/second. The assay precision was shown in obtaining clotting times having a standard deviation of ± 2.77 sec. and a coefficient of variation of 1.71%, Table III. Details of Table III are also presented to illustrate sample data collection in the assay method.

The results of comparative assays employing U.S.P. heparin lot H and the second international standard for heparin are summarized in Table IV. The U.S.P. reference standard lot H is approxi-

TABLE III—HEPARIN ASSAY (FIG. 4)[a]

Heparin source	CCT	Clotting Times (HCT)												CCT
		U.S.P.						Second International						
Order of determination	1	2	4	6	8	10	12	3	5	7	9	11	13	14
Final unit concn./ml.	—	0.11	0.12	0.13	0.14	0.15	0.16	0.11	0.12	0.13	0.14	0.15	0.16	—
	92.7	133.3	148.2	151.7	158.8	186.2	191.3	124.2	134.8	143.7	150.8	163.2	177.3	97.7
t, sec.	89.8	130.7	149.8	147.2	159.8	187.8	194.7	125.8	132.8	141.3	161.7	170.7	180.7	96.2
	88.8	138.3	148.8	150.8	158.8	190.8	203.2	123.8	132.3	143.3	152.7	167.2	185.7	94.2
	87.3			157.7			198.7							
Av.	89.6	134.1	148.9	151.8	159.1	188.2	196.9	124.6	133.3	142.8	155.1	167.0	181.2	96.0
CT (HCT–CCT[b])	—	41.3	56.1	59.0	66.3	95.4	104.1	31.8	40.5	50.0	62.3	74.2	88.4	—

[a] Date: 12/19/66; U.S.P. lot H, tablet weight: 22.1 mg. (61.9 units); second international standard, sample weight: 12.5 mg. (1625 units); sheep plasma, lot 3; Fibrometer No. 03695; assay starting time: 10:00 a.m., completion time: 1:23 p.m., time lapse: 3 hr. and 23 min. [b] Average CCT time: 92.8 sec.; combined average standard deviations: ±2.46 sec (CCT: ±2.02, HCT^U: ±2.77, HCT^I: ±2.58 sec.); combined average coefficient of variation: 1.92% (CCT: 2.19; HCT^U 1.71; HCT^I 1.86%).

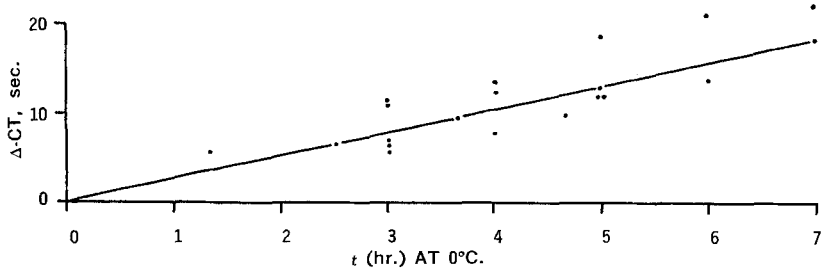

Fig. 3—Spontaneous inactivation curve (change in clotting time: Δ CT) of citrated sheep plasma, thawed, then stored at 0° in an ice bath. The slope of the curve shows a change of 0.0418 sec. in clotting time per minute of storage. The curve, illustrated, represents the average of seven assays, the slopes being 0.0309, 0.0342, 0.0366, 0.0437, 0.0445, 0.0474, and 0.0560 sec./min., respectively.

mately 10% higher in potency when compared to the second international standard for heparin.

To arrive at the percentage difference, shown in Table IV, the assumed potency in units/ml., of the unknown is divided into the difference in units/ml., between the unknown and the extrapolated potency derived from the standard curve. Reference to Fig. 4 will show that an assumed heparin potency of 0.120 units/ml. (curve U) is equivalent to an extrapolated value of 0.132 units/ml. (curve I), or a difference of 0.012 units representing a 10% higher potency.

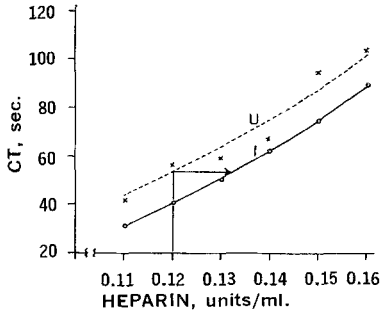

Fig. 4—Comparative assay of heparin potency. The U.S.P. heparin reference standard, curve U. *The second international standard for heparin, curve* I. *Clotting time (CT) in seconds, ordinate, were obtained by subtracting the plasma control clotting time (CCT) from the clotting time values obtained on the addition of heparin (HCT).*

TABLE IV—PER CENT POTENCY DIFFERENCE (U.S.P. HEPARIN *Versus* SECOND INTERNATIONAL STANDARD HEPARIN)

Assay Date	U.S.P. % Higher
8/2/66	18.0
8/3/66	11.3
8/4/66	8.0
8/5/66	6.4
8/25/66	7.2
9/2/66	11.0
12/15/66	11.5
12/19/66	10.0
	Av. 10.4%

Finally, it was of interest to determine the effect of autoclaving and irradiation on heparin potency. Fifty-milliliter solutions containing commercial samples of sodium heparin, in concentrations ranging from 0.5 mg./ml. to 16.0 mg./ml., were autoclaved at 121° for 45 min. Appropriate dilutions were made and the assays of these solutions revealed a negligible change from the control heparin activity.

Vacutainer[3] specimen tubes (3200KA), each containing about 140 units of dry sodium heparin, were irradiated by high energy electrons in a Hughes linear electron accelerator at dosages of 5 and 8 Mrads, at 6 to 7 Kw., 9 Mev., and 500 pulses per second. The assay revealed a loss in potency of approximately 15% as compared to appropriate controls.

[3] Vacutainer is a registered trademark of Becton, Dickinson and Co., Rutherford, N. J.

DISCUSSION

The accepted assay procedures for heparin are generally complex and subjective. The results of this investigation have led to the development of a rapid, simple, and objective *in vitro* procedure. Considerable effort was expended in obtaining plasma having reproducible and consistent control clotting times. It was found that, employing the precision coagulation timer and a diatomaceous silicate modified sheep plasma reagent, clotting times of approximately 100 sec. were obtained with a standard deviation of ± 2.5 sec. and a coefficient of variation of 2.0%. This was accomplished by the addition of 0.025 *M* $CaCl_2$, containing 1.5% of the diatomaceous silicate, to citrate sheep plasma. Just as in the Struver and Bittner method for the assay of thromboplastin activity (8), the diatomaceous silicate was found to serve as an excellent anion glass-activator for obtaining the useful clotting times. The effect of the diatomaceous silicate was not only to accelerate the clotting time but also to yield reproducible and predictable clotting time behavior without apparent interference with the heparin assay. Citrated sheep plasma, when stored in the frozen state for as long as 1 year, was found to be useful in the assay.

The spontaneous inactivation curve of citrated sheep plasma (Fig. 3) stresses the importance of assaying "standard" heparin preparations when comparisons are being made with unknown heparin preparations. The daily variations in clotting times, within the same lot of plasma, are difficult to interpret; however, these variations could be attributed to foreign contaminants, to subtle difference in concentrations of platelets, or to changes in other clotting factors.

In order to assay heparin, the differences in clotting time between the control and the samples containing an unknown heparin were obtained. A calibration plot was made of the differences in clotting time *versus* the heparin concentration in units/ml. (Fig. 4). No attempt was made to plot either curve by regression analysis because of the curvilinear characteristic. Curve I, based on observed CT values obtained with the second international standard for heparin, was amenable to visual plotting. No explanation, however, can be offered for the scatter of the observed U.S.P. heparin standard CT values used to draw curve U. This scatter, although random (Fig. 4), was observed with several of the comparative assays employing the U.S.P. sodium heparin reference standard; the second international standard for heparin gave very little scattering. Final potency values calculated from the illustrated curves, or from linear curves based upon a semilog plot, were the same.[4] Linearity was achieved as a function of heparin concentration in the range of from 0.11 to 0.16 units/ml. At lower concentrations of heparin, the curves become asymptotic and hence not suitable for assay, while at concentrations higher than 0.16 units/ml. there was an exponential increase in clotting times, rendering the curves inaccurate. Assay of heparin concentrations higher than 0.16 units/ml. required that dilutions be made to achieve clotting time values in the range of 110 to 250 sec.

U.S.P. heparin lot H exhibited a potency 10% greater than the second international standard for heparin by the method described; however, Burck (11), using the U.S.P. procedure for sodium heparin assay, noted a similar difference. It should be pointed out that the U.S.P. heparin reference standard is dispensed in the form of tablets with the units based on the weight of the tablet. A small variation in the composition of each tablet mixture may also contribute to the difference in reported activity as shown in Table IV. This observation was also noted by Burck (11).

It was of interest, in the course of this study, to determine the effect of autoclaving and irradiation on the stability of heparin preparations. Assays of sodium heparin solutions, autoclaved at 121° for 45 min., demonstrated no significant deterioration in the potency. This corroborates the findings of Pritchard (12) and Hejgard (13), who reported that autoclaving had little effect on the anticoagulant activity of heparin. Dry sodium heparin, irradiated at dosages of 5 and 8 Mrads, showed a 15% loss in potency indicating the relative stability of the material to high energy electrons.

REFERENCES

(1) Walton, P. L., Ricketts, C. R., and Bangham, D. R., *Brit. J. Haematol.*, **12**, 310(1966).
(2) Reinert, M., and Winterstein, A., *Arch. Intern. Pharmacodyn.*, **62**, 47(1939).
(3) Adams, S. S., *J. Pharm. (London)*, **2**, 836(1950).
(4) Howell, W. H., *Am. J. Physiol.*, **71**, 553(1925).
(5) Elliott, F. R., *J. Lab. Clin. Med.*, **40**, 766(1952).
(6) Barnett, R. N., and Pinto, C. L., *Am. J. Clin. Pathol.*, **46**, 148(1966).
(7) Fletcher, H. P., Burnham, R. J., Cole, R. J., and Dunham, N. W., *J. Pharm. Sci.*, **55**, 689(1966).
(8) Struver, G. P., and Bittner, D. L., *Am. J. Clin. Pathol.*, **38**, 473(1962).
(9) Bangham, D. R., and Mussett, M. V., *Bull. World Health Organ.*, **20**, 1201(1959).
(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(11) Burck, K. T., personal communication, 1966.
(12) Pritchard, J., *J. Pharm. Pharmacol.*, **16**, 487(1963).
(13) Hejgard, J. J., *Arch. Pharm. Chemi*, **72**, 1(1965).

[4] *Note:* The data, Table III, when plotted as log $\frac{HCT - CCT}{CCT}$ as a function of heparin concentration, show a linear relationship, reflecting a pseudo first-order dependence on heparin concentration.

Determination of Calcium in Official Calcium Phosphates

By JUNG SOOK KIM and MURRAY M. TUCKERMAN

The assay of official calcium phosphates for calcium can be brought into the general method for calcium determination by titration with EDTA using hydroxynaphthol blue indicator by dissolving the sample in a strictly limited amount of hydrochloric acid, passing an aliquot of the solution through a column of anion-exchange resin in the chloride form, washing out the calcium ion with water, and determining calcium in the eluate. The method is comparable in precision with the official method for calcium in dibasic calcium phosphate, but requires much less time.

In 1965 Butler, Maurina, and Morecombe (1) showed the utility of hydroxynaphthol blue as an indicator in the direct titration of calcium with disodium ethylenediaminetetraacetate (EDTA). All of the pharmaceutical substances official in U.S.P. XVII and N.F. XII which are assayed for calcium are assayed by direct titration with standard EDTA and this indicator with the exception of dibasic calcium phosphate U.S.P., which is assayed by the titrimetric determination of oxalate in a precipitated calcium oxalate with standard permanganate. This duality in methods arises from the present impossibility of a direct complexometric titration of calcium in the presence of large amounts of phosphate.

Calcium has been determined complexometrically in the presence of phosphate by addition of excess complexing agent and determination of the excess (2–9) and also after separation of calcium from phosphate by ion exchange (10–20). From a review of the literature it appeared that a simple, rapid, elegant, direct titrimetric determination of calcium in calcium phosphates was possible.

EXPERIMENTAL

Reagents—All reagents employed were reagent grade chemicals. All standard solutions employed were prepared and standardized according to the official compendia.

Methods—*A*—N.F. XI method (21).

B—Proposed Method—Charge a glass column about 37 cm. long and 2.5 cm. in diameter, fitted with a stopcock and delivery tip, with about 40 Gm. of Amberlite IRA-400 (Cl) resin previously hydrated for about 1 hr. Place about 500 mg. of dibasic calcium phosphate, previously ignited at 800° to constant weight and accurately weighed, in a 100-ml. beaker and dissolve it in 2.5 ml. of 1:2 hydrochloric acid by heating. Transfer the solution to a 100-ml. volumetric flask, dilute to volume, and mix well. Pass a 20.00-ml. aliquot of the sample solution through the ion-exchange column at a flow rate of about 0.5 ml. per minute, collecting the effluent in a 500-ml. conical flask. Wash the column with 175 ml. of water, passing the first 25 ml. at a rate of 0.5 to 1 ml. per minute, the next 50 ml. at about 4 ml. per minute, the next 50 ml. at about 8 ml. per minute, and the last 50 ml. at free flow, collecting the effluents in the same conical flask as the sample aliquot. Add 15 ml. of sodium hydroxide T.S. and about 300 mg. of hydroxy naphthol blue, A.R. indicator, and mix well. The pH of the solution should be between 12.5 and 13.0. Titrate the solution with standard 0.05 *M* disodium ethylenediaminetetraacetate to a change of color from purple to deep blue.

After passage of three samples the ion-exchange column is regenerated by passing through it about 200 ml. of 1 *M* sodium chloride solution at about 5 ml. per minute and washing with 3 to 4 L. of water until there is little free chloride present as shown by testing the effluent with silver nitrate.

RESULTS

Five determinations of a single sample by method *A* gave an average result of 98.9 ± 0.1%. Five determinations of the same sample by method *B* gave an average result of 99.1 ± 0.8%. The standard deviations were calculated from the range of values obtained by the method of Dean and Dixon (22).

Method *B* was also applied to tribasic calcium phosphate N.F. using an ignited sample of about 200 mg. Five determinations gave an average value for calcium of 37.5 ± 0.3%.

DISCUSSION

This assay is of interest from the philosophical standpoint of development of standard methods of assay for specific ions and also from the physical chemistry of the ion-exchange process. For reasons of convenience and economy, it was decided to attempt to use the anion-exchange resin in the commercially available chloride form rather than in the hydroxyl form which is expected to be more efficient. The resin has less affinity for the dihydrogen phosphate anion than for chloride (23).

Since the reactions of ion exchange are stoichiometric and reversible, one may write the equilibrium $aM_1/bR + bN_1/aX \rightleftarrows aN_1/aR + bM_1/bX$, in which M is the anion attached to the univalent resinate cation R and N is the anion added with the univalent

Received April 14, 1967, from the School of Pharmacy, Temple University, Philadelphia, PA 19122

Accepted for publication June 22, 1967.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by Jung Sook Kim to the Graduate School, Temple University, Philadelphia, Pa., in partial fulfillment of Master of Science degree requirements.

cation X. If these letter symbols are allowed to stand for concentration, and the subscripts r and w are used to indicate the resin and the outside solution, respectively, then one can write the classical law of mass action $N_r{}^a M_w{}^b / M_r{}^a N_w{}^b \simeq K$. If K is approximately constant for a given exchange, the numerical value is a measure of preference of the resin for the ion. Note, however, that K is dimensionless only if a and b are equal, so that comparison of ion affinities can be made only for exchanges of ions of the same type, that is, univalent-univalent, univalent-bivalent, *etc.* Despite the limitations of the theory, there is general knowledge that for similar ions affinity increases with increasing ionic charge.

On the basis of the foregoing discussion it is seen that the concentration of hydrochloric acid is critical. There must be enough to dissolve the sample in a reasonable time but not so much chloride ion present as to prevent complete exchange of phosphate. Additionally, a low hydrogen-ion concentration favors the multivalent anion in the equilibrium $H_2PO_4^- \rightleftarrows H^+ + HPO_4^{-2}$.

The importance to be attached to the difference in standard deviations between methods A and B is difficult to evaluate. The standard deviation for the assay of dibasic calcium phosphate is expected to be about 0.4 for either method. A collaborative study with sufficient replicates is needed to establish any real difference in the reproducibility of the two methods. The two methods yield the same result within the expected standard deviation.

No comparisons can be made in the assay of tribasic calcium phosphate as the official method is the determination of phosphate.

CONCLUSIONS

The proposed method is much more rapid than the official method of assay for dibasic calcium phosphate, has acceptable reproducibility, and yields the same result as the official method.

REFERENCES

(1) Butler, A. Q., Maurina, F. A., and Morecombe, F. A., *J. Pharm. Sci.*, **54**, 134(1965).
(2) Banks, J., *Analyst*, **77**, 484(1952).
(3) Betz, J. D., and Noll, C. A., *J. Am. Water Works Assoc.*, **42**, 49(1950).
(4) Biedermann, W., and Schwarzenbach, G., *Chimia*, **2**, 1(1948).
(5) Botha, G. R., and Webb, M. M., *J. Inst. Water Engrs.*, **6**, 459(1952).
(6) Brooke, M., and Holbrook, M., *Chem. Analyst*, **41**, 80(1952).
(7) Brunisholz, G., Genton, M., and Plattner, E., *Helv. Chim. Acta*, **36**, 782(1953).
(8) Conners, J. J., *J. Am. Water Works Assoc.*, **42**, 33 (1950).
(9) Marcy, V. M., *Power*, **94**, 105(1950).
(10) Mustafin, I. S., and Kruchkova, E. S., *Zh. Anal. Khim.*, **15**, 20(1960).
(11) Sobel, A. E., and Hanok, A., *Proc. Soc. Exptl. Biol. Med.*, **77**, 737(1951).
(12) Banewicz, J. J., and Kermer, C. T., *Anal. Chem.*, **24**, 1186(1952).
(13) Cheng, K. L., Kurtz, T., and Bray, R. H., *ibid.*, **24**, 1640(1952).
(14) Cheng, K. L., and Bray, R. J., *Soil Sci.*, **72**, 449(1951).
(15) Budo, Y., and Takagi, H., *Japan Analyst*, **9**, 998 (1960).
(16) Graue, G., and Zahler, A., *Angew. Chem.*, **65**, 532 (1953).
(17) Schafer, H. N. S., *Anal. Chem.*, **35**, 53(1963).
(18) Kruchkova, E. S., and Mustafin, I. S., *Zabod. Lab.*, **27**, 668(1961).
(19) Cluley, H. L. J., *Analyst*, **79**, 561(1954).
(20) Nielsch, W., *Glas-Emsil-Keramo-Tech.*, **6**, 73(1955).
(21) "The National Formulary," 11th ed., J. B. Lippincott Co., Philadelphia, Pa., 1960, p. 69.
(22) Dean, R. B., and Dixon, W. J., *Anal. Chem.*, **23**, 636(1951).
(23) Peterson, S., *Ann. N. Y. Acad. Sci.*, **57**, 144(1953).

Technical Articles

Pharmaceuticals Stored in Plastic Containers

By HAROLD M. BEAL, ROBERT J. DICENZO, PAUL J. JANNKE, HENRY A. PALMER, JULES PINSKY*, MORRIS SALAME*, and TULLY J. SPEAKER

IN THE DEVELOPMENT of light weight highly mobile combat support hospitals and medical treatment facilities, the U. S. Army Medical Research and Development Command recognizes the need for the standardization of pharmaceutical containers in addition to the reduction of their weight, cube, and breakage. The following excerpt from the USAMRDC bulletin, "Development Requirements," describes the general characteristics for nonglass containers which are sought for drugs, biologicals, and reagents:

"Purpose: The purpose of this requirement is to develop a family of immediate containers for drugs, biologicals and reagents that will materially reduce the packaged bulk of such items. These containers are to be compatible with contents and be suitable for use in any field environment and under all tactical conditions. They will be of standard size and shape, light weight, small cube and chemical-gas-temperature-breakage resistant."

Quite logically, the question arose as to the stability of drugs packaged in containers other than glass. Since certain plastics possess, at least in part, the characteristics sought, a project

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Accepted for publication July 14, 1967.
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This investigation was supported by the Medical Research and Development Command, U.S. Army Surgeon General's Office, under contract DA-49-193-MD-2568, Washington, D.C.

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REFERENCES

(1) Butler, A. Q., Maurina, F. A., and Morecombe, F. A., *J. Pharm. Sci.*, **54**, 134(1965).
(2) Banks, J., *Analyst*, **77**, 484(1952).
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(21) "The National Formulary," 11th ed., J. B. Lippincott Co., Philadelphia, Pa., 1960, p. 69.
(22) Dean, R. B., and Dixon, W. J., *Anal. Chem.*, **23**, 636(1951).
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concerned with the stability of pharmaceuticals packaged in polyethylene containers was jointly conducted by the School of Pharmacy, University of Connecticut, and the Monsanto Co., under the auspices of the U. S. Army Medical Research and Development Command. Because a package is a two-phase system consisting of a container and its contents, and since both phases can react with their environment, either individually or jointly, it was deemed essential for the performance of the containers to be evaluated by specialists in the properties, manufacture, and testing of plastic bottles.

Extensive experience has shown that virtually all pharmaceuticals undergo deterioration of some order with the passage of time. Both the rate and the extent of deterioration vary widely among drugs, and the rate usually is influenced by environmental conditions. The basic question which then arises is, "Do these same drugs decompose more rapidly when packaged in plastic than they do when packaged in glass?" One of the primary features of the study, therefore, becomes the comparison of the stability of a given pharmaceutical which has been packaged in both types of containers. Attention was not directed to studying the basic stability *per se* of any one drug. Because of their inherent nature, however, data which have been accumulated can and will serve as a basis for recommending that certain drug preparations should not be stored at all, or at most for limited intervals at certain temperature-humidity conditions, irrespective of what the containers might be.

A second feature of the study is the evaluation of the performance of the plastic containers, both separately and in conjunction with their contents, leading ultimately to the drafting of chemical and physical specifications for the polymer resin (and additives) and for the plastic containers as well.

EXPERIMENTAL DESIGN

Pharmaceuticals Used

The pharmaceuticals upon which the study is based include 96 of those used in greatest quantity by the U.S. Army Medical Service in the treatment facilities of its 400 bed evacuation hospitals. Representing a typical variety of medicinal agents, the samples include 45 kinds of tablets, 6 of capsules, 17 of liquids, 19 of powders, and 9 of ointments. The drugs to be used were designated by the Army Medical Research and Development Command, and were purchased by the Defense Medical Supply Center from the usual suppliers of the respective drugs to the army. Taken from single manufacturing lots which were less than 6 months old, the drugs were shipped directly to the project laboratories at the University of Connecticut. The pharmaceuticals are identified by nonproprietary name in Table I.

Containers and Closures

The choice of polyethylene as test container was based on the recommendation of Mr. Jules Pinsky. It was pointed out that high density polyethylene (0.95–0.96) was being used extensively in the packaging of foods and household commodities, and that the polymer resin formulations could be selected from those which had been accepted by the Food and Drug Administration for the packaging of foods and related items. This was not intended to imply that FDA acceptance of polyethylene for the packaging of foods predetermined the acceptability of the plastic for packaging drugs. Such limited acceptance, however, did serve as a logical point of departure because foods and drugs have much in common with regard to their complexity, gross stability, *etc.*

The plastic containers used for packaging experimental samples initially were 125-ml. oblong, amber (meeting U.S.P. requirements for light resistance) bottles of 0.96 d. polyethylene supplied and recommended by the Monsanto Co. as being best suited for this purpose. Their weight was approximately 15 Gm., and the nominal wall thickness was 0.030 in. The high density polyethylene bottles were used preferentially because of their rigidity and lower permeability characteristics. They are, however, more subject to stress cracking than are the lower density, more pliable containers. Therefore, when cracking occurred or when excessive loss of volatiles was a problem, samples were repackaged in either 0.95 d. or epoxy-lined polyethylene containers, as conditions warranted. Identical drug samples intended to serve as controls were packaged in 4-oz., wide mouth, square amber jars manufactured by Armstrong Cork Co. of U.S.P. type III glass.

Both types of containers were fitted with white coated tinplate caps in which the liner was a 0.035-in. pulpboard faced with white pigmented Vinyliner. In addition, each bottle was fitted with another laminated cap liner which consisted of polyethylene-coated aluminum foil on a Kraft paper backing. This liner is identified as Flexkin 200F. Mil B-131, Acme Backing Co. In the case of the polyethylene bottles, this liner was heat sealed onto the bottles immediately after they were filled with drug samples, thereby producing a sealed and continuous enclosure for the contents. This was regarded as an effective means of limiting the loss of volatile components to the permeation process. Sealing with an adhesive was tested, but found to be less desirable. Heat seals applied with a thermostatically controlled sealing iron were found by positive pressure testing to be uniformly effective. The polyethylene laminate was not sealed onto the glass bottles. Screw caps were applied to all bottles with a torque of 15 in.-lb. Thus, the polyethylene bottles were heat-sealed, reinforced by a metal cap, while the glass bottles had a comparable pressure seal only. Thereupon, the loss of weight by the drug in polyethylene could be attributed to permeation, whereas loss by the drug in glass was a function of liner-cap leakage.

Storage Conditions

Reserves of all commodities are subjected to various storage conditions, the nature of which are dependent upon numerous factors. These conditions, supplemented by the conditions encountered

in transit, represent what might be designated as the "storage history" of the commodity. The storage history of commodities for the civilian market within the continental United States reflects only rarely conditions which are beyond control, if and when control is desired. Military materiel, on the other hand, can be subjected to indefinite transportation and storage conditions in domestic and foreign depots where regulated environmental conditions may be desired, but are wholly impossible. For many reasons, therefore, the environmental conditions to which drugs in both types of containers were exposed include and extend beyond those usually applied in conventional stability testing programs involving products for civilian use. It should be recognized, therefore, that these broad extremes tend to establish stability requirements which generally embrace and extend beyond the performance demanded for civilian market needs.

Automatically controlled walk-in type environmental chambers were specially constructed for the project by Harris Refrigeration Co., Cambridge, Mass. Each of the six units bore recording devices such that the constancy of the respective environments (temperature and humidity) could be noted and recorded. The environmental conditions employed throughout the storage intervals were as follows: 140°F., 14% relative humidity (ambient); 100°F., 27% relative humidity (ambient); 100°F., 80% relative humidity (controlled); 73°F., 50% relative humidity (controlled); 73°F., 80% relative humidity (controlled); 40°F., 80% relative humidity (ambient); −65°F., ambient relative humidity cycling between −65°F. and 140°F.

The condition described as 73°F., 50% relative humidity is that which prevailed throughout the air-conditioned laboratory in which the project was conducted. Samples of pharmaceuticals exposed to this environment were stored on open metal shelving, protected from direct sunlight, yet exposed to the prevailing complement of daylight and fluorescent light.

The environment called "cycling" was programmed so that each sample was subjected to a 6-day cycle consisting of 2 days at 140°F., then 2 days at −65°F., and finally another 2 days at 140° F., whereafter the samples were equilibrated to room temperature for evaluation. Samples were prepared for storage under all other environmental conditions described for intervals of 6, 12, 24, and 48 weeks. If gross container failure was noted at the end of any one interval, in any one environment, that drug was repackaged in 0.95 d. or in epoxy-lined polyethylene bottles, as conditions warranted; ideally, the lower density bottles to overcome stress cracking, and the epoxy-lined bottles to minimize loss of volatiles from the formulation.

If any sample showed physical or chemical decomposition slightly beyond the tolerable limits at the end of an exposure interval, other than 48 weeks, it was carried through the next exposure interval as a matter of assurance. Gross physical or chemical decomposition, on the other hand, was cause for immediate termination of further testing.

Preparation of Samples for Storage

At the initiation of this study, it was recognized that the rate of progress was substantially predetermined by the rate of assaying, which in turn was dependent on analytical methods, personnel, and equipment. Programming of the samples throughout the test period was therefore developed with these controlling factors in mind.

Six samples of each pharmaceutical product, 3 in polyethylene and 3 in glass to serve as controls, were packaged for storage in each of the environments for the intervals stated. (This amounted to 16,704 individual sample bottles, not counting repacks necessitated by container failure. For the latter reason, an additional 528 sample bottles were packaged.) Samples approximating 100 Gm. were weighed to the nearest milligram into tared test bottles, such that changes in net weight during exposure could be recorded. This information was of interest to the subcontractor, whose responsibility it was to evaluate the significance of the loss of volatiles by permeation. Simultaneous with the transfer of pharmaceuticals from their bulk containers to test bottles, moisture content and alcohol content of each sample were determined where applicable.

Stability Evaluation

Testing the stability of pharmaceuticals is designed to determine quantitatively and/or qualitatively the changes which the products undergo during storage. The changes can involve chemical composition and physical characteristics, both of which are usually well defined and are of a proper order in a newly made drug product of high quality. Changes in these features are signs of deterioration or instability of the drug, and measurement or evaluation of them from time to time provides an insight into the stability of the product.

Chemical Testing—Assay procedures were drawn from three sources. When a procedure given in the "United States Pharmacopeia" or in the "National Formulary" was both stability-indicating and applicable on a practical basis to large numbers of samples, the official procedure was used. Some official methods were modified slightly for the simple purpose of improvement or to facilitate multiple runs. Forty official methods were employed; 26 methods were furnished (on a confidential basis) by the manufacturers of the respective drug products; 32 additional stability-indicating procedures were developed in the project laboratories. The remainder were modified official procedures.

Physical Testing—Quite aside from their aesthetic features, the physical characteristics of drug products serve a very important function in therapeutic efficacy. Changes in these characteristics can alter the intended performance in many instances. Furthermore, any drug which has undergone change in physical characteristics is regarded with suspicion as to its wholesomeness, security, and efficacy by both the dispenser and the consumer.

Evaluation of the physical stability of the pharmaceuticals was carried out by the usual methods. Uncoated tablets were subjected to friability, hardness, and disintegration or dissolution tests. Friability was determined with a Roche Friabilator. Tablet hardness was measured with a modified Strong-Cobb hardness tester. Disintegration testing involved the use of the U.S.P. basket-rack assembly. Dissolution tests were carried out on those tablets intended for extemporaneous preparation of solutions. Moisture content was determined

by the Karl Fischer method, where applicable. In a small number of instances, moisture determination by desiccation was necessary. Coated tablets, plain or enteric, were examined for cracked, crazed, or dulled coatings, and were also tested for disintegration, using the appropriate U.S.P. method. Gelatin capsules were examined for cracks, leaks, embrittlement, and for changes in the moisture level of the contents. Liquid pharmaceuticals were examined for crystal growth, precipitation, particulate matter, color change, and solvent loss. Suspension stability was evaluated by degree and ease of resuspension. The consistency of ointments was determined by means of a Haake Roto-Visco viscosimeter and by observation of the extent of separation. Powders were examined for evidence of color change and caking. Finally, all samples were subjected to the usual organoleptic tests, where applicable.

Container Testing—The performance of polyethylene bottles used in the study was evaluated by two criteria—namely, visible and invisible. As samples were removed from the environmental chambers, it was unavoidable that physical changes in some of the polyethylene bottles should be noted. Indeed, in a small number of instances, rupturing of a bottle and loss of contents was evidenced by leakage residues in the chambers even before those samples were scheduled for removal.

The visible types of change included rupturing of the bottle due to one factor or another (see under *Container Failure*), and the swelling or collapse of a bottle. The former is a very positive change and cannot possibly be misjudged. The latter (swelling or collapse), however, occurred to different extents ranging from the obvious to the debatable. For this reason, swelling or collapse of polyethylene bottles is seldom mentioned in the summary report in Table I.

The search for invisible changes in the polyethylene bottles was conducted by the Packaging Division, Monsanto Co., Bloomfield, Conn. At scheduled intervals, plastic bottles from each of the test sets were emptied of their contents and forwarded to the subcontractor, who analyzed them for changes in impact strength, elongation, melt index, tensile strength, evidence of carbonyl oxidation, environmental stress cracking, and/or tensile rupture. In addition, the Monsanto Co. conducted infrared analyses for product absorption by the test bottles and they evaluated the permeability characteristics of the bottles, based on weight changes due to loss of volatiles or gain of moisture during the storage periods. The IBM runs on these data, performed at Monsanto's Data Center in St. Louis, are on file at the project laboratories.

Definitions of Failure

Product Failure—Product stability must be judged on the basis of chemical and physical characteristics and the changes which they undergo. Before a given product can be declared stable or unstable, it is necessary to establish working definitions of these terms, because they are relative rather than absolute. As originally conceived, the purpose of this study was to compare the stability of a drug packaged in plastic with the stability of that drug packaged in glass. A criterion for chemical failure was adopted wherein it was regarded as a loss of more than 10% of the active ingredients initially present; that is to say, a drug was regarded as stable if, after a given storage interval, it retained at least 90% of its zero-time assay value.

The criteria for judging physical test data cannot be defined so neatly as that used in determining chemical failure. This is due to the subjective nature of the physical tests. In these cases, sound judgment based on experience with good practice and an appreciation for consumer acceptance became the sole criteria. Of the more objective physical tests, the only one with official limits is that for disintegration time; if the limit was exceeded, the tablet in question was considered to have failed. Wherever applicable, military specifications for disintegration time limits were applied to tablets having no official status.

Container Failure—A polyethylene container was considered to have failed when it did not protect its contents to the same extent as did glass, except for the 10% tolerance limit adopted. In addition, failure took place when the container ruptured due to a combination of temperature and contents acting as stress cracking agents and/or generating high internal pressure that exceeded the long-term tensile strength of the bottle. Serious loss of volatiles by permeation was also regarded as a form of container failure, as were exudation of lipoidal substances through the bottle wall and excessive absorption of the product by the polyethylene.

SUMMARY OF EXPERIMENTAL RESULTS

The extensive data accumulated during the study are summarized in Table I. Even in abstract form, the tabulation is of necessity lengthy; consequently, repetition has been eliminated as much as possible, without risking misinterpretation, and abbreviation has been utilized extensively. The full meaning of abbreviations and symbols and the identification of specific parameters are given herewith, such that the tabulation conveys the full description of the circumstances which attended the stored drug samples and their containers.

Storage Conditions—Temperatures designated in the identification of all storage conditions are given in degrees Fahrenheit. Relative humidity (R.H.) is associated only with those environments in which the humidity level was under positive control. In all other instances, humidity was ambient. The duration of storage is indicated by an expression such as ". . . /24 wk. at 140°," which means that after being stored for 24 weeks at 140°F., 14% R.H., the product in question was found to have undergone some kind of failure or change. Likewise, the expression ". . . at 140°/6 wk." means that the product was stored at 140°F., 14% R.H., for 6 weeks.

Chemical Stability—Chemical stability is a variform expression of chemical failure. The latter has already been described under *Product Failure* as a loss of more than 10% of the initial concentration of active ingredient. Within the project, it was necessary to differentiate between comparative changes in percentage concentration and the types of container in which the changes occurred. The degrees of failure and the related containers are identified as follows.

Type (C-1): no loss in glass, loss in polyethylene

TABLE I—PRODUCT STABILITY AND CONTAINER PERFORMANCE

Product[f]	Chemical Stability	Physical Stability	Container Performance
1, Sulfadiazine tablets U.S.P., 0.5 Gm.	Passed under all storage conditions/48 wk.	(P-1)/24 wk. at 140° and (P-3)/48 wk. at 140°; both exceed U.S.P. disintegration time	0.96; passed under all storage conditions/48 wk.; permeability negligible; slight absorption of product at 140°/48 wk.; no effect on container function
2, Acetylsalicylic acid tablets U.S.P., 0.325 Gm.	Passed 12 wk. at 140°; see physical failure; passed all other storage conditions/48 wk.	(P-3)/12 wk. at 140°; (P-4)/6 wk. at 140° in glass; salicylic acid crystals on tablets; samples exceed U.S.P. disintegration time	0.96; bottle tensile rupture/12 wk. at 140°; passed all other storage conditions up to and including 100°/48 wk.; permeability negligible
3, Acetylsalicylic acid, phenacetin, and caffeine tablets N.F.	Failed (C-3) within 6 wk. at 140°; passed all other storage conditions/48 wk.	(P-3)/6 wk. at 140°; salicylic acid crystals on tablets	0.96; passed under all storage conditions/48 wk.; permeability negligible
4, Sodium chloride tablets U.S.P., 0.65 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
5, Deleted from study			
6, Deleted from study			
7, Meprobamate tablets U.S.P., 0.4 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
8, Sulfisoxazole tablets U.S.P., 0.5 Gm.	Passed/48 wk. at 140°; see physical failure; passed under all other storage conditions/48 wk.	(P-3)/12 wk. at 140°; samples exceed U.S.P. disintegration time	0.96; passed under all storage conditions/48 wk.; permeability negligible
9, Calcium lactate tablets N.F., 0.65 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; moisture permeability 4.6% gain/48 wk. at 100°; glass control showed 2.5% gain; product permeability negligible
10, Ferrous sulfate tablets U.S.P., 0.325 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
11, Penicillin G tablets U.S.P., 250,000 units	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
12, Deleted from study			
13, Dried aluminum hydroxide tablets U.S.P., 0.325 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; moisture permeability 6.6% loss/48 wk. at 100°; product permeability negligible
14, Sodium chloride for solution tablets, 2.25 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
15A, Erythromycin tablets U.S.P., 0.25 Gm.	Passed under all conditions between 40° and 100°/48 wk., and under cycling; failed (C-3) within 6 wk. at 140°; passed/6 wk. at −65°; see physical failure	(P-3)/6 wk. at 140°; (P-3)/6 wk. at −65°; (P-3)/cycling; in all instances, coating dull, with extensive surface cracking; this failure terminated assaying of −65° samples after 6 wk.	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] product permeability negligible; moisture permeability 1.1% gain/48 wk. at 100°; glass control also showed 1.1% gain
15B, Erythromycin tablets U.S.P., 0.25 Gm.	Passed under all conditions between −65° and 100°/24 wk.; 140° and cycling samples not assayed; see physical failure	(P-3)/6 wk. at 140°; (P-3)/cycling; pitted, peeling, and dulled coatings responsible for no assays on 140° and cycling samples	0.96; passed under all storage conditions/24 wk.;[b] permeability negligible
16, Chloroquine phosphate tablets U.S.P., 0.5 Gm.	Passed/24 wk. at 140°; passed under all other storage conditions/48 wk.	(P-3)/12 wk. at 140°; (P-3)/12 wk. at −65°; cracked coatings	0.96; passed under all storage conditions/48 wk.; permeability negligible
17, Sodium salicylate tablets U.S.P., 0.325 Gm.	Passed under all storage conditions up to and including 100°; physical failure precluded assay of 140° samples	(P-3)/6 wk. at 140°; extensive cracking; (P-3)/48 wk. at −65° and (P-3)/cycling due to crazing and cracking	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] permeability negligible
18, Chlorpheniramine maleate tablets U.S.P., 4 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; slight absorption of product at 140°/48 wk.; none at 100°; no effect on container function
19, Chlorpheniramine, phenylephrine, aspirin, phenacetin, and caffeine tablets	Failed (C-3)/6 wk. at 140°; passed under all other storage conditions/48 wk.	(P-3)/6 wk. at 140° and (P-3)/cycling; discoloration and excessive disintegration time	0.96; passed under all storage conditions/48 wk.; slight absorption of product at 140°/48 wk.; none at 100°; no effect on container function
20, Tripelennamine hydrochloride tablets U.S.P., 50 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
21, Methocarbamol tablets, 0.5 Gm.	Passed under all storage conditions/48 wk.	(P-3)/6 wk. at 140° and (P-3)/cycling; marked discoloration and strong amine odor	0.96; passed under all storage conditions/48 wk.; permeability negligible
22, Chlorothiazide tablets N.F., 0.5 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
23, Sodium bicarbonate, charcoal, and peppermint tablets	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; bottles lost heat seal at 140°/6 wk., due to internal pressure; otherwise passed all storage conditions/48 wk.; permeability negligible

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
24, Sodium *para* - aminobenzoate, sodium salicylate, and ascorbic acid tablets	Passed/12 wk. at 140° and at −65°; storage terminated —see physical failure; passed all other storage conditions/48 wk.	(P-3)/12 wk. at 140° and (P-3)/12 wk. at −65°; cracked tablet coatings	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] permeability negligible
25, Acetylsalicylic acid – ethoheptazine tablets	Failed (C-3)/6 wk. at 140°; passed under all other storage conditions/48 wk.	(P-3)/6 wk. at 140° due to crystal growth and discoloration; (P-3)/24 wk. at 100°, 27% R.H., at 100°, 80% R.H., −65° and at cycling; tablets split into two layers during friability test	0.96; bottle tensile rupture after 12 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible
26, Isoniazid tablets U.S.P., 100 mg.	Passed under all storage conditions/48 wk.	(P-3)/48 wk. at 140°; excessive darkening in color	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 1.3% gain/48 wk. at 73°; glass same; slight product absorption/48 wk. at 140°; less at 100°; no effect on container function
27, Alkaline aromatic soln. tablets	Passed under all storage conditions/48 wk.	(P-1)/12 wk. at 140°; loss of aromatics	0.96; passed under all storage conditions/48 wk.; permeability negligible
28, Propantheline bromide tablets U.S.P., 15 mg.	Passed under all storage conditions/48 wk.	(P-3)/24 wk. at 140°; crystal growth on tablet surface	0.96; passed under all storage conditions/48 wk.; permeability negligible
29, Benzethonium chloride tablets, 0.25 Gm.	Failed (C-3)/24 wk. at 140°; passed under all other storage conditions/48 wk.	(P-3)/12 wk. at 140°; tablets discolored and produced discolored solutions	0.96; passed under all storage conditions/48 wk.; permeability negligible; slight container collapse/24 wk. at 140°; slight absorption of product/48 wk. at 140°; none at 100°; no effect on container function
30, Urine sugar test tablets	Failed (C-3)/12 wk. at 140°; passed under all other storage conditions/48 wk.	(P-3)/24 wk. at 140°; tablets discolored	0.96; passed under all storage conditions/48 wk.; permeability negligible; extensive damage to container *after* storage beyond 48 wk. test period; due to tablet reaction with moisture when bottles were opened for sampling; containers burst from heat and pressure
31, Mephenesin tablets N.F., 0.5 Gm.	Passed under all storage conditions/48 wk.	(P-3)/48 wk. at 140°; crystal growth on tablet surface	0.96; bottle rupture due to stress cracking at 140°/12 wk.; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible
32, Phenobarbital tablets U.S.P., 30 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
33, Deleted from study			
34, Potassium chloride tablets U.S.P., 0.325 Gm.	Passed under all storage conditions up to and including 100°/48 wk.; 140° samples passed/6 wk.; see physical failure	(P-3)/6 wk. at 140°; (P-3)/6 wk. at −65° and/cycling; coatings cracked and/or crazed	0.96; passed under all storage conditions/48 wk.; permeability negligible
35, Aluminum acetate soln. tablets	Failed (C-3)/6 wk. at 140° and/cycling; failed (C-3)/12 wk. under all other storage conditions	(P-3)/6 wk. at 140° and/48 wk. at 100°, R.H. 27%, and 100°, R.H. 80%; tablets crumble to powder	0.96; passed under all storage conditions/48 wk.; permeability of moisture 8.6% gain/48 wk. at 100°; glass control gained 5.5%; permeability product 3.5% loss; glass control lost 6.0%; moderate product absorption/48 wk. at 100°; no effect on container function
36, Ascorbic acid tablets	Failed (C-3)/24 wk. at 140°; passed under all other storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.95; passed under all storage conditions/48 wk.; permeability negligible; slight product absorption at 140°, but less at 100°; no effect on container function
37, Sodium cyclamate and sodium saccharin tablets N.F.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
38, Chloroquine and primaquine phosphate tablets	Passed under all storage conditions/48 wk.	(P-3)/6 wk. at 140°; marked discoloration and foul odor	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] product permeability negligible; moisture permeability 1.1% gain/48 wk. at 100°; glass control same
39, Reserpine tablets U.S.P., 0.25 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
40, Methenamine mandelate tablets U.S.P., 0.5 Gm.	Passed under all storage conditions/48 wk.	(P-3)/6 wk. at 140°; tablets dull and cracked	0.96; passed under all storage conditions/48 wk.; permeability negligible
41, Deleted from study			

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
42, Griseofulvin tablets U.S.P., 0.25 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
43, Belladonna alkaloids with phenobarbital tablets	Failed (C-5)/24 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.	(P-6)/24 wk. at 140°; samples darken in color	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 2.1% loss/48 wk. at 100°; glass control 1.7% loss
44, Prednisone tablets U.S.P., 5 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 1.8% gain/48 wk. at 100°; glass control 1.1% gain
45, Ammonium chloride tablets U.S.P., 0.5 Gm.	Passed under all storage conditions up to and including 100°/48 wk.; 140° samples passed/24 wk.; see physical failure	(P-3)/6 wk. at 140°; (P-3)/6 wk. at −65° and/cycling; tablet coatings cracked and/or crazed	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] permeability negligible
46, Glutethimide tablets N.F., 0.5 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; slight product absorption at 140°/48 wk.; less at 100°; no effect on container function
47, Probenecid tablets U.S.P., 0.5 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
48, Sodium aminosalicylate tablets U.S.P., 0.5 Gm.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 2.3% gain/48 wk. at 100°; glass control 2.0% gain
49, Thiamine hydrochloride tablets U.S.P., 50 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; some product absorption/48 wk. at 140°; none at 100°; no effect on container function
50, Tetracycline hydrochloride tablets N.F., 0.25 Gm.	Passed under all storage conditions/48 wk.	(P-3)/48 wk. at 140°; excessive discoloration	0.96; passed under all storage conditions/48 wk.; permeability negligible; containers may show some collapse/48 wk. at 140°
51, Propoxyphene hydrochloride capsules U.S.P., 32 mg.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
52, Chloramphenicol capsules U.S.P., 0.25 Gm.	Passed under all storage conditions up to and including 100°/48 wk. and for 6 wk. at 140°	(P-3)/12 wk. at 140°; excessive discoloration	0.96; passed under all storage conditions/48 wk.; permeability negligible
53, Sodium diphenylhydantoin capsules U.S.P., 100 mg.	Passed under all storage conditions up to and including 100°/48 wk. and for 6 wk. at 140°; see physical failure	(P-3)/12 wk. at 140°; excessive discoloration	0.96; passed under all storage conditions/48 wk.; permeability negligible
54, Diphenhydramine hydrochloride capsules U.S.P., 50 mg.	Passed under all storage conditions up to and including 100°/48 wk. and for 12 wk. at 140°; see physical failure	(P-4)/24 wk. at 140°; product discolored; capsules embrittled in plastic bottles, but softened and stuck together in glass bottles	0.96; passed under all storage conditions/48 wk.; permeability negligible; increase in melt index/48 wk. at 140° about 10%; no change/48 wk. at 100° or 24 wk. at 140°; may be due to slight product absorption; no effect on container function
55, Oleovitamin A capsules, 50,000 units	Passed under all storage conditions/48 wk. except for 140° and cycling; see physical failure	(P-3)/6 wk. at 140° and/cycling; capsules fused into nearly solid mass	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] permeability negligible
56, Dextroamphetamine sulfate and amobarbital capsules, 15–97 mg.	Passed under all storage conditions up to and including 100°/48 wk.; see physical failure	(P-3)/6 wk. at 140°; discoloration and fusion of pellets within capsules	0.96; passed under all storage conditions up to and including 100°/48 wk.;[a] permeability negligible; elongation/48 wk. at 100° 170% higher than control; normal/24 wk. 100°; may be due to slight absorption of product; no effect on container function
57, Surgical soap with 5% hexachlorophene	Passed under all storage conditions between −65° and 100°/48 wk., and/6 wk. at 140°; see container failure	Passed under all storage conditions/48 wk.	0.96; bottle rupture due to stress cracking at 140°/24 wk. and under cycling; passed under all other storage conditions up to and including 100°/48 wk. may lose heat seals/6 wk. at 140°
58, Terpin hydrate elixir N.F.	Passed under all storage conditions up to and including 100°/12 wk.; see container failure	(P-3)/6 wk. at 140°; change in odor	0.96; bottle tensile rupture/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; product permeability 1.5% loss/48 wk. at 100°; moisture permeability negligible; 150% increase

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
			in resin elongation/48 wk. at 100°; normal values/24 wk. at 100° and/48 wk. at 73°; increase may be due to product absorption; no effect on container function
59, Phenylephrine hydrochloride soln. U.S.P., 1%	Passed under all storage conditions up to and including 100°/48 wk. and at 140°/12 wk.; see physical failure	(P-1)/6 wk. at 140°; (P-3)/12 wk. at 140°; (P-3)/24 wk. at 100°; discoloration	0.96; bottle tensile rupture/24 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible
60, Thimerosal tincture N.F.	Passed under all storage conditions from −65° up to and including 100°/48 wk.; see container failure	Passed under all storage conditions from −65° up to and including 100°/48 wk.	0.96; bottle tensile rupture/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 2.7% loss/48 wk. at 100°; glass control 2.5% loss; 0.95 epoxy-lined bottles passed/6 wk. at 140°
61, Piperazine citrate syrup, U.S.P.	Passed under all storage conditions up to and including 100°/48 wk. and/6 wk. at 140°; physical failure/12 wk. at 140° precluded assaying of 12-wk. samples	(P-3)/12 wk. at 140°; (P-3)/48 wk. at 100°, R.H. 27% and at 100°, R.H. 80%; darkening in color	0.96; bottle rupture due to stress cracking at 140°/6 wk.; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible; some product absorption/48 wk. at 100°; none/24 wk. at 100° or/48 wk. at 73°; no effect on container function
62, Paregoric U.S.P.	Passed under all storage conditions up to and including 73°/48 wk.; see physical failure	(P-3)/6 wk. at 140°, at 100°, R.H. 27%, at 100°, R.H. 80% and at cycling; loss of aromatics	0.95; bottle tensile rupture/12 wk. at 140°; passed under all other storage conditions up to and including 73°/48 wk.;[e] permeability negligible; 0.95 epoxy-lined bottles passed/6 wk. at 140°
63, Alcohol U.S.P.	Passed under all storage conditions up to and including 100°/48 wk.; see container failure	Passed under all storage conditions up to and including 100°/48 wk.	0.95; bottle tensile rupture/12 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeabibity 2.2% loss at 100°/48 wk.; bottles may lose heat seals/6 wk. at 140°
64, Phenolated and mentholated calamine lotion	Product not assayed	(P-1)/6 wk. at 140°, at 100°, R.H. 27%, at 100° R.H. 80%, and at cycling; loss of menthol and phenol; (P-3)/6 wk. at 40° and at −65°; excessive separation and lowered viscosity	0.96; bottle stress crack/24 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 1.3% loss at 100°/48 wk.
65, Benzalkonium chloride soln. U.S.P., 10%	Passed under all storage conditions up to and including 100°/48 wk.; container failure precluded assay of 140° samples	Passed under all storage conditions/48 wk.	0.96; bottle stress crack/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible; 0.95 epoxy-lined bottles passed/6 wk. at 140°
66, Thimerosal soln. N.F.	Passed under all storage conditions/24 wk.; 48-wk. samples not available for assay	Passed under all storage conditions/24 wk.[d]	0.96; bottle stress crack/24 wk. at 140°; passed under all other storage conditions up to and including 100°/24 wk.; permeability negligible
67, Glacial acetic acid	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; bottle tensile rupture/12 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 7.7% loss/48 wk. at 100°; glass control 2.3% loss; some product absorption at 140°, less at 100°; no effect on container function
68, Glycerin U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
69, Compd. benzoin tincture U.S.P.	Passed (loss of volatiles) under all storage conditions up to and including 100°/48 wk.; 140° samples lost by container failure	(P-2)/6 wk. at 140°; (P-3)/12 wk. at 140°; excessive loss of volatiles	0.96; bottle tensile rupture/12 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 1.7% loss/48 wk. at 100°

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
70, Oxytetracycline oral suspension	Failed (C-3)/6 wk. at 140° and/48 wk. at 100°, passed under all other storage conditions up to and including 73°/48 wk.	(P-3)/6 wk. at 140° and/12 wk. at 100°; discoloration	0.96; passed under all storage conditions/48 wk.; permeability negligible
71, Glycyrrhiza fluid extract U.S.P.	Passed under all storage conditions up to and including 100°/48 wk.; see container failure	Passed under all storage conditions up to and including 100°/48 wk.	0.96; bottle tensile rupture/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 2% loss/48 wk. at 100°; 0.95 epoxy-lined bottles passed/6 wk. at 140°
72, Belladonna tincture U.S.P.	Passed under all storage conditions up to and including 100°/48 wk.; see container failure	Passed under all storage conditions up to and including 100°/48 wk.; see container failure	0.95; bottle stress crack/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; moisture permeability negligible; product permeability 2.5% loss/48 wk. at 100°; 0.95 epoxy-lined bottles passed/6 wk. at 140°
73, Trisulfapyrimidines oral suspension U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; bottle stress crack/12 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; permeability negligible
74, Deleted from study			
75, Sodium bicarbonate U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
76, Ammonium chloride U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
77, Talc U.S.P.	Sample not assayed	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; moderate product absorption at 140°; no effect on container function
78, Zinc oxide U.S.P.	Passed under all storage conditions/48 wk.; only 48-wk. samples assayed	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
79, Boric acid U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
80, Bismuth subcarbonate U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; 83% increase in elongation/48 wk. at 140° which is 22% greater than normal variation; 48-wk. bottles at 100° closer to normal value; may be due to product absorption and plasticization of polyethylene; no effect on container function
81, Pumice U.S.P.	Samples not assayed	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
82, Potassium phenoxymethyl penicillin for oral soln., 3,200,000 units	Passed under all storage conditions/48 wk.	(P-3)/6 wk. at 140° and during cycling; darkening in color and development of foreign odor	0.96; passed under all storage conditions/48 wk.; permeability negligible
83, Acetylsalicylic acid U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.95; passed under all storage conditions/48 wk.; permeability negligible
84, Sodium citrate U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
85, Potassium iodide U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; melt index increase 17% at 140°/48 wk., but normal at 140°/24 wk. and at 100°/48 wk.; slight absorption of product at 140°, but none at 100°; no effect on container function
86, Sodium borate U.S.P.	Failed (C-3)/24 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
87, Terpin hydrate N.F.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
88, Citric acid U.S.P.	Passed under all storage conditions/48 wk.	(P-1)/48 wk. at 140°; loss of water; (P-3)/48 wk. at 100°; samples caked	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 1.0% loss/48 wk. at 100°
89, Monohydrated sodium carbonate U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
90, Calamine U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; increase in elongation 83% which is 22% higher than normal expected at 140°; may be due to product absorption; no effect on container function
91, Magnesium carbonate N.F.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; product permeability negligible; moisture permeability 3.3% loss/48 wk. at 100°; glass control 6.2% loss
92, Magnesium oxide U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
93, Precipitated sulfur U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible
94, Green soap N.F.	Passed under all storage conditions/48 wk. on basis of pH value	(P-2)/12 wk. at 140° and at 100°, R.H. 27% and at 100°, R.H. 80%; discoloration; (P-2)/24 wk. under all other storage conditions; discoloration progressively increasing with time, ultimately dark brown	0.96; bottle stress crack/48 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.; increase in melt index 10%/48 wk. at 140°; normal value for bottles at 100°/48 wk. and at 140°/24 wk.; may be due to product absorption; no effect on container function
95, Ointment base	Product not assayed	(P-3)/12 wk. at 140° and/12 wk. at −65°; excessive separation of phases	0.96; passed under all storage conditions up to and including 100°/48 wk.; also for at least 24 wk. at 140°; borderline acceptability/48 wk. at 140°; slight to moderate increase in tensile strength and melt index/48 wk. at 140°; permeability negligible; some product absorption
96, Hydrophilic ointment U.S.P.	Product not assayed	(P-3)/6 wk. at 140°, /cycling and/12 wk. at −65°; excessive separation of phases	0.96; passed under all storage conditions up to and including 100°/48 wk.;[e] permeability negligible; increase in melt index 35% at 100°, but normal value for 73°/48 wk.; container collapse may occur in the 73° to 140° range/48 wk.; slight product absorption; no effect on container function
97, Zinc oxide ointment U.S.P.	Passed under all storage conditions/48 wk.	(P-3)/6 wk. at 140° and/cycling; excessive separation	0.96; passed under all storage conditions up to and including 100°/48 wk.;[e] permeability negligible; increase in melt index 20% at 100°; normal value/48 wk. at 73°; may be due to product absorption; no effect on container function
98, Nitrofurazone ointment N.F.	Failed (C-3)/6 wk. at 140°; passed under all other storage conditions up to and including 100°/48 wk.	(P-3)/12 wk. at 140°; darkening of color	0.96; passed under all storage conditions up to and including 100°/48 wk.;[e] permeability negligible
99, Modified tar compd. ointment	Passed under all storage conditions up to and including 73°/48 wk.; passed/6 wk. at 100°, R.H. 27% and at 100°, R.H. 80%; see physical failure	(P-3)/6 wk. at 140°; (P-3)/12 wk. at 100°, R.H. 27% and 100°, R.H. 80%; (P-3)/24 wk. at −65°; separation of phases	0.96; passed under all storage conditions up to and including 73°/24 wk.; after 48 wk. at 73° product absorption is too high; tar odor outside of bottles/6 wk. at 140° and/12 wk. at 100°; 0.95 epoxy-lined bottles passed/6 wk. at 140°; no tar odor on outside of bottles
100, Anhydrous lanolin U.S.P.	Passed under all storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; bottle stress crack/12 wk. at 140° and/48 wk. at 100°; passed under all other storage conditions up to and including 73°/48 wk.; melt index increases 90%/48 wk. at 140°; normal value/6 wk. at 140° and/48 wk. at 73°; may be due to product absorption; permeability negligible; 0.96, 0.95, and 0.95 epoxy-lined bottles showed seepage or leakage of product/6 wk. at 140°
101, Ammoniated mercury ointment U.S.P.	Passed under all storage conditions/48 wk.	(P-3)/12 wk. at 140°; excessive separation	0.96; passed under all storage conditions up to and including 100°/48 wk.;[e] in-

(*Continued on next page.*)

TABLE I—(*Continued.*)

Product[f]	Chemical Stability	Physical Stability	Container Performance
			crease in melt index 18% at 100°; normal value at 73°; slight product absorption at 100°, none at 73°; no effect on container function
102, Zinc oxide paste U.S.P.	Failed (C-3)/48 wk. at 140°; passed under all other storage conditions/48 wk.	Passed under all storage conditions/48 wk.	0.96; passed under all storage conditions/48 wk.; permeability negligible; increase in tensile strength 16% at 140°; value normal at 100°/48 wk.; increase in melt index 300% at 140° but only 10%/48 wk. at 100°; may be due to product absorption; no effect on container function

[a] Only 6 wk./140° bottles available; all passed. [b] Assay procedure difficulties delayed initiation of storage such that 48-wk. data are not available. [c] Only 12-wk./100° bottles tested; all passed. [d] Time elapsed due to failure of assay procedure prevented storage of samples for 48 wk. [e] Only 12-wk./140° bottles available; all passed. [f] Names and compendia designations for drugs are as of U.S.P. XVI and N.F. XI since the study commenced while those editions were official.

greater than 10%; type (C-2): loss in glass less than 10%, loss in polyethylene at least 15% greater; type (C-3): parallel or nearly parallel loss in glass and in polyethylene, both being in excess of 10%; type (C-4): loss in polyethylene less than 10%, loss in glass at least 15% greater; type (C-5): no loss in polyethylene, loss in glass greater than 10%; type (C-6): uncertain interpretation of data because of magnitude of experimental error.

Parenthetically, it is interesting to note that only type (C-3) and type (C-5) were encountered; the other possibilities are listed merely to emphasize their absence in the study.

The expression employed throughout to describe chemical stability—namely, "passed under all storage conditions/48 wk.," means that the samples from all environments assayed at the end of 6, 12, 24, and 48 weeks had not lost more than 10% of their initial content of active constituent. Environments and intervals for which this did not hold true are characterized by the expression "failed (C-x)/6 wk. at 140°," and variations thereon. This means that the concentration of active constituent did drop by more than 10% during the 6-week interval when stored at 140°F.

In instances when a sample underwent gross physical failure, it was not practical to assay it; attention is therefore called to the physical failure. In other instances, samples were lost because of container rupture and were not available for assay. Attention is then directed to container failure described in the same tabulation.

Physical Stability—It is noteworthy that the word "failed" is deliberately omitted from all descriptions of physical behavior of drug samples during storage. In some instances, as when coated tablets literally exploded, there is no doubt about the physical failure. In many instances, however, there was a physical change whose significance, as regards the usability of a drug which tested out well chemically, is open to debate. None of those products was declared to have failed outright; their departure from the normal condition is noted, and the reader is urged to impose his own evaluation of the significance of the change.

Physical changes occurred in different degrees and in both kinds of containers. The relationships are identified as follows.

Type (P-1): little or no change in glass, major change in polyethylene; type (P-2): some change in glass, major change in polyethylene; type (P-3): major change in both glass and polyethylene; type (P-4): some change in polyethylene, major change in glass; type (P-5): little or no change in polyethylene, major change in glass; type (P-6): uncertain applicability of other relations as in types (P-1), (P-2), (P-3), (P-4), and (P-5).

Whenever a physical change occurred (failure?), it is so indicated; for example, "(P-3)/6 wk. at 140°," which means that the product in question underwent a parallel change in glass and in polyethylene during 6 weeks of storage at 140°F. Similar expressions are to be interpreted in like manner. If specific reference is made to a physical change at a given environment, it is to be understood that the product maintained its physical integrity at all other environments which have not been so mentioned. The expression "passed under all storage conditions/48 wk." means that the product, taken from all environments, was found to be physically acceptable or intact at the end of 6, 12, 24, and 48 weeks. Variations of this are indicated where they apply. Insofar as is possible, the nature of the physical change which occurred is identified.

Container Performance—Each comment on container performance opens with the identification, *e.g.*, 0.96, of the kind of polyethylene bottle to which the subsequent comments apply. Outright and visible failure of a bottle is positively stated in each instance where it occurred. A common expression used in describing container performance is "passed under all storage conditions/48 wk." This is intended to mean that the polyethylene bottles so described were tested at the end of 48 weeks of exposure and were found to pass all of the evaluating tests described here. Any exceptions to this generalization are specifically noted. In all instances, the container testing program involved comparisons between the test bottles and unused polyethylene controls, and the results are expressed as changes from the normal control values. The specific tests which were applied include the following.

Impact Resistance—The purpose of this test is to determine whether the drug or the environment exercised any deleterious effect on the impact or breakage resistance of the bottle. Test bottles rinsed free of adhering drug were filled (120 ml.) with 50% ethanol and conditioned for 24 hr. at

−10°F., whereupon they were capped and dropped (while cold) from a height of 17 ft. Normal virgin containers do not break on impact.

Average Tensile Strength (ASTM D412-64T)—This is the standard test for the physical strength of plastics. A strip of plastic cut from the wall of a sample bottle is stretched in an Instron tester at the relative rate of 20 in. per minute. The maximum tensile force applied during stretching to the point of rupture is the tensile strength of the specimen.

Average Ultimate Elongation (ASTM 412-64T)—Conducted in conjunction with the tensile strength measurement, this value describes the maximum elongation of the specimen before rupture occurs. It is the ratio of the length of the sample at rupture to its initial length, expressed as per cent.

Melt Index (ASTM 1238-65T)—This is the standard test for measuring flow rate (decigrams/minute) of a molten plastic. It is inversely proportional to the molecular weight, provided other variables are not involved. If the plastic has absorbed any chemical which can act as a plasticizer, the flow rate, or the melt index, will not correlate with the molecular weight.

Infrared Analysis—Comparison of the I.R. absorption spectra for a sample taken from a test bottle and for the unused plastic discloses whether the polyethylene has absorbed drug components or has degraded. A Perkin-Elmer model 21 infrared spectrophotometer was used to scan the samples between 2 and 15 μ. Product absorption in the range of 1% is readily detectable. These spectrograms are on file at the University of Connecticut.

Moisture Permeability—Standard procedure for the plastics industry measures moisture permeability at 100°F., and standard acceptance limits it to 3% per year.

RECOMMENDATIONS

Based on measurements and observations made during the study, it is possible to offer recommendations concerning the use of high density polyethylene bottles for the packaging of drugs, and concerning limitations on the over-all storage of drugs. While the initial intent of the project was to draw a comparison between drug stability in glass and drug stability in plastic containers, the authors would be remiss in their professional responsibilities if they neglected to mention those obvious limitations on the storage of drugs, irrespective of container and environment identity, which they encountered. It must be understood that any recommendations which are offered are limited in their scope to such parameters as are defined in the testing program conditions. Any projection of these recommendations and any extrapolation of the test results become individually assumed responsibilities. Indeed, because of product variation from one manufacturer to another, it may be well for each to use these results merely as guidelines in the testing of his own products.

The major recommendations which have resulted from this study are grouped in two broad categories, based on whether the polyethylene bottles were found to be acceptable or unacceptable containers for the pharmaceuticals in question, strictly within the limits imposed by the test conditions. These recommendations are listed together with the item numbers to which they pertain. (See Table I for number identification and pertinent details.) An asterisk (*) preceding an item number indicates that the regular 0.96 d. polyethylene bottle failed under certain test conditions because of cracking or excessive loss of volatiles from the drug product contained therein. In these cases, epoxy-lined 0.95 d. polyethylene bottles were substituted and were tested for 6 weeks under the same environmental conditions which had caused prior failure of the 0.96 d. bottles. In most instances, the epoxy-lined bottles withstood the test for the 6-week period, but their long-term behavior at environmental extremes is not known.

The instances of acceptability of polyethylene bottles as drug containers are outlined in Table II.

TABLE II—INSTANCES OF ACCEPTABILITY OF POLYETHYLENE BOTTLES AS DRUG CONTAINERS

1. Polyethylene bottles *are* acceptable containers for these items (passed under all conditions of testing):

Items No.		
4	*39*	*79*
7	*42*	*80*
9	*44*	*81*
11	*46*	*83*
13	*47*	*84*
14	*48*	*85*
18	*49*	*87*
20	*51*	*89*
22	*75*	*90*
23	*76*	*91*
32	*77*	*92*
37	*78*	*93*

2. Polyethylene bottles *are* acceptable containers for these items. However, storage in *any* container at temperatures greater than 100° F. should be avoided.

Items No.		
1	*28*	*54*
2	*29*	*55*
3	*31*	*56*
8	*36*	*68*
10	*38*	*82*
15A	*40*	*97*
19	*43*	*98*
21	*50*	*101*
26	*52*	*102*
27[a]	*53*	

3. Polyethylene bottles *are* acceptable containers for these items. However, storage in *any* container at temperatures greater than 100°F. or less than 40°F. should be avoided.

Items No.	
15B	*34*
16	*45*
17	*95*
24	*96*

4. Polyethylene bottles *are* acceptable containers for these items. However, storage in *any* container at temperatures greater than 73°F. should be avoided.

Items No.	
35	*61*

5. Polyethylene bottles *are* acceptable containers for this item. However, storage in *any* container at temperatures greater than 73°F. or less than 40°F. should be avoided.

Item No.
25

[a] This is a tentative assignment. The sample is being retested in epoxy-lined bottles.

The instances of nonacceptability and of limited acceptability of polyethylene bottles as drug containers are outlined in Table III.

TABLE III—INSTANCES OF NONACCEPTABILITY AND LIMITED ACCEPTABILITY OF POLYETHYLENE BOTTLES AS DRUG CONTAINERS

6. Polyethylene bottles *cannot* be recommended for this item unless storage temperatures greater than 73°F. can be avoided. Storage in *any* container at temperatures greater than 73°F. or less than 40°F. should be avoided.

Item No.

*99

7. Polyethylene bottles *cannot* be recommended for these items unless storage temperatures greater than 100°F. can be avoided. Storage in *any* container at temperatures greater than 73°F. should be avoided because of changes in physical characteristics.

Items No.

59	88

8. Polyethylene bottles *cannot* be recommended for these items unless storage temperatures greater than 100°F. can be avoided.

Items No.

57	*65	*71
58	66	*72
*60	67	73
63	69	86

9. Polyethylene bottles *cannot* be recommended for these items (based on gross failure under test conditions).

Items No.

30[a]	94	*100

10. Polyethylene bottles *cannot* be recommended for these items. Storage in *any* container at temperatures greater than 73°F. should be avoided.

Items No.

*62	70

11. Polyethylene bottles *cannot* be recommended for this item. Storage in *any* container at temperatures greater than 73°F. or less than 40°F. should be avoided.

Item No.

64

[a] Throughout the various storage intervals, this item remained intact in the tightly closed containers. However, after all of the tests had been completed, the tablets in glass showed marked physical deterioration, while those in polyethylene had reacted with such intensity that the bottles were partially melted and ruptured. This deterioration was due to the hygroscopic character of the tablets which caused them to take on moisture when their original containers were opened for sampling.

CONCLUSIONS

Certain general conclusions of outstanding nature can be drawn, and numerous others can be derived, from the observations recorded in Table I. Among the most significant are the following.

1. Polyethylene bottles can be used for packaging most of the pharmaceuticals tested under the various environmental conditions. Their performance with regard to the protection of their contents is parallel to that of glass, in most instances.

2. Outright incompatibility of the polyethylene bottle with its contents was rarely encountered under the test conditions.

3. In most instances, the polyethylene bottles themselves stood up well, both chemically and physically.

4. Polyethylene bottles of 0.95 d. offered no apparent advantage over the 0.96 d. bottles where the latter failed because of tensile rupture and/or stress cracking.

5. In most instances of drug failure, whether chemical or physical, failure of the same nature and magnitude occurred both in polyethylene and in glass containers. Thereupon, such failure must have been environmentally induced.

6. The vast majority of product and/or container failures which did occur are associated with the environmental extremes, namely, −65°F., and 140°F., and cycling; the 140°F. temperature was the most damaging.

7. Product and/or container failures were few in number between 40°F. and 100°F.

8. Differences in humidity, *e.g.*, 100°F., 27% R.H., and 100°F., 80% R.H., did not play a significant part in product stability or in container performance. This may be due to the fact that polyethylene is one of the best moisture barrier plastics known.

9. The majority of product failures which did occur were of the physical type rather than of the chemical type.

10. The most common failure among coated tablets was the cracking or crazing of the coating at the very low temperature (−65°F.) and during cycling.

11. Pharmaceuticals which contain highly volatile solvents, such as acetone and alcohol, invariably cause rupture of the polyethylene bottles due to high internal pressure, when stored at elevated temperature. Packaging in 0.95 d. polyethylene bottles provides little or no advantage.

12. Aspirin tablets and formulations containing aspirin failed both chemically and physically within 6 weeks, when stored at 140°F., 14% R.H. At all other temperatures, such tablets were stable throughout the 48-week storage intervals. It is noteworthy that the raw chemical, aspirin U.S.P., is stable even at 140°F., presumably because of the nearly anhydrous character of the chemical.

Isopropanol–Water Granulating Solution Ratio Effect on Physical Tablet Parameters

By LEONARD L. KAPLAN

A chewable multivitamin tablet formulation containing dry malt solids as binder was used as the model in this study to determine the effect of varying the ratio of isopropanol–water granulating solution on hardness, thickness, and disintegration parameters of tablets. Tablet granulations were prepared using (*a*) water, (*b*) isopropanol 25 per cent–water 75 per cent, (*c*) isopropanol 50 per cent–water 50 per cent, (*d*) isopropanol 75 per cent–water 25 per cent, and (*e*) isopropanol. Granulations (*a*) through (*d*) were compressed at a target hardness of 5 to 6 Kg. and a tablet weight of 650 mg. on the Stokes model F single-punch machine. Quantitative determinations of the physical tablet parameters were made initially following the controlled compression, and at selected storage time intervals. Granule size distribution evaluation showed an empirical relationship between solution ratios and granule size distribution as the determinant of differences in tablet thickness. Statistical analysis of the model initial and storage data revealed no significant effects on tablet hardness and disintegration.

THE PURPOSE of this report is to relate the effect of selected volume to volume ratios of isopropanol–water granulating solutions on the subsequent physical properties of a chewable multivitamin tablet formulation[1] containing dry malt solids as the binder. Underlying this objective is the determinant of a satisfactory minimal quantity of water sufficient for the granulation process so as to produce granules suitable for tableting the model formulation with prescribed chewable characteristics, utilizing isopropanol as the distributant co-solvent.

Pertinent references in the recent pharmaceutical literature concerned with granulating procedures have, for the most part, discussed the evaluation of granulating agents and methods (1, 2), the evaluation of new equipment (3), and the measurement of the physical properties of the produced compressed tablets (4). The thrust of this study is to relate the influence of the selected isopropanol–water granulating solutions on the granulations formed, and on the physical properties of the resultant compressed tablets intended to be chewed. In designing this formulation, it was recognized that dry malt solids[2] assumes an adhesive property when wetted with water, and is insoluble and nonadhesive in isopropanol. This adhesive nature of dry malt solids favored its use as the binder in this formulation. It was known that it could be added to the primary blend as a dry powder, and subsequently would form granules of a compressible character when granulated with water. Furthermore, it was known that the resultant tablets would be easy to chew without adhering to the teeth. It was decided to investigate the use of isopropanol in combination with water as the granulating solution in order to determine the minimal quantity of water to achieve granules suitable for tableting and to ensure adequate distribution of the water-wetted binder throughout the multivitamin powder blend.

EXPERIMENTAL

Formulation—The model formulation for a 1000-tablet batch quantity consisted of a primary blend of thiamine mononitrate 1.2 Gm., riboflavin 2.2 Gm., calcium pantothenate 1.5 Gm., pyridoxine HCl 0.6 Gm., niacinamide 11 Gm., ascorbic acid 33 Gm., dry malt solids 25 Gm., and diluent fillers and flavors *q.s. ad.* 600 Gm. Following the granulating procedures, vitamin A–D dry (500,000 units, 50,000 units per Gm.) 10 Gm., vitamin B_{12} (1:100) 1.5 Gm., tartaric acid 5.0 Gm., magnesium stearate 5.0 Gm., and cornstarch *q.s.* were added to the granules, yielding a total batch weight for compression of 650 Gm.

Granulating Procedures—One hundred milliliters of granulating solutions was prepared of each of the following: (*a*) water, (*b*) isopropanol 25%–water 75% v/v, (*c*) isopropanol 50%–water 50%, (*d*) isopropanol 75%–water 25%, and (*e*) isopropanol.

Five batches of the primary multivitamin blend were prepared and granulated with each of the five granulating solutions in a Hobart[3]-type 4-qt. mixer set at number two speed control. The resultant agglomerated masses were spread on trays at a 0.5-in. thickness, and dried at 100°F. to a moisture content of no more than 1% as determined on the Cenco moisture balance. Following the drying process, the dried materials were reduced to granules by passing through the model D Fitzpatrick comminutor, number two screen, slow speed, knives forward. Granulations (*a*), (*b*), (*c*), and (*d*) were satisfactory for further processing and were so submitted. Granulation (*e*) prepared with isopropanol resulted in an amorphous, nonflowing powder, and was rejected as unsuitable for normal tableting procedures.

Received April 14, 1967, from Walker Laboratories, Division of Richardson-Merrell, Inc., Mount Vernon, NY 10551
Accepted for publication July 11, 1967.
Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

[1] Marketed as Viacets by Walker Laboratories, Division of Richardson-Merrell, Inc., Mount Vernon, N.Y.

[2] A mixture of soluble dextrins and malt sugars obtained from barley.

[3] Hobart Manufacturing Co., Troy, Ohio.

Granule Analyses—A granule size distribution study was conducted on each of the four primary blend granulations (*a*) through (*d*), utilizing the Cenco-Meinzer sieve shaker. The results of this analysis are shown in Table I expressed as the percentage retained on screen mesh sizes 0–16, 16–24, 24–30, 30–40, 40–60, and the residual passing through the 60-mesh screen as fines. These residual quantities are plotted against the percentage of water in the granulating solutions as shown in Fig. 1. The resultant negative, straight-line slope suggests an inverse linear relationship between the ratio of water in the granulating solution and the percentage of residual fines in the granulation.

The granule distribution data are presented in Table II on a percentage cumulative retention basis on mesh screens 16, 24, 30, 40, and 60. The plot of these data against the percentage of water in the granulating solutions is shown in Fig. 2. These slopes reveal an increasing degree of influence of the proportion of water in the granulating solutions on the per cent granules retained cumulatively on the 40- and the 60-mesh screens. The per cent granules retained on the 16-, 24-, and 30-mesh screens does not reveal an appreciably consistent pattern of increased retention with the increased granulating solution water ratios.

Tableting Procedures—Following the addition of the balance of the formula ingredients and subsequent mixing in a twin-shell type blender for 20 min. to ensure uniform dispersion of all ingredients, each of the four granulations (*a*), (*b*), (*c*), and (*d*) was compressed on the Stokes model F single-punch tablet machine, using 0.5-in. standard concave punches.

The target tablet weight was 650 mg., and the target hardness was 5 to 6 Kg. hardness, as measured with the Stokes tablet hardness tester. The thickness of the tablet thus became a variable dependent on the compression characteristics of each of the granulations. In a previously published article (5), the author studied the compression characteristics of pharmaceutical materials, *per se*. It was reported that the thickness of compressed materials varied with the volumetric fill weight of the die cavity, which in turn appeared to be a function of the density of the material subjected to compression. Following the compression of each of the four granulations (*a*), (*b*), (*c*), and (*d*), the weight, hardness, thickness, and disintegration of the tablets were determined. These results are shown in Table III.

Figure 3 represents the plots of the per cent granules cumulatively retained on the 60-mesh screen and the thickness of the compressed tablets prepared with each of the four granulations. The resultant slope is empirically suggestive of an inverse relationship between these two variables.

Friability tests were conducted, utilizing the Roche Friabilator apparatus. In this procedure, 20

TABLE I—GRANULE DISTRIBUTION

Mesh Sizes[a]	Granulations (*a*)	(*b*)	(*c*)	(*d*)
Retained on 16	5.2	2.3	2.7	1.8
Through 16, on 24	12.8	12.3	12.8	10.6
Through 24, on 30	8.2	6.0	5.2	3.9
Through 30, on 40	20.0	17.2	13.1	9.0
Through 40, on 60	14.8	14.2	9.6	8.0
Residual through 60	39.0	48.0	56.6	66.7
	100.0%	100.0%	100.0%	100.0%

[a] U.S. Standard sieve series, Newark Wire Cloth Co., Newark, N.J.

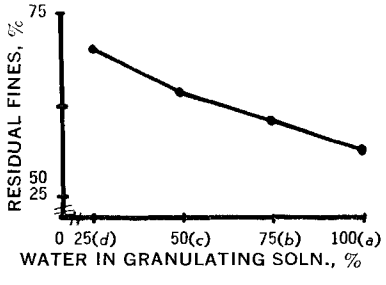


Fig. 1—Residual fines through No. 60-mesh screen.

TABLE II—GRANULE CUMULATIVE RETENTION (EXPRESSED IN PER CENT)

Granulations	Mesh Sizes 16	24	30	40	60
(*a*)	5.2	18.0	26.2	46.2	61.0
(*b*)	2.3	14.6	20.6	37.8	52.0
(*c*)	2.7	15.5	20.7	33.8	43.7
(*d*)	1.8	12.4	16.3	25.3	33.3

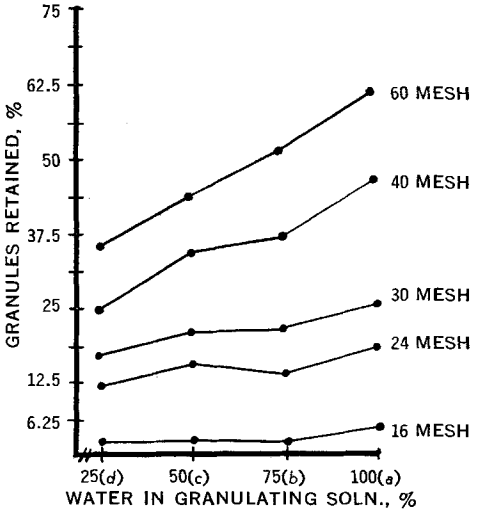


Fig. 2—Cumulative granule retention on selected mesh sizes.

TABLE III—PHYSICAL TABLET PROPERTIES

Granulations	Wt.,[a] mg.	Hardness,[a] Kg.	Thickness,[a] in.	Disintegration,[b] min.
(*a*)	647	5.1	.209	8
(*b*)	653	5.3	.214	7
(*c*)	662	5.5	.217	10
(*d*)	659	5.5	.219	12

[a] Means of 15 determinations. [b] Means of 6 determinations, U.S.P. tablet disintegration apparatus, water, without disks.

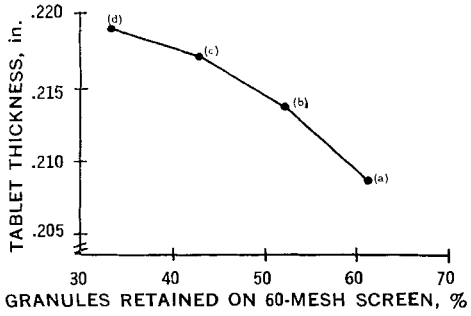


Fig. 3—Relationship of granules cumulatively retained and the resultant tablet thickness of the four test granulations (a) *through* (d).

TABLE IV—FRIABILITY STUDY DATA

Formula	3 min. Wt. Loss,[a] mg.	6 min. Wt. Loss,[a] mg.	9 min. Wt. Loss,[a] mg.
(*a*)	2	6	8
(*b*)	4	7	9
(*c*)	3	6	8
(*d*)	3	6	9

[a] Average loss of 20 tablets.

tablets of each of the four granulations were placed in the apparatus, and the apparatus rotated at 24 r.p.m. for periods of 3 min., 6 min., and 9 min. in order to introduce a time dimension in the evaluation of the relative friability of the four tablet formulations. The results obtained in terms of average tablet weight loss are presented in Table IV. No significant differences were noted among the four formulations.

Storage Data—Following storage for 4 months at ambient room temperature in amber glass bottles, samples of each of the four formulations were subjected to hardness testing and disintegration determination. The storage data are presented in Table V. Statistical computation of the difference between the initial hardness and disintegration data means presented in Table III and the storage data means revealed no statistically significant changes in these parameters.

The Student *t* test (Eq. 1) was calculated for each

$$tn1 + n2 - 2 = \frac{\bar{X}_1 - \bar{X}_2}{Sp\sqrt{1/n_1 + 1/n_2}} \quad \text{(Eq. 1)}$$

of the four formulations, at 28 degrees of freedom for the hardness data, and at 10 degrees of freedom for the disintegration data, at the p 0.05 significance level. The initial mean values of Table III are identified as $\bar{X}_1$, and the storage data means as $\bar{X}_2$. The Sp standard deviation values were computed using Eq. 2, in which S_1^2 is the estimated variance of the initial Table III data, and S_2^2 is the estimated variance of the Table V storage data.

TABLE V—FOUR MONTHS' STORAGE DATA

Formula	Hardness,[a] Kg.	Disintegration,[b] min.
(*a*)	4.9	7
(*b*)	6.0	8
(*c*)	5.9	11
(*d*)	5.7	12

[a] Means of 15 tablets. [b] Means of 6 tablets.

$$Sp = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}} \quad \text{(Eq. 2)}$$

For the hardness data, the tabled critical t value is 2.048. For the disintegration data, the tabled critical t value is 2.228. Computation of t values for the hardness data for the four formulations: (*a*) 2.02, (*b*) 2.03, (*c*) 1.95, (*d*) 2.03. Computation of t values for the disintegration data: (*a*) 2.17, (*b*) 2.04, (*c*) 1.99, (*d*) 1.97. The hypotheses that there are no differences between initial and storage data sets of means are accepted.

SUMMARY

Using a chewable multivitamin tablet formulation containing dry malt solids as the binder, a series of isopropanol–water granulating solutions was evaluated along with water alone and isopropanol alone.

It was found that the higher the ratio of water content in the granulating solution, the lesser the percentage of residual fines that passed through a 60-mesh screen. However, the percentage of granules that were 30-mesh or larger was not significantly influenced by the higher water content. It was found that the presence of some water content was necessary with this formulation to achieve a suitable granulation. The isopropanol alone granulating solution was unsatisfactory for use.

The tablets prepared were evaluated in terms of the physical parameters: weight, hardness, thickness, disintegration, and friability. No difference was noted in the tablet friability of the four compressed granulations. The thickness obtained appeared to be dependent on the percentage of residual fines in the granulations, maintaining the weight and hardness of compression as constants. There was an unexplained lengthening in the initial disintegration of the tablets prepared with the higher isopropanol content granulating solutions.

Evaluation of the 4 month's storage samples revealed no statistically significant changes occurring in the hardnesses and disintegrations of the four tablet formulations.

REFERENCES

(1) Willis, C. R., Banker, G. S., and DeKay, H. G., *J. Pharm. Sci.*, **54**, 366(1965).
(2) Fonner, D. E., Banker, G. S., and Swarbrick, J., *ibid.*, **55**, 181(1966).
(3) Scott, M. W., Lieberman, H. A., Rankell, A. S., and Battista, J. V., *ibid.*, **53**, 314(1964).
(4) Seitz, J. A., and Flessland, G. A., *ibid.*, **54**, 1353(1965).
(5) Kaplan, L. L., and Wolff, J. E., *Drug Cosmetic Ind.*, **88**, 584(1961).

Synthesis of Halogen Substituted Derivatives of 4,4-Bis(4-hydroxyphenyl)pentanoic Acid and Their Antifungal Properties

By RASHMIKANT M. PATEL*, DAVID P. CAREW, and JOHN L. LACH

Several derivatives of 4,4-bis(4-hydroxyphenyl)-pentanoic acid and 10-undecenoic hydroxamic acid were prepared. Their antifungal activity was tested against *Trichophyton mentagrophytes* (ATCC 9533). Six of the ten compounds tested exhibited greater activity than undecylenic acid under the conditions of the test.

MANY COMPOUNDS have been synthesized and evaluated for their antimicrobial activity. These include quinones, quaternary ammonium salts, sulfur containing derivatives, hydroxamic acids, halogenated phenols, and acid esters (1–6). Among these diverse chemical types, compounds having a phenol nucleus such as hexachlorophene, bithionol, and resorcinol have a prominent place in many soaps, ointment, and scrub lotions.

A previous study with the fungus *Trichophyton rubrum* and with streptococci indicated that diphenolic acid[1] [4,4-bis(4-hydroxyphenyl)pentanoic acid] possesses antifungal and antibacterial activity *in vitro* (7). Since diphenolic acid is a suitable intermediate for the preparation of other compounds with possible antifungal activity, it was of interest to prepare its halogenated derivatives and some of their esters.

Since some hydroxamic acid derivatives have been shown to exhibit antifungal activity, the hydroxamic acid of the widely used undecylenic acid was also prepared and evaluated for its antifungal property.

EXPERIMENTAL

4,4-Bis(3,5-dichloro-4-hydroxyphenyl)pentanoic Acid—A 1-L. round-bottom flask, fitted with an efficient condenser carrying a calcium chloride drying tube, was set up on a steam bath in a hood. In the flask were placed 28.6 Gm. (0.1 mole) of 4,4-bis-(4-hydroxyphenyl)pentanoic acid and 67.5 Gm. (0.5 mole) of sulfuryl chloride, and the mixture was warmed on the steam bath for 1 hr. Ten more milliliters of sulfuryl chloride was added to the reaction flask and the warming was continued for about 30 min. Excess sulfuryl chloride was removed by attaching a water pump through an empty safety flask to the reaction flask. The crude product was washed with boiling water, dried at 60°, and recrystallized from benzene.

4,4-Bis(3,5-dibromo-4-hydroxyphenyl)pentanoic Acid—In a 1-L. three-necked flask, fitted with a mechanical stirrer, dropping funnel, and a condenser, were placed 28.6 Gm. (0.10 mole) of diphenolic acid and 360.0 ml. of glacial acetic acid. A solution of 40.0 ml. (1.6 mole) of bromine in 40.0 ml. of glacial acetic acid was placed in the dropping funnel and added to the diphenolic acid solution during a 30-min. period and stirred continuously for 22 hr. The flask was heated on a steam bath and air was passed through the side neck to remove excess bromine. The mixture was poured into a beaker containing a saturated solution of sodium bisulfite in water. The resulting semisolid mass was washed with cold water and placed in a beaker containing boiling water, and stirred until the semisolid mass solidified. The hot water was decanted, and the crude product was dried at 60°, and recrystallized from benzene.

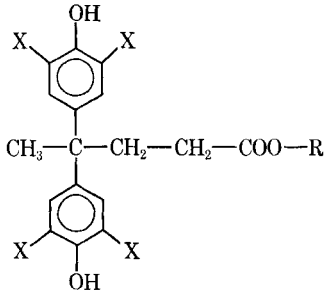
Esterification of Halogenated Acids—Methyl and propyl esters were prepared according to the following procedure. A mixture of purified halogenated diphenolic acid (0.05 mole), appropriate alcohol (75 ml.), and concentrated sulfuric acid (5 ml.) was refluxed for 5 hr. The reaction mixture was poured into a beaker containing ice, neutralized with a saturated solution of sodium bicarbonate, filtered, and washed with water. The crude ester was vacuum dried and recrystallized from benzene.

N-Decyl and cyclohexyl esters were prepared by reaction of appropriate acid chloride with the alcohol under consideration. The general procedure was as follows. In a 250-ml. conical flask, fitted with a condenser, was placed 0.05 mole of halogenated acid and 59.0 Gm. (0.5 mole) of thionyl chloride. The reaction mixture was heated on a steam bath for 0.5 hr. At the end of the reaction period, 50.0 ml. of dry benzene was placed in the flask and excess thionyl chloride distilled under vacuum. A solution of 0.05 mole of the appropriate alcohol in 30.0 ml. benzene was added to the flask containing acid chloride and refluxed for 1 hr. After removing benzene, the crude product was treated with a saturated solution of sodium bicarbonate, filtered, washed, dried, and recrystallized from an acetic acid–water mixture. The recrystallization solvents, melting points, yields, and elemental analyses are given in Table I.

10-Undecenoic Hydroxamic Acid—Separate solutions of 14.0 Gm. (0.2 mole) of hydroxylamine hydrochloride in 80.0 ml. of methyl alcohol and 16.8 Gm. (0.3 mole) of potassium hydroxide in 40.0 ml. of methyl alcohol were prepared at the boiling point of the solvent. Each was cooled to 35° and the one containing alkali was added with shaking to hydroxylamine hydrochloride solution. Any excessive rise of temperature was prevented by occasional cooling in an ice bath. After the alkali had been added the mixture was allowed to stand

Received April 3, 1967, from the College of Pharmacy, University of Iowa, Iowa City, IA 52240
Accepted for publication June 8, 1967.
* Present address: Ayerst Laboratories, Rouses Point, NY 12979
[1] Obtained from S. C. Johnson & Son, Inc., Racine, Wis.

TABLE I—DERIVATIVES OF 4,4-BIS(4-HYDROXYPHENYL)PENTANOIC ACID



No.	X	R	% Yield	M.p., °C.	Recrystn. Solvent	Anal., % Calcd. C	Calcd. H	Calcd. X	Found[a] C	Found[a] H	Found[a] X
1[b]	Cl	H	38	182–183	Benzene	48.13	3.30	33.45	48.33	3.45	32.24
2[b]	Br	H	62	159–160	Benzene	33.90	2.33	53.13	34.15	2.25	51.90
3	Cl	Me	71	139–140	Benzene	49.36	3.68	...	49.50	3.84	...
4	Br	Me	54	122–123	Benzene	35.10	2.62	...	35.36	2.86	...
5	Cl	*n*-Pr	32	112–113	Benzene	51.52	4.32	...	51.78	4.11	...
6	Br	*n*-Pr	25	123–124	Benzene–benzine	37.30	3.13	...	37.95	3.01	...
7	Br	Cyclohexyl	29	93–94	Acetic acid–water	40.37	3.53	...	40.30	3.65	...
8	Br	Decyl	20	84–85	Acetic acid–water	43.69	4.60	...	43.69	4.58	...

[a] C, H analysis was done at Clark's Microanalytical Laboratory; halogen by Parr bomb method. [b] Now sold by Emery Laboratories, Cincinnati, Ohio.

in an ice bath for 5 min. to insure complete precipitation of potassium chloride. Then 19.8 Gm. (0.1 mole) of methyl 10-undecenoate was added with thorough shaking and the mixture was filtered immediately with suction. The filtrate was placed in a conical flask and allowed to stand at room temperature. After 2 days, crystals were filtered, washed with a minimum amount of absolute ethyl alcohol, and air dried. A mixture of the potassium salt and 100.0 ml. of dilute acetic acid was stirred and heated on a steam bath until a clear solution was obtained. It was allowed to cool at room temperature and finally placed in an ice bath. The precipitated hydroxamic acid was recrystallized twice from glacial acetic acid–water, whereupon colorless crystals were obtained. The yield was 11.0 Gm. (55%), m.p. 72–73°.

Anal.—Calcd. for $C_{11}H_{21}NO_2$: C, 66.32; H, 10.62; N, 7.02. Found: C, 67.03; H, 10.92; N, 7.22.

Determination of Fungicidal Activity—A study of the fungicidal properties of the compounds whose synthesis has just been reported was conducted. The testing procedure was a modification of the methods of Burlingame (8), Golden (9), and Reddish (10). The test organism *T. mentagrophytes* (ATCC 9533) was used exclusively. Since the compounds to be tested for fungicidal activity were insoluble in water, ethanolic solutions were prepared. Solutions of 0.1%, 0.5%, and 1.0% in ethanol (95%) were made. Ten tests for each of the three concentrations were run for each compound.

Test Procedures—Petri dishes containing 20 ml. Sabouraud dextrose agar were inoculated with a culture of *T. mentagrophytes*. The inoculated plates were incubated at a temperature of 28° for 15 days. On the day of the test 1-cm. disks of agar and fungus were removed from the Petri dishes with a sterile cork borer (10 mm. diameter). Each disk was immediately aseptically transferred to a test tube containing 10 ml. of a particular concentration of the solution of the compound under test. The test tube was agitated slowly for 2 min. and the disk was transferred to a test tube containing 10 ml. sterile Sabouraud dextrose broth. This tube was agitated slightly for a period of 3 min. to free the mycelia of any soluble or miscible material. The disk was then transferred to a test tube containing 10 ml. of a 30% aqueous acetone solution where it was kept for 5 min. to remove any traces of adhering antifungal compound. The disk was transferred to a test tube of sterile Sabouraud dextrose broth for a 2-min. period in order to remove any traces of acetone. Finally the disk was removed from the broth and placed culture side down on the surface of a sterile slant of Sabouraud dextrose agar contained in a 25 × 150 mm. test tube. The inoculated slants were incubated at a temperature of 28° for 25 days. Controls were run along with each compound and these consisted of agar-fungus disks which were treated as above except that the initial alcoholic solution did not contain any chemical compound being tested. Evidence of growth in control tubes was always evident in a few days after inoculation. In addition to the newly synthesized compounds, the known antifungal agent undecylenic acid was used for comparison. Since the testing procedure was designed to determine fungicidal activity, any evidence of fungal growth after a 25-day period was regarded as a negative result. Table II lists the results of the fungicidal testing procedure.

TABLE II—RESULTS OF FUNGICIDAL TESTING

Compd. No. or Name	No. of Tubes Exhibiting Growth After 25 Days[a] 0.1%	0.5%	1.0%
1	10	8	6
2	10	10	10
3	4	4	3
4	6	4	4
5	4	4	0
6	6	4	2
7	8	6	6
8	4	4	4
Diphenolic acid	10	3	3
Undecylenic acid	8	4	2
10-Undecenoic hydroxamic acid	2	0	0

[a] The maximum number of tubes exhibiting growth for each concentration is 10.

CONCLUSION

From Table II it can be seen that 10-undecenoic hydroxamic acid exhibited the greatest amount of fungicidal activity. Propyl 4,4-bis(3,5-dichloro-4-hydroxyphenyl) pentanoate was the next most active fungicidal compound tested. Six of the ten compounds tested exhibited greater activity than undecylenic acid under the conditions of the test.

The undecylenic hydroxamic acid, because of its activity against *T. mentagrophytes*, was further tested and showed good activity against the following organisms: *Microsporum audouini* ATCC 9079, *Microsporum canis* ATCC 10241, *Trichophyton mentagrophytes* (*gypseum*) ATCC 9129, *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9972, *Candida albicans* ATCC 10231, *Trichophyton rubrum* ATCC 10281, *Stemphylium solani* ATCC 11128, and *Stemphylium species* ATCC 9569.

REFERENCES

(1) Halweg, H., *Geuzlica*, **24**, 233(1958).
(2) Walker, G. C., and Portor, C. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 77(1952).
(3) Sakai, S., and Kada, T., *J. Sci. Res. Inst.* (*Tokyo*), **46**, 113(1952).
(4) Easterly, W. D., Jr., Jordin, M. W., Dorsey, W. S., and Clark, G., *J. Pharm. Sci.*, **54**, 1358(1965).
(5) Gingras, B. A., Colin, G., and Bayley, C. H., *ibid.*, **54**, 1674(1965).
(6) Narayan, V. L., Bernstein, J., and Williams, J., *ibid.*, **55**, 217(1966).
(7) "Technical Bulletin: Derivatives of Diphenolic Acid," S. C. Johnson & Son, Inc., Racine, Wis.
(8) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 765(1939).
(9) Golden, M. J., and Oster, K. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **36**, 359(1947).
(10) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization," Lea and Febiger, Philadelphia, Pa., 1957, p. 975.

Activity of Different Segments of Rabbit Gastrointestinal Tract

By THEODORE H. EICKHOLT, ROGER H. BOX, and NOLAN D. COURVILLE

Five different segments of the rabbit intestinal tract, (duodenum, jejunum, ileum, ascending and descending colon), were used to record the normal activity of these segments using an isolated muscle bath technique. Kymograph recordings and transducer-recorder recordings were compared. Contractions per minute, interval between contractions, and amplitude of contractions were observed and evaluated. In the transducer recorded series, *t* tests indicated statistically significant difference for all comparisons of contractions per minute. From 60 comparisons of normal rabbit intestinal activity using *t* tests, only 5 comparisons were not found to be significantly different at *P* 0.05.

THE WEIGHTS and linear measurements of several segments of the gastrointestinal tract of the rabbit have been investigated by Latimer and Sawin (1). Other tissues and organs have been studied (2–5), but the variations in the normal activity of different segments of the rabbit gut so often employed in drug testing and drug experimentation require delineation.

It was, therefore, the purpose of this study to record some of the variations observed in the different segments of the rabbit intestine and subject them to statistical evaluation for reference when a similar preparation was used experimentally. Two systems, typical kymograph and transducer systems, were employed to further delineate differences in observations of activity of rabbit intestinal segments.

EXPERIMENTAL

Adult, albino rabbits were anesthetized with ether and 2–3 cm. segments of duodenum, jejunum, ileum, ascending and descending colon were expediently removed. The segments were individually maintained in oxygenated Tyrode's solution in a constant-temperature bath at 38° prior to their placement in the isolated muscle bath. The Tyrode's solution contained sodium chloride 0.8%, potassium chloride 0.02%, calcium chloride 0.02%, magnesium chloride 0.01%, sodium bicarbonate 0.1%, sodium diphosphate 0.005%, and glucose 0.1%.

The first series of experiments utilized constant-temperature muscle baths and kymographs. Tyrode's solution (100 ml.) was used to bathe the segment; temperature was maintained at 38° and the preparation was oxygenated using an air flow bubbled through the solution. The segment was attached to the muscle hooks, one stationary and the other being connected to a lever and stylus, such that the segment was completely submerged in the Tyrode's solution. The muscle lever was made to record on a slowly rotating carbon black kymograph drum. A synchronous timer recorded the time on the same tracing. The only constant tension placed upon the segment was that of the weight of the lever which in turn was centered on its fulcrum and

Received January 17, 1967, from the Department of Pharmacology, School of Pharmacy, Northeast Louisiana State College, Monroe, LA 71201
Accepted for publication June 5, 1967.

TABLE II—RESULTS OF FUNGICIDAL TESTING

Compd. No. or Name	No. of Tubes Exhibiting Growth After 25 Days[a] 0.1%	0.5%	1.0%
1	10	8	6
2	10	10	10
3	4	4	3
4	6	4	4
5	4	4	0
6	6	4	2
7	8	6	6
8	4	4	4
Diphenolic acid	10	3	3
Undecylenic acid	8	4	2
10-Undecenoic hydroxamic acid	2	0	0

[a] The maximum number of tubes exhibiting growth for each concentration is 10.

CONCLUSION

From Table II it can be seen that 10-undecenoic hydroxamic acid exhibited the greatest amount of fungicidal activity. Propyl 4,4-bis(3,5-dichloro-4-hydroxyphenyl) pentanoate was the next most active fungicidal compound tested. Six of the ten compounds tested exhibited greater activity than undecylenic acid under the conditions of the test.

The undecylenic hydroxamic acid, because of its activity against *T. mentagrophytes*, was further tested and showed good activity against the following organisms: *Microsporum audouini* ATCC 9079, *Microsporum canis* ATCC 10241, *Trichophyton mentagrophytes* (*gypseum*) ATCC 9129, *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9972, *Candida albicans* ATCC 10231, *Trichophyton rubrum* ATCC 10281, *Stemphylium solani* ATCC 11128, and *Stemphylium species* ATCC 9569.

REFERENCES

(1) Halweg, H., *Geuzlica*, **24**, 233(1958).
(2) Walker, G. C., and Portor, C. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 77(1952).
(3) Sakai, S., and Kada, T., *J. Sci. Res. Inst.* (*Tokyo*), **46**, 113(1952).
(4) Easterly, W. D., Jr., Jordin, M. W., Dorsey, W. S., and Clark, G., *J. Pharm. Sci.*, **54**, 1358(1965).
(5) Gingras, B. A., Colin, G., and Bayley, C. H., *ibid.*, **54**, 1674(1965).
(6) Narayan, V. L., Bernstein, J., and Williams, J., *ibid.*, **55**, 217(1966).
(7) "Technical Bulletin: Derivatives of Diphenolic Acid," S. C. Johnson & Son, Inc., Racine, Wis.
(8) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 765(1939).
(9) Golden, M. J., and Oster, K. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **36**, 359(1947).
(10) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization," Lea and Febiger, Philadelphia, Pa., 1957, p. 975.

Activity of Different Segments of Rabbit Gastrointestinal Tract

By THEODORE H. EICKHOLT, ROGER H. BOX, and NOLAN D. COURVILLE

Five different segments of the rabbit intestinal tract, (duodenum, jejunum, ileum, ascending and descending colon), were used to record the normal activity of these segments using an isolated muscle bath technique. Kymograph recordings and transducer-recorder recordings were compared. Contractions per minute, interval between contractions, and amplitude of contractions were observed and evaluated. In the transducer recorded series, *t* tests indicated statistically significant difference for all comparisons of contractions per minute. From 60 comparisons of normal rabbit intestinal activity using *t* tests, only 5 comparisons were not found to be significantly different at *P* 0.05.

THE WEIGHTS and linear measurements of several segments of the gastrointestinal tract of the rabbit have been investigated by Latimer and Sawin (1). Other tissues and organs have been studied (2–5), but the variations in the normal activity of different segments of the rabbit gut so often employed in drug testing and drug experimentation require delineation.

It was, therefore, the purpose of this study to record some of the variations observed in the different segments of the rabbit intestine and subject them to statistical evaluation for reference when a similar preparation was used experimentally. Two systems, typical kymograph and transducer systems, were employed to further delineate differences in observations of activity of rabbit intestinal segments.

EXPERIMENTAL

Adult, albino rabbits were anesthetized with ether and 2–3 cm. segments of duodenum, jejunum, ileum, ascending and descending colon were expediently removed. The segments were individually maintained in oxygenated Tyrode's solution in a constant-temperature bath at 38° prior to their placement in the isolated muscle bath. The Tyrode's solution contained sodium chloride 0.8%, potassium chloride 0.02%, calcium chloride 0.02%, magnesium chloride 0.01%, sodium bicarbonate 0.1%, sodium diphosphate 0.005%, and glucose 0.1%.

The first series of experiments utilized constant-temperature muscle baths and kymographs. Tyrode's solution (100 ml.) was used to bathe the segment; temperature was maintained at 38° and the preparation was oxygenated using an air flow bubbled through the solution. The segment was attached to the muscle hooks, one stationary and the other being connected to a lever and stylus, such that the segment was completely submerged in the Tyrode's solution. The muscle lever was made to record on a slowly rotating carbon black kymograph drum. A synchronous timer recorded the time on the same tracing. The only constant tension placed upon the segment was that of the weight of the lever which in turn was centered on its fulcrum and

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Accepted for publication June 5, 1967.

raised only until the attachment *via* muscle hooks was tight enough to transfer the segment's movement to the lever and recording stylus.

The conditions for the second series of experiments for comparison were the same as above except intestinal activity was recorded *via* an E & M physiograph isotonic myograph transducer on an E & M physiograph. A timer was an integral part of the recorder and the paper speed was 0.05 cm./sec. The only constant tension placed upon the segment was that of the weight of the movable armature (2.2 Gm.) and of the lever which in turn was centered on its fulcrum and raised only until the attachment *via* muscle hooks was tight enough to transfer the segment's movements to the transducer and recording physiograph. In both systems relaxation was attempted with minimal stretching to best approximate normal conditions.

The myograph had amplitude and calibration controls and all observed pen deflections were equilibrated to the standard pen deflection displacements.

In each experiment, the segments were allowed to acclimate to the muscle bath environment. Acclimation was determined by consistency and/or rhythmicity of contractions.

Following acclimation in both series of experiments, normal intestinal activity for the different intestinal segments was recorded. Five to seven different rabbits were used to provide different individual samples of duodenum, jejunum, ileum, ascending and descending colon. Tracings of a minimum of 15 min. were obtained for each of the 5 different segments in each series. From each tracing of each individual gut segment, amplitude of contraction, interval between contractions, and contractions per minute were measured. Three separate measurements of the three parameters above were made on each tracing providing an average of the three for that particular parameter of that single gut segment. These averages were in turn averaged for the 5 to 7 samples of each individual gut segment. This provided 15 to 21 actual measurements for the averages of the three parameters. Standard deviations were calculated and t tests were made between all possible combinations of intestinal segments.

RESULTS

Table I summarizes the averages and standard deviations of the amplitude of contraction, interval between contractions, and contractions per minute obtained in the first series of experiments recording on the smoked kymograph.

The t test values from the kymograph recorded series for all combinations of intestinal segments on the three parameters, contractions per minute, interval between contractions, and amplitude of contractions, indicated significant differences at P 0.05 for 28 of the total 30 comparisons.

Table II summarizes the averages and standard deviations of the amplitude of contraction, interval between contractions, and contractions per minute obtained in the second series of experiments recording with the myograph transducer.

The t test values from the transducer recorded series for all combinations of intestinal segments on the three parameters indicated significant differences at P 0.05 for 27 of the total 30 comparisons.

SUMMARY AND CONCLUSIONS

Five different segments of the rabbit intestinal tract, duodenum, jejunum, ileum, ascending and descending colon, were utilized in recording normal activity of these segments using an *in vitro* isolated muscle bath technique. One series of experiments was recorded using a typical smoked kymograph, while the second series for comparison employed an isotonic myograph transducer and multichannel recorder.

Contractions per minute, interval between contractions, and amplitude of contractions were recorded, determined, averaged, standard deviations calculated, and t tests performed on all possible segment combinations. In the kymograph recorded series, the contractions per minute decreased with progression to the lower segments of the intestinal tract. The interval between contractions increased which would correlate with the decreased contractions per minute. All t values for comparing

TABLE I—SUMMARY OF AVERAGES AND STANDARD DEVIATIONS OF MEASUREMENTS OF RABBIT INTESTINAL ACTIVITY OBTAINED FROM KYMOGRAPH RECORDINGS

	No. of Samples	Contractions/min.	Interval Between Contractions, sec.	Amplitude of Contraction, mm.
Duodenum	6	16.9 ± 2.01	3.1 ± 0.6	22.0 ± 7.40
Jejunum	6	13.8 ± 1.63	3.7 ± 0.7	36.1 ± 20.10
Ileum	6	13.3 ± 3.21	4.6 ± 1.1	12.0 ± 7.55
A. colon	6	6.2 ± 3.97	6.0 ± 4.1	2.8 ± 1.84
D. colon	6	4.9 ± 1.56	10.0 ± 4.3	19.5 ± 1.98

TABLE II—SUMMARY OF AVERAGES AND STANDARD DEVIATIONS OF MEASUREMENTS OF RABBIT INTESTINAL ACTIVITY OBTAINED FROM TRANSDUCER RECORDINGS

	No. of Samples	Contractions/min.	Interval Between Contractions, sec.	Amplitude of Contraction, mm.
Duodenum	7	16.7 ± 1.68	3.9 ± 0.1	2.56 ± 2.50
Jejunum	7	12.4 ± 2.31	5.0 ± 1.5	6.53 ± 3.46
Ileum	7	10.5 ± 1.27	6.2 ± 1.1	9.17 ± 0.68
A. colon	5	2.1 ± 0.83	15.0 ± 8.2	1.68 ± 0.57
D. colon	5	4.3 ± 1.68	13.4 ± 5.0	3.91 ± 2.38

all possible combinations of intestinal segments showed statistically significant differences at P 0.05 except two. The jejunum–ileum and A. colon–D. colon contractions per minute comparisons were not significantly different; however, the two other adjacent segments comparisons here were significantly different.

In the transducer recorded series, the contractions per minute again decreased with progression to the lower intestinal tract and the intervals between contractions again increased. The A. colon was the only exception to this trend in these two cases and may be due to more sensitive recording.

In this series the t values indicated statistically significant differences for all comparisons of contractions per minute which would be very important for drug comparison effects upon the intestinal tract.

The comparison of interval between contractions between A. colon and D. colon, however, was not significantly different. The reverse was true in the kymograph series in the comparisons between jejunum–ileum and A. colon–D. colon; the interval between contractions was significant but the contractions per minute were not significantly different. The transducer system would appear to better record the contractions per minute.

Two comparisons of amplitude of contractions in the transducer series also were not significantly different. They were duodenum–A. colon and duodenum–D. colon.

It would appear that this study and evaluation of normal rabbit intestinal activity could serve as a basis for further drug activity studies employing the rabbit intestinal tract and furthermore, in determining the preferential intestinal segment of a drug's activity.

REFERENCES

(1) Latimer, H. B., and Sawin, P. B., *Anat. Rec.*, **129**, 1(1957).
(2) DeBruyn, P. P. H., and Tornova-Svehlik, M. M., *ibid.*, **119**, 275(1954).
(3) Latimer, H. B., and Sawin, P. B., *ibid.*, **123**, 81(1955).
(4) Latimer, H. B., and Sawin, P. B., *J. Compt. Neurol.*, **103**, 513(1955).
(5) Tanner, J. M., and Sawin, P. B., *J. Anat.*, **87**, 54(1953).

Inhibition of Cortisone-Induced Cleft Palate in Mice by Cobaltous Chloride

By GILBERT KASIRSKY, RONALD F. GAUTIERI, and DAVID E. MANN, JR.

Young primipara CF-1 mice were mated overnight and those with vaginal plugs (day zero) were considered pregnant. Animals were divided into five groups of 26 each and subjected to the following treatment: group *A* received 2.5 mg. of cortisone acetate i.m. on days 11, 12, 13, and 14; group *B* received cobaltous chloride, 25 mg./Kg. i.p. on day 11 only followed by cortisone acetate, 2.5 mg. i.m. on days 11, 12, 13, and 14; group *C* received physiological saline, 0.1 ml. i.m. on days 11, 12, 13, and 14; group *D* represented untreated controls; group *E* received cobaltous chloride, 25 mg./Kg. i.p. on day 11 only followed by saline, 0.1 ml. i.m. on days 11, 12, 13, and 14. Mice were laparotomized and fetuses were examined on day 18. Group *A* (cortisone), 140 of 185 fetuses developed cleft palate (75.6 per cent incidence); group *B* (cobaltous chloride–cortisone), 24 of the 190 fetuses had clefts (12.6 per cent incidence); group *C* (saline), none of the 201 fetuses showed clefts; group *D* (untreated controls), none of the 194 fetuses had clefts; group *E* (cobaltous chloride–saline), 27 of the 208 fetuses had cleft palate (12.9 per cent incidence). Also, the incidence of cleft palate by litters was group *A*, 100 per cent; group *B*, 23.1 per cent; group *C*, 0 per cent; group *D*, 0 per cent; group *E*, 50 per cent.

DURING A recent evaluation of the relative antitumorigenic properties of cortisone (1), cobaltous chloride (2), and sodium cobaltinitrite (3, 4) in CF-1 mice subjected to the minimal carcinogenic dose$_{50}$ (MCD_{50}) of methylcholanthrene, several striking similarities became apparent in the character of the histological responses evoked by each drug, for not only was there a significant reduction of tumor incidence, but also the remarkable absence of cutaneous inflammation. The latter observation may be related to a report of an accentuation of anti-inflammatory activity of δ-1 hydrocortisone by the addition of a cobalt atom to the C-20 carbonyl function (5). Because interference with normal closure of the palatine shelves in certain strains of mice by cortisone is well documented (6), and furthermore, because previous studies in this laboratory have uncovered certain cortisone-like characteristics of two inorganic cobalt compounds, it thus became the primary objective of this investigation to discern the nature and extent of any cobalt–steroid interactions in terms of greater or lesser anomalies in the mouse fetus.

Received April 6, 1967, from the Department of Pharmacology, School of Pharmacy, Temple University, Philadelphia, PA 19122
Accepted for publication June 5, 1967.
Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by grant H D 01975-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Groups of 30 female mice of the CF-1 strain, weighing approximately 25 Gm., were individually

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REFERENCES

(1) Latimer, H. B., and Sawin, P. B., *Anat. Rec.*, **129**, 1(1957).
(2) DeBruyn, P. P. H., and Tornova-Svehlik, M. M., *ibid.*, **119**, 275(1954).
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EXPERIMENTAL

Groups of 30 female mice of the CF-1 strain, weighing approximately 25 Gm., were individually

TABLE I—INHIBITION OF CORTISONE-INDUCED CLEFT PALATE IN MICE BY COBALTOUS CHLORIDE

Group	Treatment	No. Litters	Litters with CP	No. Fetuses	No. Fetuses with CP	% with CP
A	Cortisone, 2.5 mg. i.m., days 11, 12, 13, 14	26	26	185	140	75.6
B	Cortisone, 2.5 mg. i.m., days 11, 12, 13, 14 Cobaltous chloride 25 mg./Kg. i.p., day 11	26	6	190	24	12.6
C	Physiological saline, 0.1 ml. i.m., days 11, 12, 13, 14	26	0	201	0	0
D	Untreated controls	26	0	194	0	0
E	Physiological saline, 0.1 ml. i.m., days 11, 12, 13, 14 Cobaltous chloride, 25 mg./Kg. i.p., day 11	26	13	208	27	12.9

placed in a breeding cage with a male of the same strain at 4 p.m. every Wednesday and Thursday. The following morning (9:00–9:30 a.m.), females were returned to their own cages and those with vaginal plugs were considered pregnant and put aside in individual cages. The morning of the appearance of the vaginal plug was designated day 0 (gestation period 0–19 days). Pregnancy was confirmed by the weight gain which becomes apparent on or after day 8, or the placental sign which appears on day 10. The diet consisted of Purina laboratory chow and tap water, both given *ad libitum*. Food was placed in ample amounts on the floor of each cage and water was obtained through stainless steel drinking tubes which were attached to 4-oz. bottles. Excreta pans were arranged to permit coprophagy. Care was taken to minimize drafts and noise, and environmental temperature was maintained at 25° throughout the experiment.

Pregnant animals were divided into 5 groups comprising 26 mice each and subjected to the following treatment.

Group A—Each mouse received an intramuscular injection of 2.5 mg. of cortisone acetate[1] on days 11, 12, 13, and 14.

Group B—Each animal received an intraperitoneal injection of cobaltous chloride, 25 mg./Kg. (10% aqueous solution), on day 11 only followed by cortisone acetate, 2.5 mg. i.m., on days 11, 12, 13, and 14.

Group C—Each animal received an intramuscular injection of physiological saline solution, 0.1 ml., on days 11, 12, 13, and 14.

Group D—Untreated controls.

Group E—Each animal received an intraperitoneal injection of cobaltous chloride, 25 mg./Kg., on day 11 only followed by saline, 0.1 ml. i.m., on days 11, 12, 13, and 14.

All compounds were injected with a 26-gauge, 0.5-in. needle attached to a 1-ml. syringe which had been previously rinsed with sterile water for injection.

A single injection of the cobalt compound was administered intraperitoneally at 9:30 a.m. on the eleventh day of gestation. Injections of cortisone acetate were administered 5 min. after the cobalt administration on day 11. Subsequent injections of the steroid on days 12, 13, and 14 were also given intramuscularly, alternating the sites of injections from one hind leg to the other (biceps femoris muscle).

[1] Merck Sharp and Dohme, West Point, Pa.

EXAMINATION OF FETUSES

On day 18, which is 12 to 24 hr. prior to expected delivery, the mice were sacrificed by cervical dislocation and laparotomized immediately.

The abdominal wall of the mother was incised from the vaginal opening to an area just below the xiphihumeralis along the linea alba. Incisions were also made diagonally through the rectus abdominis, obliquus externus, and transverse abdominis to form a flap.

Before opening the uterine horns, fetal swellings and resorption sites (metrial glands) were recorded and the uterus was excised for removal of the fetuses. Before weighing and sexing, the fetuses were placed on blotting paper to remove excessive amniotic fluid, whereupon they were observed under an Ednalite magnifier for the presence of gross anomalies (2 × magnification).

Following the preliminary examination, the fetuses were prepared for further study. In order to evaluate skeletal structure, every third fetus was immersed in a modified clearing and staining solution according to the Staples and Schnell modification of the Spaltheholtz method (7). The remainder of the fetuses was fixed in Bouin's solution for 2 weeks in preparation for free hand razor blade sectioning as described by Wilson (8). This method allows for the evaluation of malformations in a large number of animals with a minimum expenditure and delay.

RESULTS

The incidence of cleft palate obtained for the various groups of mice at the termination of this study is recorded in Table I as follows.

Group A—Twenty-six pregnant mice which received cortisone acetate, 2.5 mg. i.m., on days 11, 12, 13, and 14 of gestation responded with a

75.6% incidence of fetal cleft palate. All of these litters had some clefts, the total number of fetuses having been examined was 269, with 3 born dead (84 of these were placed in alizarin red S solution for bone staining). Average weights of pregnant mice and fetuses were 46.2 and 0.86 Gm., respectively; number of resorptions was 49.

Group B—Twenty-six pregnant mice which received cobaltous chloride ($CoCl_2.6H_2O$), 25 mg./Kg. i.p., on day 11 only and cortisone acetate, 2.5 mg. i.m., on days 11, 12, 13, and 14, had a 12.6% incidence of cleft palate. Only six litters in this group had clefts. The total number of fetuses examined was 259, with 3 born dead (69 of these were placed in the alizarin red S solution for bone staining). The average weights of pregnant mice and fetuses were 44.3 and 1.10 Gm., respectively; number of resorptions was 38.

Group C—Twenty-six pregnant mice received physiological saline solution, 0.1 ml. i.m., on days 11, 12, 13, and 14 of gestation. None of these litters had cleft palate; however, one fetus was exencephalic. The total number of fetuses examined was 288, with 3 born dead (87 of these were placed in alizarin red S solution for bone staining). The mean weights of pregnant mice and fetuses were 51.7 and 1.21 Gm., respectively; the number of resorptions was 13.

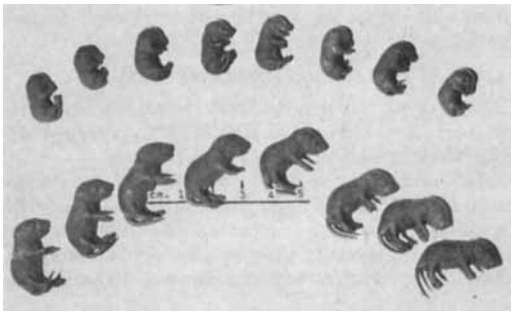


Fig. 1—Key: top, growth retardation produced by cortisone acetate, 2.5 mg. i. m.; bottom, inhibition by cobaltous chloride pretreatment, 25 mg./Kg. i. p.

Group D—This group of 26 pregnant mice was untreated and showed no incidence of cleft palate. The mean weights of pregnant mice and fetuses were 52.8 and 1.47 Gm., respectively; the number of resorptions was 13.

Group E—The final group of 26 pregnant mice received cobaltous chloride, 25 mg./Kg i.p., on day 11 only and saline solution, 0.1 ml. i.m., on days 11, 12, 13, and 14. Thirteen of these litters had some fetuses with clefts. The total number of fetuses examined was 293 (85 of these were placed in alizarin red S solution for bone staining). The incidence of cleft palate, average weights of pregnant mice and fetuses, and number of resorption sites were 12.9%, 49.6 and 0.99 Gm., and 31, respectively. A high incidence of complete fetal resorptions was observed in this group, 13 of the 26 animals.

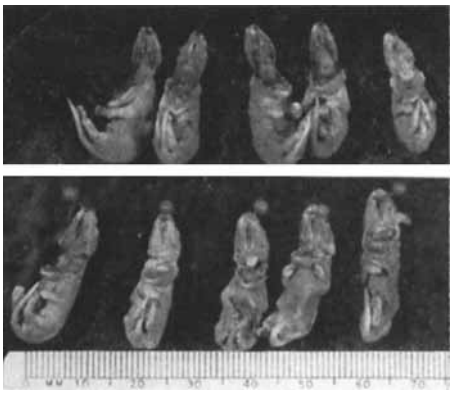


Fig. 2—Key: top, cleft palate induced by cobaltous chloride, 25 mg./Kg. i. p.; bottom, cleft palate induced by cortisone acetate, 2.5 mg. i. m.

DISCUSSION

The original premise which prompted this study concerning cobalt–steroid interrelationships in modifying fetal development was based upon recent findings during experiments with methylcholanthrene carcinogenesis in mice that two inorganic cobalt compounds, cobaltous chloride (2) and sodium cobaltinitrite (3, 4), manifested cortisone-like characteristics (*i.e.*, anti-inflammatory and antitumorigenic actions). The results (Table I) reveal additional similarities such as growth retardation, as denoted by group *A* (cortisone) and group *E* (cobaltous chloride–saline) having the lowest mean fetal weights, and cleft palate induction (Fig. 2 and Table I) by cobaltous chloride alone, and the prevention of both of these events by the cobalt compound in the presence of cortisone (Fig. 1 and Table I). It appears that the cobalt–steroid interaction may occur at the same site through competitive inhibition, for each alone induces clefts while together marked inhibition occurs. It should be noted, however (Fig. 2), that cobaltous chloride alone does not produce a macroscopically identical fissure to that caused by cortisone. On the contrary, cortisone-induced cleft palate is measurably more pronounced than that produced by cobaltous chloride. Whether this reflects a dosage relationship or difference in mechanism of action from that of cortisone remains to be clarified.

REFERENCES

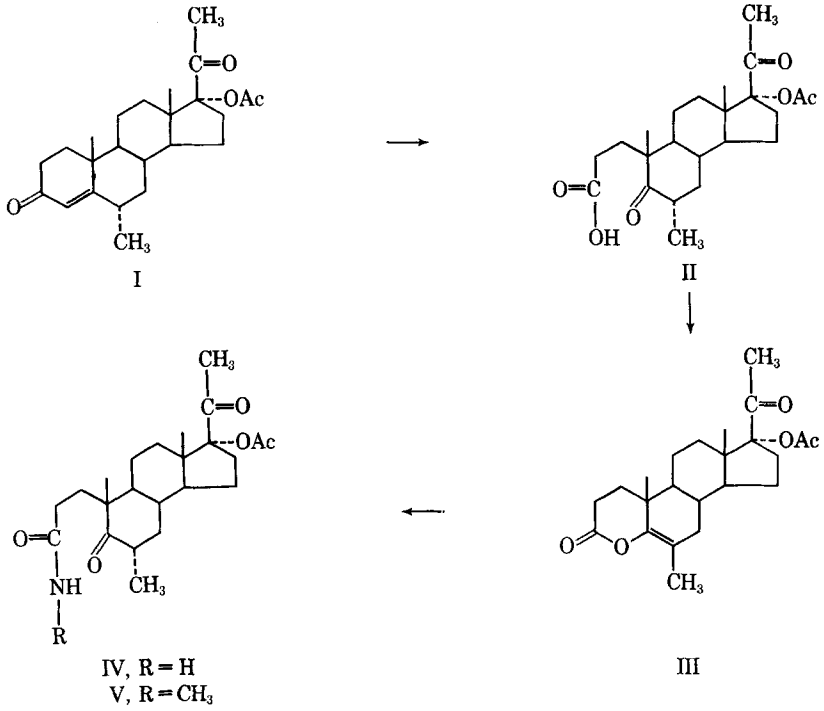
(1) Mancini, R. T., Gautieri, R. F., and Mann, D. E., Jr., *J. Pharm. Sci.*, **53**, 385(1964).
(2) Kasirsky, G., Gautieri, R. F., and Mann, D. E., Jr., *ibid.*, **54**, 491(1965).
(3) Thompson, R. S., Gautieri, R. F., and Mann, D. E., Jr., *ibid.*, **54**, 595(1965).
(4) Orzechowski, R. F., Gautieri, R. F., and Mann, D. E., Jr., *ibid.*, **54**, 64(1965).
(5) Fisher, J. W., *J. Pharmacol. Exptl. Therap.*, **132**, 232(1961).
(6) Fraser, F. C., and Fainstat, T. D., *Pediatrics*, **8**, 527(1951).
(7) Staples, R. E., and Schnell, V. L., *Stain Technol.*, **39**, 61(1964).
(8) Wilson, J. G., and Warkany, J., "Teratology, Principles and Techniques," University of Chicago Press, Chicago, Ill., 1965, pp. 271–277.

Secosteroids as Progestational Agents

By ARVIN P. SHROFF

This paper describes the synthesis of a few secosteroids. Their proton magnetic resonance spectra in $CDCl_3$ and C_6H_6 were investigated to evaluate the Δ values of the C-18, C-19, and C-6 methyl groups. The progestational activity of these compounds as evaluated in the Clauberg test is also described.

PROGESTATIONAL AGENTS have come into prominence in the last decade because of greatly increased potency. This has been accomplished through alteration of the basic structure of the progesterone or testosterone molecule. These include dehydrogenation, demethylation, or introduction of a methyl group, hydroxyl group, acetoxy group, and/or halogen atoms. At first interest was in the replacement of a carbon atom of the cyclopentanoperhydrophenanthrene system by nitrogen. A number of compounds resulting from these types of modification have been reported to be of potential biological interest (1).



Scheme I

Ozonization of the α,β-unsaturated ketone, 17α-acetoxy-6α-methylpregn-4-ene-3,20-dione (I), followed by oxidative decomposition of the ozonide with hydrogen peroxide gave 17α - acetoxy - 6α-methyl-3,5-seco-4-norpregna-5,20- dion - 3 - oic acid (II). (Scheme I.) The configuration of C-6 methyl was confirmed by PMR. The resonance peaks for C-6 methyl in deuterochloroform and benzene (Table 1) did not show any shift ($\Delta = 0$) and this indicates equatorial (α) conformation (2). The C-19 axial methyl, on the other hand, shows a significant shift ($\Delta = 0.38$) as observed by Williams and Baccha (2). The keto acid (II) was refluxed in a mixture of acetic anhydride and acetyl chloride (3) to give the lactone, 4 - oxa - 17α - acetoxy - 6 - methylpregn - 5 - ene-3,20-dione (III). The C-6 methyl group was deshielded, as expected by 0.70 p.p.m. (in $CDCl_3$). Shielding of C-19 methyl to the extent of 0.33 p.p.m. indicates that the benzene–solute complex formation occurred to the same extent as in keto acid (II). Treatment of lactone (III) with ammonia and methylamine gave the open chain amides (IV and V). The Δ values for C-19 and C-6 methyl groups suggest that the C-6 methyl group is equatorial. The infrared and ultraviolet data, along with PMR, confirm the assigned structures for IV and V.

Biological Data—Progestational activity of these compounds (II–V) was quantitatively evaluated in the Clauberg test as described earlier (4). The dose and the McPhail index are recorded in Table II.

Received April 14, 1967, from the Division of Organic Chemistry, Ortho Research Foundation, Raritan, NJ 08869
Accepted for publication June 28, 1967.
Presented to the Medicinal Chemistry Section, A. PH. A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The author thanks Dr. R. P. Blye, Division of Pharmacology, Ortho Research Foundation, for biological data and Miss Elizabeth Warren for technical assistance.

TABLE I—Δ VALUES ($CDCl_3$–C_6H_6)[a] OF SECOSTEROIDS

Compd.	C_{18} $CDCl_3$	C_{18} C_6H_6	Δ	C_{19} $CDCl_3$	C_{19} C_6H_6	Δ	C_6 $CDCl_3$	C_6 C_6H_6	Δ
II	0.72	0.55	0.17	1.10	0.72	0.38	0.97	0.97	0
III	0.67	0.53	0.14	1.08	0.76	0.32	1.67	1.65	0.02
IV	0.63	0.56	0.07	1.05	0.77	0.28	0.97	0.95	0.02
V	0.62	0.60	0.02	1.03	0.70	0.33	0.84	0.91	0.13

[a] Values are in p.p.m. units.

TABLE II—PROGESTATIONAL POTENCY OF SECOSTEROIDS

Compd.	Total Dose, mg.	McPhail Index
II	5.0	0
III	5.0	0.3
IV	5.0	3.17
V	5.0	0.1

EXPERIMENTAL

All melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected. The ultraviolet and infrared data were obtained on Cary model 11 and Beckman IR-5 spectrophotometers, respectively. The proton magnetic resonance spectra were determined on a Varian A-60 spectrometer, in deuterochloroform and benzene, using tetramethylsilane as an internal standard (0 p.p.m.). All p.p.m. values are the centers of the signals. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

17α - Acetoxy - 6α - methyl - 3,5 - seco - 4 - norpregna-5,20-dion-3-oic Acid—A solution of 5.0 Gm. (0.013 mole) of 17α-acetoxy-6α-methylpregn-4-ene-3,20-dione in 30 ml. of ethyl acetate and 100 ml. of glacial acetic acid was cooled in an ice-water bath and ozonized for 1.5 hr. To this 5 ml. of 30% hydrogen peroxide was added, followed by 2000 ml. of water. The mixture was refrigerated for 2 hr. before adding an additional 100 ml. of water. The white semisolid was collected by filtration and dissolved in ether. The ethereal solution was extracted several times with 5% sodium hydroxide. The extracts were combined and acidified with concentrated hydrochloric acid. The cloudy solution was once again extracted with ether and the ether layer was washed several times with water until neutral to litmus. It was dried over sodium sulfate and evaporated to give a semisolid which could be recrystallized from ether–hexane to give 4.5 Gm. (79%) of 17α-acetoxy-6α-methyl-3,5-seco-4-norpregna-5,20-dion-3-oic acid, m.p. 216–218°. $[\alpha]_D$ +8.6° (1.0 c $CHCl_3$). $\lambda_{max.}^{CHCl_3}$ 2.81 μ, 5.77 μ, 5.85 μ. PMR in $CDCl_3$, 0.72 p.p.m. (C-18); 1.10 p.p.m. (C-19); 0.97 p.p.m. (C-6).

Anal.—Calcd. for $C_{23}H_{34}O_6$: C, 67.95; H, 8.43. Found: C, 68.13; H, 8.44.

4 - Oxa - 17α - acetoxy - 6 - methylpregn - 5 - ene-3,20-dione—A solution of 1.0 Gm. (0.0025 mole) of 17α-acetoxy-6α-methyl - 3,5 - seco - 4 - norpregna-5,20-dion-3-oic acid in 12 ml. of acetic anhydride and 6 ml. of acetyl chloride was refluxed for 64 hr. The solvent was removed under reduced pressure and the solid residue was dissolved in ether. The ethereal solution was washed several times with 5% potassium hydroxide solution followed by water until neutral to litmus. It was then dried over sodium sulfate and evaporated. The solid residue was recrystallized from ether to give 800 mg. (84%) of 4-oxa-17α-acetoxy-6-methylpregn-5-ene-3,20-dione, m.p. 204–206°. $[\alpha]_D$ −77.9° (1.0 c $CHCl_3$) $\lambda_{max.}^{KBr}$ 5.75 μ, 5.82 μ. PMR in $CDCl_3$, 0.67 p.p.m. (C-18); 1.08 p.p.m. (C-19); 1.67 p.p.m. (C-6).

Anal.—Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.83; H, 8.37.

17α - Acetoxy - 6α - methyl - 3,5 - seco - 4 - norpregna - 5,20 - dione - 3 - carboxamide — 4 - Oxa-17α - acetoxy - 6α - methylpregn - 5 - ene - 3,20-dione (1.0 Gm., 0.0025 mole) was dissolved in 10 ml. of absolute alcohol saturated with ammonia and allowed to remain in the icebox for 3 days. The solution was added to a large amount of ice and water and extracted with ethyl acetate. The ethyl acetate layer was washed several times with water, dried over sodium sulfate, and evaporated to give a semisolid which was recrystallized from ethyl acetate–hexane. Analytically pure 17α-acetoxy-6α-methyl-3,5 - seco - 4 - norpregna - 5,20 - dione - 3-carboxamide (650 mg., 63%), m.p. 120–123°, was obtained by recrystallization from hexane–acetone. $[\alpha]_D$ +41.5° (1.0 c $CHCl_3$), $\lambda_{max.}^{KBr}$ 2.95 μ, 5.78 μ, 5.84 μ, 5.03 μ. PMR in $CDCl_3$, 0.63 p.p.m. (C-18); 1.05 p.p.m. (C-19); 0.97 p.p.m. (C-6).

Anal.—Calcd. for $C_{23}H_{35}NO_5$: C, 68.12; H, 8.70; N, 3.45. Found: C, 68.08; H, 9.03; N, 3.18.

17α - Acetoxy - 6α - methyl - 3,5 - seco - 4 - norpregna - 5,20 - dione - 3 - carboxmethylamide—4 - Oxa - 17α - acetoxy - 6 - methylpregn - 5 - ene-3,20-dione (600 mg., 0.0015 mole) was dissolved in 10 ml. of absolute alcohol saturated with methylamine and allowed to stand in the icebox for 3 days. The solution was poured into ice and water and extracted with methylene chloride. The organic layer was washed with dilute hydrochloric acid, followed by water, dried over sodium sulfate, and evaporated to give an oil. This was recrystallized from ethyl acetate–pentane to give 394 mg. (61%) of 17α - acetoxy - 6α-methyl-3,5-seco-4-norpregna- 3,20-dione-3-carboxymethylamide, m.p. 90–92°. $[\alpha]_D$ +29.7° (1.0 c $CHCl_3$). $\lambda_{max.}^{KBr}$ 2.95 μ, 5.76 μ, 5.86 μ, 6.05 μ. PMR in $CDCl_3$, 0.62 p.p.m. (C-18); 1.03 p.p.m. (C-19); 0.91 p.p.m. (C-6); 2.80 p.p.m. (N-CH_3); 5.95 p.p.m. (NH). No absorption in the ultraviolet.

Anal.—Calcd. for $C_{24}H_{37}NO_5$: C, 68.70; H, 8.89; N, 3.34. Found: C, 68.92; H, 8.83; N, 3.76.

REFERENCES

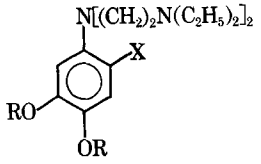
(1) Doorenbos, N. J., *Drug Trade News*, September 3, 1962; Martin-Smith, M., and Sugrue, M. F., *J. Pharm. Pharmacol.*, **16**, 569(1964) and references therein.
(2) Williams, D. H., and Bhacca, N. S., *Tetrahedron*, **21**, 2021(1965).
(3) Turner, R. B., *J. Am. Chem. Soc.*, **72**, 579(1950).
(4) Shroff, A. P., *J. Med. Chem.*, **8**, 881(1965).

Synthesis and Physical Properties of the Antimalarial Agent 4-(2-Bromo-4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine (RC-12) (WR-27,653)

By LESLIE M. WERBEL, EDWARD F. ELSLAGER, MARLAND P. HUTT, and J. M. VANDENBELT

Several synthetic routes to the antimalarial agent 4-(2-bromo-4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine (RC-12) (WR-27,653) were evaluated. The preferred scheme involved alkylation of 4-aminoveratrole with excess 2-chlorotriethylamine in dimethylformamide at 140–145° to give 4-(4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine, which was brominated in acetic acid to give the desired product. The physical and biological properties of the drug are summarized.

SEVERAL DECADES ago it was discovered that certain pyrocatechol amine derivatives possess interesting antimalarial properties (1, 2). Among them, dimeplasmin (I*a*) was reported to be as active as pamaquine against trophozoite-induced *Plasmodium cathemerium* and *P. relictum*, and to be substantially less toxic for the mouse, rat, dog, and cat. However, dimeplasmin was clearly less active than quinine in controlling parasitemia and fever resulting from *P. falciparum* infections in man (3–5). Results



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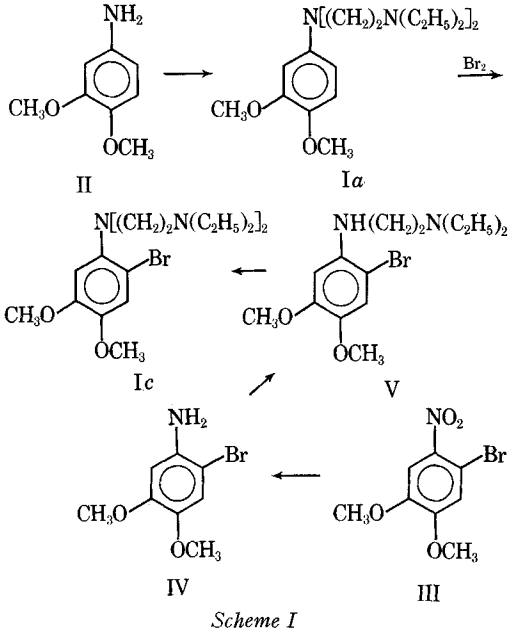
a, X = H; R = CH_3 (dimeplasmin)
b, X = Br; R = CH_2CH_3 (diapromin)
c, X = Br; R = CH_3 (RC-12)

of limited clinical studies with the related compound diapromin (I*b*) were likewise disappointing (6). Therefore, interest in this class of compounds waned.

More recently, interest in the antimalarial properties of the pyrocatechol amines was reawakened by the demonstration that 4-(2-bromo-4,5-dimethoxyphenyl) - 1,1,7,7 - tetraethyldiethylenetriamine (I*c*) (RC-12) had a pronounced effect on the exo-erythrocytic stages of *P. cathemerium* in canaries (7, 8). This observation stimulated an appraisal of the antimalarial effects of this agent against *P. cynomolgi* in the monkey (9). Although the drug *per se* had little promise as a schizonticidal or suppressive agent in monkeys, it showed significant promise as a prophylactic or radical curative agent. These authors concluded that RC-12 might find use when: (*a*) the combination of chloroquine–primaquine is not effective in causal prophylaxis, as appears to be the case where there is chloroquine resistance; (*b*) there is a need for a curative agent which can produce benefits in less than 10–14 days; and (*c*) there are fears of enhanced susceptibility to the hematotoxicity of primaquine (9). In anticipation that RC-12 might prove useful in connection with malaria problems encountered by the armed forces in Southeast Asia, the U. S. Army Medical Research and Development Command requested that suitable methods be developed for the laboratory synthesis and characterization of this substance. (Scheme I.)



Scheme I

General methods for the preparation of various pyrocatechol amines were described many years ago by Schulemann and Kropp (1), but to our knowledge the synthesis and properties of I*c* have not been described in the literature. In the present work several synthetic approaches to I*c* were evaluated. Among them, the following route proved to be most useful: alkylation of 4-aminoveratrole (II) with excess 2-chlorotriethylamine in dimethylformamide at 140–145° gave, in 57% yield, 4-(4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine (dimeplasmin) (I*a*) which was 98–100% homogeneous by vapor phase chromatographic analysis. Attempted bromination of I*a* with bromine in carbon tetrachloride or with *N*-bromosuccinimide was unsuccessful; however, bromine in acetic acid at 55-60° afforded

Received April 19, 1967, from the Research Laboratories Parke, Davis and Co., Ann Arbor, MI 48106
Accepted for publication July 5, 1967.
This investigation was supported by contract DA-49-193-MD-2754 from the U. S. Army Medical Research and Development Command, Washington, D. C.
WR-27,653 is the code number assigned to this drug by the Walter Reed Army Institute of Research.
The authors thank Mr. C. E. Childs and his colleagues for the microanalytical and vapor phase chromatography data.

essentially homogeneous *Ic* in 41% yield. The material thus obtained was identical in all respects with an authentic sample of RC-12 base. The amounts of *Ia* and *Ic* formed in these reactions, as indicated by GLC analyses, were considerably larger than he amounts of pure materials isolated, and the use of more sophisticated distillation equipment would undoubtedly increase yields substantially.

Alternatively, alkylation of 2-bromo-4,5-dimethoxyaniline (IV) (10) with a slight excess of 2-chlorotriethylamine at 90–100° for 2.5 hr. (1) gave a mixture of 37% unchanged aniline (IV), 58% of the monoalkylated product (V), and 5% of *Ic* based on vapor phase chromatographic analysis. Extending the heating time to 27 hr. gave a mixture of 90% V and 10% *Ic*. Treatment of the latter mixture with 2-chlorotriethylamine at 130–140° for 10 hr. increased the amount of *Ic* in the mixture only to 25%. An attempt to bisalkylate IV directly with an excess of 2-chlorotriethylamine at 140–150° for 18 hr. gave a complex mixture which contained between six and eight components based on VPC analysis. The 2-bromo-4,5-dimethoxyaniline (IV) (10) used as starting material was prepared by bromination of either 4-amino-1,2-dimethoxyaniline or the corresponding acetamide, or preferably by catalytic reduction of 4-bromo-5-nitro-1,2-dimethoxybenzene (III) (11).

The infrared and NMR spectra of RC-12 appear quite normal. The absence of any obvious coupling of the aromatic protons, *cf.* clean doublet at 6.71, 6.91 p.p.m. in the NMR spectrum (in CCl_4 with tetramethylsilane as internal standard), supports the existence of 2 *para* protons and thus the structural orientation as proposed (*Ic*).

The ultraviolet absorption spectrum of RC-12 is similar to that of simpler analogous structures (Table I). In methanol, the spectrum has a secondary band (*13*) at lower wavelength and lower intensity than the corresponding amine IV. This shift to lower wavelength reflects the well known phenomenon of inhibition of resonance interaction between the tertiary aromatic amine and the benzene ring by the bromine in the *ortho* position (12). The primary band is not resolved. The authors have observed that bromobenzenes substituted in the *ortho* position by very weak donor groups characteristically show intense absorption at lower wavelength, in which this band may not be resolved.

The spectra of the cationic forms of compounds IV and *Ic* should be like that of 4-bromoveratrole, since the cation cannot resonate with the ring. This is evident for compound IV which shows a characteristic shift with acid to lower wavelength, with band wavelengths and intensities similar to those of 4-bromoveratrole. RC-12 (*Ic*), on the other hand, shifts very little with acid, indicating that the aromatic amine has not been affected. Attempts to protonate the aromatic amine in very strong acid were only partially successful. In 25% concentrated sulfuric acid, the secondary band shifted several millimicrons, and resolution of the primary band occurred. In still stronger acid, a slight further shift occurred, but other effects appeared also, including darkening of the solutions.

The basicity of the aromatic amine in RC-12 is thus seen to be strongly decreased as a result of its s ructural orientation. The ionization constants determined experimentally are in accord with this conclusion. Introduction of bromine *ortho* to the amine in II reduces the pK'a from 4.6 to 2.6 (IV) (Table I). Introduction of the basic side chain also decreases the basicity of the aromatic amine, as indicated by a pK'a of about −0.1 for compound *Ia*, determined spectrophotometrically. Both these effects would be operative in *Ic*. The predicted pK'a is as low as −2, a value beneath the pH to which the compound could be subjected without development of other effects.

EXPERIMENTAL

Melting points are corrected. Vapor phase chromatograms were run on an F & M No. 810 instrument using a 6-ft. × 1/4-in. 5% SE-30 on Anakrom ABS 90-100 mesh column at 250° with a nitrogen carrier gas flow rate of 60 ml./min.

Preparation of 2-Bromo-4,5-dimethoxyaniline (IV)—A solution of 13.1 Gm. (0.05 mole) of 4-bromo-5-nitro-1,2-dimethoxybenzene (Eastman Kodak) in 150 ml. of tetrahydrofuran was hydrogenated over 2 Gm. of Raney nickel at 25° and an initial pressure of 49.5 psig. The catalyst was removed by filtration and the solvent removed *in vacuo*. The residue was taken up in ether, the ether solution was filtered to remove some insoluble material, and dry hydrogen chloride was bubbled into the ether filtrate. The white precipitate was removed by filtration, washed with ether, and dried *in vacuo* at 50°

TABLE I—ULTRAVIOLET[a] AND pK'a DATA ON RC-12 AND INTERMEDIATES

	Solvent	λ	ε	λ	ε	pK'a[b]
4-Bromoveratrole	MeOH	283	2,800	232	8,900	...
II	MeOH	292	3,000	236	7,900	4.6
ortho-Bromoaniline	MeOH	293	2,600	236	8,200	
IV·HCl	MeOH, 0.05 *N* in NaOH	304	4,100	237	8,800	2.6
	MeOH, 0.05 *N* in HCl	284	3,300	234	9,200	
Ia	MeOH	309	2,800	256	10,900	7.6, 9.1 ~−0.1[c]
Ic	MeOH	292	3,300	Unresolved		7.7, 9.2
	MeOH, 0.05 *N* in HCl	290	3,300	Unresolved		
	MeOH, 25% Conc. H_2SO_4	286	3,600	238	8,500	

[a] Ultraviolet spectra were determined on a Cary model A recording spectrophotometer. [b] By potentiometric titration in 50% methanol (v/v). [c] By spectrophotometric determination in 20% methanol.

to give 10.2 Gm. (76%) of 2-bromo-4,5-dimethoxyaniline hydrochloride, m.p. 203-204° dec. A portion recrystallized from ethanol gave m.p. 202.5–203.5° dec.

Anal.—Calcd. for $C_8H_{10}BrNO_2 \cdot HCl$: C, 35.78; H, 4.13; N, 5.22. Found: C, 36.11; H, 4.30; N, 5.10.

Preparation of 4-(4,5-Dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine (Ia)—A mixture of 99.2 Gm. (0.647 mole) of 4-aminoveratrole (Hi Laboratories), 387.7 Gm. (2.59 moles) of freshly distilled chlorotriethylamine (generated from the hydrochloride salt),[1] and 165 ml. of dimethylformamide was heated with stirring for 20.5 hr.[2] by means of an oil bath kept at 140–145°. After cooling to room temperature, the reaction mixture was treated with 10% aqueous sodium hydroxide solution[3] and chloroform.[4] The chloroform extract was dried over sodium sulfate and the chloroform removed on a rotary evaporator. The resulting oil was suspended in 10% aqueous sodium hydroxide solution[5] and extracted with ether.[6] The ether solution was dried over sodium sulfate and the ether removed on a rotary evaporator. The residual crude product (196 Gm.) was distilled under vacuum through a 6-in. Vigreaux column[7] to give 128.8 Gm. (57%) of 4-(4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine, b.p. 163–165°/0.17–0.20 mm., shown by vapor phase chromatography to be 98–100% homogeneous.

Anal.—Calcd. for $C_{20}H_{37}N_3O_2$: C, 68.33; H, 10.61; N, 11.95. Found: C, 67.99; H, 10.54; N, 11.89.

Preparation of 4-(2-Bromo-4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine (Ic)—To a solution of 82.2 Gm. (0.234 mole) of 4-(4,5-dimethoxyphenyl)-1,1,7,7-tetraethyldiethylenetriamine in 500 ml. of glacial acetic acid was added dropwise with stirring at room temperature 41.0 Gm. (0.256 mole) of bromine.[8] The addition required 50 min., and the final temperature of the solution was 41°. The mixture was stirred for 1 hr. at room temperature and then for 1 hr. at 55–60°. The acetic acid was removed on the rotary evaporator. The cooled residue was made alkaline with a 50% aqueous sodium hydroxide solution[9] and extracted first with ether and then with benzene.[10] The extracts were washed with water[11] and dried over sodium sulfate. The solvents were removed *in vacuo* leaving about 82 Gm. of crude product which was subjected to distillation with an oil diffusion pump.[12] After removing the forerun up to 150°/0.005 mm., two fractions, b.p. 150–151°/.005–.001 mm. (28.0 Gm.), were collected which were shown by vapor phase chromatography to contain about 95% of Ic. Two additional fractions, b.p. 149–150°/<.001 mm. (28.5 Gm.) and 151.5–149°/.001– <.001 mm. (13.1 Gm.), were collected and shown to be essentially homogeneous Ic (98–99%). These two fractions represent a yield of 41%. The product is a yellow-brown viscous liquid with a retention time of 12.25 min. under the conditions of vapor phase chromatography indicated above.

Anal.—Calcd. for $C_{20}H_{36}BrN_3O_2$: C, 55.81; H, 8.43; Br, 18.57; N, 9.76. Fraction b.p. 149–150°/<.001 mm. Found: C, 55.75; H, 8.41; Br, 18.75; N, 9.68. Fraction b.p. 151.5–149°/.001– <.001 mm. Found: C, 55.96; H, 8.61; Br, 18.80; N, 9.62.

The NMR spectrum of RC-12 in CCl_4 was determined on a Varian A-60 using tetramethylsilane as an internal standard. The peaks (p.p.m.) integrate for the following relative intensities: singlet 6.71 (1H), singlet 6.91 (1H), singlet 3.75 (6H), multiplet 3.00 (4H), multiplet 2.43 (12H), triplet 0.93 (12H).

[1] Shirley, D. A., "Preparation of Organic Intermediates," John Wiley & Sons, Inc., New York, N. Y., 1951.

[2] Attempted fusion of a model monoalkylated material with 2 equivalents of diethylaminoethyl chloride at 140° up to 14 hr. resulted in only 56% conversion to the bisalkylated material.

[3] About 200–300 ml. of 10% sodium hydroxide was used.

[4] The mixture was extracted four times with 250-ml. portions of chloroform.

[5] About 200 ml. was used. This is to remove any inorganic material which has been carried into the chloroform extract. The presence of small amounts of this material leads to difficulties in the distillation.

[6] Extracted four times with 250-ml. portions of ether.

[7] The pot was heated with an oil bath (silicone) maintained at 200–250°. The oil bath was heated by an Agit-Therm magnetic stirrer which also served to agitate a stirring bar in the distillation pot.

[8] The beaker used to weigh out the bromine was rinsed with 10 ml. of acetic acid and this was added to the bromine in the dropping funnel. Room temperature was approximately 26°.

[9] About 50 ml. was used.

[10] Two extractions using about 250 ml. of ether each followed by two extractions with 250-ml. portions of benzene.

[11] Two portions of approximately 250 ml. each.

[12] A 4-in. Vigreaux column heated with an electric heating tape was used for this distillation. The pot was heated (200–250°) and stirred as described in *Footnote 7*.

REFERENCES

(1) Schulemann, W., and Kropp, W., U. S. pat. 1,757,394 (May 6, 1930).
(2) Schönhöfer, F., "Chemotherapy," *Office Tech. Services Rept.*, PB-85033, 1948; FIAT, *Review of German Science*, 43.
(3) Green, R., *Lancet*, **217**, 1137(1929).
(4) Green, R., *Bull. Inst. Med. Res. Federation Malaya*, **28**, 29(1929).
(5) Sinton, J. A., *Indian J. Med. Res.*, **17**, 815(1930).
(6) Coppock, J. B. M., "The Pharmaceutical Industry in Germany During the Period 1939–1945," *Office Tech. Services Rept.*, PB-101472 1950; BIOS, Rept., No. 24.
(7) Schulemann, W., World Health Organization, unpublished report, 1963.
(8) Bruce-Chwatt, L. J., *Trans. Roy. Soc. Trop. Med. Hyg.*, **59**, 105(1965).
(9) Schmidt, L. H., Rossan, R. N., Fradkin, R., Woods, J., Schulemann, W., and Kratz, L., *Bull. World Health Organ.*, **34**, 783(1966).
(10) Simonsen, J. L., and Rau, M. G., *J. Chem. Soc.*, **1918**, 782.
(11) Pettit, G. R., and Piatak, D. M., *J. Org. Chem.*, **25**, 721(1960).
(12) Remington, W. R., *J. Am. Chem. Soc.*, **67**, 1838(1945).
(13) Doub, L., and Vandenbelt, J. M., *ibid.*, **69**, 2714 (1947).
(14) Parke, T. V., and Davis, W. W., *Anal. Chem.*, **26**, 642(1954).

Bioassay of Different Formulations of Aspirin by Means of Human EEG

By CARL C. PFEIFFER, LEONIDE GOLDSTEIN, HENRY B. MURPHREE, and MARTHA HOPKINS

Several commercial formulations of aspirin were compared for potency and duration of action. Quantitative human EEG was employed as the bioassay. The effects of aspirin as determined by this technique are highly reproducible and dose-related, resembling qualitatively the effects of common "antianxiety" drugs. The results show that differences in formulation lead to differences in pharmacological effect.

INNUMERABLE claims appear continually about the relative efficacy and speed of action of different formulations of aspirin. Careful evaluation of these is important, because aspirin is probably the most heavily consumed of all drugs. Serum salicylate concentrations give information about absorption from the gastrointestinal tract into the blood stream, but are not directly informative about the time course of the concentrations of aspirin at its pharmacological sites of action. The latter can be determined only by means of some kind of bioassay, preferably in humans, since man is the species of interest where the consumption of this drug is concerned. Aspirin does not reliably influence pain thresholds or other varieties of experimentally induced pain which can be employed in humans, although some information has been gained from dogs by means of the intra-arterial bradykinin technique of Lim (1). Clinical pain can be demonstrably influenced by aspirin, but not with sufficient precision to differentiate among various formulations. Therefore, a new approach has been needed to delineate with sensitivity and accuracy the time course and potencies of aspirin preparations in their pharmacological actions in humans.

The authors have reported previously (2) that aspirin has characteristic, highly reproducible effects upon the quantitative electroencephalograms (EEG) of normal human volunteers. The present report describes the results obtained when the earlier trials were replicated with larger numbers of subjects given varying doses and when different commercial preparations of aspirin were compared. Some related drugs were again studied concurrently for comparison.

METHODS

The method employed quantitative analysis of the EEG, recorded "monopolarly" from the left occipital area. Fifty-four subjects, 31 male reformatory inmates and 16 male and 7 female members of the laboratory staff, ages 18 to 30, took part in a total of 279 experimental sessions. During the first hour of a standard 7-hr. session, the electrodes were affixed and a 10-min. recording was obtained. Immediately after this, the drug (or placebo) was administered orally. Thereafter, 10-min. recordings were made every hour for 6 hr. During all recordings the subjects were reclining with eyes closed in a dimly lighted, quiet room. Between recordings, the subjects were free to move around or to sit and read in a lounge adjoining the EEG recording room.

Quantitation of the EEG was performed with an electronic integrator (3, 4). This device transforms the complex EEG signals into pulses which are recorded concomitantly with the direct tracings. The number of pulses for any given time period is directly proportional to the cumulated electrical energy. Calibration is by the application of known energy constants, and the values obtained therefore can be related to fixed standards. The basic time-unit chosen for analysis was 20 sec. (that is, 2 pages of standard EEG); thus any 10-min. recording yielded 30 successive measurements. The number of pulses during each 20-sec. period was automatically totalized and printed on paper tape. Artifacts were easily removed from the counts since each set of numbers could be related to the page of direct recording from which it was obtained.

All the corresponding values from each predrug and postdrug run, as obtained from all subjects involved in each study, were averaged, and the mean energy content (MEC) for the group was thus determined. Observed differences in MEC were tested for statistical significance by means of the t test. In addition, a careful analysis of variability within subjects was performed for each time period. It has been previously shown (5) that this parameter of quantitated EEG data is highly informative, not only for the detection and characterization of drug effects, but also for baseline features. For example, it has been found that male chronic schizophrenics have much less EEG variability than nonpsychotics (5). Statistical significances of the differences in variability were determined from F ratios. The within-subject variabilities are shown in the tables as coefficients of variation (CV). This is to allow averaging of the variability in the records of subjects who have different MEC values.

Since the drugs were ordinary commercial preparations obtained locally, it was not always possible to have identical-appearing placebo and active medication tablets. However, in most cases the tablets were similar in form and color. Strict double-blind design was followed in all other respects. Cross-over of subjects from one study to another served to minimize possible biases.

RESULTS

Table I gives the results obtained with effective doses of the different forms of commercially avail-

Received April 10, 1967, from the Neuropharmacology Section, Bureau of Research in Neurology and Psychiatry, New Jersey Neuropsychiatric Institute, Princeton, NJ 08540
Accepted for publication June 19, 1967.
This work was supported in part by grants from the U. S. Public Health Service and the Geschickter Fund for Medical Research.
The authors are grateful for permission to employ selected men from the New Jersey Reformatory, Bordentown, as volunteers.

TABLE I—EFFECTS OF VARIOUS FORMS OF ASPIRIN (AND OF PLACEBO) ON THE ELECTRICAL ENERGY LEVELS AND VARIABILITY OF THE LEFT OCCIPITAL EEG IN NORMAL SUBJECTS

Drug, Dose, and No. of Subjects		Predrug Control	hr. Following Drug Administration[d] 1	2	3	4	5	6
Generic aspirin	MEC[a]	61	63	59	53*	54*	65	64
1.0 Gm., N[c] = 20	CV[b]	20	24	26	40*	40*	27	27
Brand aspirin	MEC	70	68	60*	62*	63*	72	71
1.0 Gm., N = 10	CV	25	27	34*	41*	27	29	30
Generic buffered aspirin	MEC	76	64	67	56*	66	72	63
1.0 Gm., N = 10	CV	25	39*	37*	43*	37*	41*	31*
Brand buffered aspirin	MEC	49	45	38*	38*	41*	36*	41*
1.0 Gm., N = 10	CV	27	35	43*	42*	42*	38*	37*
Brand buffered aspirin	MEC	83	75	73*	74*	75	86	82
660 mg., N = 10	CV	27	32	35*	32	34	29	34
Effervescent aspirin	MEC	84	74	72*	72*	96	95	81
1.0 Gm., N = 10	CV	22	33*	34*	36*	23	24	33*
Placebo	MEC	62	63	65	65	65	62	67
N = 20	CV	18	21	23	26	23	25	19

[a] MEC, mean integrated energy levels of the EEG. [b] CV, coefficient of variation (standard deviation × 100/mean). [c] N, number of subjects. (The actual number of measurements used to compute the variance was ($N \times 30$) − N; thus 580 in the case of generic aspirin.) [d] The asterisk denotes statistically significant departure from control levels (t tests for MEC, F ratios for variances) at the 0.05 level or better.

able aspirin. In most of the experimental trials the effective dose was 3 tablets or about 1.0 Gm. As can be seen, two significant changes occurred after all the dosage forms, namely, a decrease in MEC and an increase in CV. This is the characteristic effect of aspirin. The times of onset and peak of action were different, but the dual trend was present in all cases. With brand aspirin, the shifts occurred sooner and lasted longer than with generic aspirin. The most pronounced effects were observed following the administration of brand buffered aspirin. With this drug a significant dose-response relationship was shown in that 2 tablets had a relatively smaller and shorter-lasting action than 3 tablets. This is illustrated in Fig. 1. Placebo did not produce significant changes. The threshold dose for significant EEG effects was found to be 2 tablets; 1 tablet (330 mg.) was ineffective.

It will be noticed that the initial levels of the MEC varied in magnitude among the different groups. This is due to random assortment of the subjects with high or low amplitude of the so-called "α-waves." On the other hand, the coefficient of variation was found to be constant (within the limits of statistical variation) from group to group.

From the standpoint of visual inspection of records, the changes in MEC and CV corresponded to what is sometimes called "EEG drowsiness," *i.e.*, flattening of the amplitude of α-waves with sporadic occurrence of waves corresponding to light sleep and even some high amplitude slow waves.

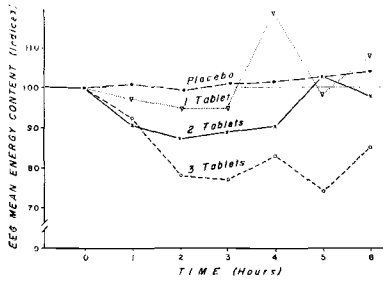


Fig. 1—Dose-response curves of the effect of brand buffered aspirin on the quantitative EEG of 4 groups of 10 subjects each.

Of the other antipyretic compounds studied, sodium salicylate, salicylamide, and acetaminophen had negligible effects (Table II). Significant increases in the CV occurred 1, 2, 4, 5, and 6 hr. after phenacetin, but there was no significant shift in the MEC. The lack of effect of sodium salicylate might be a result of its slow rate of absorption (6), since the time course of the EEG changes after aspirin correlates with previously reported curves of the serum levels of salicylates following oral administration (7). Another kind of analgesic, codeine, was studied at 2 dose levels, 32 and 64 mg. orally. After 32 mg., a dose which is commonly employed clinically for analgesic effect, no changes in the EEG were detected (Table II). The larger dose of codeine, 64 mg., caused significant increases in variability with no alteration in the MEC; thus it resembled phenacetin in effect but was unlike aspirin.

When the aspirin data are compared with those obtained from other classes of compounds, a striking resemblance to the effects (8) of those drugs which are variously called "minor tranquilizers" or "anti-anxiety" drugs is observed. In Table III, data from experiments performed with normal subjects given 3 such agents are presented. Although differences in time course and extent of change occurred, the characteristic decrease in MEC and increase in CV were present nonetheless. It should be emphasized that these changes are characteristic of the anti-anxiety class of drugs only; neither stimulants nor potent depressants nor any other kind of psychotropic agent yet tested produces the same kind of action. For example, stimulants decrease both the MEC and the variability of the EEG; major tranquilizers increase the MEC and decrease the CV. It would thus appear that in normal subjects aspirin produces changes in occipital electrical activity very similar to those caused by anti-anxiety agents. Since this effect is not mimicked by a more potent analgesic, codeine, it is probably not related to analgesia *per se*.

DISCUSSION

The drugs used in these studies were purchased

TABLE II—EFFECTS OF AN ANALGESIC (CODEINE) AND OF DRUGS RELATED TO ASPIRIN ON THE ELECTRICAL ENERGY LEVELS AND VARIABILITY OF THE LEFT OCCIPITAL EEG IN NORMAL SUBJECTS[a]

Drug, Dose and No. of Subjects		Predrug Control	hr. Following Drug Administration 1	2	3	4	5	6
Codeine, 32 mg.	MEC	62	66	67	69	67	68	68
$N = 10$	CV	18	19	19	22	19	18	24
Codeine, 64 mg.	MEC	76	75	75	72	78	72	77
$N = 10$	CV	19	28*	31*	26*	24	30*	25
Sodium salicylate	MEC	68	73	70	70	71	70	65
1.3 Gm., $N = 10$	CV	17	15	23	18	17	21	22
Phenacetin, 1.0 Gm.	MEC	60	62	62	61	58	63	59
$N = 21$	CV	21	33*	38*	26	48*	32*	31*
Salicylamide, 1.0 Gm.	MEC	38	47*	42	39	42	38	43
$N = 13$	CV	21	25	25	20	20	21	21
Acetaminophen	MEC	63	63	64	63	63	62	59
1.0 Gm., $N = 10$	CV	18	20	24	23	19	29*	25

[a] See Table I for explanation of the symbols used.

TABLE III—EFFECTS OF ANTIANXIETY DRUGS (AND PLACEBO CONTROLS) ON THE ELECTRICAL ENERGY LEVELS AND VARIABILITY OF THE LEFT OCCIPITAL EEG IN NORMAL SUBJECTS[a]

Drug, Dose and No. of Subjects		Predrug Control	hr. Following Drug Administration 1	2	3	4	5	6
Chlordiazepoxide	MEC	43	46	43	34*	33*	34*	32*
20 mg., $N = 9$	CV	19	23	28*	23	24	19	33*
Meprobamate	MEC	60	58	43*	46*	53	54	64
800 mg., $N = 10$	CV	19	29*	32*	38*	47*	56*	39*
Phenobarbital	MEC	71	60*	63	65	56*	51*	61
20 mg., $N = 20$	CV	19	29*	22	19	25*	25*	27*
Placebo	MEC	58	57	56	64	65	57	61
$N = 10$	CV	28	25	27	32	29	35	19*

[a] See Table I for explanation of the symbols used.

from local commercial sources. The identification of the brands used is not germane to this scientific report. The authors believe that this study indicates that careful pharmaceutical formulation produces a greater "aspirin effect" on the human brain. The careful selection of buffers and binders is important. It is well known that acetylsalicylic acid in the presence of moisture undegoes hydrolysis to salicylic and acetic acids. The question can be raised as to whether the reported differences between "generic" and "brand" aspirin are due to the relative commercial turnover of these different preparations, in other words, to the time duration between production and consumption; this could be faster in the case of "brand" products, with the result of a higher concentration of acetylsalicylic acid. No attempt was made to determine the extent to which this assumed difference could have biased the data, primarily because the aim was to reproduce as nearly as possible the conditions under which the different forms of aspirin are used in everyday life. Various commercial firms claim that bottled aspirin will remain stable for many years. This is probably true, since no date limit is required for its medical use.

The results of this study raise questions about the extent to which people take aspirin for effects other than relief of pain, fever, or inflammation, and their reasons for doing so. Because the EEG effects of aspirin are similar to those of compounds such as phenobarbital, meprobamate, and chlordiazepoxide, it is possible that other effects may also be similar and may thus account for the popularity of aspirin which, unlike the other drugs, can be purchased over the counter and taken without any pejorative connotations. Even if aspirin is not obviously effective in marked anxiety, it may be sufficiently active to relieve the lesser anxieties and tensions which occur in everyday life. Some support for this has appeared in two reports (9, 10) of studies which employed psychological rating scales to assess the effects of aspirin in groups of hospital attendants, nurses, and other employees. Significant reductions in "depression" and "fearfulness" occurred after aspirin administration. The authors have thus confirmed the psychological findings by an independent and objective study of human brain waves.

REFERENCES

(1) Lim, R. K. S., Guzman, F., Rodgers, D. W., Goto, K., Braun, C., Dickerson, G. D., and Engle, R. J., *Arch. Intern. Pharmacodyn.*, **152**, 25(1964).
(2) Pfeiffer, C. C., Goldstein, L., and Murphree, H. B., *J. New Drugs*, **5**, 260(1965).
(3) Drohocki, Z., *Rev. Neurol.*, **80**, 619(1948).
(4) Goldstein, L., and Beck, R. A., in "International Review of Neurobiology," Pfeiffer, C. C., and Symthies, J. R., eds., Academic Press Inc., New York, N. Y., 1965, p. 265.
(5) Goldstein, L., Sugerman, A. A., Stolberg, H., Murphree, H. B., and Pfeiffer, C. C., *Electroenceph. Clin. Neurophysiol.*, **19**, 350(1965).
(6) Seed, J. C., *Clin. Pharmacol. Therap.*, **6**, 354(1965).
(7) Hollister, L., and Kanter, S. L., *Proc. Western Pharmacol. Soc.*, **7**, 73(1964).
(8) Pfeiffer, C. C., Goldstein, L., Murphree, H. B., and Jenney, E. H., *Arch. Gen. Psychiat.*, **10**, 446(1964).
(9) Krumholz, W. V., Sheppard, C., and Merlis, S., *Clin. Pharmacol. Therap.*, **5**, 691(1964).
(10) *Ibid.*, **6**, 359(1965).

Dissolution Kinetics of Benzoic Acid in High Concentrations of Surface-Active Agents

By E. L. PARROTT and V. K. SHARMA

The dissolution kinetics of benzoic acid has been studied in the presence of certain surface-active agents. The addition of tyloxapol, polysorbate 80, sodium lauryl sulfate, and poloxakol to the dissolving fluid increased the apparent solubility of benzoic acid. In considering the experimentally determined dissolution rate as a function of solubility, it appears that the dissolution process in solutions of tyloxapol, polysorbate 80, and sodium lauryl sulfate concurs with the diffusion layer concept, while the dissolution process in the presence of poloxakol is best described by an interfacial barrier model.

IT IS WELL RECOGNIZED that the dissolution rate can be the rate-limiting step in the biological availability of drugs administered in the solid state. Wagner (1) has discussed the relation of dissolution kinetics to biological availability. Wurster and Taylor (2) have reviewed factors which influence the dissolution rate. In a recent review Higuchi (3) presented models for three mechanisms of dissolution rate behavior of solids.

Since surface-active agents are often a constituent of liquid and solid pharmaceuticals, a more complete understanding of the effect of surface-active agents on the dissolution rates of solids is of interest in the design of these dosage forms. Limited information has been published regarding the effect of surface-active agents on dissolution rate (4–7). Generally these investigations have been conducted in the region of the critical micelle concentration (CMC) of the surface-active agent employed. The purpose of this work is to investigate the effect of high concentrations of surface-active agent on dissolution rate of a relatively water-insoluble compound.

EXPERIMENTAL

Materials—The surface-active agents were commercial lots which were used without further purification. Poloxakol[1] is an oxyethylene polymer which contains 80% polyoxyethylene and has an approximate molecular weight of 8700. Tyloxapol[2] is a condensation product of *tert*-octylphenol with ethylene oxide having an average molecular weight of 2800. U.S.P. polysorbate 80[3] and sodium lauryl sulfate;[4] benzoic acid, citric acid, and disodium hydrogen phosphate were reagent grade.

Dissolution Rate Determinations—The procedure for tablet production and the dissolution apparatus were similar to that previously described (8). Spherical $^3/_8$-in. diameter tablets of pure benzoic acid were used. All determinations were made at 25° and at a stirrer speed of 324 r.p.m. At hourly intervals the 2 L. of dissolving fluid was changed, and the tablet was weighed and its diameter measured with a micrometer. At each concentration of surface-active agent, the dissolution rate was determined in triplicate with the rates falling within a 3% range.

Solubility Studies—An excess of benzoic acid and aqueous solutions of the surface-active agent were rotated in 50-ml. amber bottles in a constant-temperature bath at 25 ± 0.1° for 24 hr. An aliquot was withdrawn from each bottle using a pipet fitted with a filter. After diluting with distilled water, the absorbance was measured with a Beckman DU spectrophotometer, and the solubility of the benzoic acid was determined by means of a standard curve.

In the analysis of samples containing polysorbate 80, sodium lauryl sulfate, and poloxakol, distilled water was used as a blank since the samples were diluted 400-fold and there was no absorption by the surface-active agents. The absorbance was measured at 272.5 mμ. The concentration of benzoic acid was determined from a standard curve prepared by measuring in known concentrations of surface-active agents the absorbances of known concentrations of benzoic acid which had been diluted in a similar manner.

The strong absorption of tyloxapol in the region of 280 mμ necessitated the use of an extraction procedure. In preparing a standard curve using chloroform as the solvent it was found that there was a shift in the absorption maximum of benzoic acid to 273.5 mμ. In the analytical procedure a 10-ml. sample was diluted 100-fold with distilled water, and 25 ml. of this dilution was extracted with three 25-ml. portions of chloroform. The combined chloroform extract was filtered into a 100-ml. volumetric flask and adjusted to 100 ml. with chloroform. The absorbance was measured at 273.5 mμ. The concentration of benzoic acid was determined from a standard curve prepared by measuring in known concentrations of tyloxapol the absorbances of known concentrations of benzoic acid which had been extracted in the manner described.

RESULTS AND DISCUSSION

Solubilization—In dilute aqueous solutions in which the surface-active agent is molecularly dispersed, compounds of low water solubility, such as benzoic acid, do not dissolve to a greater extent than they would in water; however, at concentrations of surface-active agent in excess of the CMC the total solubility is increased. The amount of benzoic acid solubilized by micellar interaction ($C_s - C_0$) is represented by the difference between the total solubility (C_s) in a solution of a surface-active agent and the solubility (C_0) in water (9). The quantity ($C_s - C_0$) is linearly dependent on the concentration of the surface-active agent.

Received April 19, 1967, from the College of Pharmacy, University of Iowa, Iowa City, IA 52240

Accepted for publication June 21, 1967.

Abstracted from a thesis submitted by V. K. Sharma to the Graduate School, University of Iowa, in partial fulfillment of Master of Science degree requirements.

[1] Pluronic F-68, Wyandotte Chemical Corp., Wyandotte, Mich.

[2] Triton WR-1339, Winthrop Laboratories, New York, N. Y.

[3] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

[4] Marketed as Duponol C by E. I. du Pont de Nemours and Co., Wilmington, Del.

Table I—Dissolution Rate and Solubility of Benzoic Acid at 25° in Various Concentrations of Aqueous Tyloxapol

% Surfactant	Dissolution Rate, mg./cm.²/hr.	C_s, Total Solubility (mg./ml.)	$C_s - C_0$, Micellar Solubility (mg./ml.)
0	42.80	3.23	...
0.01	41.79	3.31	...
0.03	42.96	3.11	...
0.07	44.18	3.35	0.12
1.00	50.88	4.35	1.12
2.00	53.40	5.62	2.39
5.00	59.38	10.22	6.99
6.00	59.25	11.60	8.37
8.00	55.80	14.85	11.62
10.00	53.55	17.48	14.25

The data for tyloxapol given in Table I and plotted in Fig. 1 are typical of the experimental results for the solubilization of benzoic acid at 25° in various concentrations of surface-active agent. The linear relationship between solubilization and concentration of surface-active agent was also found to exist in solutions of polysorbate 80, sodium lauryl sulfate, and poloxakol, as shown in Figs. 2 to 4.

Dissolution Rate in Concentrations of Surface-Active Agent Exceeding the CMC—When a single component, one phase solid of macroscopic size dissolves without disintegration and without chemical reaction in an agitated solvent, the dissolution rate (R) at a given temperature may be expressed by the Noyes and Whitney equation:

$$R = \frac{dw}{dt}\frac{1}{S} = k\,(C_s - C) \qquad \text{(Eq. 1)}$$

where dw/dt is the amount of solute dissolved per unit time, S is the surface area, C_s is the solubility, C is the concentration of the solute, and k is a constant.

When $C_s \gg C$ the dissolution rate will be:

$$R = k\,C_s \qquad \text{(Eq. 2)}$$

Numerous reports have substantiated this relationship which states that the dissolution rate is proportional to the solubility of a compound (8, 10–12).

Since any dissolution interaction may affect C_s and consequently alter the dissolution rate, it was thought that micellar solubilization, which increased the solubility of benzoic acid, would increase its dissolution rate. The experimentally determined dissolution rate and the corresponding quantity $(C_s - C_0)$ for benzoic acid in various concentrations of tyloxapol are given in Table I and Fig. 1. With an increase in the concentration of tyloxapol, there is an increase in the solubility and an increase in the dissolution rate. The dissolution rate attains its maximum value at 5% tyloxapol, and the dissolution rate decreases upon further increases in the concentration of the surface-active agent.

Similarly, as shown in Fig. 2 an increase in the concentration of polysorbate 80 results in an increased solubility and an increased dissolution rate. A maximum dissolution rate is reached at 9% polysorbate 80.

Qualitatively the experimental results are in accordance with Eq. 2; however, with micellar solubilization the micelle phase must be considered and according to the diffusion layer concept (3):

$$R = \frac{D}{h}C_0 + \frac{D'}{h}(C_s - C_0) \qquad \text{(Eq. 3)}$$

where h is the effective diffusion layer thickness, D is the diffusion coefficient, C_0 is the solubility of the solute molecule, and D' is the diffusion co-

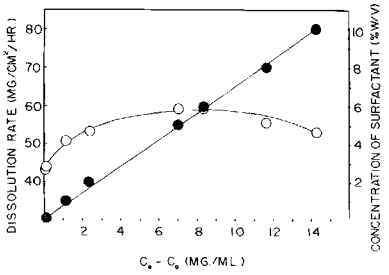


Fig. 1—The relationship of the solubility of benzoic acid at 25° to its dissolution rate and the concentration of tyloxapol. Key: ○, rate; ●, concentration.

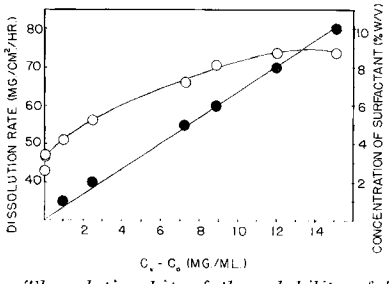


Fig. 2—The relationship of the solubility of benzoic acid at 25° to its dissolution rate and the concentration of polysorbate 80. Key: ○, rate; ●, concentration.

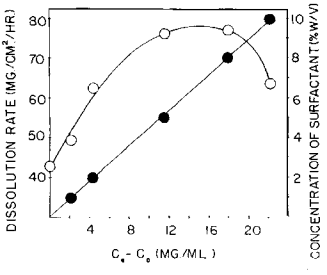


Fig. 3—The relationship of the solubility of benzoic acid at 25° to its dissolution rate and the concentration of sodium lauryl sulfate. Key: ○, rate; ●, concentration.

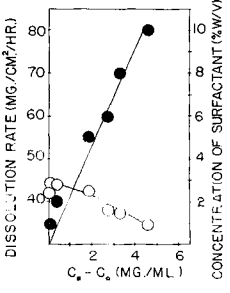


Fig. 4—The relationship of the solubility of benzoic acid at 25° to its dissolution rate and the concentration of poloxakol. Key: ○, rate; ●, concentration.

efficient of the micelle-solubilized solute and (C_s − C_0) is the solubility increase due to solubilization.

As the concentration of tyloxapol and polysorbate 80 is increased, the increase in dissolution rate becomes progressively less, and at high concentrations the dissolution rate is decreased. If this effect were due to the influence of viscosity, it would be reflected in Eq. 3 in the diffusion coefficients which by the Stokes-Einstein equation are inversely proportional to the viscosity (13). If dissolution were an interfacially controlled process, viscosity should have little effect on the dissolution rate. If dissolution were a diffusion-controlled process, an increase in viscosity should decrease the dissolution rate (2). The fact that both tyloxapol and polysorbate 80 in high concentrations cause a decrease in dissolution rate suggests that the dissolution process is primarily diffusion controlled and would support the diffusion layer concept as the model of their dissolution behavior; however, a further study is necessary to substantiate this suggestion.

The effect of sodium lauryl sulfate on the dissolution rate of benzoic acid and prednisolone in the region of the CMC has been reported (14, 15). In this region the solubilities and dissolution rates of both compounds were increased. In the present study the effect on solubility and dissolution rate was determined in concentrations greatly in excess of the CMC. The system was buffered to pH 3 with McIvaine's buffer in order to suppress ionization and minimize salt formation which would contribute to the solubility of benzoic acid by a mechanism other than micellar solubilization.

As shown in Fig. 3, the dissolution rate of benzoic acid is increased with increased concentrations of sodium lauryl sulfate; however, a maximum dissolution rate is reached at 9% sodium lauryl sulfate. Further increases in the concentration of sodium lauryl sulfate result in a decrease in dissolution rate.

The dissolution rate may be considered to be determined by 2 consecutive reaction rates: the rate of reaction at the interface and the rate of diffusion from the interface. If the reaction at the interface is infinitely fast, the Nernst and Brunner concept of dissolution by diffusion from a saturated film existing at the interface is acceptable. This appears to be the dissolution model of benzoic acid in solutions of sodium lauryl sulfate. If the surface reaction is the rate-limiting step, the dissolution kinetics would be different from that expressed by Noyes and Whitney.

The solubility of benzoic acid is a linear function of the concentration of poloxakol. It might be postulated that poloxakol is too hydrophilic to form micelles, but that it increases the solubility through association or hydrogen bonding of the benzoic acid to its polyoxyethylene groups. As shown in Fig. 4 the dissolution rate of benzoic acid in poloxakol is not proportional to the concentration of the surface-active agent. This suggests that the dissolution process occurs by a mechanism other than a primarily diffusion-controlled process.

Dissolution Rate in Concentrations of Surface-Active Agent Less Than the CMC—As shown in Table I, in concentrations of tyloxapol less than the CMC, the dissolution rate of benzoic acid is not significantly increased by the addition of the surface-active agent. Since no solubilization was shown to occur in this region, any increase in dissolution rate may be attributed to a lowered interfacial tension which improved the wetting of the surface and increased the effective surface area (13). Similar results were found with poloxakol.

The greatest effect of wetting the surface was shown with polysorbate 80. Although the solubility was not increased, at 0.001, 0.002, and 0.003% polysorbate 80, the dissolution rates were 45.9, 46.8, and 47.5 mg./cm.^{2}/hr., respectively.

SUMMARY

A linear relationship between solubilization of benzoic acid and concentration of a surface-active agent was found to exist in solutions of polysorbate 80, sodium lauryl sulfate, tyloxapol, and poloxakol. At concentrations of these surface-active agents less than the CMC, the dissolution rate was only slightly increased due to improved wetting of the surface. At concentrations exceeding the CMC, the dissolution rate was increased to a maximal rate by an increase in concentration of tyloxapol, polysorbate 80, and sodium lauryl sulfate. This behavior agrees with the diffusion layer theory. At higher concentrations the dissolution rate decreased; however, this may be rationalized by the diffusion layer theory when the micelle phase and its diffusional coefficient are considered. The dissolution rate of benzoic acid was retarded by poloxakol.

REFERENCES

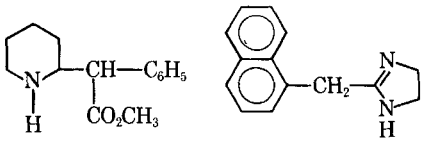
(1) Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961).
(2) Wurster, D. E., and Taylor, P. W., *ibid.*, **54**, 169 (1965).
(3) Higuchi, W. I., *ibid.*, **56**, 315(1967).
(4) Mukerjee, P., and Mysels, K., *J. Am. Chem. Soc.*, **77**, 2937(1955).
(5) Harkins, W., and Oppenheimer, H., *J. Chem. Phys.*, **16**, 1000(1948).
(6) Ekwall, P., and Danielson, I., *Acta Chem. Scand.*, **5**, 973(1951).
(7) McBain, J., and Huff, H., *J. Am. Chem. Soc.*, **70**, 3838(1948).
(8) Parrott, E. L., Wurster, D. E., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 269(1955).
(9) Goodhart, F. W., and Martin, A. N., *J. Pharm. Sci.*, **51**, 50(1962).
(10) Nelson, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 607(1957).
(11) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.*, **47**, 376(1958).
(12) Hamlin, W. E., Northam, J. I., and Wagner, J. G., *J. Pharm. Sci.*, **54**, 1651(1965).
(13) Einstein, A., *Ann. Phys.*, **17**, 549(1905).
(14) Wurster, D. E., and Seitz, J., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 335(1960).
(15) Taylor, P. W., and Wurster, D. E., *J. Pharm. Sci.*, **54**, 1654(1965).

Phenylalkylamines

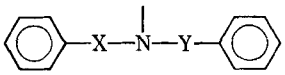
By JOSEPH SAM*

The syntheses of 1- and 2-aralkylpyridines and piperidines and phenylalkylamines are described.

THE ARRAY of compounds which contain the phenylalkylamine moiety and which possess useful biological properties is impressive. The investigation with various phenylalkylamines has culminated in the preparation of compounds with a narrow spectrum of sympathomimetic activity, useful in specific conditions. For example, the incorporation of the alkylamine portion in a heterocyclic molecule such as methyl α-(2-piperidyl)phenylacetate (I) and 2-(1-naphthylmethyl)-2-imidazoline (II) provides compounds with specific central nervous system stimulant (1, 2) and nasal decongestant activity (3, 4), respectively. The preparation of di-(phenylalkyl) amines represented by structure III has led to the development of compounds useful in the management of bronchoconstrictor disorders (5, 6) and in vascular constrictive diseases (7).

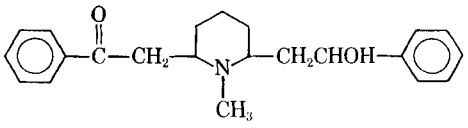


I II



III

Because of the pronounced biological effects exhibited by di-(phenylalkyl)amines (III) and because of the desirable properties elicited by the incorporation of the alkylamine portion in a cyclic structure (8), it was of interest to prepare both mono- and di-(phenylalkyl)amines containing the pyridine and piperidine ring (Tables I and III). The compounds listed in Table III also bear some structural similarity to the alkaloid lobeline (IV) (9).



IV

The pyridine derivatives listed in Table I were prepared by (*a*) the reaction of picolinealdehyde with acetophenones (10), or (*b*) by the reaction of 3,4-dimethoxybenzaldehyde with α-picoline (11), or (*c*) by the reaction of 2-vinylpyridine with *N*-methyl-β-hydroxyphenethylamine (V) (13, 14), or (*d*) by the Pd-C catalyzed reduction of the ethylenic groups of the products obtained in (*a*) and (*b*). (Scheme I.)

The piperidine derivatives (VIII) of Table III were prepared by the catalytic reduction of pyridinium bromides (VII) (Table II). (Scheme II.)

Hypotensive activity (15) and neurosedative activity (16) have been observed recently in similar compounds. Included also in this study are several nonpiperidine derivatives (Table IV). Preliminary pharmacological evaluation did not indicate any pronounced biological activity in the compounds that were studied.

EXPERIMENTAL[1]

α - {([2 - (5 - Ethyl - 2 - pyridyl)ethyl]methylamino)methyl}benzyl Alcohol (VI)—A mixture of 40 Gm. (0.3 mole) of 5-ethyl-2-vinylpyridine, 46 Gm. (0.3 mole) of *N*-methyl-β-hydroxyphenethylamine, 18 Gm. of glacial acetic acid, and 200 ml. of methanol was refluxed for 18 hr. The methanol was distilled *in vacuo*. The residual oil was added to ice and neutralized with 10% sodium hydroxide. An ether extract of the mixture was dried over anhydrous sodium sulfate. Evaporation of the ether and distillation of the oil gave product, n_D^{26} 1.5489.

1 - (4 - Chlorophenyl) - 3 - (2 - pyridyl) - 2 - propene-1-one (IX)—To 2 Gm. of piperidine cooled in an ice bath were added 2 Gm. of glacial acetic acid, 15 Gm. (0.1 mole) of 4′-chloroacetophenone, and 11 Gm. (0.1 mole) of picolinealdehyde, respectively. The resulting mixture was heated on a steam bath for 2 hr. and then cooled. An ether extract of the oily material was washed thoroughly with water and dried over anhydrous potassium carbonate. Evaporation of the solvent *in vacuo* left a dark brown oil which solidified on standing.

1,5 - Bis - (4 - chlorophenyl) - 3 - (2 - pyridyl)-pentane-1,5-dione (XII)—A solution of 4 Gm. (0.1 mole) of sodium hydroxide in 40 ml. of water and 20 ml. of ethyl alcohol was treated with 15.5 Gm. (0.1 mole) of 4′-chloroacetophenone. The mixture was cooled in an ice bath and treated with 10.7 Gm. (0.1 mole) of picolinealdehyde in one lot. The mixture was shaken intermittently during a 1-hr. period. The oil that separated solidified; it was removed by filtration, washed with methanol, and recrystallized.

1 - (4 - Nitrophenyl) - 3 - (2 - pyridyl) - 2 - propene-1-one (XI)—The procedure for the preparation of 1 - (4 - chlorophenyl) - 3 - (2 - pyridyl) - 2 - propene-1-one was followed using 16.5 Gm. (0.1 mole) of 4′-nitroacetophenone and 11 Gm. (0.1 mole) picolinealdehyde.

4′ - Chloro - 3 - (2 - pyridyl)propiophenone (X)—A solution of 44 Gm. (0.18 mole) of 1-(*p*-chlorophenyl)-3-(2-pyridyl)-2-propene-1-one in 200 ml. of warm methanol was hydrogenated at 52 p.s.i. in the presence of 1 Gm. of 5% Pd-C catalyst. Reduction was complete in 30 min. The catalyst was removed by filtration and the solvent was distilled under reduced pressure.

Received May 3, 1967, from Bristol Laboratories, Syracuse, NY 13210
Accepted for publication July 5, 1967.
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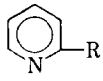
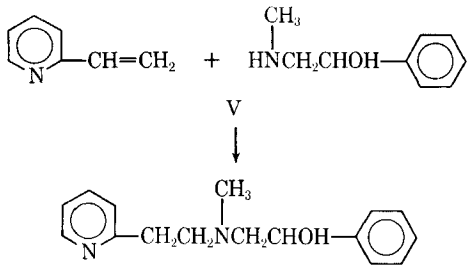
[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

TABLE I—2-SUBSTITUTED PYRIDINES



No.	R	% Yield	B.p., °C. (mm.) M.p., °C.	Molecular Formula	Anal., % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
VI	$C_6H_5CHOHCH_2N(CH_3)(CH_2)_2$—	29	178 (0.4 mm.)	$C_{18}H_{24}N_2O$[a]	76.0	8.51	...	76.3	8.70	...
IX	4-ClC_6H_4COCH=CH—	41	85–86[b]	$C_{14}H_{10}ClNO$[c]	69.0	4.14	5.75	69.3	3.95	5.75
X	4-$ClC_6H_4COCH_2CH_2$—	66	169–172 (1 mm.)	$C_{14}H_{12}ClNO$	68.4	4.92	...	68.7	5.17	...
XI	4-$NO_2C_6H_4COCH$=CH—	51	150–151[d]	$C_{14}H_{10}N_2O_3$	66.1	3.97	...	66.2	3.97	...
XII	$(4\text{-}ClC_6H_4COCH_2)_2CH$—	33	136–137[d]	$C_{22}H_{17}Cl_2NO_2$	66.3	4.30	3.51	66.4	4.39	3.63
XIII	3,4-di$CH_3OC_6H_3CH$=CH—	38	195–200 (0.2 mm.)	$C_{15}H_{15}NO_2$	74.7	6.27	5.81	74.5	6.01	6.05
XIV	3,4-di$CH_3OC_6H_3(CH_2)_2$—	90	152–155 (0.2 mm.)	$C_{15}H_{17}NO_2$	74.0	7.05	...	74.1	7.45	...

[a] Contains a 5-ethyl group in the pyridine ring. [b] Recrystallized from *n*-hexane. [c] *Reference 10.* [d] Recrystallized from methanol.

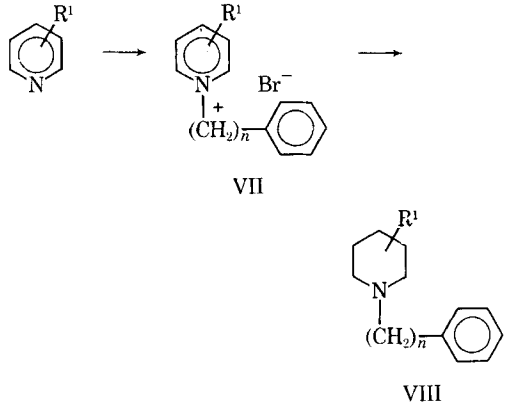


Scheme I

2-(3,4-Dimethoxystyryl)pyridine (XIII)—A solution of 93 Gm. (1 mole) of 2 picoline, 166 Gm. (1 mole) of veratric aldehyde in 204 Gm. (2 moles) of acetic anhydride was refluxed for 66 hr., cooled, and poured into ice water. The mixture was extracted with ether and dried over anhydrous sodium sulfate. Evaporation of the solvent and distillation of the residual oil gave product.

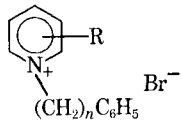
2-(3,4-Dimethoxyphenethyl)pyridine (XIV)—The procedure described for the preparation of X was followed using 93 Gm. (0.385 mole) of 2-(3,4-dimethoxystyryl)pyridine; reduction was complete after 18 hr.

Pyridinium Bromides (VII, Table II)—A solution of 0.2 mole of desired pyridine and 0.2 mole of phenylalkyl bromide in 100 ml. of acetonitrile was heated under reflux on a steam bath for 48 hr. and then cooled. The solid was removed by filtration, washed with cold acetone, and thereafter recrystallized from a suitable solvent. In some cases ether was added to the cooled acetonitrile solution to precipitate the pyridinium bromide.



Scheme II

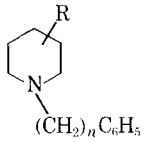
TABLE II—PYRIDINIUM BROMIDES



No.	R	n	% Yield	M.p., °C.	Molecular Formula	Anal., % Calcd. C	Calcd. H	Found C	Found H
VII*a*	3-HO	2	90	193–194[a]	$C_{13}H_{14}BrNO$	55.7	5.04	55.8	5.17
VII*b*	2-[4-$CH_3OC_6H_4(CH_2)_2$]	2	75	148–150[b]	$C_{22}H_{24}BrNO$	66.3	6.07	66.5	6.25
VII*c*	2-[3,4-di$CH_3OC_6H_3(CH_2)_2$]	2	57	[d]	$C_{23}H_{26}BrNO_2$	...	...	...	...
VII*d*	2-$C_6H_5CH_2$	2	66	164–166[a]	$C_{20}H_{20}BrNO$	67.8	5.69	68.0	5.45
VII*e*	4-[3-$HO(CH_2)_3$]	3	..	[c]	$C_{17}H_{22}BrN$	...	...	...	...
VII*f*	2-$C_6H_5CH_2$	3	76	85–90[d]	$C_{21}H_{22}BrN$	...	...	...	...
VII*g*	3-$HOCH_2$	2	85	144–145[a]	$C_{14}H_{16}BrNO$	57.2	5.48	57.2	5.55
VII*h*	4-CO_2CH_3	2	..	[c]	$C_{15}H_{16}BrNO_2$	...	...	...	...
VII*i*	3-$CO_2C_2H_5$	2	88	188–190	$C_{16}H_{18}BrNO_2$	57.1	5.40	57.2	5.44

[a] Recrystallized from acetonitrile. [b] Recrystallized from an acetonitrile–acetone solution. [c] Isolated as an oil and used in the preparation of the piperidine derivative (Table III). [d] Not purified, used in the preparation of the piperidine derivative (Table III).

TABLE III—PHENYLALKYLPIPERIDINES

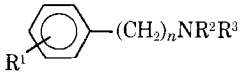


VIII

No.	R	n	Method	% Yield	B.p., °C. (mm.) M.p., °C.	Molecular Formula	Anal., % Calcd. C	Calcd. H	Found C	Found H
VIII*a*	3-ClCH$_2$	2	*D*	87	124–126 (0.3)	$C_{14}H_{20}ClN$	70.7	8.48	70.8	8.65
VIII*b*	3-HO	2	*A*	53	130 (0.5)	$C_{13}H_{19}NO$[a]	76.1	9.33	76.2	9.52
VIII*c*	2-(4-CH$_3$OC$_6$H$_4$CH$_2$CH$_2$)	2	*A*	87	138–139	$C_{22}H_{30}BrNO$[b]	65.3	7.48	65.5	7.67
VIII*d*	2-(3,4-diCH$_3$OC$_6$H$_3$CH$_2$CH$_2$)	2	*A*	89[c]	154–156	$C_{23}H_{32}BrNO_2$[b]	63.6	7.43	63.5	7.36
VIII*e*	2-C$_6$H$_5$CH$_2$	2	*A*	83[c]	166–168	$C_{20}H_{26}BrN$[b]	66.8	7.27	66.7	7.20
VIII*f*	4-(HOCH$_2$CH$_2$CH$_2$)	3	*B*	83[c]	172–175 (0.3)	$C_{17}H_{27}NO$	78.1	10.41	77.8	10.20
VIII*g*	2-C$_6$H$_5$CH$_2$	3	*C*	85[c]	160–161	$C_{21}H_{28}BrN$[b]	67.4	7.54	67.5	7.74
VIII*h*	4-CH$_3$OCO	2	*C*	54[c]	142 (0.3)	$C_{15}H_{21}NO_2$	72.8	8.56	73.0	8.45
VIII*i*	4-NH$_2$NHCO	2	*E*	70	154–155	$C_{14}H_{21}N_3O$	68.0	8.56	67.9	8.50
VIII*j*	3-C$_2$H$_5$OCO	2	*A*	81	137 (0.5)	$C_{16}H_{23}NO_2$	73.5	8.87	73.4	8.56

[a] Kralt, T., Asma, W. J., and Moed, H. D., *Rec. Trav. Chim.*, **80**, 330(1961). [b] Hydrobromide. [c] Yield is based on the tertiary amine used.

TABLE IV—PHENYLALKYLAMINES



No.	R^1	n	R^2	R^3	Recrystn. Solvent[a]	% Yield	B.P., °C. (mm.) M.p., °C.	Molecular Formula	Anal., % Calcd. C	Calcd. H	Found C	Found H
XV	3,4-diCH$_3$O	2	C$_9$H$_9$O[b]	H	M	71	126–127	$C_{19}H_{23}NO_3$	72.8	7.40	73.2	7.60
XVI	H	1	C$_{10}$H$_9$O[c]	H	M	53	192–193	$C_{17}H_{18}ClNO$[d]	71.0	6.30	71.3	6.60
XVII	2-HO-4-(1-indanyl)	1	CH$_3$	CH$_3$	M–W	40	63–64	$C_{18}H_{21}NO$	80.9	7.92	81.1	8.11
XVIII	2-HO-3-CH$_3$O	1	CH$_3$	H	B	96	90–91	$C_9H_{13}NO_2$	64.7	7.84	64.8	7.68
XIX	H	1	C$_{10}$H$_{11}$[e]	CH$_3$	...	79	152–156 (1 mm.)	$C_{18}H_{21}N$	...	...	...	...
XX	H	1	C$_{10}$H$_{11}$[e]	CH$_3$	MEK	90	193–194	$C_{18}N_{22}ClN$[d]	75.1	7.71	75.4	7.80

[a] M, Methanol; M–W, methanol–water; B, benzene; MEK, methyl ethyl ketone. [b] 2-Hydroxy-1-indanyl. [c] 1-Oxo-2-indanylmethyl. [d] Hydrochloride. [e] 2-Indanylmethyl.

Hydrogenation of Pyridines to Piperidines (Table III)—*Method A*—A solution of 0.1 mole of the desired 1-substituted pyridinium bromide in 200 ml. of water was hydrogenated at 50–60 p.s.i. at approximately 40–50° in the presence of 0.4 Gm. of platinum oxide catalyst until the theoretical amount of hydrogen was absorbed (5–18 hr.). The mixture was concentrated *in vacuo* on a water bath, treated with methanol, and thereafter filtered to remove the catalyst. The filtrate was concentrated *in vacuo* on a water bath and the residual material either was recrystallized from a suitable solvent or was converted to the base, isolated in the usual manner, and distilled.

Method B—A mixture of 0.2 mole of desired pyridine and 0.2 mole of phenylalkyl bromide was heated on a steam bath for 1 hr. The residual oil was dissolved in water and hydrogenated as described under *Method A*.

Method C—A solution of 0.2 mole of desired pyridine and 0.2 mole of phenylalkyl bromide in 100 ml. of acetonitrile was refluxed on a steam bath for 20–30 hr. and thereafter cooled and diluted with ether. The mixture was extracted with 200 ml. of water. The aqueous solution was warmed on a steam bath to expel any ether present and then hydrogenated as described under *Method A*.

1-Phenethyl-3-piperidylmethyl Chloride (VIIIa)—*Method D*—A solution of 28 Gm. (0.13 mole) of 1-phenethyl-3-piperidylcarbinol in 100 ml. of chloroform was added dropwise to a solution of 30 ml. of thionyl chloride in 100 ml. of chloroform. The solution was allowed to remain at room temperature for 18 hr. and then refluxed for 3 hr. The solvent and excess thionyl chloride were distilled *in vacuo*. The residual solid was dissolved in ice water, neutralized with 10% sodium hydroxide, extracted with chloroform, and dried over anhydrous potassium carbonate. Evaporation of the solvent and distillation of the residual oil gave product, n_D^{26} 1.5303.

1-Phenethylisonipecotohydrazide (VIIIi)—*Method E*—A solution of 10 Gm. (0.04 mole) of methyl 1-phenethylisonipecotate and 10 Gm. (0.2 mole) of hydrazine hydrate in 30 ml. of water was refluxed 3 hr. The solvent was distilled under reduced pressure and the residual solid was recrystallized.

α - {[2 - (5 - Ethyl - 2 piperidyl)ethyl]methylamino)methyl}benzyl Alcohol—The procedure described under *Method A* for the preparation of *N*-substituted piperidines (VIII) was followed. From 13 Gm. (0.05 mole) of the corresponding pyridine derivative (VI) there was obtained 12 Gm. (88%) of product, b.p. 178–180° (0.6 mm.), $n_D^{24.5}$ 1.5218.

1-(3,4-Dimethoxyphenethyl)-2-indanol (XV)—A mixture of 13.2 Gm. (0.1 mole) of indene oxide, 18.1

Gm. (0.1 mole) of 3,4-dimethoxyphenethyl amine, and 150 ml. of benzene was refluxed on a steam bath for 6 hr. The benzene was evaporated and thereafter the dark brown oily residue was heated on a steam bath for 6 hr. The residual material solidified on cooling.

2-Benzylaminomethyl-1-indanone (XVI)—To a mixture of 14 ml. of concentrated hydrochloric acid and 14 Gm. (0.13 mole) of benzylamine were added 18.5 Gm. (0.14 mole) of 1-indanone and 12 ml. (4.1 Gm., 0.14 mole) of 37% formaldehyde solution. The resulting mixture was heated on a steam bath for 40 min. and thereafter cooled and filtered. The residual material was washed with two 50-ml. portions of methanol to give 20 Gm. (53%) of white solid, m.p. 190–193° dec.

2 - Dimethylaminomethyl - 4 - (1 - indanyl)-phenol (XVII)—A solution of 21 Gm. (0.1 mole) of 4-(1-indanyl)phenol in 100 ml. of isopropanol was treated with 18 ml. of 25% aqueous dimethylamine solution and 10.5 ml. of a 37% formaldehyde solution and thereafter refluxed on a steam bath for 1.5 hr. The solvent was distilled *in vacuo*. The residue was treated with dilute hydrochloric acid and extracted with ether. The acid solution was neutralized with ammonium hydroxide and extracted with ether. The latter ether extract was dried over anhydrous magnesium sulfate and then evaporated to dryness. The residual material (10 Gm., 40%), which melted at 51–56°, was recrystallized.

2-Hydroxy-3-methoxybenzylmethylamine (XVIII)—The procedure for the preparation of X was followed using 31 Gm. (0.7 mole) of 2-hydroxy-3-methoxybenzalmethylamine. Reduction was complete after 18 hr.

N - Benzyl - N - (2 - indanylmethyl)methylamine (XIX)—A mixture of 10.6 Gm. (0.05 mole) of 2-bromomethylindan and 25 Gm. of *N*-benzylmethylamine was refluxed for 6 hr. Thereafter the mixture was cooled and extracted with dilute hydrochloric acid. The acid solution was neutralized and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and then distilled to give product.

A hydrochloride (XX) was prepared in the usual manner and recrystallized.

REFERENCES

(1) Scholz, K., and Panizzon, L., *Helv. Chim. Acta*, **37**, 1605(1954).
(2) Meier, R., Gross, F., and Tripod, J., *Klin. Wochschr.*, **32**, 445(1954); through *Chem. Abstr.*, **48**, 8945*e*(1954).
(3) Voss, E., *Am. Profess. Pharmacist*, **19**, 722, 727(1953).
(4) Legier, J. F., *Arch. Intern. Pharmacodyn.*, **115**, 201 (1958); through *Chem. Abstr.*, **52**, 20615*e*(1958).
(5) Biel, J. H., Friedman, H. L., Leiser, H. A., and Sprengeler, E. P., *J. Am. Chem. Soc.*, **76**, 3149(1954).
(6) Claassen, V., "First International Pharmacology Meeting," Brunings, K. J., ed., Academic Press, Inc., New York, N. Y., 1963, vol. 7, p. 265.
(7) Kulz, F., and Schneider, M., *Klin. Wochschr.*, **28**, 535(1950); through *Chem. Abstr.*, **45**, 1252*h*(1951).
(8) Belleau, B., *J. Med. Pharm. Chem.*, **2**, 553(1960).
(9) Henry, T. A., "The Plant Alkaloids," P. Blakiston Co., Philadelphia, Pa., 1949, p. 22.
(10) Marvel, C. S., Coleman, L. E., Jr., and Scott, G. P., *J. Org. Chem.*, **20**, 1785(1955).
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(12) Reich, H. E., and Levine, R., *J. Am. Chem. Soc.*, **77**, 4913, 5434(1955).
(13) Profft, E., *Chemiker-Ztg.*, **81**, 427(1957); through *Chem. Abstr.*, **52**, 2011*h*(1958).
(14) Minor, W. F., Hoekstra, J. B., Fisher, D., and Sam, J., *J. Med. Chem.*, **5**, 96(1962).
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Gm. (0.1 mole) of 3,4-dimethoxyphenethyl amine, and 150 ml. of benzene was refluxed on a steam bath for 6 hr. The benzene was evaporated and thereafter the dark brown oily residue was heated on a steam bath for 6 hr. The residual material solidified on cooling.

2-Benzylaminomethyl-1-indanone (XVI)—To a mixture of 14 ml. of concentrated hydrochloric acid and 14 Gm. (0.13 mole) of benzylamine were added 18.5 Gm. (0.14 mole) of 1-indanone and 12 ml. (4.1 Gm., 0.14 mole) of 37% formaldehyde solution. The resulting mixture was heated on a steam bath for 40 min. and thereafter cooled and filtered. The residual material was washed with two 50-ml. portions of methanol to give 20 Gm. (53%) of white solid, m.p. 190–193° dec.

2 - Dimethylaminomethyl - 4 - (1 - indanyl)-phenol (XVII)—A solution of 21 Gm. (0.1 mole) of 4-(1-indanyl)phenol in 100 ml. of isopropanol was treated with 18 ml. of 25% aqueous dimethylamine solution and 10.5 ml. of a 37% formaldehyde solution and thereafter refluxed on a steam bath for 1.5 hr. The solvent was distilled *in vacuo*. The residue was treated with dilute hydrochloric acid and extracted with ether. The acid solution was neutralized with ammonium hydroxide and extracted with ether. The latter ether extract was dried over anhydrous magnesium sulfate and then evaporated to dryness. The residual material (10 Gm., 40%), which melted at 51–56°, was recrystallized.

2-Hydroxy-3-methoxybenzylmethylamine (XVIII)—The procedure for the preparation of X was followed using 31 Gm. (0.7 mole) of 2-hydroxy-3-methoxybenzalmethylamine. Reduction was complete after 18 hr.

N - Benzyl - N - (2 - indanylmethyl)methylamine (XIX)—A mixture of 10.6 Gm. (0.05 mole) of 2-bromomethylindan and 25 Gm. of *N*-benzylmethylamine was refluxed for 6 hr. Thereafter the mixture was cooled and extracted with dilute hydrochloric acid. The acid solution was neutralized and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and then distilled to give product.

A hydrochloride (XX) was prepared in the usual manner and recrystallized.

REFERENCES

(1) Scholz, K., and Panizzon, L., *Helv. Chim. Acta*, **37**, 1605(1954).
(2) Meier, R., Gross, F., and Tripod, J., *Klin. Wochschr.*, **32**, 445(1954); through *Chem. Abstr.*, **48**, 8945*e*(1954).
(3) Voss, E., *Am. Profess. Pharmacist*, **19**, 722, 727(1953).
(4) Legier, J. F., *Arch. Intern. Pharmacodyn.*, **115**, 201 (1958); through *Chem. Abstr.*, **52**, 20615*e*(1958).
(5) Biel, J. H., Friedman, H. L., Leiser, H. A., and Sprengeler, E. P., *J. Am. Chem. Soc.*, **76**, 3149(1954).
(6) Claassen, V., "First International Pharmacology Meeting," Brunings, K. J., ed., Academic Press, Inc., New York, N. Y., 1963, vol. 7, p. 265.
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TABLE I—EFFECTS OF SALINE INJECTION ON LOCOMOTOR ACTIVITY OF SIX STRAINS OF MICE TESTED SINGLY OR IN GROUPS OF FOUR

Mean 30-min. Activity Count[a] (Log-Transformed)					
Single Mice			Grouped Mice		
Strain	No Inject.	Saline	Strain	No Inject.	Saline
NLW	2.990	2.810	NLW	3.522	3.409
C57BL/6	2.948	2.904	C57BL/6	3.452	3.360
DBA/2	2.934	2.855	C3H/An	3.381	3.331
BALB/c	2.913	2.905	CBA	3.335	3.353
C3H/An	2.847	2.796	DBA/2	3.324	3.235
CBA	2.778	2.714	BALB/c	3.322	3.300

[a] $N = 12$ in all cases.

RESULTS

Locomotor activity values under the several treatment conditions are shown in Table I. Results of the analysis of variance performed on the log-transformed data[4] are shown in Table II. The analysis revealed significant main effects of both saline injection ($p < 0.005$) and of strains ($p < 0.005$), and quite expectedly, of grouping ($p < 0.005$).

In view of the nonsignificant interaction of injection and strains, further analysis of the injection effects within strains was unwarranted. Further details of the between-strains differences contributing to the significant strains effect were determined by means of Duncan's multiple range test (8). This test was performed separately on the data for singly-tested and group-tested mice because of the significant interaction of strains and groupings, but after combining injected and noninjected groups in view of the nonsignificant interaction of injection with strains. Results of these multiple comparisons between strains are given in Table III. The significant interaction between grouping and strains, along with obvious difference in ordering of the strains under single and group tested conditions (Table III) suggested also the application of Duncan's test to the differences for each strain between values of single and grouped mice. The results of this test (Table IV) indicate that in addition to the differences in motility between strains as shown in Table III there is a significant difference in the effect of grouping on motility in different strains. The obvious and expected increase in counts when four mice are tested rather than one was in fact significantly more pronounced in the CBA, NLW, and C3H/An strains than in the DBA/2 and BALB/c. This suggests that the increase of counts with grouping is, for some strains at least, not merely a physical phenomenon as a consequence of the increased number of mice in the actometer, but also a behavioral phenomenon involving social factors.

TABLE II—ANALYSIS OF VARIANCE ON LOG-TRANSFORMED ACTIVITY DATA

Term	Degrees of Freedom	Sum of Sq.	Mean Sq.	F	p
Treatments	(23)	19.381	0.842	30.07	<.005
Injection	1	0.280	0.280	10.00	<.005
Grouping	1	17.763	17.763	634.39	<.005
Strains	5	0.695	0.139	4.96	<.005
Injection × grouping	1	0.001	0.001	<1	N.S.
Injection × strains	5	0.115	0.023	<1	N.S.
Grouping × strains	5	0.493	0.098	3.50	<.01
Injection × grouping × strains	5	0.034	0.006	<1	N.S.
Error	264	7.482	0.028	...	...
Total	287	26.863	0.093	...	...

jection on locomotor activity of mice seemed desirable. Since spontaneous locomotor activity is well known as a behavioral phenotype which is influenced by genetic differences between strains of laboratory mice (5, 6), it is of particular interest whether the effect of saline injection would occur independently of genotype. As all the previous reports of this phenomenon were based on observations of individual mice, it also seemed desirable to test for the effect in the case of small groups as are often used in studying locomotor effects of drugs. In summary, locomotor activity of groups of four mice and of single mice from six strains under noninjection and saline-injection conditions have been compared.

EXPERIMENTAL

Observations of locomotor activity were made in three commercially manufactured[1] photocell activity devices (7). All actometer units were exposed to each level of all the experimental variables. The number of counts, each representing the interruption of one of the six light beams distributed symmetrically around the ring-shaped track, was recorded by digital counters.

Inbred male mice of the C57BL/6, DBA/2, C3H/An, CBA, and BALB/c strains[2] and random-bred male mice of a Swiss-Webster albino strain (NLW)[3] were received at about 30 days of age and were between 8 and 12 weeks old when 60 from each strain were used in these experiments. A 0.9% saline solution in distilled water was administered intraperitoneally in a volume of 0.01 ml./Gm. Locomotor activity recording was begun immediately after injection and continued for 30 min. The handling of noninjected mice was approximately the same as for the saline group, but sham-injection (*i.e.*, needle penetration) was not performed as Schnitzer and Ross (1) had found needle insertion had little or nothing to do with reduction of activity.

DISCUSSION

The earlier reports (1, 2, 4) of the activity-inhibiting effect of intraperitoneal saline treatment in mice are further confirmed and extended by these positive results. The question of age as a factor in the effect which was suggested by one negative report (3) is contradicted since the mice used in this case were also considerably younger than those of the initial report (1). An additional attribute of this

[1] Woodard Research Corp., Herndon, Va.
[2] Cumberland View Farms, Clinton, Tenn.
[3] National Laboratory Animal Co., Creve Couer, Mo.

[4] Log-transformation was performed in order to eliminate the proportionality between the means and variances of single mice *versus* groups of mice that occurred in the untransformed data.

TABLE III—RANKING AND COMPARISON OF MEAN LOCOMOTOR ACTIVITY OF SIX STRAINS OF MICE COMBINED ACROSS NONINJECTION AND SALINE TREATMENTS

	Mice Tested Singly[a]					
Strain	CBA	C3H/An	DBA/2	NLW	BALB/c	C57BL/6
Activity	2.733	2.821	2.894	2.900	2.908	2.926

	Mice Tested in Groups[a]					
Strain	DBA/2	BALB/c	CBA	C3H/An	C57BL/6	NLW
Activity	3.820	3.311	3.344	3.356	3.406	3.465

[a] Values not sharing underline differ significantly ($p < 0.05$).

TABLE IV—COMPARATIVE EFFECTS OF GROUPING ON ACTIVITY IN SIX STRAINS MEASURED AS MEAN DIFFERENCES FROM SINGLY TESTED MICE[a]

Strain	DBA/2	BALB/c	C57BL/6	C3H/An	NLW	CBA
Difference	0.386	0.403	0.480	0.535	0.565	0.611

[a] Values not sharing underline differ significantly ($p < 0.05$).

phenomenon indicated by these data is its occurrence in mice tested in groups, as well as those tested singly. Although the present observations include four of the same inbred strains utilized in the study by Meier *et al.* (4), the data do not confirm their implication that the effect of saline treatment varies between strains. While there was a superficial suggestion in the untransformed data that the degree of response to saline might differ between strains, the nonsignificant injection x strain interaction term failed to support such an inference. Any such trend surely was not consistent enough to be detected by the analysis.

The pharmacological basis for the phenomenon of saline inhibition of activity remains quite unknown. The authors have termed it a placebo effect following the definition of Wolf (9), "... any effect attributable to a pill, potion or procedure, but not to its pharmacodynamic or specific properties." It surely cannot have the same explanation as that given for a placebo effect of saline injection upon locomotor activity of rats (10), *i.e.*, simple Pavlovian conditioning of the depressant effect of a previously administered pharmacologically active agent, scopolamine.

REFERENCES

(1) Schnitzer, S. B., and Ross, S., *Psychol. Rept.*, **6**, 351 (1960).
(2) Meier, G. W., *ibid.*, **11**, 333(1962).
(3) Schnitzer, S. B., and Ross, S., *ibid.*, **8**, 142(1961).
(4) Meier, G. W., Hatfield, J. L., and Foshee, D. P., *Psychopharmacologia*, **4**, 81(1963).
(5) Thompson, W. R., *Can. J. Psychol.*, **7**, 145(1953).
(6) Thompson, W. R., *J. Heredity*, **47**, 147(1956).
(7) Wright, L. S., Jr., Horn, H. J., and Woodard, G., *Federation Proc.*, **21**, 420(1962).
(8) Duncan, D. B., *Biometrics*, **11**, 1(1955).
(9) Wolf, S., *Pharmacol. Rev.*, **11**, 689(1959).
(10) Herrnstein, R. J., *Science*, **138**, 677(1962).

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The *in situ* isolated brain, supplied by an extracorporeal pump-oxygenator system, shows a drug metabolizing activity. The tested substances (aminopyrine and oxazepam) show a disappearance from the extracorporeal blood of dog and monkey related to the metabolic transformations (demethylation, acetylation, or glucurono-conjugation) and to the fixation at the cerebral tissues.

IN A PREVIOUS PAPER (1) the authors described a method to investigate *in situ* the metabolizing activity of the liver connected normally with the body, except for blood circulation, which was supplied by a pump-oxygenator system. In such conditions, the liver functional tests remained within normal limits throughout the experiment, and it was possible to study the rate of some hepatic metabolizing activities, such as demethylation, acetylation, and glucurono-conjugation. In the course of systematic investigations on drug metabolism and tissue distribution (2–4) the research was extended to the metabolic activity of the brain, isolated *in situ* in the

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living animal. A cat's brain perfusion method *in vivo* was described in 1947 by Geiger and Magnes (5) and modified by Geiger and associates (6, 7). Experiments of brain perfusion were performed either on the severed head (8–10) or with incomplete separation of the brain circulation from the head circulation (11) and from the systemic circulation *in situ* (12–17).

According to Geiger (6), the choice of experimental animals depends on the vascular anatomy of the head and the reasonable availability. The monkey is more suitable for the isolation of the arterial side of the circulation, since no major communication exists between the brain and the rest of the body, except *via* the internal carotid and the vertebral arteries; nevertheless, the blockage of the vertebral sinuses is difficult. The dog is suitable for availability and size, but it shows a *rete mirabile* between the brain and the extracerebral circulation, and a rather large anterior spinal artery. It is impossible to separate the arterial side of the cerebral circulation from that of the extracranial circulation in the cat, because the blood from the carotid arteries reaches the Willis' circle through a *rete mirabile*. Thus, the Geiger procedure (18) consists mainly of the isolation and the occlusion of all the venous outlets of the cat's brain and of opening a new outlet by tapping the cerebral sinuses. In view of the possibility of extending the research to the isolated fetus (19), the dog and monkey were chosen because of their size.

To test the metabolizing activity of the brain isolated *in situ*, some transformations of aminopyrine (demethylation and acetylation) and oxazepam (glucuronide-conjugation) have been examined.

METHOD

The experiments were carried out on 18 dogs (10.3 to 19.7 Kg.) and 6 monkeys (*C. hamadryas*, 8.2 to 12.8 Kg.) preanesthetized with urethan (0.4 Gm./Kg. i.p.). Anesthesia was induced and maintained in closed circuit by nitrous oxide, cyclopropane, or ethyl ether. The animals were given artificial ventilation after tracheal intubation by Warne tube, following succinylcholine chloride (1 mg./Kg. i.v.) administration. Arterial blood pressure was measured from a cannula inserted into a femoral artery. Extradural electrodes were set in place some days before.

The operative procedure consists mainly of the isolation of the jugular external veins in the dog or the jugular internal veins in the monkey, and the isolation of the common carotid arteries and ligature of all their branches except the internal carotid arteries. The vertebral vessels are ligated before their entrance into the transverse foramen of C2 or C3. The numerous muscular branches arising from the vertebral vessels, the anastomosis between vertebral and carotid arteries, the anastomosis between vertebral and jugular veins, the internal jugular veins in the dog or the external jugular veins in the monkey, the vascular muscular branches of the neck, the vessels running under the carotid arteries and vagus nerves, the zygomatic, maxillary, auricular, and supra-orbital vessels are occluded by ligature or compression. The occlusions of the sinus columnae vertebralis and of the anterior spinal artery were made (*a*) by opening the rachis in C2, ligating the dog's spinal artery according to the method of Greeley and Greeley (20), and compressing the venous vessels; (*b*) by opening the rachis between C2 and C3, and compressing all the vessels around the spinal cord.

Both isolated jugular veins were ligated, cannulated, and connected with the venous reservoir of the pump-oxygenator system through the gravitational flow. Both the isolated carotid arteries were cannulated and connected with the pump-oxygenator system; at this point the perfusion started.

Hematocrit, clotting-time, and arterio-venous blood oxygen were measured from samples of both extracorporeal and systemic circulation.

The brain perfusion apparatus employed consists of a venous reservoir, an oxygenator with gasmeter, a roller-type pump with flowmeter, a blood filter, an apparatus to eliminate blood foam, a perfusion pressure regulator with manometer, and a blood exchanger with telethermometer.

Before the extracorporeal brain perfusion, the pump-oxygenator system was filled with 400–500 ml. of heparinized, defibrinated blood. This blood was previously obtained from the animal infused with blood from donor animals. The collected blood was filtered through cloth, preserved by ampicillin (1:10,000), and stored in a refrigerator. Before the perfusion, the blood was filtered, diluted with Tyrode's solution (to O_2 capacity = 13 ± 1 vol. %), and added to glucose (10%). The priming blood was circulated through the pump-oxygenator system fully oxygenated and warmed. A flow of O_2–CO_2 mixture (95:5) into the oxygenator was maintained at the rate of 4–8 L./min.; during the extracorporeal brain perfusion, the blood flow rate was kept very high (8–10 ml./min./Kg.). In most experiments, it was possible to maintain the blood pressure constantly equal to the initial systemic pressure of the animal (100 to 140 mm. Hg).

The time of brain perfusion was limited to 60 or 90 min. and was related to the presence of cerebral electric activity.

The eventual leakage of perfusate into the systemic circulation was evaluated (*a*) during the metabolic research, by taking samples from the general circulation of the blood and of the lymph to verify the absence of the tested substance or its metabolites; (*b*) at the end of the metabolic research by adding either a dye or a radiopaque substance to the blood of the extracorporeal circuit.

The metabolizing activity of the brain was investigated *in situ* by (*a*) the demethylation, by evaluating the transformation of aminopyrine to 4-aminoantipyrine using the method of Brodie and Axelrod (21) after addition of 50–100 mcg./ml. of aminopyrine into the extracorporeal circuit; (*b*) the acetylation, by evaluating the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine under the above-mentioned conditions; (*c*) the glucuronoconjugation, by evaluating the transformation of oxazepam to glucuronide, using the method of Walkenstein *et al.* (22) after addition of 10–20 mcg./ml. of oxazepam into the extracorporeal circuit.

RESULTS

The *in situ* isolated brain shows a metabolizing activity. In the extracorporeal circuit of both the dog and monkey, the decrease of aminopyrine plasma levels is 16% (14 to 21%) in 60 min., and 21% (17 to 23%) in 90 min.; the decrease of oxazepam plasma

levels is 22% (20 to 25%) in 60 min., and 25% (23 to 30%) in 90 min. The drugs' disappearance is partially replaced by their metabolites.

The affinity of aminopyrine and oxazepam for the brain tissues seems similar. Furthermore, no significant difference is noted on the brain metabolizing activity in the two different ways of occlusion of the sinus columnae vertebralis.

Figure 1 is a typical example of the rate of transformation of aminopyrine in two metabolites. The demethylation and the acetylation are quite similar in the dog and monkey.

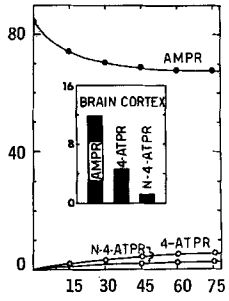


Fig. 1—Aminopyrine metabolism studied by the isolated brain perfusion in situ *in a monkey* (C. hamadryas) *weighing 12.8 Kg. Blood in extracorporeal circuit = 500 ml.; blood flow rate of the extracorporeal circuit = 115 ml./min.; initial concentration of aminopyrine in the plasma of the extracorporeal circuit = 86.5 mcg./ml. An ordinate, the plasmatic concentration (mcg./ml.) of aminopyrine* (AMPR), *4-aminoantipyrine* (4-ATPR), *and N-acetyl-4-aminoantipyrine* (N-4-ATPR), *assayed for 75 min. (in abscissae) after aminopyrine addition. Insert shows the brain cortex concentration (mcg./Gm. in ordinate) of aminopyrine and its two metabolites, assayed after 75 min. of the extracorporeal brain perfusion with addition of aminopyrine.*

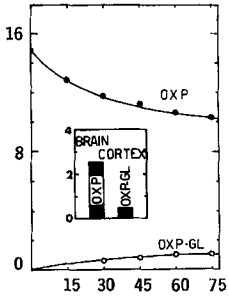


Fig. 2—Oxazepam metabolism studied by the isolated brain perfusion in situ *in a monkey* (C. hamadryas) *weighing 10.7 Kg. Blood in extracorporeal circuit = 480 ml.; blood flow rate of extracorporeal circuit = 95 ml./min.; initial concentration of oxazepam in the plasma of the extracorporeal circuit = 15.3 mcg./ml. An ordinate, the plasmatic concentrations (mcg./ml.) of oxazepam* (OXP) *and its glucuronide* (OXP-GL) *assayed for 75 min. (in abscissae) after oxazepam addition. Insert shows the brain cortex concentration (mcg./Gm. in ordinate) of oxazepam and its glucuronide, assayed after 75 min. of the extracorporeal brain perfusion with addition of oxazepam.*

Figure 2 is a typical example of the glucurono-conjugation of the isolated brain; also the glucurono-conjugation is quite similar in both species.

CONCLUSIONS

The brain isolated *in situ* shows a drug metabolism evaluated by studying the demethylation and the acetylation of aminopyrine and the glucurono-conjugation of oxazepam. In dogs and monkeys, the decrease of aminopyrine plasma levels is 16% and of oxazepam is 22% in 60 min. In studies (1) on the isolated liver *in situ* it was found that the half-time for aminopyrine ranged from 10 to 14 min. in the dog, and from 16 to 21 min. in the monkey, and for oxazepam from 7 to 10 min. in both animals.

These great differences on drug metabolism between brain and other tissues *in situ* seem to be interesting, particularly when the action on the nervous system is not induced by the drug itself, but by its metabolic products.

SUMMARY

The cerebral drug metabolism of dog and monkey has been investigated by the *in situ* isolated brain supplied by an extracorporeal pump-oxygenator system.

To test the brain metabolizing activity, some transformation of aminopyrine (demethylation and acetylation) and oxazepam (glucuronide-conjugation) was examined.

The drugs disappeared from the extracorporeal blood at the rate of 16% for aminopyrine and of 22% for oxazepam in 1 hr.; at the same time their metabolic products appeared. The concentration in the brain cortex of the substances has been evaluated also.

REFERENCES

(1) Benzi, G., Berté, F., and Crema, A., *J. Pharm. Sci.*, **56**, 893(1967).
(2) Mascherpa, P., *Actualités Pharm.*, **16**, 121(1963).
(3) Mascherpa, P., Atti 20° Conv. Farmitalia, Milan, Italy, 1965, p. 458.
(4) Mascherpa, P., and Berté, F., *Biochem. Pharmacol.*, **5**, 200(1966).
(5) Geiger, A., and Magnes, J., *Am. J. Physiol.*, **149**, 517 (1947).
(6) Geiger, A., *Physiol. Rev.*, **38**, 1(1958).
(7) Geiger, A., Magnes, J., Taylor, R. M., and Veralli, M., *Am. J. Physiol.*, **177**, 138(1954).
(8) Bouckaert, J. J., and Jourdan, F., *Arch. Intern. Pharmacodyn.*, **53**, 523(1936).
(9) Chute, A. L., and Smyth, D. H., *Quart. J. Exptl. Physiol.*, **29**, 379(1939).
(10) Heymans, C., Bouckaert, J. J., Jourdan, F., Nowak, S. J. G., and Faber, S., *Arch. Neurol. Psychiat.*, **38**, 304 (1937).
(11) Malméjac, J., Neverre, G., and Rambert, R., *Compt. Rend. Soc. Biol.*, **150**, 382(1956).
(12) Schmidt, C. F., *Am. J. Physiol.*, **84**, 223(1928).
(13) Jensen, J. M., and Parkins, W. M., *Federation Proc.*, **13**, 75(1954).
(14) Delorme, E. J., *Lancet*, **II**, 914(1952).
(15) Lougheed, W. M., and Kahn, D. S., *J. Neurosurg.*, **12**, 226(1952).
(16) Woodhall, B., and Reynolds, D. H., *Proc. Soc. Exptl. Biol. Med.*, **97**, 194(1958).
(17) Woodhall, B., Hall, K., Mahaley, M. S., Jr., and Jackson, J., *Ann. Surg.*, **150**, 640(1959).
(18) Geiger, A., *Methods Med. Res.*, **9**, 248(1961).
(19) Alexander, D. P., Britton, H. G., and Nixon, D. A., *J. Physiol., London*, **175**, 113(1964).
(20) Greeley, C. E., and Greeley, P. O., *Am. J. Physiol.*, **95**, 382(1930).
(21) Brodie, B. B., and Axelrod, J., *J. Pharmacol. Exptl. Therap.*, **99**, 171(1950).
(22) Walkenstein, S. S., Wiser, R., Gudmundsen, C. H., Kimmel, H. B., and Corradino, R. A., *J. Pharm. Sci.*, **53**, 1181 (1964).

Xenon Trioxide Oxidation of Certain Alcohols

By HAROLD J. RHODES and MARTIN I. BLAKE

A series of alcohols, aliphatic and aromatic, was oxidized with xenon trioxide. Oxidation to carbon dioxide and water was effected by adding a known excess of xenon trioxide to the reaction mixture. The excess reagent was determined iodometrically. Of the alcohols tested only triphenylcarbinol resisted oxidation. Essentially quantitative recoveries were obtained for ethanol, normal, secondary, and tertiary butyl alcohols, benzyl alcohol, benzohydrol, and cinnamyl alcohol.

THE PREPARATION and oxidizing properties of xenon trioxide in aqueous solution have been described by Williamson and Koch (1) and Appelman and Malm (2). Jaselskis and his associates have studied the xenon trioxide oxidation of a variety of organic structures including vic-diols, certain primary and secondary aliphatic alcohols, and a number and variety of acids. Quantitative analytical procedures sensitive in the microgram range were developed for these compounds. Those studies have been referred to in an earlier paper (3) which described the xenon trioxide oxidation of benzyl alcohol and benzaldehyde.

The present investigation concerns the determination and stoichiometry involved in the oxidation of certain aliphatic and aromatic alcohols. These include ethanol, normal, secondary, and tertiary butyl alcohols, benzyl alcohol, benzohydrol, triphenylcarbinol, and cinnamyl alcohol. This selection of alcohols permitted a correlation of chemical structure and oxidation capacity.

EXPERIMENTAL

Solutions and Reagents—Xenon trioxide solution (0.0199 *M* to 0.0493 *M*) was prepared by diluting a concentrate (0.2 *M*) with water. The preparation of the concentrated xenon trioxide solution by hydrolysis of xenon hexafluoride has been described by Appelman and Malm (2).

The following stock solutions of the different alcohols were prepared: 0.157 *M* ethanol, 0.253 *M* *n*-butyl alcohol, 0.101 *M* *sec*-butyl alcohol, 0.0915 *M* *tert*-butyl alcohol, 0.0100 *M* and 0.0020 *M* benzyl alcohol, 0.0020 *M* benzohydrol, 0.0020 *M* cinnamyl alcohol, and a suspension of triphenylcarbinol in water (50.0×10^{-3} mmoles in 10 ml. of water).

Sulfuric acid, 10% w/v, and starch test solution. All chemicals and solvents were reagent grade.

Procedure—Aliquots of the alcohol stock solution containing between 0.5×10^{-3} and 20×10^{-3} mmole of the alcohol were added by micropipet to a series of 50-ml. glass-stoppered conical flasks. Ten milliliters of distilled water was added to each flask. Xenon trioxide solution in known excess was added to each solution. The flasks were stoppered and swirled gently to assure homogeneous distribution of the reactants. The reaction mixtures containing the aliphatic alcohols were allowed to stand at room temperature for at least 17 hr. The oxidation of the aromatic alcohols was permitted to continue for longer periods of time; 5 days for cinnamyl alcohol, 6 days for benzyl alcohol, 8 days for benzohydrol, and 30 days for triphenylcarbinol. In each series one flask contained no alcohol and served as a blank. At the end of the reaction period 5.0 ml. of 10% sulfuric acid solution and 1.0 Gm. of potassium iodide were added to each flask. The liberated triiodide was titrated with standard sodium thiosulfate solution using a 10-ml. microburet. As the end point was approached 3 drops of starch solution was added and the titration was continued until the solution turned colorless. The number of millimoles of xenon trioxide consumed in the oxidation was determined from the normality of the thiosulfate solution and the difference in the volumes consumed by the blank and the sample. The millimoles of xenon trioxide consumed in the oxidation was plotted against the millimoles of alcohol added. The slope and *y*-intercept were determined from the data by the least squares method.

In preliminary studies reaction mixtures were prepared of all alcohols and xenon trioxide in minimum excess. The rate of oxidation was followed by analyzing for xenon trioxide consumed after varying periods of time.

Because of the low solubility of triphenylcarbinol in water, 50.0×10^{-3} mmole of the alcohol was dispersed in 10 ml. of water. An excess (493.0×10^{-3} mmole) of xenon trioxide solution was added and the solution was stirred with a magnetic stirrer for 30 days at room temperature. After varying periods of time, an aliquot of the supernatant was analyzed for unspent xenon trioxide.

Gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) were employed to detect whether reaction products other than carbon dioxide and water were formed under the reaction conditions employed. The details of these procedures were described in the previous paper (3); however, pertinent GLC and TLC data are included here.

RESULTS AND DISCUSSION

A series of alcohols of varying structure were oxidized with xenon trioxide. Oxidation of the aliphatic alcohols proceeded rapidly forming carbon dioxide and water as the only reaction products. Although preliminary studies indicated that for the aliphatic alcohols reaction was complete in less than 4 hr., a 17-hr. reaction period was selected for these compounds. The pH of all reaction mixtures was about 4. Analysis of the reaction mixtures by GLC and TLC indicated that oxidation was complete; neither starting alcohol nor intermediate oxidation products were detected. With the water-soluble aromatic alcohols, benzyl alcohol, benzohydrol, and cinnamyl alcohol, a 17-hr. reaction period gave incomplete oxidation, as evidenced by the detection of

Received April 14, 1967, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Accepted for publication July 5, 1967.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors gratefully acknowledge E. H. Appelman and J. G. Malm, Argonne National Laboratory, Argonne, Ill., for supplying the xenon trioxide used in this study and thank Miss Regina Shiau for technical assistance in certain aspects of this study.

TABLE I—RESULTS OF VAPOR PHASE AND THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF REACTION MIXTURES OF BENZOHYDROL, CINNAMYL ALCOHOL, AND TRIPHENYLCARBINOL

Compd.	Vapor Phase Chromatography Col. Temp., °C.	Vapor Phase Chromatography Retention Time, sec.	Thin-Layer Chromatography[a] $R_f \times 100$
Benzohydrol	190	154	60
Benzophenone	190	92	86
Cinnamyl alcohol	200	308	22
Cinnamaldehyde	200	180	52
Cinnamic acid	200	1440	16
Triphenylcarbinol	190	206	71

[a] Silica Gel HF_{254}; two ascending developments with chloroform for 67 min. each; detection by scanning with ultraviolet light.

oxidation products and unreacted alcohol employing GLC and TLC methods of analysis. The benzyl alcohol reaction was found to contain benzaldehyde, benzoic acid, and unreacted benzyl alcohol. Complete oxidation was noted, however, after 6 days. Benzophenone, benzoic acid, and unreacted benzohydrol were detected in the benzohydrol reaction mixture. However, after 8 days no detectable quantities of organic compounds were observed. The cinnamyl alcohol reaction mixture showed the presence of the corresponding aldehyde and acid along with the unreacted alcohol. After 5 days oxidation was complete. TLC and GLC data characterizing benzyl alcohol, benzaldehyde, and benzoic acid were reported in the previous paper (3). The results for the other alcohols and their corresponding oxidation products are shown in Table I.

Because of poor solubility, a heterogeneous system was used for studying the oxidation of triphenylcarbinol. The xenon trioxide content of the reaction mixture remained unchanged even after a 30-day reaction period. The reaction mixture was extracted with ether and the ether extract was examined by GLC and TLC. Triphenylcarbinol was the only compound identified in the extract. This alcohol appears to be resistant to xenon trioxide oxidation. GLC and TLC data for triphenylcarbinol are shown in Table I.

The results of the oxidation studies are shown in Table II. The concentrations of the alcohol and xenon trioxide before reaction, the amount of xenon trioxide actually consumed in the reaction, and the ratio of xenon trioxide to alcohol involved in the reaction are recorded in the table. A plot of alcohol concentration *versus* xenon trioxide concentration consumed in the oxidation gives a straight line which intersects the ordinate above the origin. The y-intercept and slope, obtained by least square treatment of the data shown in Table II, and the theoretical slope are recorded in Table III. A similar approach was used by Jaselskis and Warriner (4) for the oxidation of methanol, ethanol, and 2-propanol. They attributed the nonstoichiometric nature of the reaction to secondary processes which occur in the oxidation. Theoretically, the curve should pass through the origin ($c = 0$) and the slope of the line should correspond to the ratio of xenon trioxide to alcohol, assuming complete oxidation to CO_2 and H_2O. Such a plot is useful as a calibration curve for determining

TABLE III—RESULTS OF LEAST SQUARE TREATMENT OF DATA FOR EACH ALCOHOL

Alcohol	y-Intercept, mmoles $\times 10^3$	Slope, m	Theoretical Slope,[a] m'
Ethanol	1.29	2.02	2.00
n-Butyl alcohol	2.82	3.75	4.00
sec-Butyl alcohol	1.38	3.76	4.00
tert-Butyl alcohol	2.88	3.76	4.00
Benzyl alcohol	0.27	5.75	5.67
Benzohydrol	2.83	8.84	10.33
Cinnamyl alcohol	2.41	6.84	7.33

[a] Based on alcohol being completely oxidized to carbon dioxide and water.

TABLE II—RESULTS OBTAINED FROM XENON TRIOXIDE OXIDATION OF ALCOHOLS

Alcohol	Alcohol Taken, mmoles $\times 10^3$	Alcohol[a] Found, mmoles $\times 10^3$	Xenon Trioxide Taken, mmoles $\times 10^3$	Xenon Trioxide Consumed, mmoles $\times 10^3$	Xenon Trioxide to Alcohol Ratio
Ethanol	15.70	15.56	57.60	34.90	2.22
	7.85	8.18	57.60	18.90	2.42
	1.57	1.44	57.60	11.90	2.74
n-Butyl alcohol	12.60	12.58	58.00	50.00	3.97
	5.10	5.17	58.00	20.50	4.05
	2.50	2.45	58.00	11.89	4.66
sec-Butyl alcohol	10.10	10.08	40.00	39.30	3.89
	5.10	5.14	40.00	20.71	4.10
	2.00	1.94	40.00	8.66	4.28
tert-Butyl alcohol	9.50	9.47	40.00	38.50	4.04
	4.76	4.86	40.00	21.15	4.44
	1.90	1.84	40.00	9.79	5.60
Benzyl alcohol	2.32	2.29	20.00	16.04	6.90
	1.55	1.61	20.00	12.13	7.83
	0.77	0.74	20.00	7.13	9.20
Benzohydrol	1.50	1.49	20.00	15.99	10.66
	1.00	1.02	20.00	11.83	11.83
	0.50	0.49	20.00	7.20	14.40
Cinnamyl alcohol	1.53	1.51	20.00	12.77	8.35
	1.02	1.05	20.00	9.58	9.40
	0.51	0.50	20.00	5.80	11.38

[a] Calculated from the equation: $y = c + mx$, from data for each alcohol, where y is mmoles of xenon trioxide consumed, c is the y-intercept, m is the slope, and x is mmoles of alcohol.

the alcohol content in a sample for analysis as suggested by Jaselskis and Warriner, or the equation:

$$y = c + mx$$

may be solved; where c is the y-intercept, m is the slope, y is the millimoles of xenon trioxide, and x is the millimoles of alcohol in the sample. This technique was applied to the alcohol samples listed in the first column of Table II. The alcohol concentrations found by substituting in the above equation are listed in the second column. Per cent recovery was better than 97% in most cases.

REFERENCES

(1) Williamson, S. M., and Koch, C. W., "Noble Gas Compounds," Hyman, H. H., ed., University of Chicago Press, Chicago, Ill., 1963, pp. 158–166.

(2) Appelman, E. H., and Malm, J. G., *J. Am. Chem. Soc.*, **86**, 2141(1964).

(3) Rhodes, H. J., Kluza, R., and Blake, M. I., *J. Pharm. Sci.*, **56**, 779(1967).

(4) Jaselskis, B., and Warriner, J. P., *Anal. Chem.*, **38**, 563(1966).

Synthesis of Ketoximino Esters as Antihistaminics

By S. L. LEE, B. B. WILLIAMS, and M. M. KOCHHAR*

In a search for new antihistamines, a series of oximino esters of 2-, 3-, and 4-benzoylpyridine oxime and 2-benzoylthiophene oxime was prepared. The respective ketones were oximinated in pyridine with hydroxylamine hydrochloride. The esterification was conducted in benzene with equimolar quantities of acetyl chloride, oxime, and a basic reagent. Twelve new oximino esters were synthesized and evaluated for their antihistaminic activity. All synthesized compounds exhibited antihistaminic activity with almost no anticholinergic action. The propionyl analogs showed the most significant antihistaminic effectiveness.

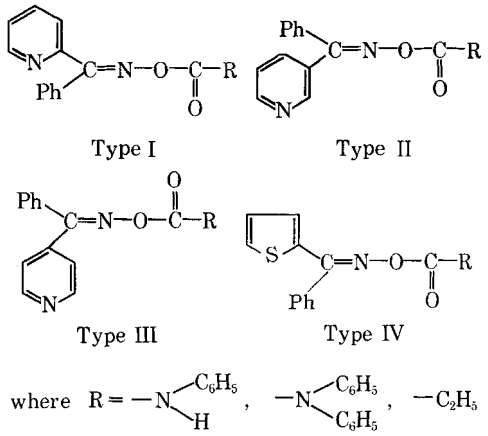
BOVET (1) issued preliminary reports concerning the first effective synthetic antihistaminic drugs. Since that time, a multitude of compounds have been prepared and tested for antihistaminic activity. The chemical structures of these antihistaminic drugs vary, yet the prominent compounds exert similar pharmacological and therapeutic action. Several review articles (2–5) have appeared in the literature. Some of the many useful antihistaminic agents differ sufficiently to hold out hope of attaining drugs which would be more specific for a given allergic manifestation.

Most commonly used drugs can be classified as derivatives of ethanolamine, ethylenediamine, aminopropane, phenothiazine, or piperazine (6). Almost all of these compounds contain a terminal tertiary nitrogen, generally having dimethyl substitution or part of the heterocyclic structure. In the ethanolamine series, the most effective group attached to the oxygen atom has been found to be the benzhydryl radical (7); whereas in the ethylenediamine series, several different radicals on the second nitrogen of the chain have led to active compounds. Several physical properties of antihistaminics have been studied in an effort to relate them to activity of the various drugs (8), but no direct relationship can be made between physical properties and antihistaminic activity.

These observations prompted the investigations reported in this paper. The object of the present work was to prepare and examine some of the oximino esters of benzoylpyridines and benzoylthiophene and to determine whether they possessed significant antihistaminic action.

DISCUSSION

The compounds which were selected for synthesis are the oximino esters of benzoylpyridines and benzoylthiophenes (types I, II, III, and IV).



The preparation of 12 new esters of type I, II, III, or IV, starting with respective benzoylpyridines and 2-benzoylthiophene, was accomplished by modifying the methods described in the literature (9, 10).

The oximes selected in this study included representatives of both syn- (types I and III) and anti- (types II and IV) configuration. The esters were not geometrically confirmed since it was assumed that esterification did not alter configuration. The purpose of including compounds of both configurations was to allow some comparison of antihistaminic effect of the isomers. Pharmacological screening provided no evidence of effect of geometric configuration on intensity of antihistaminic action.

The basic unit for all effective agents contains the ethylamine skeleton in one form or another. It is interesting that the ethylamine skeleton also corresponds to the side chain of the histamine molecule and to part of the imidazole ring. With this in mind the authors attempted replacement of the

Received April 14, 1967, from the School of Pharmacy, Auburn University, Auburn, AL 36830

Accepted for publication July 12, 1967.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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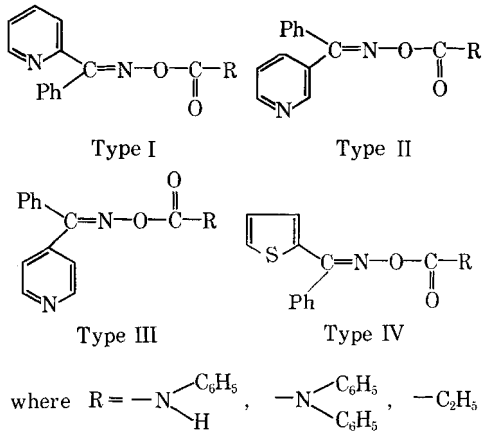

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ethylamine side chain with oximino linkage to see its effect on antihistamine activity. The terminal nitrogen is an amide nitrogen having one or two phenyl substituents (compounds I, II, IV, V, VII, VIII, X, and XI). These compounds exhibited antihistaminic activity ranging from 0.5 to 0.1 of diphenhydramine. This very clearly indicates that the ethylamine side chain is not an essential feature. The authors feel that a region of high electron density in that neighborhood is essential for the binding of drug to the receptor. This is further substantiated by synthesis of the propionyl derivatives (III, VI, IX, and XII) in which the carbonyl oxygen exhibits a higher electron density. These exhibited higher antihistaminic activity, 0.5 to 0.2 of diphenhydramine. This also indicates that the terminal nitrogen is not absolutely essential for biological activity. The oximino group should certainly alter the partition coefficient of the compound and thus affect its distribution in the body. Additionally, the oximino linkage should affect the intrinsic reactivity and stereochemistry of a given compound as well as its susceptibility toward metabolic degradation.

Most of the antihistaminic drugs have a minimum of two aryl or aralkyl groups or their equivalent in a polycyclic ring system or one aryl and one heterocyclic ring system. The authors' experimental compounds have benzoylpyridines or 2-benzoylthiophene. This moiety of the drug is the flat portion and this might occupy the flat area on the receptor.

Another very striking similarity among some of the known antihistaminics is the interatomic distances.[1] The interatomic distances between the terminal nitrogen atom of histamine and the 1 and 3 nitrogens of the imidazole ring are 6.12 and 4.52 Å., respectively. The interatomic distance between the pyridine nitrogen and the dimethylamino nitrogen of mepyramine, 2-[(2-dimethylaminoethyl) (*p*-methoxybenzyl) amino] pyridine, is 6.12 Å. The distance between the methoxy oxygen and the terminal nitrogen of mepyramine is 4.52 Å. In methapyrilene,2-[(2-dimethylaminoethyl)-2-thenylamino]-pyridine, the distance between pyridine *N* and terminal nitrogen is 6.12 Å. The distances in some of the more potent antihistaminics are very close to the above-mentioned compounds. The interatomic distances in the synthesized compounds are very comparable to histamine, mepyramine, methapyrilene, diphenhydramine, *etc.* In 2-diphenylcarbamyloximino benzoylpyridine (II) and 2-phenylcarbamyloximino benzoylpyridine (I), the interatomic distances between the pyridine nitrogen and the carbamyl nitrogen and between the pyridine nitrogen and the ether oxygen are 6.16 Å. and 4.2 Å., respectively. Similarly the interatomic distances in compounds IV, V, VII, VIII, X, and XI are very comparable with some of the known antihistaminics.

Studies of spatial dispositions, bond angles, and other physicochemical properties might possibly reveal correlations.

PHARMACOLOGIC EVALUATION

Antihistaminic potency was estimated by use of isolated strips of guinea pig ileum. The lowest concentration which blocked the spasmogenic effect of histamine was determined for the 12 test compounds and for diphenhydramine according to the procedure of Zielenski (11). A Phipps and Bird isolated tissue apparatus was used which provided for temperature maintenance of reservoirs of physiological solution and provided also for upward draining and replacement of physiological solution in the tissue chamber. Contractions were recorded with an E and M myograph photoelectric force transducer and physiograph recording system. Histamine phosphate (0.2 ml. of a 0.055% solution) was added to give a concentration of 5.5×10^{-6} in the tissue chamber. The test compound in water or aqueous propylene glycol was added followed in 1 min. by histamine in the same quantity as the first addition. Failure of needle deflection on addition of the challenge dose of histamine was considered the end point. Strips of ileum were washed after presentation of each concentration of test drug and replaced after two tests or in event of failure of a strip to respond to histamine after washing. For most of the end point concentrations at least one replication was done. Table I presents data from the antihistaminic activity tests.

TABLE I—ANTIHISTAMINIC ACTIVITY OF KETOXIMINO ESTERS ON EXCISED ILEUM SEGMENTS OF THE GUINEA PIG

Compd.	Lowest Histamine Blocking Concn., Gm./ml.
I	3.12×10^{-5}
II	6.25×10^{-5}
III	1.56×10^{-5}
IV	3.12×10^{-5}
V	1.25×10^{-4}
VI	6.25×10^{-6}
VII	7.81×10^{-6}
VIII	3.12×10^{-5}
IX	1.56×10^{-5}
X	3.12×10^{-5}
XI	3.12×10^{-6}
XII	7.81×10^{-6}
Diphenhydramine	3.91×10^{-6}

EXPERIMENTAL[2]

Synthesis of Oximes

2-Benzoylpyridine Oxime—2-Benzoylpyridine (5.49 Gm., 0.03 mole), hydroxylamine hydrochloride (3.5 Gm., 0.05 mole), and pyridine (20 ml.) in ethanol were refluxed for 12 hr. The bulk of the ethanol was removed under reduced pressure and the mixture was poured into cold water. The crude product thus obtained was recrystallized from ethanol which afforded the oxime, m.p. 151–152.5°. [Lit. (12) m.p. 150.5–152.5°.]

3-Benzoylpyridine Oxime—The above procedure was adapted to this preparation. A pure sample of this compound melted at 160.5°. [Lit. (13) m.p. 161°.]

4-Benzoylpyridine Oxime—The method of Kochhar *et al.* (10) was adapted to this oxime. The oxime

[1] Interatomic distances were estimated from Dreiding

[2] Reported melting points are uncorrected. A Thomas-Hoover Unimelt apparatus was used for melting point determination. Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., conducted the elemental analyses.

TABLE II—PHYSICAL CONSTANTS AND ANALYTICAL DATA

Compd.	R	Method of Prepn.	M.p.	Recrystn. Solvent	% Yield (Crude)	Empirical Formula	Anal., % Calcd.	Anal., % Found.
				Type I				
I	$-N(C_6H_5)H$	*A*	167–168° dec.	Ethanol	95	$C_{19}H_{15}N_3O_2$	C, 71.90 H, 4.76	C, 72.22 H, 4.76
II	$-N(C_6H_5)_2$	*B*	165° dec.	Benzene	74	$C_{25}H_{19}N_3O_2$	C, 76.31 H, 4.86	C, 76.20 H, 4.91
III	$-C_2H_5$	*C*	67°	Methanol	82	$C_{15}H_{14}N_2O_2$	C, 70.84 H, 5.55	C, 71.01 H, 5.71
				Type II				
IV	$-N(C_6H_5)H$	*A*	153–154°	Benzene	79	$C_{19}H_{15}N_3O_2$	C, 71.90 H, 4.76	C, 72.22 H, 4.93
V	$-N(C_6H_5)_2$	*B*	193° dec.	Ethanol–methanol	52	$C_{25}H_{19}N_3O_2$	C, 76.31 H, 4.86	C, 76.34 H, 4.97
VI	$-C_2H_5$	*C*	82–83.5°	Ethanol	42	$C_{15}H_{14}N_2O_2$	C, 70.84 H, 5.55	C, 70.60 H, 5.42
				Type III				
VII	$-N(C_6H_5)H$	*A*	198–199° dec.	Benzene	91	$C_{19}H_{15}N_3O_2$	C, 71.90 H, 4.76	C, 71.90 H, 4.76
VIII	$-N(C_6H_5)_2$	*B*	173° dec.	Ethanol	64	$C_{25}H_{19}N_3O_2$	C, 76.31 H, 4.86	C, 76.13 H, 4.83
IX	$-C_2H_5$	*C*	100–101°	Propanol–methanol	74	$C_{15}H_{14}N_2O_2$	C, 70.84 H, 5.55	C, 71.12 H, 5.62
				Type IV				
X	$-N(C_6H_5)H$	*A*	128°	Ethanol	51	$C_{18}H_{14}N_2O_2S$	C, 67.03 H, 4.37	C, 66.90 H, 4.28
XI	$-N(C_6H_5)_2$	*B*	211–212° dec.	Ethanol–methanol	48	$C_{24}H_{18}N_2O_2S$	C, 72.33 H, 4.55	C, 71.93 H, 4.44
XII	$-C_2H_5$	*C*	112–113°	Methanol	38	$C_{14}H_{13}NO_2S$	C, 64.83 H, 5.05	C, 64.94 H, 4.98

exhibited m.p. of 153°. [Lit. (14) m.p. 152–155°.]

2-Benzoylthiophene Oxime—The procedure mentioned under 2-benzoylpyridine oxime was followed. The pure sample gave a m.p. of 113°. [Lit. (14) m.p. 113–114°.]

Synthesis of Oximino Esters[3]

2-Phenylcarbamyloximino Benzoylpyridine (I)—*Method A*—2-Benzoylpyridine oxime (1.98 Gm., 0.01 mole), pyridine (1 Gm. 0.0126 mole), and phenylisocyanate (2.38 Gm., 0.02 mole) were refluxed for 1 hr. A brownish solid residue was obtained on cooling the mixture. The solid residue was washed with distilled water until there was no pyridine odor. Three repeated recrystallizations from ethanol afforded a pure analytical sample, m.p. 167–168° dec. Strong bands in the I.R. at 5.8 μ ($-\overset{\overset{O}{\|}}{C}-O-$) and 6.2 μ ($-C=N-$) are indicative of $-C-O-$ and $-C=N$, respectively; yield was 95% of theory.

3-Diphenylcarbamyloximino Benzoylpyridine (V)—*Method B*—In a three-neck flask, fitted with a stirrer, condenser, and dropping funnel, were placed 3-benzoylpyridine (1.98 Gm., 0.01 mole), pyridine (25 ml., the minimum amount which will dissolve the oxime), and benzene (100 ml.). The mixture was stirred and diphenylcarbamylchloride (2.31 Gm., 0.01 mole) in benzene (100 ml.) was added dropwise. After the completion of addition, the mixture was refluxed for 12 hr. with stirring. The mixture was cooled, washed with 10% potassium bicarbonate (5 times), and then washed with water (4 times), and the benzene phase was dried over anhydrous magnesium sulfate. On evaporation of benzene under reduced pressure a solid residue was obtained which on recrystallization from a mixture of ethanol–methanol afforded a pure sample, m.p. 193° dec. The I.R. gave a sharp band at 5.75 μ indicative of ester linkage. Yield was 52% of theory.

2-Propionyloximino Benzoylthiophene (XII)—*Method C*—2-Benzoylthiophene oxime (2.03 Gm., 0.01 mole) dissolved in the minimum quantity of pyridine was placed in a round-bottom flask. The mixture was diluted with benzene (200 ml.). On addition of propionylchloride (1.15 Gm., 0.016 mole) dropwise while stirring, a white suspension formed. This mixture was stirred for 4 hr. at room temperature, refluxed for 12 hr., cooled, and benzene washed with 10% potassium bicarbonate and water successively, and dried as in method *B*. On evaporation of benzene, under reduced pressure, an oily residue was obtained which on repeated washing

[3] See Table II for physical constants and analytical data.

with cold water gave a solid residue. The crude mixture was recrystallized from methanol, and yielded a pure sample, m.p. 112–113°. The I.R. gave a sharp peak at 5.70 μ indicative of ester. Yield was 38% of theory.

REFERENCES

(1) Bovet, D., and Staub, A. M., *Compt. Rend. Soc. Biol.*, **124**, 547(1937).
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(7) Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharmacol. Exptl. Therap.*, **86**, 1(1946).
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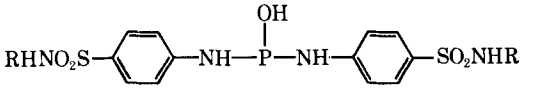
The synthesis of some phosphorus–nitrogen compounds *via* the condensation of phosphorus trichloride with a number of sulfa drugs is described. With sulfacetamide, sulfathiazole, and sulfadimidine three molecules of the sulfa compound condensed with one molecule of the acid (sulfamethazine) chloride although the molecular ratio of the reactants was 2 of the former to 1 of the latter. On the other hand, with sulfanilamide and sulfapyridine the condensation took place according to the ratio mentioned earlier.

THE INTEREST in the synthesis of phosphorus–nitrogen compounds arises from the fact that some of these compounds are of potential medicinal value. Cates *et al.* in a series of publications (1–5) reported the synthesis of nearly 60 such compounds and those containing substituted *p*-toluidine and 2-aminopyridine moieties were prepared for evaluation as antineoplastic agents. Likewise, it has been reported that the *N*-arylsulfonylphosphinimide derivatives synthesized by Oyamada have tumor inhibiting activity against mammary carcinoma (6).

Several attempts were made to condense sulfanilamide with phosphorus trichloride. Condensation in reagent dioxane according to the method of Cates (1, 4) proved to be unsatisfactory due to the immediate formation of an insoluble addition complex which did not dissolve even when the reflux was continued for several hours. The addition product afforded the starting sulfa compound after being worked up. The use of other nonpolar solvents such as carbon tetrachloride (6–8), absolute

TABLE I—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORODIAMIDOUS ACID



R	Solvent of Crystallization[a]	M.p.,[b] °C.	Formula	Anal.,[c] % N Calcd.	N Found	S Calcd.	S Found	P Calcd.	P Found
H	A	178–180 230 dec.	$C_{12}H_{15}N_4O_5PS_2$	14.35	13.90	16.42	16.50	7.93	7.94
2-Pyridyl	B	195–200 250 dec.	$C_{22}H_{21}N_6O_5PS_2$	15.44	15.07	11.78	11.35	...	...

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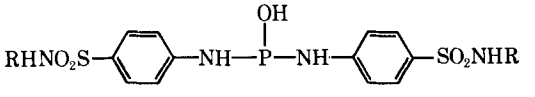

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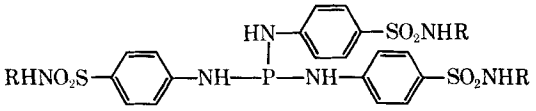
TABLE II—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORUS TRIAMIDE



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4,6-Dimethyl-2-pyrimidinyl	B	248–250[b] 270 dec.	$C_{36}H_{39}N_{12}O_6PS_3$	19.48	19.78	11.14	11.68	3.58	3.50

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That the phosphorus atom in the synthesized compounds is linked to the N^4 of the sulfa drug was shown qualitatively by the failure of all the condensation products to diazotize and by their solubility in dilute alkali and insolubility in dilute mineral acids.

EXPERIMENTAL

The condensation was carried out according to the following general procedure.

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The synthesis of some sulfazopyridine derivatives is described.

SINCE THE discovery of sulfamidochrysoidine[1] (1), a large number of sulfamoyl phenylazo dyes was made and their chemotherapeutic action was studied against various bacterial infections. In recent years combinations of sulfonamides and azo dyes are widely used as urinary disinfectants. The authors now report the synthesis of some azo dyes containing a sulfonamido moiety together with a diaminopyridyl moiety (I).

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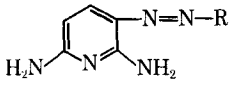

I

Derivatives having the above general formula were synthesized by coupling diazotized sulfonamides with 2,6-diaminopyridine hydrochloride. Attempts to prepare 2,6-diaminopyridine by amination of pyridine with sodamide in the presence of solvents were unsuccessful; in the absence of solvents, a 20% yield was obtained. Two methods were applied for diazotizing the sulfa compounds: (*a*) the standard procedure and (*b*) the alternate one, which consists of dissolving the sulfonamide in sodium hydroxide first. The diazotized sulfa compound

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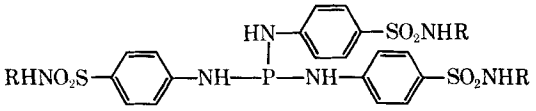

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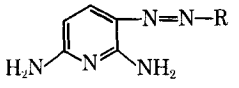
By H. Y. ABOU-ELENEIN, M. KHALIFA, and Y. M. ABOU-ZEID

The synthesis of some sulfazopyridine derivatives is described.

SINCE THE discovery of sulfamidochrysoidine[1] (1), a large number of sulfamoyl phenylazo dyes was made and their chemotherapeutic action was studied against various bacterial infections. In recent years combinations of sulfonamides and azo dyes are widely used as urinary disinfectants. The authors now report the synthesis of some azo dyes containing a sulfonamido moiety together with a diaminopyridyl moiety (I).

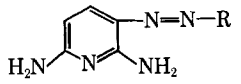
Received February 6, 1967, from the Faculty of Pharmacy, Cairo University, Cairo, United Arab Republic.
Accepted for publication May 4, 1967.
The products at present are under preliminary screening for possible antimicrobial action or any useful pharmacological activity.
[1] Prontosil.



I

Derivatives having the above general formula were synthesized by coupling diazotized sulfonamides with 2,6-diaminopyridine hydrochloride. Attempts to prepare 2,6-diaminopyridine by amination of pyridine with sodamide in the presence of solvents were unsuccessful; in the absence of solvents, a 20% yield was obtained. Two methods were applied for diazotizing the sulfa compounds: (*a*) the standard procedure and (*b*) the alternate one, which consists of dissolving the sulfonamide in sodium hydroxide first. The diazotized sulfa compound

TABLE I—SULFAZOPYRIDINE DERIVATIVES



M.p.,[b] °C.	Yield, %	Solvent of Crystallization[a]	Method of Diazotization	Formula	Anal.,[c] % Calcd. N	Calcd. S	Found N	Found S
R = N-(2-Phenyl-3-pyrazolyl)-p-sulfamoyl Phenyl								
134–135	95	B	(*a*)	$C_{20}H_{18}N_8O_2S$	25.77	7.36	25.16	7.14
R = N-(2,4-Dimethyl-6-pyrimidinyl)-p-sulfamoyl Phenyl								
250	89–92	B	(*a*)	$C_{17}H_{18}N_8O_2S$	28.1	8.04	28.66	8.20
R = N-(3,4-Dimethyl-5-isoxazolyl)-p-sulfamoyl Phenyl								
250	90	A	(*a*)	$C_{16}H_{17}N_7O_3S$	25.31	8.53	25.12	8.55
R = N-(2,4-Dimethoxy-6-pyrimidinyl)-p-sulfamoyl Phenyl								
220	89	A	(*a*)	$C_{17}H_{18}N_8O_4S$	26.04	7.44	25.99	7.29
R = N-(3,4-Dimethylbenzoyl)-p-sulfamoyl Phenyl								
210	88	A	(*b*)	$C_{20}H_{20}N_6O_3S$	19.82	7.55	19.52	7.57
R = N-(6-Methoxy-3-pyridazinyl)-p-sulfamoyl Phenyl								
180	90	A	(*b*)	$C_{16}H_{16}N_8O_3S$	27.98	8.00	27.82	8.48

[a] A, aqueous ethanol; B, absolute ethanol. [b] All melt with decomposition. [c] Analyses were performed by Janssen Pharmaceutica, Beerse, Belgium.

thus obtained was then coupled with 2,6-diaminopyridine hydrochloride and in all cases the azo dye was isolated by adjusting the pH of the reaction mixture to 6.5–7.

EXPERIMENTAL[2]

2,6-Diaminopyridine—Sodium amide was obtained by dissolving freshly cut sodium (27.6 Gm.) in anhydrous liquid ammonia (600 ml.) in the presence of 0.5 Gm. ferric nitrate as catalyst. The flask containing the suspension of sodium amide was fitted with a dropping funnel containing 39.4 Gm. of dry pyridine which was added cautiously while the ammonia was allowed to escape through the soda-lime tube. After all the ammonia was driven out (by removal of the dry ice–ether bath) and after the temperature of the apparatus reached the laboratory temperature, the dry ice condenser was replaced by a vertical condenser protected by a calcium chloride tube, and the funnel was replaced by a thermometer which dipped into the reaction mixture. The flask was heated in an oil bath, the temperature of the reaction mixture being maintained at 170° until hydrogen ceased to evolve (9–10 hr.). The solid cake which separated on cooling was treated with 10% sodium hydroxide solution until the vigorous decomposition stopped and then with water to complete the hydrolysis of the sodium salt. The product which was obtained in 20% yield (10 Gm.) melted at 120–122° as reported (2) after one crystallization from benzene.

Diazotization Procedures—(*a*) The sulfonamide (0.01 mole) was dissolved in 20 ml. of 25% hydrochloric acid by gentle warming, diluted with 100 ml. of water, and cooled to 0–5°. This solution was diazotized by the dropwise addition of a cold solution of 0.012 mole sodium nitrite in water. It was set aside for 15 min. at a temperature not exceeding 5° after complete addition of the nitrite.

(*b*) The sulfonamide (0.01 mole) was dissolved in 20 ml. of 25% sodium hydroxide solution, 0.012 mole of sodium nitrite was added with continuous stirring and then acidified by the dropwise addition of 25 ml. of hydrochloric acid. It was left for 10 min. after complete addition of the acid, the temperature being maintained at 0–5° during the diazotization.

Coupling Procedure—The diazonium salt solution prepared above was gradually added with continuous stirring to a solution of 2,6-diaminopyridine (0.01 mole) dissolved in 10% hydrochloric acid (temperature should not exceed 5° during the addition), and left aside for 15 min. for complete reaction. The pH of the medium was then adjusted to about 6.5–7 by the addition of dilute sodium hydroxide solution with stirring. The azo dye which precipitated was filtered at the pump and crystallized either from aqueous ethanol or absolute ethanol. (See Table I.)

[2] Melting points were performed by the capillary tube method and are uncorrected.

REFERENCES

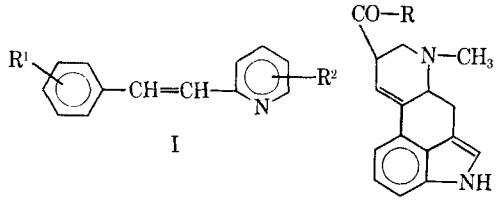
(1) Domagk, G., *Deut. Med. Wochschr.*, **61**, 250(1935).
(2) Shreve, R., Riechers, E., Koenig, H., and Goodman, A., *Ind. Eng. Chem.*, **32**, 173(1940).

New Compounds: Pyridylacrylonitrile Derivatives

By JOSEPH SAM*

The preparation of several pyridylacrylonitriles (V), the hydrolysis to an acid (VII), and subsequent conversion to an ester (VIII) are described.

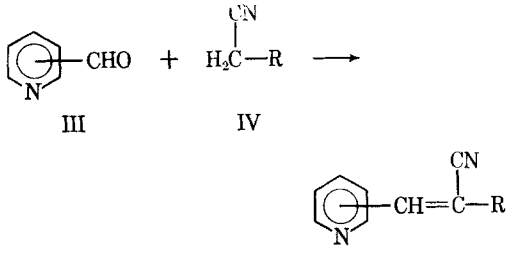
THE AVAILABILITY of pyridinealdehydes prompted the investigation of a number of their derivatives for potentially useful biological agents. Others (1–9) have described the preparation of similar compounds. Substituted 2-stilbazoles (I) or the corresponding hydrogenated derivatives bear some structural similarity to the ergot (II), veratrum, and reserpine alkaloids and also to the synthetic and steroidal estrogens. The nitriles (V) also have some structural similarity to the ergot alkaloids.



I

II

The reaction of pyridinealdehydes (III) with substituted acetonitriles (IV) was catalyzed by piperidine and provided yields of 64 to 96% of the desired acrylonitriles (V). (Scheme I.)



III IV V

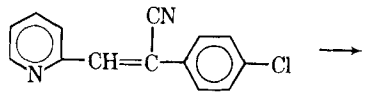
Scheme I

The hydrolysis of 2-(*p*-chlorophenyl)-3-(2-pyridyl)acrylonitrile (VI) with concentrated sulfuric acid provided the corresponding acid (VII) which was subsequently reacted with ethanolic hydrogen chloride to yield the ester (VIII). (Scheme II.)

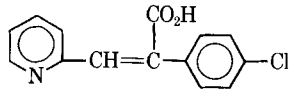
On the basis of the successful reductive cyclization of α-(2′-nitro-4′,5′-dimethoxyphenyl)-β-phenylacrylonitrile (IX) to 3-benzyl-5,6-dimethoxyindole (X) (6) (Scheme III), a number of unsuccessful attempts were made to cyclize β-cyano-4′,5′-dimethoxy-2′-nitro-2-stilbazole (XI) to 5,6-dimethoxy-3-(2-pyridylmethyl)indole (XII) by the same procedure. (Scheme IV.)

The reductive cyclization of XI to 3-cyano-2-(pyridyl)indoles (XIII) has been reported (7).

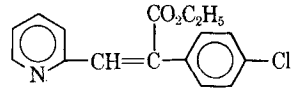
Table I summarizes the physical characteristics of the compounds that were investigated.



VI

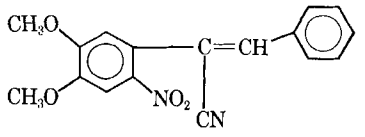


VII

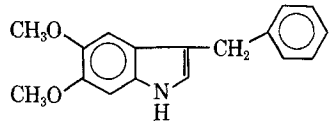


VIII

Scheme II

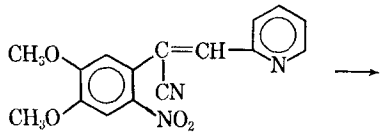


IX

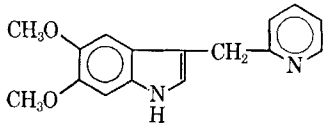


X

Scheme III

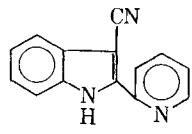


XI



XII

Scheme IV



XIII

Preliminary pharmacological evaluation did not indicate any pronounced biological activity in the compounds that were studied.

EXPERIMENTAL[1]

Synthesis—The starting materials for the preparation of the compounds described in Table I (with

Received March 30, 1967, from Bristol Laboratories' Syracuse, NY 13201
Accepted for publication June 7, 1967.
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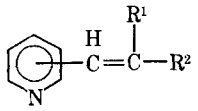
[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

TABLE I—PYRIDYLACRYLONITRILE DERIVATIVES



Pyridyl Isomer	R^1	R^2	% Yield	Recrystn. Solvent[a]	M.p., °C.	Molecular Formula	Anal. Calcd. C	Calcd. H	Found C	Found H
2	3,4-$(CH_3O)_2$-6-$NO_2C_6H_2$	CN	96	EA	192.5–193.5	$C_{16}H_{13}N_3O_4$	61.7	4.21	62.0	4.28
2	4-$NO_2C_6H_4$	CN	96	M	199–201	$C_{14}H_8N_3O_2$[b]	66.9	3.61	67.1	3.70
2	4-ClC_6H_4	CN	67	M	128–129	$C_{14}H_9ClN_2$	69.9	3.77	69.9	3.81
2	4-ClC_6H_4	CO_2H	90	B	147–148 dec.	$C_{14}H_{10}ClNO_2$	64.8	3.88	64.9	3.86
2	4-ClC_6H_4	$CO_2C_2H_5$	90	A	140–142	$C_{17}H_{17}ClINO_2$[c]	47.5	3.99	47.4	4.19
2	4-ClC_6H_4	$CO_2C_2H_5$	86	E	134–135	$C_{16}H_{14}ClNO_2$	66.8	4.90	67.0	5.19
2	$C_2H_5O_2C$	CN	64	M	94.5–96	$C_{11}H_{10}N_2O_2$[d]	65.3	4.99	65.5	4.87
3	$C_2H_5O_2C$	CN	83	M	87.5–88.5	$C_{11}H_{10}N_2O_2$	65.3	4.99	65.2	4.74

[a] EA, ethyl acetate; M, methanol; B, benzene; A, acetone; E, ethanol. [b] *Reference 8.* [c] Methiodide. [d] *Reference 9.*

the exception of 3,4-dimethoxy-6-nitrophenylacetonitrile) were purchased from commercial sources. The latter nitrile was prepared according to the procedure described in the literature (6).

Pyridylacrylonitriles (Table I)—A cooled solution of 0.1 mole of desired nitrile and 0.1 mole of pyridinealdehyde in 100–150 ml. of absolute ethanol or methanol was treated with 8.5 Gm. (0.1 mole) of piperidine and thereafter refluxed for 1–5 hr. The solution was concentrated to a small volume. The solid was removed by filtration and recrystallized from a suitable solvent.

α-(p-Chlorophenyl)-β-(2-pyridyl)acrylic Acid—A carefully prepared solution of 7 Gm. (0.03 mole) of α-(*p*-chlorophenyl)-β-(2-pyridyl)acrylonitrile in 100 Gm. of concentrated sulfuric acid was heated on a steam bath for 0.5 hr. Thereafter, the solution was cooled and poured into 500 ml. of water. The solid (7 Gm., 90%) was removed by filtration and dissolved in 10% aqueous sodium hydroxide. The resulting solution was filtered and the filtrate was neutralized with acetic acid. The solid was removed by filtration and recrystallized from benzene, m.p. 147–148° dec.

Ethyl α-(p-Chlorophenyl)-β-(2-pyridyl)acrylate—A solution of 50 Gm. of hydrogen chloride in 200 ml. of absolute ethanol was treated with 26 Gm. (0.1 mole) of α-(*p*-chlorophenyl)-β-(2-pyridyl)acrylic acid. The resulting solution was refluxed for 4 hr. and thereafter concentrated *in vacuo* to dryness. The residual material was washed with 300 ml. of water and recrystallized from ethanol.

A methiodide was prepared by refluxing the compound above with excess methyl iodide in acetonitrile for 30 hr.

REFERENCES

(1) Hartmann, M., and Bosshard, W., *Helv. Chim. Acta*, **24**, 24E(1941).
(2) Castle, R. N., and Seese, W. S., *J. Org. Chem.*, **20**, 987 (1955).
(3) Katritzky, A. R., and Monro, A. M., *J. Chem. Soc.*, **1958**, 150.
(4) Beard, J. A. T., and Katritzky, A. R., *Rec. Trav. Chem.*, **78**, 592(1959).
(5) Clarke, F. H., Felock, G. A., Silverman, G. B., and Watnick, C. M., *J. Org. Chem.*, **27**, 533(1962).
(6) Walker, G. N., *J. Am. Chem. Soc.*, **77**, 3844(1955).
(7) Suh, J. T., and Puma, B. M., *J. Org. Chem.*, **30**, 2253 (1965).
(8) Walker, G. N., *J. Med. Chem.*, **8**, 583(1965).
(9) Mechino, J. A., U. S. pat. 3,185,707(1965).

New Compounds: Synthesis of *N*-Haloacyl Analogs of 1-(*N*-β-Hydroxyethyl-4-piperidyl)-3-(4-piperidyl)propane as Potential Antineoplastic Agents

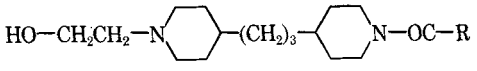
By WILLIAM D. ROLL

A series of haloamide analogs of 1-(*N*-β-hydroxyethyl - 4 - piperidyl) - 3 - (4 - piperidyl)propane has been synthesized for the purpose of studying their potential anticarcinogenic activity.

DURING THE past few years a number of potential mono- and difunctional alkylating agents have been prepared in this laboratory for the purpose of studying chemotherapeutic activity-structure relationships in regard to their value as antineoplastic agents. Based upon the experimental observations obtained in this laboratory (1–4) and those of other investigators (5–9), the synthesis of haloacetyl and halopropionyl derivatives of 1-(*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (4 - piperidyl)propane was undertaken.

It is theoretically possible that these compounds interfere with vital enzymatic systems or nucleic acids within a cancer cell through reversible and/or irreversible bonding to amine, sulfhydryl, or phosphoryl groups under physiological conditions. As noted by Triggle (10), the specificity of chemotherapeutic alkylating agents probably will reside in the carrier portion of the structure rather than in the alkylating moiety. Through this and following studies it is hoped that some additional information regarding the definition of the carrier moiety and specificity of action will be forthcoming, and that a structure will evolve which will possess a selective action against tumor cells.

TABLE I—*N*-HALOACYL ANALOGS OF 1-(*N*-β-HYDROXYETHYL)-4-PIPERIDYL-3-(4-PIPERIDYL)PROPANE



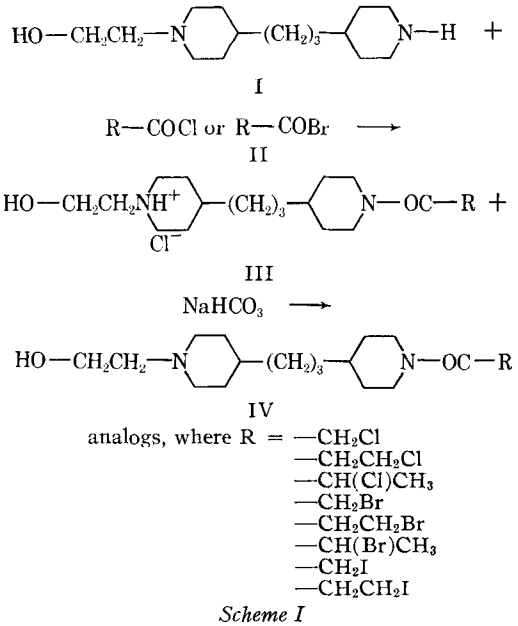
R	M.p., °C.	Yield, %	Anal. Calcd.	Anal. Found	Infrared cm.⁻¹ (KBr) (C=O Amide)	Infrared cm.⁻¹ (KBr) (OH)
—CH_2Cl	55–56	85	C, 61.70 H, 9.44 Cl, 10.71 N, 8.46	C, 61.65 H, 9.40 Cl, 10.80 N, 8.40	1650	3450
—CH_2CH_2Cl	88–89	79	C, 62.68 H, 9.64 Cl, 10.28 N, 8.12	C, 62.75 H, 9.69 Cl, 10.35 N, 8.02	1630	3450
—$CH(Cl)CH_3$	49–50	68	C, 62.68 H, 9.64 Cl, 10.28 N, 8.12	C, 62.60 H, 9.59 Cl, 10.30 N, 8.19	1640	3450
—CH_2Br	58–59	84	C, 54.40 H, 8.32 Br, 21.28 N, 7.46	C, 54.51 H, 8.40 Br, 21.19 N, 7.40	1640	3450
—CH_2CH_2Br	96–97	80	C, 55.52 H, 8.54 Br, 20.52 N, 7.19	C, 55.50 H, 8.60 Br, 20.45 N, 7.09	1630	3450
—$CH(Br)CH_3$	47–48	72	C, 55.52 H, 8.54 Br, 20.52 N, 7.19	C, 55.60 H, 8.50 Br, 20.59 N, 7.26	1640	3450
—CH_2I	59–60	80	C, 48.34 H, 7.40 I, 30.04 N, 6.64	C, 48.42 H, 7.45 I, 29.95 N, 6.59	1640	3450
—CH_2CH_2I	91–92	75	C, 49.54 H, 7.62 I, 29.08 N, 6.42	C, 49.51 H, 7.59 I, 29.19 N, 6.29	1640	3450

EXPERIMENTAL

The synthetic scheme for the preparation of the analogs of 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(4-piperidyl)propane[1] may be summarized as follows. (Scheme I.) To a solution of 5.02 Gm. (0.02 mole) of the parent amine (I) in 15 ml. of anhydrous

Received April 14, 1967, from the College of Pharmacy, University of Toledo, Toledo, OH 43606
Accepted for publication June 19, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

[1] Supplied by Reilly Tar and Chemical Corp., Indianapolis, Ind.



Scheme I

chloroform was added 0.02 mole of the haloacyl halide (II) (in anhydrous chloroform). An exothermic reaction occurred as the acylating agent was added dropwise at room temperature. When the addition of the acylating agent (II) was complete, the reaction mixture was refluxed for 30 to 60 min. The chloroform was evaporated *in vacuo*, and the solid remaining was dissolved in a minimum amount of absolute ethanol and the *in vacuo* evaporation operation repeated. The solid (III), a hygroscopic hydrochloride or hydrobromide salt, was treated with sodium bicarbonate and chloroform to give the crude product. The crude haloamides were recrystallized twice from aqueous ethanol to give the pure products listed in Table I. The iodoamides were synthesized as previously reported (1).

The infrared spectra of the compounds were obtained with a Perkin-Elmer Infracord. The melting points were determined with a Fisher-Johns melting point apparatus and are corrected values.

DISCUSSION AND RESULTS

The series of eight derivatives prepared in this work are: 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(*N*-chloroacetyl - 4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - bromoacetyl-4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - bromoacetyl - 4 - piperidyl)-propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - iodoacetyl - 4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - β - chloropropionyl - 4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - β - bromopropionyl - 4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - β - iodopropionyl-4 - piperidyl)propane, 1 - (*N* - β - hydroxyethyl - 4-piperidyl) - 3 - (*N* - α - chloropropionyl - 4 - piperidyl)propane, and 1 - (*N* - β - hydroxyethyl - 4 - piperidyl) - 3 - (*N* - α - bromopropionyl - 4 - piperidyl)propane.

REFERENCES

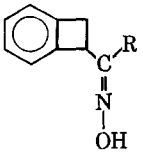
(1) Roll, W. D., *J. Pharm. Sci.*, **54,** 269(1965).
(2) *Ibid.*, **54,** 1385(1965).
(3) *Ibid.*, **55,** 529(1966).
(4) *Ibid.*, **55,** 1151(1966).
(5) Carbon, J. A., Brehm, S. M., and Ratajczyk, J. S., "Abstracts," American Chemical Society, St. Louis meeting, March 1961, p. 11-N.
(6) Peck, R. M., Breuninger, E. R., Miller, A. J., and Creech, H. J., *J. Med. Chem.*, **7,** 480(1964).
(7) Sass, S., Williamson, C. E., Kramer, S. P., Goodman, L. E., Ulfohn, A., Seligman, A. M., and Witten, B., *ibid.*, **8,** 14(1965).
(8) Gaozza, C. H., *ibid.*, **8,** 400(1965).
(9) Johnston, T. P., McCaleb, G. S., Opliger, P. S., and Montgomery, J. A., *ibid.*, **9,** 892(1966).
(10) Triggle, D. J., *J. Theoret. Biol.*, **7,** 241(1964).

Stereochemical Assignments of 1-Benzocyclobutenyl Ketoximes

Sir:

The 1-benzocyclobutenyl ketoximes, listed in Table I, exhibit muscle relaxant characteristics (1). No assignments of the oxime configurations were made for the compounds used in the pharmacological tests. This study was undertaken to establish the configurations of the oximes.

TABLE I—1-BENZOCYCLOBUTENYL KETOXIMES



Compd.	R	Oxime Configuration Relative to R
I	Methyl	*syn*
II	Phenyl	*anti*
III	*n*-Butyl	*syn*
IV	Benzyl	*syn*
V	Cyclohexyl	*syn*

Initially, NMR appeared to be useful for assigning the stereochemistry of I. Oximes of structurally related ketones (benzyl methyl ketone and α-phenethyl methyl ketone) were prepared. Only a single isomer (*syn* to the methyl group) could be detected for each compound on thin-layer chromatography using benzene–ethyl acetate (10:1) as the solvent system. Only a single isomer is reported in the literature for each compound (2). Silica gel was the adsorbent for this experiment and all other chromatography in the study. Since both isomers of the model compounds were not available, no definite assignment could be made for I based upon comparisons with the NMR spectra of the model oximes.

A Beckmann rearrangement was run on I with phosphorus pentachloride in anhydrous ether (3). The resulting amide had a peak in the infrared spectrum at 7.35 μ which is characteristic of CH_3CO—groups (4). This amide is consistent with a *syn* assignment for I, since the group *anti* to the oxime hydroxyl migrates in the Beckmann rearrangement (3). Basic hydrolysis and ether extraction gave a solid amine which had aromatic and aliphatic C-H absorption in its infrared spectrum, confirming the stereochemistry of I.

Compound II, after Beckmann rearrangement, basic hydrolysis, and ether extraction, afforded an amine which had a R_f value identical to aniline on thin-layer chromatography with benzene–methanol (95:5). In this solvent system, the amine from I failed to migrate from its origin. Thus, II was assigned an *anti* configuration.

When III, IV, and V were treated with the same series of reactions, the amines produced had the same R_f value as the amine from I on thin-layer chromatography with *n*-butanol–acetic acid–water (4:1:1). The amines failed to migrate in the benzene–methanol system. A tentative *syn* assignment for III, IV, and V was made. The hydrolysis residues from II, III, IV, and V were acidified and extracted with ether. These acids exhibited individual R_f values on thin-layer chromatography with benzene–dioxane–acetic acid (90:25:4). The acids from III, IV, and V had R_f values identical to valeric, phenylacetic, and cyclohexane carboxylic acids, respectively. This observation confirmed the assignments of the stereochemical configuration of III, IV, and V.

(1) Skorcz, J. A., Suh, J. T., Judd, C. I., Finkelstein, M., and Conway, A. C., *J. Med. Chem.*, **9**, 656(1966).
(2) "Dictionary of Organic Compounds," Heilbron, I., ed., Oxford University Press, New York, N. Y., 1946.
(3) Donaruma, L. G., and Heldt, W. Z., "Organic Reactions," vol. 11, Cope, A. C., ed., John Wiley & Sons, Inc., New York, N. Y., 1960, p. 1.
(4) Nakanishi, K., "Infrared Absorption Spectroscopy," Holden-Day, Inc., San Francisco, Calif., 1962, p. 21.

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Received April 25, 1967.
Accepted for publication August 2, 1967.

The author is indebted to Lakeside Laboratories, Inc., Milwaukee, Wis., for providing the 1-benzocyclobutenyl ketoximes used in this study and to Dr. Wendel Nelson, University of Washington, Seattle, Wash., for the NMR spectra.

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Interpretation of *In Vitro* Dissolution Data Relative to the Gastrointestinal Absorption Characteristics of Drugs in Tablets

Sir:

The recognition that the gastrointestinal absorption of drugs administered in the form of compressed tablets is frequently rate-limited by the process of dissolution (1–3) has led to numerous studies of the effect of tablet formulation factors on the *in vitro* dissolution rate(s) of the active ingredient(s). The difficulty and expense of clinical trials, and the desirability of utilizing the eventually marketed dosage form as early as possible in clinical studies of a new drug, have resulted in the use of *in vitro* dissolution tests as a means for selecting the most promising tablet formulation(s) from among several experimental preparations. It is the purpose of this communication to outline some hazards in the interpretation of results obtained from *in vitro* dissolution test procedures which have not been calibrated exactly on the basis of *in vivo* data.

Two experimental tablet formulations of aspirin (0.32 Gm. per tablet) were recently made available to the authors.[1] These were conventional compressed tablets, differing essentially only in the particle size of aspirin, and designed to yield gastrointestinal absorption characteristics in the upper and lower range of rates ordinarily encountered with commercial products. The tablets designated as type *A* were prepared with finely milled aspirin (80-mesh and finer) while the type *B* tablets contained relatively coarse aspirin (all particles larger than 20-mesh). Gastrointestinal absorption studies were carried out on 6 ambulatory subjects (trial *1*); the early phase of this clinical trial was repeated once with these 6 subjects and 2 others (trial *2*) in order to obtain additional data and to assess the degree of intrasubject variability. The tablets were swallowed whole, with 200 ml. water, in the morning on an empty stomach. No food was permitted for 2 hr. following tablet ingestion. Blood samples were obtained at various times and the concentration of total salicylate in the plasma was determined by the method of Brodie *et al.* (4) after heating the plasma with 2 *N* hydrochloric acid at 100° for 10 min. The results are summarized in Table I[2] and indicate that aspirin in type *A* tablets is absorbed about 3 times as rapidly *on the average* as it is when given in type *B* tablets.

TABLE I—TOTAL SALICYLATE CONCENTRATION (mg./L.) IN THE PLASMA AS A FUNCTION OF TIME AFTER ORAL ADMINISTRATION OF 0.65 GM. ASPIRIN AS TWO TABLETS OF TYPE *A* AND TYPE *B* IN TWO TRIALS

Time, min.	Tablets *A* Mean	S.D.[a]	Tablets *B* Mean	S.D.
		Trial 1[b]		
10	24.7	13.9	5.5[c]	1.8
20	41.0	17.8	12.8[c]	4.7
30	46.1	14.8	18.7[c]	6.2
60	48.7	10.7	32.0[c]	7.1
120	48.0	9.0	43.8	8.2
240	34.7	8.0	34.5	6.0
360	23.0	6.5	24.2	6.2
		Trial 2[d]		
10	17.8 (15.5)[e]	8.3	7.2[c] (7.8)[e]	4.1
20	41.0 (38.5)	16.5	15.0[c] (16.7)	5.7
30	51.4 (49.7)	19.9	21.5[c] (24.3)	9.0

[a] Standard deviation (the meaningfulness of this value is limited due to inter-subject variations in apparent volume of distribution). [b] Average of 6 subjects (5 F, 1 M); av wt., 67.5 Kg.; av. age, 27.7 yr. [c] Significantly different from tablets *A* ($p < 0.01$). [d] Average of 8 subjects (5 F, 3 M); av. wt., 70.0 Kg.; av. age, 28.0 yr. [e] Figures in parentheses are data for the 6 subjects who participated in trial *1*.

In vitro dissolution rates were determined by the beaker method of Levy and Hayes (5) with recently described modifications (2), using the precision stirring apparatus of Levy and Tanski (6). The times required for dissolution of one third, one half, and two thirds of the drug content of one tablet (*i.e.*, $t_{1/3}$, $t_{1/2}$, and $t_{2/3}$) were determined graphically from cumulative dissolution plots. These measurements were made at stirring rates of 50, 60, and 75 r.p.m., using an average of six tablets each. The results of these experiments are shown in Fig. 1. The inset in the figure shows the $t_{1/3}$ ratios (type *B*:type *A*) for the tablets as a function of stirring rate. It can be noted that this ratio changed from less than unity at 50 r.p.m. to almost 15 at 75 r.p.m.[3]

This striking sensitivity of relative dissolution rates to small changes in stirring intensity has profound implications. Only at about 55 r.p.m. (by interpolation) is the ratio of *in vitro* dissolution rates similar to the ratio of the *average in vivo* absorption rates. A small change in agitation intensity (such as is obtained by a change in stirring rate by ±10 r.p.m. or by a change in the dimensions of the dissolution apparatus) can yield results either contrary to those obtained *in vivo* or showing much greater differences from those observed in the clinical

[1] The authors thank Dr. John H. Wood and the Bristol-Myers Products Division for preparing the experimental tablet preparations used in this study.

[2] A tabulation of individual data is available upon request.

[3] The reader is referred to *References 7* and *8* for a discussion of the physicochemical basis of these observations.

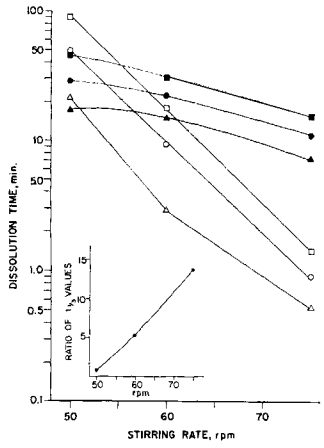

Fig. 1—Dissolution rate of aspirin from type A *(open symbols) and type* B *(solid symbols) tablets as a function of stirring rate. Triangles,* $t_{1/3}$; *circles,* $t_{1/2}$; *squares,* $t_{2/3}$. *Inset: ratio of* $t_{1/3}$, *type* B : *type* A *tablets, as a function of stirring rate.*

study. It must be emphasized that the preparations studied were conventional type tablet preparations rather than specialty dosage forms such as sustained-release or "buffered" formulations. The results of the investigation described here show clearly that dissolution test procedures which have not been calibrated *exactly* on the basis of *in vivo* measurements are unlikely to reflect the gastrointestinal absorption characteristics of the preparations tested and may in fact yield very misleading data.

The pronounced sensitivity of the dissolution rate of a drug in tablet form to agitation intensity has some interesting clinical implications. It suggests that subjects who are consistently rapid or slow absorbers, such as were noted in a previous study from this laboratory (2), may be individuals who by reason of certain anatomic or physiological characteristics of their gastroin testinal tract cause ingested tablets to be subjected to relatively intensive or mild agitation, respectively. As a corollary, one would expect to find the same type of differences in *in vivo* absorption kinetics with rapid and slow absorbers as were noted in *in vitro* dissolution kinetics at high and low stirring rates, respectively. Accordingly, plasma salicylate levels obtained after administration of type *A* and type *B* tablets were compared in a consistently rapid and a consistently slow absorber (as judged on the basis of data obtained in two tests with type *A* tablets) from among the group of subjects who participated in this study. The results, expressed as ratios of plasma salicylate concentration after type *A* to those obtained after type *B* tablets, are listed in Table II. They show, as predicted, that

Table II—Relative Absorption Rate of Aspirin from Type *A* and Type *B* Tablets in a Rapid and a Slow Absorber[a]

Subject	Ratio of Salicylate Concn. in Plasma Type *A* : Type *B* Time, min. 10	20	30
Rapid absorber	2.7	3.1	2.6
Slow absorber	0.8	1.0	1.1

[a] Based on data obtained in 2 trials per subject per tablet type.

differences in absorption rate of drug in the two types of tablets are clearly evident in the rapid absorber, but are essentially absent in the slow absorber.

Extending this reasoning one step further, one would expect less inter-subject variation in the rate of drug absorption from type *B* tablets (which show very little dissolution rate dependency on agitation intensity) than from type *A* tablets (which show a pronounced dissolution rate dependency on agitation intensity). These effects are indeed evident in the clinical results obtained from the cross-over study in 6 subjects. The time of occurrence of peak salicylate levels in the plasma following administration of type *B* tablets was 120 min. in 5 subjects and 240 min. in the sixth subject. On the other hand, these times varied markedly following administration of type *A* tablets: 20 min. (1 subject), 30 min. (1 subject), 60 min. (2 subjects), and 120 min. (2 subjects). It should be noted, however, that there may be some bias in these results because of the longer sampling intervals at the later times.

The *in vitro* results illustrate the limitations and possible hazards in the use and interpretation of dissolution rate data derived from tests carried out under arbitrary conditions. It is now evident that small differences in agitation intensity or in the pH of the solvent (9) can have a pronounced effect on the relative dissolution rate of a drug in different tablet formulations. These problems are of course obviated by administering drugs such as aspirin in solution (10). Where it becomes necessary to select the most promising tablet formulation from among several experimental formulations available for clinical study, it would appear prudent to base this selection on dissolution rate data obtained over a range of agitation intensities and solvent pH.

The clinical data suggest that differences in absorption rates may, in the case of dissolution rate limited drug absorption, be due to differences in the intensity of agitation of the gastrointestinal content. Therefore, disease and environmental conditions (including stress, physical activity, food, and medication) which affect gastro-

intestinal motility (and gastric pH) may have a corresponding effect on dissolution rate limited drug absorption. It is possible, therefore, that 2 types of tablet formulations of a given drug may show pronounced dissolution rate differences in one kind of population or clinical environment, but not in another. However, it must be emphasized that the conclusions concerning these *in vivo* implications are clearly preliminary and require confirmation in a large population sample containing significant numbers of consistently rapid and slow absorbers.

(1) Levy, G., *J. Pharm. Sci.*, **50**, 388(1961).
(2) Levy, G., and Hollister, L. E., *ibid.*, **53**, 1446(1964).
(3) Levy, G., Leonards, J. R., and Procknal, J. A., *ibid.*, **54**, 1719(1965).
(4) Brodie, B. B., Udenfriend, A., and Coburn, A. F., *J. Pharmacol. Exptl. Therap.*, **80**, 114(1944).
(5) Levy, G., and Hayes, B. A., *New Engl. J. Med.*, **262**, 1053(1960).
(6) Levy, G., and Tanski, W., Jr., *J. Pharm. Sci.*, **53**, 679(1964).
(7) Levy, G., *ibid.*, **52**, 1039(1963).
(8) Levy, G., Antkowiak, J. M., Procknal, J. A., and White, D. C., *ibid.*, **52**, 1047(1963).
(9) O'Reilly, R. A., Nelson, E., and Levy, G., *ibid.*, **55**, 435(1966).
(10) Leonards, J. R., *Proc. Soc. Exptl. Biol. Med.*, **110**, 304(1962).

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Received June 12, 1967.
Accepted for publication August 8, 1967.

Diffusional Model for Transport Rate Studies Across Membranes

Sir:

There has been much in the literature in recent years concerning the so-called "three-phase" model of drug absorption. Absorption studies in this area have concentrated mainly on the passive or diffusional transport of drugs. Most models currently proposed are kinetic outgrowths of the original Brodie-Shore-Hogben pH-partition thesis. In recent communications to this journal authors (1, 2) have proposed equations based on first-order kinetics, which give the amount of drug in each of the three phases *versus* time.

The importance of diffusion in drug absorption has been recognized (3). However, no strictly diffusional model of drug absorption has been proposed for the three-phase multicomponent diffusion problem that can physically describe, for example, pH effects.

The authors in these laboratories are exploring general diffusional models for gastrointestinal absorption of ionizing and nonionizing drugs. A proposed steady-state model, shown in Fig. 1 for the case of a weak base, RN, which can become protonated to give RNH^+, allows for intestinal and blood buffers. It is assumed here also that only the uncharged drug species can diffuse in the lipid phase. An important feature of this model is the inclusion of aqueous diffusional barriers on each side of the lipoidal membrane.

Preliminary results (Fig. 2) obtained for the steady-state case *via* computer correlate the combined effects of varying pKa and the partition coefficient (KLH). The units of G, the transport rate, are mmoles cm.$^{-2}$ sec.$^{-1}$. TH1 and TH3, the effective aqueous diffusion layer thicknesses on the left and right side of the membrane, respectively, are taken as equal for these calculations. TH2 is the effective thickness of the lipid phase. For low to moderate agitation conditions, TH1 should be around 50 to 200 μ (4). The authors would estimate TH2 to be effective somewhere between tens of microns to few millimeters.

In Fig. 2 most noticeable is the fact that steady-state transport rates are linear with increasing partition coefficients only up to a certain point

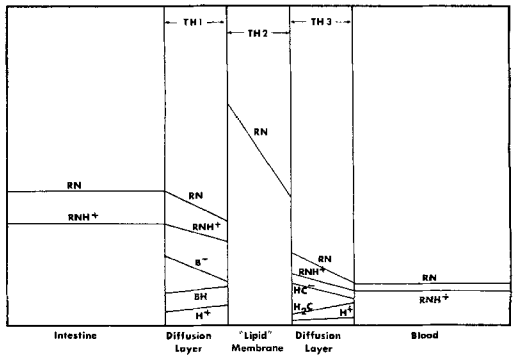

Fig. 1—The steady-state diffusional model for the transport of a weak amine drug, RN, *across the intestinal barrier. Concentration profiles are schematically given for all species:* RNH^+ *= protonated drug;* BH *and* B^- *= the buffer molecule and its anion in the intestinal fluids;* H^+ *= hydrogen ion;* H_2C *and* HC^- *= carbonic acid and bicarbonate in the blood.* TH1, TH2, *and* TH3 *are the diffusion layer thicknesses in the three phases.*

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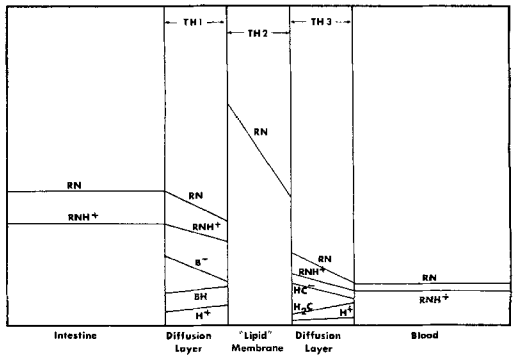

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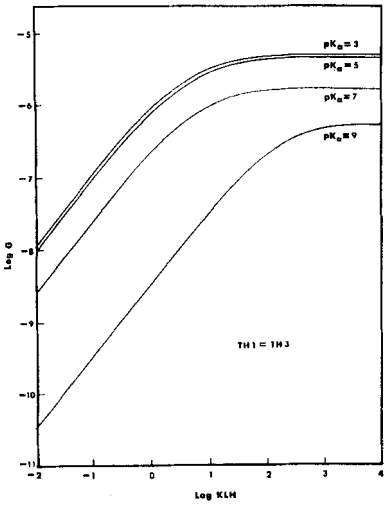

Fig. 2—The results of drug transport rate computations for the steady-state model for basic drugs of various pKa's. G, the transport rate, is in mmoles cm.$^{-2}$ sec.$^{-1}$. All diffusion coefficients are assumed to be D = 1×10^{-5} *cm.^{2} sec.$^{-1}$. Intestinal pH = 6.5. The total drug concentration,* (RN) + (RNH^+), = 10^{-2} M. *The total buffer concentration,* (B^-) + (HB), = 10^{-2} M. *Total serum carbonate,* (H_2CO_3) + (HCO_3^-), = 2.6×10^{-2} M. TH1 *and* TH3 *were taken to be* 10^{-2} *cm., while* TH2 *was taken as* 10^{-1} *cm.*

(KLH ~1), beyond which the aqueous diffusion layers give a limiting effect. Experimental data given by Hogben *et al.* (3) indicate that this leveling off effect does occur in rat intestine absorption studies. Hogben and his associates indicated in the same paper that the observed pattern of intestinal absorption demonstrated the crucial importance of drug diffusion to the intestinal wall when absorption conditions (high partition coefficient, drug present mostly in uncharged form) were otherwise favorable. We feel that this phenomenon should also be observed in *in vitro* absorption studies. The collodion-phospholipid membranes used by Misra *et al.* (5) averaged 20 μ in thickness. In this case the aqueous diffusion layers (50 to 200 μ) would probably be greater than the thickness of the membrane by the order of magnitude of 2.5 to 10. The diffusional leveling off effect, if a 20-μ membrane were to be inserted in the above model, would occur at partition coefficient values of about 10^{-2}. If the diffusion coefficient of the drug in the lipid is less than that in these calculations, the leveling off would occur at correspondingly larger KLH values.

The data curves (Fig. 2) are quite close at low pKa values until the pKa of the drug approaches the intestinal pH, at which point they become increasingly separated with increasing pKa. The linearity of *G* with increasing partition coefficient for a base with a pKa of 9 is seen to extend over a much wider range than for weak bases having lower pKa values. This indicates that, due to the lower amount of the uncharged species present per amount of charged species, the diffusion process is still dependent on the permeability of the lipid layer at partition coefficients in the neighborhood of unity.

An expression for the nonionizing drug case, under the same conditions as above, fits point-for-point the pKa = 3 curve in Fig. 2. Further work is underway concerning the case of weak acids in the intestine. Due to the generality of the model, additional factors such as micellar transport and complexation can be considered. Further studies should also treat the nonsteady-state case and the heterogeneous nature of the lipid barrier.

(1) Doluisio, J. T., and Swintosky, J. V., *J. Pharm. Sci.*, **53**, 597(1964).
(2) Perrin, J., *ibid.*, **56**, 411(1967).
(3) Hogben, C. A. M., Tocco, D. J., Brodie, B. B., and Schanker, L. S., *J. Pharmacol. Exptl. Therap.*, **125**, 275(1959).
(4) Glasstone, S., "An Introduction to Electrochemistry," D. Van Nostrand & Co., New York, N. Y., 1942, pp. 448, 452.
(5) Misra, A. L., Hunger, A., and Keberle, H., *J. Pharm. Pharmacol.*, **18**, 531(1966).

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Received May 1, 1967.
Accepted for publication July 18, 1967.

This investigation is supported by fellowship 1-F1-GM-30, 048-02 and by grant GM-13368 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

Nomenclature Correction of Certain Plant *n*-Alkanes

Sir:

Recently we initiated phytochemical studies on certain *Euphorbia* species because of the reported folkloric use of members of this genus (*E. ingens*, *E. bupleurifolia*, *E. clavarioides*, and *E. gorgonis*) as anticancer remedies (1). Indeed, certain of the euphorbias (*E. amygdaloides*, *E. drummondii*, *E. marginata*, *E. pilulifera*, and *E. resinifera*) have been shown to elicit antitumor activity in laboratory animals (2). During the course of one of our investigations, a considerable amount of waxy material was encountered, which readily deposited white plates from the usual crystallizing solvents. These plates gave a sharp melting point, indicative of either *n*-nonacosane or *n*-hentriacontane, and elemental analyses were in agreement with an identification as either of these *n*-alkanes. This prompted us to request samples of these two compounds from other investigators for comparative purposes. Samples of *n*-nonacosane isolated from *Chaerophyllum bulbosum* L. (3), *Euphorbia watanabei* Makino[1] (4), and *Arctostaphylos patula* Greene[1] (5), as well as *n*-hentriacontane from *Euphorbia lathyris* L. (6) and *Plantago asiatica* L. (7) were obtained.

Prior to using these samples for comparative purposes, they were each subjected to a gas chromatographic analysis using the conditions previously described (8). Of the five samples analyzed, only the *n*-nonacosane from *C. bulbosum* (3) was reasonably pure (about 97%). The *n*-nonacosane from *E. watanabei* (4) was actually 61% *n*-hentriacontane, and the *n*-nonacosane from *A. patula* (5, 8) was 60% *n*-hentriacontane. Samples of *n*-hentriacontane isolated from *E. lathyris* (6) and *P. asiatica* were complex mixtures, but each was more than 50% of the labeled hydrocarbon (Table I).

These studies re-emphasize the need for subjecting all *n*-alkanes isolated from plant sources to a gas chromatographic and/or mass spectrometric analysis before any identification of these compounds can be made, and their presence in plant material reported.

TABLE I—COMPOSITION OF *n*-ALKANES FROM SELECTED PLANTS

n-Alkane Reported	Plant Source	Ref.	*n*-Alkanes Found[a]									
			C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}
n-Nonacosane	*Chaerophyllum bulbosum* L.	(3)	...	...	1	2	97	...	...	...	...	...
n-Nonacosane	*Euphorbia watanabei* Makino	(4)	<1	1	3	4	20	5	61	...	5	...
n-Nonacosane	*Arctostaphylos patula* Greene	(5)	<1	<1	3	4	17	5	60	6	5	...
n-Hentriacontane	*Euphorbia lathyris* L.	(6)	...	...	2	2	17	6	53	5	15	...
n-Hentriacontane	*Plantago asiatica* L.	(7)	<1	1	2	1	16	6	61	6	3	5

[a] As determined by gas chromatography (8), expressed to the nearest 1.0%. Estimations were made by planimetry.

[1] Tentative identifications, based solely on melting points, were reported in the original papers (4, 5).

(1) Watt, J. M., and Breyer-Brandwijk, M. G., "The Medicinal and Poisonous Plants of Southern and Eastern Africa," 2nd ed., E.&S. Livingstone Ltd., Edinburgh, Scotland, and London, England, 1962.
(2) Farnsworth, N. R., *J. Pharm. Sci.*, **55**, 225(1966).
(3) Huneck, S., *Naturwissenschaften*, **21**, 498(1960).
(4) Takemoto, T., and Ishiguro, T., *Yakugaku Zasshi*, **86**, 530(1966).
(5) Constantine, G. H., Jr., *J. Pharm. Sci.*, **55**, 1378 (1966).
(6) Ludwiczak, R. S., Trzebny, W., and Zycznska, I., *Roczniki Chem.*, **39**, 1233(1965).
(7) Torigoe, Y., *Yakugaku Zasshi*, **85**, 176(1965).
(8) Wagner, H., Hörhammer, L., Farnsworth, N. R., and Hörhammer, H.-P., *J. Pharm. Sci.*, unpublished data.

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Received June 30, 1967.
Accepted for publication August 21, 1967.

The authors thank Drs. T. Takemoto, S. Huneck, R. S. Ludwiczak, Y. Torigoe, and G. H. Constantine, Jr., for supplying the *n*-alkanes used in this study.

REVIEWS

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This book, the first of a series, is intended to survey major topics in the area of pharmacodynamic agents acting on the nervous system; the approach is on a fundamental biochemical–pharmacologic basis. These volumes are a memorium to the late Fred Schueler who participated in the selection of authors.

There are ten chapters covering such areas as the molecular aspects of cholinergic mechanisms, muscarinic drugs, atropine-like agents, ganglionic drugs, adrenergic drugs, and others. For the most part standard drug classification has been followed. The structure-activity relationships of many compounds and series of compounds are given. In some areas the older data have been reinterpreted in the light of newer knowledge. In other areas this has not been done. Even so, one of the values of this book is the extensive documentation of the large number of chemical structures tested in each drug class. Although at least one new class of compounds is not extensively covered—the β-adrenergic blocking agents—on the whole the references are up-to-date.

Another plus value is the complete author index of the cited literature. This serves as an excellent system to locate older references in which the author's name is better known than the class of drugs he studied.

Some of the authors have ventured into predictive speculations. The chapter on cholinergic mechanisms is of this kind. Each author in this book has been intimately associated with the material presented in his chapter. Both university laboratories and drug industry laboratories are represented. The projected series, if up to the quality of this first volume, will be a worthwhile addition to any pharmacology library.

Reviewed by R. P. Ahlquist
Medical College of Georgia
Augusta, Georgia

Index to Reviews, Symposia Volumes and Monographs in Organic Chemistry. For the period 1963–1964. Compiled and edited by NORMAN KHARASCH and WALTER WOLF. Pergamon Press, Inc., 44–01 21st Street, Long Island City, New York, N. Y. 11101, 1966. x + 326 pp. 21.5 × 28 cm. Price $17.50.

This is the third volume of this "Index." The earlier two volumes covered 1940–1960 and 1961–1962, respectively. The "Index" covers works in French and German, and English translations of Russian studies, as well as those in English. Presentation is by journals, *i.e.*, the journals are listed alphabetically with the titles of the review articles contained in each listed chronologically. Eighty-three journals and periodic publications and fifty-three symposia, collective volumes, and nonperiodical publications are covered. A separate listing of monographs dealing with organic chemistry and closely related topics which have been published in English, French, and German is also given. A complete author and subject index is included.

Staff review

NOTICES

Organic Photochemistry. Vol. 1. Edited by ORVILLE L. CHAPMAN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1967. xi + 339 pp. 15.5 × 23 cm. Price $15.75.

Quelques Aspects de la Chimie des Medicaments. By A. LESPAGNOL, D. BAR, C. LESPAGNOL, and M. DAUTREVAUX. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris VI^e, France, 1966. viii + 268 pp. 16 × 23.5 cm. Price 78F. Paperbound.

Russian Drug Index. 2nd ed., 1967. By STANLEY JABLONSKI. National Library of Medicine, Bethesda, Md. May be obtained from the Supt. of Documents, U.S. Government Printing Office, Washington, DC 20402 (PHS Publication No. 814, Rev. 1967). iii + 384 pp. 21.5 × 28 cm. Price $2.25. Paperbound.

Lehrbuch der pharmazeutischen Chemie. By G. WAGNER. Veb Verlag Volk und Gesundheit Berlin, Absatzabteilung, 69 Jena, Villengang 2, Germany, 1966. 669 pp. 17 × 24.5 cm. Price Geb. MDN 59.80.

The Chemistry of Lignin. By IRWIN A. PEARL. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1967. xii + 339 pp. 15.5 × 23 cm. Price $15.75.

Pharmacological and Chemical Synonyms. 4th rev. ed. Compiled by E. E. J. MARLER. Excerpta Medica Foundation, 119–123 Herengracht, Amsterdam, The Netherlands, 1967. vii + 349 pp. 17 × 24 cm. Price $18.20 postpaid.

Toxicants Occurring Naturally in Foods. Prepared by the Food Protection Committee, National Academy of Sciences–National Research Council, 2101 Constitution Ave., N.W., Washington, DC 20418, 1966. 15.5 × 23.5 cm. 301 pp. Price $6.00.

Neurotoxicity of Drugs. Vol. VIII of the Proceedings of the European Society for the Study of Drug Toxicity—Prague, June 30–July 2, 1966. (International Congress Series No. 118). Excerpta Medica Foundation, Herengracht 119–123, Amsterdam, The Netherlands, 1967. 227 pp. 16.5 × 24 cm. Price $12.50 postpaid. Paperbound.

Gadamers Lehrbuch der chemischen Toxikologie und Anleitung zur Ausmittelung der Gifte. 3. vollig neu bearb. Aufl. Bd. II: Allgemeine Methoden. Herausgegeben von ENGELBERT GRAF. Vandenhoeck & Ruprecht, Gottingen, West Germany, 1966. xii + 720 pp. 17.5 × 24.5 cm. Price Lw.DM 198–.

Mongolism. Ciba Foundation Study Group No. 25. Edited by G. E. W. WOLSTENHOLME and RUTH PORTER. Little, Brown & Co., Boston, MA 02106, 1967. ix + 99 pp. 12.5 × 19 cm. Price $3.50.

The Effects of External Stimuli on Reproduction. Ciba Foundation Study Group No. 26. Edited by G. E. W. WOLSTENHOLME and MAEVER O'CONNOR. Little, Brown & Co., Boston, MA 02106, 1967. x + 107 pp. 12.5 × 19 cm. Price $3.50.

Synthetic Methods of Organic Chemistry. Yearbook 1967. Vol. 21. By W. THEILHEIMER. S. Karger, Basel, Switzerland. Available from Albert J. Pheibig, P. O. Box 352, White Plains, NY 10602. xx + 540 pp. 15.5 × 23 cm. Prices Fr./DM 19S.—U. S. $47.80

Problèms Actuels de Biochimie Appliquée. Edited by MAURICE-LOUIS GIRARD. Premier Serie. Masson & Cie, Editeurs, 120 Boulevard Saint-Germain, Paris VI°, France, 1967. vi + 368 pp. 16.5 × 24.5 cm. Price 90 F.

Problems in Laboratory Evaluation of Antianginal Agents. Edited by M. M. WINBURY. North-Holland Publishing Co., P. O. Box 103, 68–70 N. Z. Voorburgwal, Amsterdam, The Netherlands, 1967. v + 65 pp. 15.5 × 23 cm. Price $4.50.

Black Market Medicine. By MARGARET KREIG. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1967. x + 304 pp. 15.5 × 24 cm. Price $5.95. [A review of this book appeared in *J. Am. Pharm. Assoc.*, **NS7**, 364(1967).]

Development of the Lung. Ciba Foundation Symposium. Edited by A. V. S. DE REUCK and R. PORTER. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1967. xv + 408 pp. 14 × 21 cm. Price $13.00.

Endocrinology of the Testis. Ciba Foundation Colloquia on Endocrinology, Vol. 16. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1967. xiii + 331 pp. 14 × 21 cm. Price $12.50.

Thiamine Deficiency: Biochemical Lesions and Their Clinical Significance. Ciba Foundation Study Group No. 28. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1967. x + 163 pp. 12 × 18.5 cm. Price $3.50.

Caste and Race: Comparative Approaches. Ciba Foundation Symposium. Edited by A. DE REUCK and J. KNIGHT. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1967. xi + 348 pp. 14 × 21 cm. Price $12.00.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

November 1967 volume 56, number 11

Review Article

Some Pharmaceutical Applications of Ultrasonics

By DONALD M. SKAUEN

IT IS DIFFICULT to imagine the scope of ultrasonics until one searches through the recent literature. On gathering material for this review the author has covered approximately 9000 references, a great many of which are allied to pharmacy. It is obvious that a complete review of the literature is impossible in this journal. References were selected which were, in the opinion of the author, most likely to stimulate interest and to illustrate the broad scope of ultrasonic technology.

It is apparent that much less attention has been paid to ultrasonics in pharmacy in the United States than in biology, medicine, chemistry, and engineering. This is brought out in the relatively few research papers in this field which have been published in the past two decades. It is also evident that many more research reports have appeared in foreign literature and in particular in the Russian literature than in our own country.

It would appear that part of the reluctance on the part of the pharmaceutical researcher to use ultrasonics in solving some of his problems has been the lack of standardized equipment or satisfactory standardizing techniques. It is now possible to obtain equipment which will indicate output on a meter and thus aid in standardizing procedures in so far as energy requirements are concerned. With the development of several colorimetric standardizing techniques, it is also possible to standardize procedures even though meters are not supplied with the equipment.

In addition, modern equipment in the ultrasonic field has kept pace with power requirements and actually has much more power than is presently needed in the pharmaceutical field.

For the purpose of this review, the author has divided the references into more or less distinct interest areas. In certain instances, there seems to be some overlapping but it does not appear to be very extensive.

EMULSIFICATION

One of the earliest reports of the use of ultrasound for dispersions of oil and water and mercury in water was by Wood and Loomis (1) in 1927. Richards (2) studied the emulsifying action of ultrasound shortly thereafter and came to the conclusion that the emulsification is due largely to the action of transverse waves in the walls of the glass vessel. He observed clouds of emulsion coming from the sides of the container when he used low intensity sound. He concluded from this that compressional waves within the liquid were not primarily responsible for the emulsifying action.

Bondy and Sollner (3–6) performed a series of experiments to clarify the mechanism of emulsification by ultrasonic waves. It was their conclusion that a so-called "cavitation" at the interface of water and oil caused emulsification. They found (3) that emulsions were obtained when con-

Received from the School of Pharmacy, University of Connecticut, Storrs, CT 06268

The author acknowledges the assistance afforded to him by several graduate students past and present.

ditions were such that cavities were formed and collapsed. Liquids irradiated in vacuum formed cavities, but the cavities did not collapse because the vapor pressure inside the cavities was practically in equilibrium with the outside pressure. If liquids were sealed under a gas and exposed to ultrasonic vibration, they were easily emulsified.

Bondy and Sollner (4) concluded from their observations that the mechanism of emulsification of mercury was quite different from that of oils and did not involve cavitation. They confirmed Richards' (2) mechanism, in which it was thought that droplets of water are thrown into the mercury, thin films of mercury form around them, and on bursting, a gray cloud of emulsion is driven into the water. Since this happened even *in vacuo*, cavitation was ruled out as the cause of emulsification.

Campbell and Long (7) were unable to form emulsions of mineral oil, peanut oil, and light liquid petrolatum at concentrations much above 10% with or without emulsifying agents at frequencies of 1000 and 500 kc. From this they concluded that the higher ultrasonic frequencies do not favor the formation of emulsions other than the dilute ones. When they used a generator at a 15-kc. frequency they were able to make a 50% peanut oil-in-water emulsion using triethanolamine stearate as the emulsifying agent. It was found that the globule size of this emulsion was smaller than the same emulsion prepared by hand shaking or by homogenization.

Audouin and Levavasseur (8) experimented with emulsification of peanut, linseed, olive, and mineral oils in an effort to determine the effect of frequency on results. They found that frequency was a predominant factor with regard to the possibility of formation and orientation of an emulsion in a certain direction (w/o or o/w) and only in this direction. The presence of an emulsifying agent normally thought to influence emulsification in the opposite direction did not change the type of emulsion. At a frequency of 960 kc., orientation occurred in the direction of water-in-oil even in the presence of sodium oleate, which normally orients in an oil-in-water direction. At 187 kc. and 320 kc., orientation occurred in the oil-in-water direction in spite of the presence of barium oleate, normally a water-in-oil director. They pointed out that an emulsion begun in a direction with a certain frequency continued to be formed in this direction, even if submitted to another frequency which normally gives an emulsion in the opposite direction. Frequencies of 576 kc. and 720 kc. seemed to have little tendency to form emulsions with most oils. Using a frequency of 25 kc. they first obtained an oil-in-water emulsion which was abruptly converted to a very concentrated water-in-oil emulsion after 15 min. insonation.

Audouin and Levavasseur (8) found that an elevation in temperature is unfavorable to emulsification and favorable to coagulation, due to lessened cavitation. With a small rise in temperature, there was a reduction in viscosity which favored emulsification. Beal and Skauen (9) came to the same conclusion since a temperature rise did not hinder emulsification; on the contrary, it rendered viscous oils more easily emulsified.

Esche and Wenke (10) state that the formation of an emulsion in an ultrasonic field occurs in cases where the viscosity of the dispersing medium is low—less than 120 cps. As the viscosity of the medium increases, there is greater absorption of acoustic energy with diminished cavitation.

While studying emulsifying agents under the influence of ultrasound, Tamas (11) found that the viscosities of tragacanth and polyethylene glycol 400 solutions were diminished by 50% and 20%, respectively. The particle size of emulsions was first reduced and then increased. They presumed that every kind of oil has an optimum frequency at which emulsions form. In addition, as ultrasonic intensity increases, cavitational forces increase, and the rate of emulsification increases.

There is some disagreement in relation to the relative importance of high- *versus* low-frequency equipment for emulsification purposes. Relative intensity of insonation has likewise been discussed by numerous investigators. Mounier *et al.* (12) prepared emulsions at frequencies from 958 kc. to 288 kc. and state that a stable oil-in-water emulsion is always formed. An emulsion prepared at one frequency is not destroyed at another frequency regardless of the oil.

According to Lifshits (13), homogeneous emulsions were formed at a sonic frequency of 7.6 kc. The dispersion of the emulsions increased with increase in the duration of irradiation. There was a limit to the decrease in mean globule size.

Neduzhii (14–16) studied the relationship of sound intensity to emulsification. At a frequency of 22 kc. with a water–dibutylphthalate system he found that a threshold intensity of sound was required to initiate emulsification and was different depending upon whether it was a water-in-oil or an oil-in-water emulsion. As the sound intensity was raised, the rate of emulsification increased. He suggests that the particle size of the dispersed phase is independent of the sound intensity. Neduzhii (16) also indicates that on

continued insonation there is a tendency for the particles to begin to coalesce.

Beal (9) investigated the effect of exposure time on a liquid petrolatum emulsion and reported that up to 30 min. there was a continued decrease in globule size.

While reports that highly dispersed and concentrated emulsions have been produced by ultrasonic means without emulsifiers (17), there is general agreement that the presence of emulsifying agents improves the stability and quality of emulsion systems.

Beal and Skauen (9) showed that hard soap was the emulsifier of choice for a mineral oil emulsion, with polyethylene glycol 400 monostearate and polysorbate 80 following in that order. This study proves the necessity to examine the effect of ultrasonics on various components of emulsions, since tragacanth when used as an emulsifier appeared to suffer degradation. Tamas (11) reported a 50% loss in viscosity with tragacanth and a 20% loss with polyethylene glycol 400 monostearate. Haavisto and Hagner (18) attempted to prepare emulsions of peanut oil and water without the aid of emulsifiers and found them to be nonhomogeneous and unstable. Using a 5% total sorbitan monoloeate–polyoxyethylene 20 sorbitan monopalmitate[1] (1:1) emulsifier, they found that superior emulsions could be made with 5, 20, and 40% peanut oil and an ultrasonic step-horn transducer.

Emulsions of mineral oil were prepared by Myers and Goodman (19) using a liquid whistle type of apparatus called a Rapisonic.[2] Liquids are pumped through an orifice at high pressures and velocities against a steel blade, causing the blade to vibrate at ultrasonic frequencies. When the system reaches a steady state, a cavitational field is produced. Results reported by the authors indicated that on microscopic examination the diameter of the globules was generally in the range of 1–3 μ.

Singiser and Beal (20) carried out a study comparing the efficiency of several ultrasonic generators and a colloid mill for the production of an emulsion. Their evaluation covered emulsions of heavy mineral oil, peanut oil, and safflower oil, and four emulsifiers—acacia, polyoxyethylene 20 sorbitan monopalmitate–sorbitan monooleate, poloxalkol[3]–sorbitan monooleate, and sodium lauryl sulfate. The Minisonic Mark 2[4] hydrodynamic generator produced emulsions superior to those made by any other apparatus. It would appear that the hydrodynamic apparatus is the best of the ultrasonic generators for large volume liquid processing. Major advantages, including faster through-put, no premix, faster emulsification, and reduced temperature requirements, are suggested by Marshall (21) and McCarthy (22) for this type of generator.

With the recent development of the step-horn type of ultrasonic transducer, it is possible to study various emulsion systems where small batches are involved and where power output is important. One of the advantages of this type of transducer is that the ultrasonic horn is in direct contact with the liquid being processed with the net transfer of maximum energy. Another advantage considered important for such devices is the availability of a variety of transducers with different power output. It is even possible for instruments to be made to match requirements.

Kann and Tester (23) used a step-horn transducer called the Sonifier[5] to produce an emulsion for a parenteral contrast medium for lymphography. It was their desire to prepare a 50% emulsion with a particle size less than 0.5 μ with a viscosity of 15–20 cps. at 30°. They reported satisfactory results with both the Sonifier and the Mini-Mill.

EXTRACTION

El'piner (24) has reviewed the literature very thoroughly in regard to viral and bacterial extraction and associated action of ultrasonic waves. He has, in addition, reported on the action of ultrasonic waves on unicellular and multicellular organisms.

This review therefore includes only those references which seem to be most significant with respect to the field of pharmacy.

Much of the early work in extraction was accomplished with equipment of low power output and relatively high frequency. With the recent development of the step-horn transducers with low frequency and high power output, it is expected that many more studies on effects of ultrasound on cells of pharmaceutical interest will appear.

Yamada and Ishida (25) studied the mechanism of fish oil extraction by means of ultrasonic waves. They reported that the muscle protein absorbed the ultrasonic energy selectively, for the temperature rise of the protein always preceded that of the oil layer by about 10°. The protein was coagulated by heat and water was released from

[1] Marketed as Span 80 and Tween 40, respectively, by Atlas Chemical Industries, Wilmington, Del.
[2] Ultrasonics Ltd., Westgate, Otley, York, England.
[3] Pluronic F-68.
[4] Sonic Engineering Corp., Norwalk, Conn.
[5] Branson Sonic Power Co., Danbury, Conn.

the muscle. The lipids of the connective tissue within the muscle were emulsified along with the water and flowed out.

Gomez and Matas (26) applied ultrasonic waves to seeds of *Strophanthus gratus* that had lost 50% of its strophanthin on storage. The yield of alkaloid was not improved and was actually diminished in alcoholic solution. The same authors (27) studied the observed loss of activity of extracts of *S. gratus* in terms of alkaloidal degradation and suggested hydrolytic effects due to ultrasonic stimulation.

Head *et al.* (28) studied the effect of ultrasonic waves on the extraction of *Cinchona succirubra*. Ultrasonic extractions at 20 kc. and 450 kc. were compared with conventional Soxhlet extraction under similar conditions. The ultrasonic extraction was more efficient than Soxhlet extraction, and there was no destructive action by the ultrasound on the alkaloids.

Adamski *et al.* (29) also treated cinchona species and showed an increase of 50% in the extraction yield when the bark was treated with ultrasound prior to maceration or percolation. They mention that the keeping qualities of the extracts were good but that decomposition of quinine was noticed with treatment lasting more than 30 min.

Using the Sonifier, Ovadia and Skauen (30) were able to increase not only the rate but the extent of extraction of cinchona bark. No destruction of alkaloids was noted even after 3 hr. insonation.

Colian and Tomas (31) reported that ultrasonic extraction of *Atropa belladonna* at 800 kc. produced 36.7% more alkaloids than mechanical agitation. The yield was only 2.4% superior to solvent extraction but extraction time was shorter. The amount of alkaloids did not increase after 15 min. insonation nor was decomposition observed. Wray and Small (32) also studied the effect of ultrasonic treatment on the extraction of belladonna leaf. Samples of maceration mixtures were put in surface treatment vessels and were subjected to ultrasonic treatment at 500 kc. for 20 min. Then Soxhlet extraction and ultrasonic extraction in a submersion treatment vessel were effected. The ultrasonic maceration shortened the maceration time from 8 hr. to 20 min., and the ultrasonic maceration followed by ultrasonic extraction resulted in considerable saving of time. Analysis of the extract showed no decomposition products of the alkaloids.

El'piner and Stekol'nikov (33) subjected various alkaloids to ultrasonic treatment, among them atropine and quinine. These alkaloids, they state, are attacked by ultrasound. Alkaloidal salts were treated for 15–90 min. at 800 kc. under saturated air, hydrogen, argon, or helium. The pH of all solutions decreased, with the greatest change in contact with air. Atropine lost its activity by such treatment under air but retained it in a hydrogen, helium, or argon atmosphere. Quinine also showed little change after exposures except in those cases where it was heated for 60–90 min. in contact with air.

Rauwolfia serpentina roots were extracted under ultrasonic irradiation by Bose *et al.* (34). Samples were subjected to ultrasonic maceration at 25 kc. for 5, 10, and 15 min. It was found that the maceration time was reduced from 8 hr. to 15 min. for the complete extraction of the alkaloids.

DeMaggio *et al.* (35) examined the effect of change in frequency on extraction yield of *Datura stramonium*. They state that a 15% increase in alkaloidal yield results when the frequency is increased during a 1–2 hr. insonation period.

DeMaggio and Lott (36) studied the influence of ultrasound on the maceration process as well as during the extraction of *D. stramonium*. The application of ultrasound during short periods of maceration was effective in producing a greater yield of alkaloids than conventional methods. When ultrasound was applied during the process of continuous solvent extraction, it proved to be slightly more efficient in liberating the desired alkaloids from the drug.

Shinyans'kii and his co-workers (37) exposed the dry leaves, seeds, seed pods, and stalks of the poppy plant to ultrasonic waves at 500 kc. for 15–17 min. in an aqueous medium. The extent of extraction of morphine was equivalent to 24 hr of conventional liquid extraction. A similar study was reported (38) using 22, 300, and 750 kc. on pods of opium poppies in an aqueous environment. Approximately 60% of the morphine was extracted into the aqueous phase during the first 10 min. At each frequency studied, there seemed to be no change in the amount extracted. Length of exposure to ultrasound was an important factor, since the yield decreased with increased exposure time because of decomposition of morphine. Changing the solvent system effected a corresponding increase in morphine yields.

Paradissis and Maney (39) irradiated ergot at 400 kc. and found that ergotoxin was evidently destroyed. When cationic surfactants and polyethoxylated aliphatic amine[6] were used extraction was improved. They also found that a frequency of 400 kc. was superior to 10 kc. for extraction of ergot.

[6] Ethomeen C-25, Armour Industrial Chemical Co., Chicago, Ill.

Pivnenko and Sotnikova (40) used a 500-kc. generator for the extraction of ergot alkaloids and found that extraction time was three times more rapid than by the USSR Pharmacopoeia method.

The efficiency of extraction of jaborandi leaf and ipecac root with the Sonifier was investigated by Ovadia and Skauen (30). The amount of alkaloids obtained by ultrasonic extraction of ipecac root was higher than that obtained by the U.S.P. XVI method, while the amount obtained by ultrasonic extraction of jaborandi under temperature controlled conditions was also greater than the B.P.C. 1949 extraction method. The amount of alkaloids extracted from ipecac root in 30 sec. was greater than the amount produced by Soxhlet extraction in 5 hr. Fifteen seconds of ultrasonic treatment resulted in greater yields of from jaborandi leaf than 5 hr. of Soxhlet extraction. After 0.5-min. extraction, jaborandi leaf alkaloids showed degradation, with almost complete degradation after 15 min. Kusano (41) reports no such loss of pharmacological activity of pilocarpine or atropine after ultrasonic irradiation.

Kazarnovskii (42) used ultrasonics as part of an extraction technique for obtaining epinephrine from adrenal glands. Ultrasound was helpful in obtaining complete extraction of all the adrenaline present.

Grochowska (43) reports on the use of ultrasound to aid in removal of barbiturates from human tissue. He states that the time of extraction is considerably shortened.

CRYSTALLIZATION

The use of ultrasonic energy during crystallization of supercooled melts is well documented. Danilov (44) found that the appearance of the first crystallization center of *o*-chloronitrobenzene was accelerated by ultrasound. When salol, naphthalene, and camphor were treated, an increase in rate of crystallization resulted (45). Danilov further reported a large increase in the number of nuclei in piperine after insonation (46). Ultrasonics has been applied to the crystallization of betol (47), thymol (48), benzophenone (48), and piperonal (48).

Supersaturated solutions have been treated with high-frequency sound resulting in smaller crystals and more uniform size distributions. The crystallization of tartaric acid was accelerated by ultrasound, and the crystals were found to be more finely dispersed and homogeneous (49). Thorium oxalate, when prepared by precipitation under ultrasonic treatment, yielded more uniform crystals with smaller particle size (50).

The production and growth of crystals of thymol, potassium alum, salol, Rochelle salt, and hyposulfite from supersaturated solutions treated with ultrasound was investigated by Chormonov (51). He stated that spontaneous crystallization was accelerated by ultrasound.

One of the most energetic workers in the field of ultrasonic crystallization of sugars has been Van Hook (52). He has been able to improve the crystal size distribution of the final crop as well as induce crystallization in such normally refractory supersaturated solutions as arabinose, fructose, sorbitol, glucose, and melibiose.

Progesterone was prepared as microcrystals by insonating a saturated solution of the hormone (53). Cohn (54) investigated the ultrasonic crystallization of hydrocortisone. He developed a procedure which allowed him to prepare low micron particles of hydrocortisone by direct insonation of a saturated solution. Fincher (55) insonated a supersaturated solution of sulfisoxazole and successfully prepared 1.7-μ crystals.

It has been stated (56) that the effect of ultrasound is due to the acoustic wind and to the mechanical vibrations of the crystal. This was based on the observations that the growth rate of a crystal in an ultrasonic field increases with the ultrasonic intensity and that the velocity of growth increases with decreasing supersaturation.

Berlaga (57, 58) reported that ultrasound increased the number of nuclei in betol, which caused a great increase in crystallization rate. He explained this by assuming that the field separates the fresh nuclei from ones formed previously. Levin (45) uses the same theory to explain the effect of ultrasound on the crystallization of salol, naphthalene, and camphor. Smaller crystals were obtained with increased intensity of vibration. It was stated that the decrease in size of primary crystals and the increased rate of crystallization are caused by the cavitation bubbles which separated the growing crystals at the crystal solution interface and carried them into solution. Kudryavtsev (59) suggested that the increased crystallization rate in ultrasonic crystallization is due to an increase in the number of crystallization centers as a result of ultrasonic scattering of the microcrystals forming in the melt.

Kapustin (48) and Polotskii (60) have felt that ultrasound increased the number of crystal nuclei by shattering or eroding the nuclei that occur in a normal manner. Sokolov (61) states that the mechanism is associated with the initiation of the cavitation process which disrupts the

growing crystal. After studying the effect of ultrasound on crystallization of potassium bitartrate, it was concluded that ultrasound augmented the formation of crystalline centers in the beginning stages of the process (62). Kapustin (63) indicates that ultrasonics breaks off microcrystals which act as fresh nuclei. Teumin (64) explains that the mechanism is due to "the formation of cavitation processes, which disintegrate the growing crystals, to radiation pressure, which transports the crystal fragments to the body of the melt where they form fresh crystallization centers, and to agitation, which results in temperature equalization throughout the entire volume, thereby assisting bulk crystallization." Babikov (65) in general agrees with Teumin but also includes the possible effect of ultrasound on the origination of primary crystallization centers, while Meleshko (66) attributes the crystallization effects to the dispersing action of the ultrasonic field.

Kapustin (67, 68) reported that ultrasound effects crystallization by producing many fresh nuclei from any seed. A small crystal of thymol fixed in a wooden holder was immersed in water in a tube. Liquid thymol was carefully layered on the water. No crystals arose on standing, but crystallization began when ultrasonics was applied to the bottom of the tube. He observed the field to break off minute particles from the seed which were carried to the interface. Identical tests without the seed did not initiate crystallization. Sokolov (69) also found that insonation produced more rapid crystallization when solutions of beet sugar and zinc sulfate were seeded prior to insonation.

Kapustin (70) studied the process of relieving supersaturation in an aqueous solution of potassium alum by ultrasonics and reports desaturation occurs in the presence and absence of nucleation.

Polotskii (71) presented photographic evidence to support the belief that ultrasound acts by dispersing nuclei. He observed cavitation at the crystal–melt interface of supercooled, chemically pure salol to cause strong dispersion of the newly formed crystals. In addition Polotskii (72) photographed the shattering of acicular crystallites of salol and a naphthalene–camphor eutectic shortly after their formation. Without ultrasound, this did not occur.

Van Hook (73) found that the number of crystallization centers increases with the duration of insonation in an autocatalytic way. Due to the limiting effect of prolonged treatment, it was suggested that ultrasound acted chiefly on newly formed crystallites. Nickus (74) makes a similar observation, stating that insonation at 25–50 kc. accelerated the formation of crystallization centers as well as increasing the crystallization rate of molten menthol. Kapustin (75) stated that ultrasonics brought about the formation of new crystallization centers only when a crystal reaches a certain size.

A number of workers have felt that ultrasound generates crystal nuclei. An investigation of the effect of ultrasound on supersaturated solutions of thymol, Rochelle salt, salol, and hyposulfite proved that ultrasound significantly accelerates the process of spontaneous crystallization and plays an essential role during the formation of nuclei (51).

Kapustin (76) stated that ultrasound affects the production of the first crystallization center. This was based on the action of ultrasound on *o*-chloronitrobenzene. The compound was heated to 100°, slowly cooled, and exposed to 10 sec. of 700 kc. ultrasound of varying intensities. The field did not affect the production of the first crystallization center above 20° but caused "spontaneous crystallization" at 18 to 20°. He explained that a system which is macroscopically homogeneous has a certain micro nonhomogeneity which varies little with time. Thus ultrasound could cause a redistribution of the micro nuclei and alter the probability of spontaneous production of crystals.

Brown (77) states that "the effect of ultrasonic irradiation on the crystallization process is similar to its effect on precipitation, *i.e.*, the cavitation produced offers nucleation centers for the crystals to initially form around, and it also breaks up any large crystals." Mazhul (78) studied ultrasonic crystallization and especially the electric fields associated with cavitation. His experiments led him to conclude that nucleation in the melt cannot be explained by the electric fields associated with cavitation. Rather the sites of cavitation themselves act directly as nuclei. He adds that cavitation cannot be taken as the sole cause of the effects, for weak fields which do not cause cavitation still increase the nucleation rate. After experimenting with betol, Mazhul (47) later stated that cavitational ruptures may themselves become crystallization centers under varying external pressures. Glinskii (79) agrees in reporting that the effect of ultrasound on primary crystallization is associated with a decrease of grain size in the melt under pressure. He shows on a theoretical basis that equal compression and rarefaction cause the same effect as to the number of crystal nuclei arising. The effect of ultrasound increases with lower supercoolings.

During the ultrasonic crystallization of sulfisoxazole (80) it was explained that ultrasonics aids crystallization "by supplying cavitation bubbles, which act as the site of nucleation."

In his work on the ultrasonic crystallization of sugar, Van Hook (81) proposed both a heterogeneous and a homogeneous mechanism. He appeared to agree with Mazhul and Brown when he stated that "if one irradiates a strong syrup with a bar transducer, cavitation loci appear at definite points according to the size, shape, and arrangement of the system. The crystal nuclei are generated, presumably within these small volumes. They may then be dispersed and grow to visible size." He also reported that irradiation of a "sterile, isolated solution" gave poorer results or no improvement in grain at all. This suggested to him a heterogeneous mechanism rather than the spontaneous generation of nuclei by the ultrasonic field.

Hem (82) reported reproducible reduced crystal size distributions from insonating a supersaturated solution, whose background particle count was controlled, at a fixed temperature. The crystallization temperature of both insonated and uninsonated samples was dependent on the number of heterogeneous nuclei present.

DISPERSION

The application of ultrasonic waves for the dispersion of coherent solid materials dates back to the classical work of Richards (2) in 1929. He reported unsuccessful attempts to disperse such solids as glass and quartz in liquids. Sollner (83) commenting on this work says that only brittle and soft substances of high cleavage seem to offer promise. He irradiated mica, gypsum, steatite, and hematite in water, producing colloidal or semicolloidal solutions.

Most of the early work was done with coherent solid bodies, but it has been shown that suspended particles can be dispersed. In all systems containing preformed particles the latter may be separated from one another by insonation. Sound-dispersed preparations do not show any special features, the sound-induced cavitation being merely an efficient mechanical dispersing agent (83).

The sonic peptization of gels and gel-like substances has been investigated (84–86). Using a liquid whistle, Ramaszeder (87) dispersed potato starch and suggests that the particle size of the starch granules can be reduced by increased time of insonation. He suggests the use of ultrasonic dispersion of starch granules as an alternative to mechanical homogenization (88).

The dispersion of dyestuffs and pigments by ultrasonics has been reported in the literature (89–91). Fischer (92) stated that the disintegrating effects of ultrasound is negligible, and the changes produced cannot be distinguished from those caused simply by heating and stirring of the pigment. However, since more powerful equipment has become available, other workers have reported some success in dye dispersion. Lifshits (93) compared dye dispersion using piezoquartz, magnetostrictor, and hydrodynamic ultrasonic devices and a mechanical mixer. He states that a higher degree of dispersion was obtained by ultrasound than by mechanical mixing. Prakash (94–97) has studied the fading of colors of various dyes such as Congo red, rhodamine B, bromocresol green, and safranine O. He explains the decolorization on the basis that the high cavitation energy is responsible for a molecular breakdown of the dye.

Chambers (98) reported the preparation of microcrystals of sulfathiazole using ultrasound. Direct sonic vibration of suspensions of the preformed crystals produced no measurable effect. An indirect method was developed consisting of the neutralization of a solution of the sodium salt of the drug while the reaction mixture was kept in violent agitation by sonic vibrations. The size of the resulting crystals was regulated by controlling the speed of admixture or the temperature of the reactants or both. The microcrystals produced were fairly uniform in size. Gartner (99) investigated the action of ultrasound at frequencies of 500, 800, and 1500 kc. on aqueous suspensions of magnesium carbonate, lead carbonate, and barium sulfate. Best results were obtained at a frequency of 500 kc. and at suspension concentrations of 0.002 Gm./ml. Misek (100) studied the suitability of ultrasonic energy for dispersing progesterone in suspensions. He used a surface-active agent to obtain a satisfactory dispersion and states that the degree of dispersion increased as the ultrasonic intensity increased. Several other parameters were also studied (101). It was found that the extent of dispersion decreased as the viscosity of the suspending liquid increased. The application of 15 lb. of pressure upon the suspension during insonation rendered dispersion most complete. Such dispersions showed that 85% of the suspended material was 10μ or less in size. At the frequencies studied, 300, 500, and 750 kc., the frequency of the ultrasonic vibrations had no effect on the dispersion. Araujo (102) insonated suspensions of zinc oxide, bismuth subcarbonate, sulfathiazole, and procaine penicillin G at 400 kc. In all the cases studied, they report that ultrasonic energy

reduces the particle size of the suspended medicament and retards its rate of sedimentation, providing a more stable preparation. The dispersive action of ultrasound on crystals of potassium dichromate in ethanol was observed by Kosolapov (103). Sheikh (104) suspended polyethylene spheres in water and subjected them to the action of ultrasonics at 100 kc. He found that there was a significant reduction in particle size only if a surfactant was present. Increasing insonation time brought about reduction up to 2 hr.

When dealing with the action of ultrasound on suspended material, one must be concerned with the fact that dispersion is not the only effect that can be obtained. Coagulation can also be brought about by ultrasonic irradiation of suspensions. Bondy and Sollner (6) have explained the action of ultrasound on dispersions. They suggest that dispersion requires an energy level exceeding a certain limit, and coagulation may therefore result below this energy limit. Dispersion may also be precluded by prevention of cavitation.

Thompson and Vilbrandt (105) investigated the effect of ultrasonic energy on the coagulation of clay particles and reported that mild intensity insonation (below cavitation threshold) increased the rate of coagulation, while intense insonation (above cavitation threshold) retarded settling of the clay due to dispersion and disruption of the aggregates.

Kruglitskii (106) obtained a more uniform disperse phase when montmorillonite was insonated at 19.5 kc. He reports cavitational breaking of bonds between the disperse phase and the dispersion medium. After 8.5 min., dispersion is maximum, and the clay structure formed has maximum bond energy and stability. Further treatment caused coagulation with the coagulated particles so stable that further treatment does not destroy them. Kruglitskii (107) reports that more satisfactory thixotropic properties and greater stabilities can be obtained by treating clay suspensions with ultrasound, colloids like carboxymethylcellulose, and sodium chloride or calcium chloride than by using any single one or combination of any two of these means.

OTHER PHYSICAL-CHEMICAL AND CHEMICAL EFFECTS

For a very thorough and instructive discourse the interested person is urged to read the text of El'piner (180).

The effect of ultrasonic waves on the degradation of natural and synthetic polymer solutions has been well established.

In 1949, Prudhomme and Graber (108) claimed that the rupture of high polymer chains was due entirely to cavitation. Time required for depolymerization was inversely proportional to the intensity of sound and directly proportional to the concentration, but the final degree (limiting degree) of depolymerization was independent of these factors. They also claimed that polymers of the same composition but different molecular weights approach the same limiting chain length with longer periods of insonation. Later, Prudhomme (109) confirmed his claim that the rupture of the polymers was due entirely to cavitation by insonating 1% aqueous solutions of carboxymethylcellulose at 960 kc. under vacuum, air, and CO_2. He showed that the forces of friction between solvent and polymer molecules are not the basis of the degradation process. Weissler (110) observed that the depolymerization of polystyrene in toluene occurred only in the presence of cavitation. A sample degassed by boiling under vacuum showed no cavitation and no appreciable molecular weight change. This study revealed that oxidants produced by ultrasonic waves cannot be responsible for degradation. Jellinek and White (111) tried to study the number and weight-average molecular weight distributions and number and weight-average chain lengths of polystyrene after insonation and found satisfactory agreement between calculated values for an assumed degradation mechanism and experimental results.

Head and Lauter (112) investigated the ultrasonic depolymerization of a number of natural and semisynthetic polymers. By relating viscosity measurements to molecular weights, they showed that most of the polymers followed a first-order rate equation for degradation. Okuyama (113) explained polymer rupture by ultrasound by stating that at the contracting step of a violently vibrating bubble, the polymer molecule takes a linearly extended configuration owing to the suction force of the bubble occurring by slipping and friction between solvent molecules and the extended part of the polymer. The partial chain is subject to a tensile force arising from friction, which is maximum at the middle carbon atom of the chain. If this force is greater than the critical tensile strength of the C—C bond, the polymer degrades. Zorina and El'piner (114) exposed an aqueous solution of dextran (mol. wt. 35,000) to 840 kc. ultrasound in atmospheres of oxygen, nitrogen, and argon. After 3–4 hr. of insonation the molecular weight decreased to a minimum value of about 20,000. When irradiated in an alkaline solution (pH 13) dialyzable particles were formed. They were also able to produce low molecular weight substances by ir-

radiating solutions of mono-, di-, and trisaccharides at pH 13. El'piner and Pyshkina (115) exposed a 5% solution of sodium carboxymethylcellulose solution to 740 kc. ultrasonics and found that in the presence of oxygen a decrease of molecular weight and viscosity occurred, while in the presence of hydrogen the molecular weight increased and the degree of hydration and viscosity decreased.

Kukoz (116) discussed some of the literature on the chemical action of ultrasonics. The results of experimental studies confirming the cavitation–photoelectrochemical theory of chemical action of ultrasound and experiments demonstrating the need of improving or modifying this theory are given. He mentions that free radicals, ions, and excited molecules of the medium can all play a definite role in ultrasonic action. Information is presented showing the contradictory view that chemical action of ultrasound is explainable by local increase in temperature or the mechanical–chemical effects. He makes the important point that the chemical action of ultrasound on an aqueous medium may be due to many factors, and the role of each of these factors must be analyzed separately in each concrete case.

Vaisman (117) investigated the stability of some medicinal solutions toward ultrasound. The resistance of a series of inorganic and organic pharmaceuticals in aqueous solutions of different concentrations to the action of ultrasound was studied with respect to the visual changes of the solutions, the pH changes, and in some cases the decomposition products. At 800 kc. for 15 min. insonation, sodium chloride, potassium chloride, ascorbic acid, and phenol did not undergo a change. Potassium iodide, sodium salicylate, resorcinol, epinephrine, and procaine HCl were oxidized, and magnesium sulfate and zinc sulfate were partially hydrolyzed.

Maffei (118) treated pure expressed olive oil with ultrasonic waves. He noticed a shift of fluorescence from yellow to blue and attributes this to trace amounts of substances formed by decomposition of the glycerides of the oil. He concluded that the alteration of the visible spectrum is connected with a partial decomposition of carotenoids and chlorophyll.

Faust (119) used a 400-kc. generator to observe its effects on glutathione showing oxidation of the reduced glutathione. Nearly one half of the tripeptide is reported to have been lost after 41 min. exposure.

Examination of protein fragments produced by ultrasonic treatment of aqueous solutions of serum albumin or trypsin showed that the ultrasonic treatment did indeed produce protein fragments. Such fragments are much more prevalent immediately following the irradiation than with the passage of time and disappear completely within 30–40 min. (120). Myosin and deoxyribonucleic acid specimens also showed this effect, yet gelatin failed to do so. Negative results were also obtained with substances such as glycine, alanine, cystine, glucose, and succinic acid. It was concluded that the fragmentation of these macromolecules caused by ultrasound produces macroradicals with half-life of the order of some minutes. A semiquantitative estimation showed that the molecules of serum albumin are cleaved into three parts, while those of trypsin are cleaved into two parts.

Miyagawa (121) reports acetates being hydrolyzed twice as rapidly as normal under the influence of 540 kc. ultrasonic waves. Mario (122) followed the rate of hydrolysis of aspirin with and without the influence of ultrasound. He states that there is an increase in the rate of hydrolysis using ultrasound that is comparable to the effect obtained by increasing the reaction temperature. On the other hand, Fenn (123) reported no significant difference in the rates of hydrolysis of procaine and butethamine hydrochlorides when insonated at 400 kc.

El'piner (124) followed the decomposition of sucrose in an ultrasonic field. The sucrose decomposed, as shown by the appearance in solution of reducing substances which increase in concentration with increased duration of exposure. The presence of carbon tetrachloride in buffered sucrose solutions enhances the rate of decomposition. Buchnea (125) subjected solutions of sucrose, glucose, and fructose to ultrasonic waves at various frequencies for times up to 100 hr. under various conditions. With increasing frequency the temperature of the solution increased, favoring splitting of sucrose. In the presence of oxygen, sucrose decomposed more rapidly with the formation of carbon dioxide and formic acid. With hydrogen, only slight inversion occurred without acid formation. The rate of inversion increased with sucrose concentration, but none resulted if the solution was cooled. The decomposition of the hexoses depended mainly on oxidation. Zapior (126) also reported an increase in rate of sucrose hydrolysis especially in strongly acid solutions but stresses the fact that the temperature was verified indirectly by use of a thermometer in the water bath.

El'piner (127–129) subjected insulin to ultrasonic irradiation and reported changes in the molecule which are influenced by the nature of the atmosphere in which treatment was performed. In the presence of oxygen or argon the

hormonal activity decreases, while in a hydrogen atmosphere the activity is increased. Chromatographic analysis showed that the hormone lost different substituent groups depending on the atmosphere used.

After ACTH in aqueous solution was exposed to 800 kc. ultrasound for 1 to 4 hr., a solution of yellow color and distinctive odor was produced (130). End-group analysis showed that methionine rather than serine became the end group. Longer exposure produced a solution which failed to respond to the usual end-group analysis.

Gramicidin-S was treated with ultrasound in ethyl alcohol by El'piner (131). This resulted in the formation of glyoxalic acid, but no great change in spectrophotometric analysis was noted in 8 hr. whether it was conducted in an atmosphere of oxygen or argon. However, the biological activity of the antibiotic was increased in these atmospheres. No such increase in activity was observed after treatment in a hydrogen atmosphere.

Gerraughty (132) explored the possibility of using the energy of ultrasound to effect the direct esterification of sterically hindered acids. The methyl, ethyl, *n*-propyl, and *n*-butyl esters of *o*-thymotic and ursolic acids were prepared. Branched chain esters of the acids, however, could not be made.

A number of investigators have studied the effect of ultrasonic irradiation on distilled water. Akiya (133) showed that irradiated water forms H_2O_2, HNO_2, and HNO_3. The amount of these formed is related to the intensity of the ultrasonic waves. At maximum intensity the amount of these substances varies with the length of radiation. He also reported the liberation of iodine from an acid potassium iodide solution under ultrasonic activity. Grabar (134) also mentions the decomposition of water by supersonics into H_2 and O_2. When water is subjected to ultrasonic waves at frequencies of 404 and 960 kc. in the dark, a nonuniform blue luminescence is produced which is independent of the pH or the presence of oxygen (135). Even in the absence of oxygen, water treated with ultrasonics develops oxidizing powers which are retained after treatment is stopped. A photochemical reaction probably occurs by which water is converted to H_2O_2, H, and O.

Bhar (136) observed that when a solution of potassium iodide was insonated, decomposition resulted with the amount of iodine liberated being proportional to the strength of the ultrasonic field and inversely proportional to the strength of the iodide solution. Others (137–139) studied various aspects of the oxidation of potassium iodide solutions, such as the effect of frequency, the kinetics of oxidation, and the mechanism of oxidation.

Weissler (140) developed a simple colorimetric procedure to measure cleaning efficiency of ultrasound. This was based on the liberation of chloride ion from carbon tetrachloride in an aqueous solution. Lur'e (141) indicates that the equation for the above reaction at a pH of 12 is as follows: $CCl_4 + H_2O \rightarrow Cl_2 + 2HCl + CO$. As the duration of insonation increased from 10–60 min., the degree of decomposition went from 60.3% to 100%. Liu (142) suggests the use of the reaction cited above as a technique to test for cavitation-activity measurements. He reports that chlorine is liberated from carbon tetrachloride in a linear relationship with time. A linear relationship to cavitation can also be plotted. Prakash (143) observed a similar result when he irradiated chloroethane in aqueous solution. He suggests that the reaction is governed by ultrasonic cavitation.

MISCELLANEOUS

Cleaning by high frequency sound is now a standard procedure in many industries. Central sterile supply departments are routinely washing glassware, surgical instruments, syringes, and needles in automatic ultrasonic equipment.

Clayton (144), Mamer (145), and McKay (146) discuss the use of ultrasound in the hospital environment. Beal and Skauen (147) were able to show that hypodermic syringes could be cleaned in a satisfactory manner by high-frequency sound. They later compared the cleaning efficiency of ultrasound with other methods used in hospital practice using radioactive iodide-^{131}I soils (148).

Palmer and Skauen (149) compared various cleaning methods for catheters using an iodide-^{131}I contaminated soil. Ultrasound cleaning was proven to be unfavorable in this study and was attributed to the "soft" nature of rubber catheters. Gligorijevic (150) found this to be a factor in a similar study, and he concludes that decontamination with ultrasound depends on the surface and the material.

Witekowa (151) felt that ultrasound might be used in various absorption phenomena. Accordingly, he treated a sample of charcoal with an aqueous solution of methylene blue and irradiated it with ultrasound. The suspension was treated with water, then filtered. The concentration of the methylene blue in the filtrate was determined colorimetrically. The quantity of absorbed methylene blue was 8–10% higher in the insonated sample than the control and increased

with power and frequency of ultrasound. He explains the increase on the basis of increased degree of fineness and a change of electrochemical properties of the charcoal.

Soloff (152) reviews the use of ultrasound for the sealing of various types of plastics. While this is a recent development, it would appear to offer certain benefits to the pharmaceutical industry.

Kuppusami (153) offers the suggestion that ultrasound can be used for determining the critical micelle concentration of surfactants. In his experiments he measured ultrasonic velocity in solutions of sodium dodecyl sulfate, cetyltrimethylammonium bromide, potassium laurate, and tetrahydroanacardol ammonium monosulfate. Each showed a reversal in slope of the velocity *versus* concentration curve in the region of the critical micelle concentration in agreement with the literature. This decrease in ultrasonic velocity he explains on the basis of internal pressure.

A list of reviews and books pertinent to the subject is included under *References* (154–185).

REFERENCES

(1) Wood, R. W., and Loomis, A. L., *Phil. Mag.*, **4**, 417 (1927).
(2) Richards, W. T., *J. Am. Chem. Soc.*, **51**, 1724(1929).
(3) Bondy, C., and Sollner, K., *Trans. Faraday Soc.*, **31**, 835(1935).
(4) *Ibid.*, **31**, 843(1935).
(5) *Ibid.*, **32**, 556(1936).
(6) *Ibid.*, **32**, 616(1936).
(7) Campbell, H., and Long, C. A., *Pharm. J.*, **163**, 127(1949).
(8) Audouin, A., and Levavasseur, G., *Oleagineux*, **4**, 95(1949).
(9) Beal, H. M., and Skauen, D. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 490(1955).
(10) Esche, R., and Wenke, P., *Elektrotech. Z.*, **5**, 97(1960); through *Reference 180*, p. 232.
(11) Tamas, G., *Acta Pharm. Hung.*, **32**, 225(1962); through *Chem. Abstr.*, **58**, 2325*b*(1963).
(12) Mounier, J., Blanquet, P., Piffault, G., and Dallies, G., *Bull. Soc. Pharm. Bordeaux*, **94**, 161(1955); through *Chem. Abstr.*, **51**, 1537*i*(1957).
(13) Lifshits, A. T., *Tekstil. Prom.*, **1**, 52(1960); through *Reference 180*, p. 234.
(14) Neduzhii, S. A., *Akust. Zh.*, **7**, (1), 99(1961); through *Chem. Abstr.*, **55**, 25402*g*(1961).
(15) Neduzhii, S. A., *Kolloid Zh.*, **23**, 448(1961); through *Chem. Abstr.*, **56**, 2904*h*(1962).
(16) Neduzhii, S. A., *Akust. Zh.*, **8**, 481(1962); through *Chem. Abstr.*, **58**, 7393*a*(1963).
(17) Rzhevkin, S. I., and Ostrovskii, E. P., *Zh. Fiz. Khim.*, **1**, 73(1935); through *Reference 180*, p. 233.
(18) Haavisto, M. L., and Hagner, R., *Farm. Aikakauslehti*, **75**, 75(1966); through *Chem. Abstr.*, **64**, 15677*f* (1966).
(19) Myers, J. A., and Goodman, J. E., *Pharm. J.*, **173**, 422(1954).
(20) Singiser, R. E., and Beal, H. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 478(1960).
(21) Marshall, K., *Mfg. Chemist*, **34**, 562(1963).
(22) McCarthy, W. W., *Drug Cosmetic Ind.*, **94**, 821 (1964).
(23) Kann, D. E., and Tester, W. W., *Am. J. Hosp. Pharm.*, **22**, 606(1965).
(24) *Reference 180*, pp. 243–295, 296–331.
(25) Yamada, M., and Ishida, M., *Science (Japan)*, **21**, 467(1951); through *Chem. Abstr.*, **45**, 10619*b*(1951).
(26) Gomez, C. G., and Matas, L. C., *Med. Colonial (Madrid)*, **15**, 401(1950); through *Chem. Abstr.*, **44**, 9634*b* (1950).
(27) Gomez, C. G., and Matas, L. C., *Farmacognosia (Madrid)*, **12**, 153(1952); through *Chem. Abstr.*, **48**, 3632*i* (1954).
(28) Head, W. F., Beal, H. M., and Lauter, W. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 239(1956).
(29) Adamski, R., and Mizgalsk W., *Acta Polon. Pharm.*, **14**, 119(1957); through *Chem. Abstr.*, **51**, 14206*a* (1957).
(30) Ovadia, M. E., and Skauen, D. M., *J. Pharm. Sci.*, **54**, 1013(1965).
(31) Colian, B., and Tomas, G., *Acta Pharm. Hung.*, **26**, 67(1956); through *Chem. Abstr.*, **52**, 15829*g*(1958).
(32) Wray, P., and Small, L., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 823(1958).
(33) El'piner, I. E., and Stekol'nikov, L. I., *Dokl. Akad. Nauk S.S.S.R.*, **141**, 219(1961); through *Chem. Abstr.*, **56**, 10294*e*(1962).
(34) Bose, P. C., Sen, T. K., and Ray, G. K., *Indian J. Pharm.*, **23**, 222(1961).
(35) DeMaggio, E., Lott, J. A., and Gerraughty, R. J., *Nature*, **199**, 1292(1963).
(36) DeMaggio, E., and Lott, J. A., *J. Pharm. Sci.*, **53**, 945(1964).
(37) Shinyans'kii, L. A., Kazarnovskii, L. S., Karavai, N. Ya., and Solon'ko, V. N., *Farmatsevt. Zh.*, **15**, 48(1960); through *Chem. Abstr.*, **61**, 10533*b*(1964).
(38) Aimukhamedova, G. B., and Korneva, G. M., *Izv. Akad. Nauk Kirg. S.S.R., Ser. Estestv. i Tekhn. Nauk*, **4**, 17(1962); through *Chem. Abstr.*, **59**, 5487*e*(1963).
(39) Paradissis, G. N., and Mauey, P. V., *Mfg. Chemist Aerosol News*, **36**, 62(1965); through *Chem. Abstr.*, **64**, 2327*f* (1966).
(40) Pivnenko, G. P., and Sotnikova, O. M., *Farmatsevt. Zh.*, **20**, 39(1965); through *Chem. Abstr.*, **64**, 6403*g*(1966).
(41) Kusano, S., *Tohoku J. Exptl. Med.*, **30**, 175(1936); through *Chem. Abstr.*, **31**, 2908(1937).
(42) Kazarnovskii, L. S., Solon'ko, V. N., and Shinyanskii, L. A., *Med. Prom. S.S.S.R.*, **18**, 29(1964); through *Chem. Abstr.*, **62**, 772*b*(1965).
(43) Grochowska, Z., *Przeglad Lekar.*, **22**, 305(1966); through *Chem. Abstr.*, **66**, 44220*p*(1967).
(44) Danilov, V. I., and Teverovskii, B., *Zh. Eksperim. i Teor. Fiz.*, **10**, 11(1940); through *Reference 182*, p. 39.
(45) Levin, G. I., and Polotskii, I. G., *Izvest. Akad. Nauk S.S.S.R., Otdel. Tekh. Nauk Met. i Toplivo*, (No. 3), 167 (1961); through *Chem. Abstr.*, **55**, 22982*e*(1961).
(46) Danilov, V. I., Pluzhnik, E., and Teverovskii, B., *Zh. Eksperim. i Teor. Fiz.*, **9**, 66(1939); through *Reference 182*, p. 113.
(47) Mazhul, M. M., *Uch. Zap. Belorussk. Gos. Univ.* (No. 32), 211(1957); *Ref. Zh. Met. Abstr.*, No. 24786 (1957); through *Chem. Abstr.*, **53**, 17619*c*(1959).
(48) Kapustin, A. P., *Zh. Tekhn. Fiz.*, **22**, 765(1952); through *Chem. Abstr.*, **50**, 6123*c*(1956).
(49) Glavatskii, D. E., and Kortner, A. V., *Nauchn. Zap. Odessk. Politekhn. Inst.*, **41**, 22(1962); through *Chem. Abstr.*, **59**, 2232*g*(1963).
(50) Straughn, J. L., and Tarpley, W. B., *U. S. At. Energy Comm.*, NYO-7935 (1959); through *Chem. Abstr.*, **54**, 19025*f*(1960).
(51) Chormonov, T. Kh., *Sb. Nauchn. Tr. Kazakhsk. Politekhn. Inst.*, **1960**, 271; through *Chem. Abstr.*, **60**, 12727*b* (1964).
(52) VanHook, A., and MacInnes, M. B., *Sugar J.*, **16**, 20(1953).
(53) Principe, J. R., and Skauen, D. M., *J. Pharm. Sci.*, **51**, 389(1962).
(54) Cohn, R. M., and Skauen, D. M., *ibid.*, **53**, 1040 (1964).
(55) Fincher, J. H., Adams, J. G., and Beal, H. M., *ibid.*, **54**, 704(1965).
(56) Kavalyunaite, V., *Primenenie Ul'traakustiki k Issled. Veshchestva*, **13**, 171(1961); through *Chem. Abstr.*, **60**, 7520*f* (1964).
(57) Berlaga, R. Ya., *Zh. Eksperim. i Teor. Fiz.*, **9**, 11 (1939); through *Reference 182*, p. 14.
(58) Berlaga, R. Ya., *Zh. Eksperim. i Teor. Fiz.*, **16**, 7(1946); through *Reference 182*.
(59) Kudryavtsev, B. B., "Ultrasonic Applications in Industry," p. 34; through *Reference 184*.
(60) Polotskii, I. G., and Levin, G. I., *Kristallografiya*, **7**, 645(1962); through *Chem. Abstr.*, **57**, 14505*d*(1962).
(61) Sokolov, S. Ya., *Acta Physiochim. USSR*, **3**, 939 (1939); through *Reference 175*, p. 213.
(62) Kortnev, A. V., and Protopopov, R. V., *Novye Fiz. Metody Obrabotki Pishchevykh Produktov, Kiev, Sb.*, **1963**, 267; through *Chem. Abstr.*, **63**, 5014*g*(1965).
(63) *Reference 182*, p. 24.
(64) Teumin, I. I., "Influence of Elastic Vibrations on the Crystallization and Technical Properties of Alloys," p. 83; through *Reference 184*.
(65) *Reference 175*, p. 213.
(66) Meleshko, L. O., *Inzh. Fiz.-Zhur., Akad. Nauk Belorussk. S.S.R.*, **4**, 123(1961); through *Chem. Abstr.*, **56**, 9512*i*(1962).
(67) *Reference 182*, p. 41.
(68) *Ibid.*, p. 23.
(69) Sokolov, S. Ya., *Tech. Phys. U.S.S.R.*, **3**, 176(1936); through *Reference 182*, p. 14.
(70) Kapustin, A. P., and Kovalyunaite, V., *Kristallografiya*, **7**, 613(1962); through *Chem. Abstr.*, **57**, 15900*i* (1962).
(71) Polotskii, I. G., and Levin, G. I., *Sb. Nauchn. Rabot Inst. Metallofiz., Akad. Nauk Ukr. S.S.R.*, **1961**, No. 13, 176; through *Chem. Abstr.*, **57**, 1579*b*(1962).
(72) Polotskii, I. G., and Levin, G. I., *Kristallizatsiya i Fazovye Perekhody, Otd. Fiz. Tverd. Tela i Poloprov, Akad. Nauk Belorussk. S.S.R.*, **1962**, 372; through *Chem. Abstr.*, **58**, 9684*a*(1963).

(73) *Reference 185*, p. 207.
(74) Nickus, Ip., and Seilius, A., *Lietuvos TSR Aukstuju Mokyklu Mokslo Darbai, Chem. ir. Chem. Tech.*, **6**, 107(1965); through *Chem. Abstr.*, **63**, 17225*f*(1965).
(75) Kapustin, A. P., *Primenenie Ul'traakustiki k Issled. Veshchestva*, **1957**, 65; through *Chem. Abstr.*, **54**, 23568*e* (1960).
(76) *Reference 182*, p. 39.
(77) *Reference 178*, p. 209.
(78) Mazhul, M. M., "Cavitation Phenomena and the Formation of Crystal Nuclei," Dissertation, 1954; through *Reference 182*, p. 14.
(79) Glinskii, A. A., *Primenenie Ul'traakustiki k Issled. Veshchestva*, **20**, 3(1964); through *Chem. Abstr.*, **64**, 8999*e* (1966).
(80) Fincher, J. H., Ph. D. Dissertation, The Graduate School, University of Connecticut, Storrs, Conn., 1964, p. 48.
(81) VanHook, A., Radle, W. F., Bujake, J. E., and Casazza, J. J., *Proc. Am. Soc. Sugar Beet Tech.*, **9**, 590(1957).
(82) Hem, S. L., Skauen, D. M., and Beal, H. M., *J. Pharm. Sci.*, **56**, 229(1967).
(83) Sollner, K., *Trans. Faraday Soc.*, **34**, 1170(1938).
(84) Freundlich, H., Rogowski, F., and Sollner, K., *Kolloid-Beihefte*, **37**, 223(1933); through *Chem. Abstr.*, **27**, 3379(1933).
(85) Freundlich, H., and Sollner, K., *Trans. Faraday Soc.*, **32**, 966(1936); through *Chem. Abstr.*, **30**, 5856(1936).
(86) Srivastava, A. M., *Proc. Natl. Acad. Sci., India*, **18A**, 51(1949); through *Chem. Abstr.*, **45**, 8853*e*(1951).
(87) Ramaszeder, K., *Textil-Praxis*, **17**, 1231(1962); through *Chem. Abstr.*, **59**, 12968*b*(1963).
(88) Ramaszeder, K., *Magy. Textiltech.*, **14**, 403(1962); through *Chem. Abstr.*, **58**, 11512*e*(1963).
(89) Zezyulinski, V. M., and Tumanski, S. S., *J. Phys. Chem. (USSR)*, **11**, 801(1938); through *Chem. Abstr.*, **33**, 6116(1939).
(90) Tumanski, S. S., and Maksimova, Ch., *Colloid J. (USSR)*, **5**, 517(1939); through *Chem. Abstr.*, **34**, 922(1940).
(91) Tumanski, S. S., *Colloid J. (USSR)*, **6**, 603(1940); through *Chem. Abstr.*, **35**, 7791(1941).
(92) Fischer, E. K., "Colloidal Dispersions," John Wiley & Sons, Inc., New York, N. Y., 1950, p. 265.
(93) Lifshits, A. G., *Nauchn. Issled. Tr., Tsentr. Nauchn.-Issled. Inst. Khlopchatobumazhn. Prom.*, **2**, 43(1959); through *Chem. Abstr.*, **59**, 3334*f*(1963).
(94) Prakash, S., and Prakash, S., *J. Acoust. Soc. Am.*, **32**, 128(1960); through *Chem. Abstr.*, **60**, 13919*h*(1964).
(95) Prakash, S., and Prakash, S., *Z. Physik. Chem. (Leipzig)*, **221**, 145(1962); through *Chem. Abstr.*, **58**, 3918*e* (1963).
(96) Prakash, S., and Prakash, S., *Kolloid. Z.*, **188**, 59 (1963); through *Chem. Abstr.*, **58**, 11974*g*(1963).
(97) Prakash, S., and Prakash, S., *Kolloid Z.*, **187**, 53 (1963); through *Chem. Abstr.*, **59**, 1115*d*(1963).
(98) Chambers, L. A., Harris, T. N., Schumann, F., and Ferguson, L. K., *J. Am. Med. Assoc.*, **119**, 324(1942).
(99) Gartner, W., *Akust. Beih.*, **1953**, 124; through *Chem. Abstr.*, **47**, 11895*e*(1953).
(100) Misek, B., and Skauen, D. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 32(1958).
(101) *Ibid.*, **47**, 36(1958).
(102) Araujo, O. E., and Belcastro, P. F., *ibid.*, **47**, 309 (1958).
(103) Kosolapov, A. T., *Primenenie Ul'traakustiki k Issled. Veshchestva*, **1958**, 143; through *Chem. Abstr.*, **53**, 19507*c* (1959).
(104) Sheikh, M. A., Prince, J. C., and Gerraughty, R. J., *J. Pharm. Sci.*, **55**, 1048(1966).
(105) Thompson, D., and Vilbrandt, F. C., *Ind. Eng. Chem.*, **46**, 1172(1954).
(106) Kruglitskii, N. N., Simurov, V. V., Ovcharenko, F. D., and Nichiporenko, S. P., *Dokl. Akad. Nauk S.S.S.R.*, **159**, 1367(1964); through *Chem. Abstr.*, **62**, 9839*b*(1965).
(107) Kruglitskii, N. N., Simurov, V. V., Ovcharenko, F. D., and Nichiporenko, S. P., *Ukr. Khim. Zh.*, **31**, 669 (1965); through *Chem. Abstr.*, **63**, 12353*f*(1965).
(108) Prudhomme, R. O., and Graber, P., *J. Chim. Phys.*, **46**, 667(1949); through *Chem. Abstr.*, **44**, 7623*a*(1950).
(109) Prudhomme, R. O., and Graber, P., *J. Chim. Phys.*, **47**, 795(1950); through *Chem. Abstr.*, **45**, 6901*h*(1951).
(110) Weissler, A., *J. Appl. Phys.*, **21**, 171(1950); through *Chem. Abstr.*, **44**, 7150*g*(1950).
(111) Jellinek, H. H. G., and White, G., *J. Polymer Sci.*, **6**, 745(1951); through *Chem. Abstr.*, **45**, 6901*i*(1951).
(112) Head, W. F., and Lauter, W. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 617(1957).
(113) Okuyama, M., and Hirose, T., *J. Appl. Polymer Sci.*, **7**, 591(1963); through *Chem. Abstr.*, **58**, 12699*h*(1963).
(114) Zorina, O. M., and El'piner, I. E., *Biokhimiya*, **28**, 781(1963); through *Chem. Abstr.*, **60**, 4379*a*(1964).
(115) El'piner, I. E., and Pyshkina, N. I., *Vysokomolekul. Soedin.*, **2**, 243(1960); through *Chem. Abstr.*, **54**, 20433*d* (1960).
(116) Kukoz, L. A., *Tr. Novocherk. Politekhn. Inst.*, **133**, 129(1962); through *Chem. Abstr.*, **59**, 8144*e*(1963).
(117) Vaisman, G. A., Gurevich, M. I., and Skvirskaya, E. S., *Aptechnoe Delo*, **10**, 11(1961); through *Chem. Abstr.*, **56**, 10294*b*(1962).
(118) Maffei, F., *Olearia*, **7**, 79(1953); through *Chem. Abstr.*, **47**, 12842*d*(1953).
(119) Faust, R. E., and Cwalina, G. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 234(1955).
(120) El'piner, I. E., and Zorina, O. M., *Dokl. Akad. Nauk S.S.S.R.*, **134**, 1472(1960); through *Chem. Abstr.*, **55**, 8494*g* (1961).
(121) Miyagawa, I., *J. Soc. Org. Syn. Chem. (Japan)*, **7**, 167(1949); through *Chem. Abstr.*, **47**, 4831*e*(1953).
(122) Mario, E., and Gerraughty, R. J., *J. Pharm. Sci.*, **54**, 321(1965).
(123) Fenn, G. D., and Belcastro, P. F., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 105(1960).
(124) El'piner, I. E., and Surova, M. D., *Dokl. Akad. Nauk S.S.S.R.*, **90**, 1083(1953); through *Chem. Abstr.*, **48**, 7951*h*(1954).
(125) Buchnea, D., *Z. Zuckerind.*, **11**, 197(1961); through *Chem. Abstr.*, **55**, 24061*e*(1961).
(126) Zapior, B., and Litynska, A., *Zesz, Nauk. Uniw. Jagielon., Pr. Chem.*, **10**, 151(1965); through *Chem. Abstr.*, **66**, 30200(1967).
(127) El'piner, I. E., and Stekol'nikov, L. I., *Dokl. Akad. Nauk S.S.S.R.*, **146**, 700(1962); through *Chem. Abstr.*, **58**, 7097*h*(1963).
(128) El'piner, I. E., and Stekol'nikov, L. I., *Dokl. Akad. Nauk S.S.S.R.*, **146**, 929(1962); through *Chem. Abstr.*, **58**, 7098*b*(1963).
(129) El'piner, I. E., and Stekol'nikov, L. I., *Biokhimiya*, **28**, 501(1963); through *Chem. Abstr.*, **59**, 7818*b*(1963).
(130) El'piner, I. E., and Stekol'nikov, L. I., *Dokl. Akad. Nauk S.S.S.R.*, **153**, 710(1963); through *Chem. Abstr.*, **60**, 8297*h*(1964).
(131) El'piner, I. E., and Sutokskaya, I. V., *Dokl. Akad. Nauk S.S.S.R.*, **157**, 729(1964); through *Chem. Abstr.*, **61**, 9908*h*(1964).
(132) Gerraughty, R. J., and Jannke, P. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 350(1960).
(133) Akiya, S., and Okui, S., *J. Pharm. Soc. Japan*, **67**, 232(1947); through *Chem. Abstr.*, **45**, 6447*i*(1951).
(134) Grabar, P., and Prudhomme, R. C., *Compt. Rend.*, **226**, 1821(1948); through *Chem. Abstr.*, **42**, 7142*b*(1948).
(135) Mellaerts, J. F., *Tech. eau (Brussels)*, **4**, 15(1950); through *Chem. Abstr.*, **47**, 8444*e*(1953).
(136) Bhar, J. N., *J. Sci. Ind. Res. (India)*, **1**, 106(1943); through *Chem. Abstr.*, **37**, 5910(1943).
(137) Busnel, R. G., and Picard, D., *Compt. Rend.*, **235**, 1217(1952); through *Chem. Abstr.*, **47**, 4710*i*(1953).
(138) Jankovic, A., *Acta Vet. Yugoslavia*, **7**, 81(1957); through *Chem. Abstr.*, **54**, 23659*i*(1960).
(139) Levinson, M. S., and Kovrov, B. G., *Izvest. Sibir. Otdel. Akad. Nauk S.S.S.R.*, **1960**, 67; through *Chem. Abstr.*, **55**, 13971*g*(1961).
(140) Weissler, A., *Intern. Conv. Record*, **10**, 24(1962); through *Chem. Abstr.*, **58**, 5054*c*(1963).
(141) Lur'e, Y. Y., Kandzas, P. F., and Mokina, A. A., *Zh. Fiz. Khim.*, **37**, 13(1963); through *Chem. Abstr.*, **59**, 48*c*(1963).
(142) Liu, S. P., *J. Acoust. Soc. Am.*, **38**, 817(1965).
(143) Prakash, S., Pandey, J. D., and Ichhaporia, F. M., *Indian J. Chem.*, **2**, 76(1964); through *Chem. Abstr.*, **60**, 14357*h*(1964).
(144) Clayton, R. T., *Hospitals*, **31**, 68(1957).
(145) Mamer, L. J., *Hospital Management*, **86**, 84(1958).
(146) McKay, J. H., *Scot. Med. J.*, **5**, 209(1960).
(147) Beal, H. M., and Skauen, D. M., *Am. J. Hosp. Pharm.*, **15**, 222(1958).
(148) Beal, H. M., and Skauen, D. M., *Hospital Management*, **87**, 78(1959).
(149) Palmer, H. A., and Skauen, D. M., *Am. J. Hosp. Pharm.*, **18**, 182(1961).
(150) Gligorijevic, J., Pavlovic, B., and Novcic, D., *Glasnik Hem. Drustva, Beograd*, **27**, 65(1962); through *Chem. Abstr.*, **59**, 3504*a*(1963).
(151) Witekowa, S., and Kaminski, W., *Zeszyty Nauk. Politech. Lodz., Chem.*, **12**, 55(1962); through *Chem. Abstr.*, **61**, 6429*g*(1964).
(152) Soloff, R. S., *Mod. Plastics*, **41**, 125(1964).
(153) Kuppusami, J., and Suryanarayana, C. V., *Nature*, **208**, 780(1965).
(154) Alexander, P., *Mfg. Chemist.*, **22**, 5–8, 12(1951).
(155) Baisio, M. L., *Boll Chimicofarm*, **93**, 21(1954).
(156) Curry, B., Hsi, E., Ambrose, J., and Wilcox, F., "Bibliography, Supersonics or Ultrasonics," 1926 to 1949, with supplement to 1950, Research Foundation, Okla. Agr. Mech. Coll., Stillwater, Okla., 1951.
(157) El'piner, I. E., *Usp Sovrem. i Biol.*, **25**, 161(1948).
(158) *Ibid.*, **30**, 113(1950).
(159) Graber, P., *Advances Biol. Med. Physics*, **3**, 191 (1953).
(160) Hall, J. P., *J. Pharm. Pharmacol.*, **6**, 577(1954).
(161) Horioka, M., and Tatsuhara, T., *Yakuzaigaku*, **25**, 1(1965).
(162) Komar, C. A., and Pasini, J., *U. S. Bur. Mines, Inform. Circ. No. 8146*, 1963, 31 pp.
(163) Lesser, M. A., *Drug Cosmetic Ind.*, **74**, 192(1954).
(164) Littlewood, K., *Roy. Inst. Chem. J.*, **86**, 78(1962).
(165) Marignan, R., *Prod. Pharm.*, **6**, 490(1951).
(166) Myers, J. A., and Goodman, J. E., *Pharm. J.*, **173**, 422(1954).
(167) Myer, W., *Scientia Pharm.*, **9**, 49(1938).
(168) Naimark, G., Klair, J., and Mosher, W. A., *J. Franklin Inst.*, **251**, 279(1951).
(169) Neduzhii, S. A., *Akust. Zh.*, **7**, 275(1961); through *Chem. Abstr.*, **56**, 6682*i*(1962).
(170) Oblensky, G., *Anee Biol.*, **33**, 465(1957).
(171) Prakash, S., Pandey, J. D., and Kapur, Km. S. L., *Univ. Allahabad Studies (Chem. Sect.)*, **1965**, 1.
(172) Renaud, P., *Chim. Ind.*, **94**, 241(1965).

(173) Speyer, K., *Seifen-Ole-Fette-Wachse*, **78,** 153(1952).
(174) Yeager, E., *J. Soc. Cosmetic Chemists*, **8,** 139(1957).
(175) Babikov, O. I., "Ultrasound and Its Industrial Applications," Consultants Bureau, New York, N. Y., 1960.
(176) Bergmann, L., "Der Ultraschall," Hirzel Verlag, Stuttgart, Germany, 1954.
(177) Blitz, J., "Fundamentals of Ultrasonics," Butterworths & Co., London, England, 1963.
(178) Brown, B., and Goodman, I. E., "High Intensity Ultrasonics," D. Van Nostrand and Co., Princeton, N. J., 1965.
(179) Dogo, A., "Les Ultrasons et leurs Applications," Paris Presses Universite, Paris, France, 1953.
(180) El'piner, I. E., "Ultrasound Physical, Chemical, and Biological Effects," Consultants Bureau, New York, N. Y., 1964.
(181) Goldman, R. G., "Ultrasonic Technology," Reinhold Publishing Corp., New York, N. Y., 1962.
(182) Kapustiu, A. P., "The Effects of Ultrasound on the Kinetics of Crystallization," Consultants Bureau, New York, N. Y., 1963.
(183) Nosov, V. A., "Ultrasonics in the Chemical Industry," Consultants Bureau, New York, N. Y., 1965.
(184) Nozdreva, V. F., "Ultrasound in Industrial Processing and Control," Consultants Bureau, New York, N. Y., 1964.
(185) VanHook, A., "Crystallization Theory and Practice," Reinhold Publishing Corp., New York, N. Y., 1961.

Research Articles

Anti-Inflammatory Evaluation of Cryogenine

By HARVEY R. KAPLAN*, RICHARD E. WOLKE†, and MARVIN H. MALONE

Cryogenine was effective in limiting the development of artificially induced inflammatory responses in acute and chronic experiments in rats. Cryogenine altered the acute edematous reaction to plantar injection of carrageenin and inhibited development of increased foot thickness induced by plantar injection of nonviable mycobacterial adjuvant in chronic studies. Histopathologic examinations did not suggest actions that would interfere with the anti-inflammatory evaluations. Cryogenine demonstrated a low order of analgesic (rat tail flick) and antipyretic (peptone fever) activity, and was capable of partially reversing edema and pain produced by silver nitrate injections in the ankle joints of rats. Cryogenine blocked serotonin, bradykinin, and histamine responses in guinea pig ileum; and pretreatment limited serotonin-induced pedal edema in rats and blocked histamine-induced intradermal wheals in the rabbit. Cryogenine was ineffective as a fibrinolytic agent. Prototype nonsteroidal anti-inflammatory compounds as well as agents with structural similarities to cryogenine were also investigated. Cryogenine may, like aspirin, manifest its anti-inflammatory activity through a combination of selective central and nonspecific peripheral mechanisms.

CRYOGENINE (vertine) was first isolated from *Heimia salicifolia* Link and Otto in 1963 by Blomster *et al.* (1), and its chemical structure has been proposed (2) and confirmed (3). The folklore concerning this Mexican plant is interesting and includes mention of its abilities to induce bizarre central nervous system reactions (4–6). The initial pharmacologic studies of Robichaud *et al.* (7) on both plant extracts and purified cryogenine alkaloid revealed qualitative and quantitative similarities between the two with the most interesting activity being the production of a selective central nervous system depression in unanesthetized animals. The general pharmacodynamics of cryogenine and other of the naturally occurring alkaloids have been reported in the literature (8, 9). Preliminary evaluation in this laboratory also indicated that cryogenine possessed activity in an acute anti-inflammatory screen in rats. These initial data suggested that cryogenine could be a new chemical prototype with nonsteroidal anti-inflammatory activity. Most recently Jiu has reported (10) that ethanol extractives from the plant showed depressant central nervous system activity, anti-atherogenic potential, and anti-inflammatory ability.

Received April 14, 1967, from the Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs, CT 06268
Accepted for publication July 26, 1967.
Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Cryogenine base was supplied by the Division of Pharmacognosy, University of Connecticut, while carrageenin (Sea-Kem type I) was courtesy of Seaplant Chemical Corp., New Bedford, Mass., and synthetic bradykinin (BRS-640) from Sandoz Pharmaceuticals, Hanover, N. J. The pharmacological and histopathological studies were partially supported by grants AM-11861 and TIGM 985-05, respectively, from the U. S. Public Health Service, Bethesda, Md.
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EXPERIMENTAL

General—During the investigations cryogenine was employed either as an aqueous suspension of

(173) Speyer, K., *Seifen-Ole-Fette-Wachse*, **78,** 153(1952).
(174) Yeager, E., *J. Soc. Cosmetic Chemists*, **8,** 139(1957).
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(176) Bergmann, L., "Der Ultraschall," Hirzel Verlag, Stuttgart, Germany, 1954.
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EXPERIMENTAL

General—During the investigations cryogenine was employed either as an aqueous suspension of

free base in 0.25% agar or as a solution of the acetate salt prepared by dissolving the base in 0.5% v/v of glacial acetic acid. As is often the case with the pharmacologic evaluation of natural products, the relatively limited availability of test alkaloid restricted extensive replication of the experimental procedures. The young adult albino rats used in this study were of the Wistar strain and obtained from the E. G. Steinhilber Co., Oshkosh, Wis. Unless otherwise specified here, the animals were allowed continuous access to Purina laboratory chow and tap water.

Acute Anti-Inflammatory Evaluation Using Carrageenin-Induced Edema—Nonfasted male and female rats were employed and the experimental procedures were similar to those described by Winter *et al.* (11). Test drugs were administered orally (10 ml./Kg.) in order to minimize counter-irritation phenomena often seen after parenteral injection. To insure a uniform degree of hydration among the individual rats, each animal received a supplementary amount of water so that the total volume administered to each rat was 5.0 ml. One hour following the oral administration, 0.1 ml. of the phlogistic agent (1% suspension of carrageenin in sterile 0.9% saline) was injected aseptically into the plantar aponeurosis of the left hind paw using a 27 gauge 0.5 in. needle attached to a 0.25 ml. capacity syringe. The width or volume of the injected paws was determined immediately after the carrageenin injection and 3 hr. later. For width determinations, a vernier caliper was used with medial foot thickness measured to the nearest 0.1 mm. When volume measurements were obtained, a modified plethysmographic apparatus similar to that described by Harris and Spencer (12) was employed by which foot volume was determined to the nearest 0.01 ml. Rectal temperatures were recorded throughout the test period using a precalibrated thermistor-type thermometer.

Assay of Cryogenine Versus Phenylbutazone—Plethysmographic measurements were made following the administration of 0.05 ml. of 1% carrageenin. A randomized complete block experimental design was utilized with a low (50 mg./Kg.) and a high (100 mg./Kg.) level for both cryogenine acetate and sodium phenylbutazone. Each treatment consisted of three experimental units per day with the assay conducted on 3 separate days resulting in a final number of 9 units for each of the four treatments. On any one day, the rats were equally but randomly assigned among the four test treatments. The statistical evaluation was patterned after the methods of Bliss and Calhoun (13).

Multiple Drug Comparisons—The plethysmographic technique was employed with 0.05 ml. of 1% carrageenin injected into the paw as the phlogistic substance. Young unfasted adult rats (2 male and 2 female) were assigned to the treatment groups in a random fashion. Drugs were administered orally as suspensions in 0.25% agar. The treatment effects were analyzed employing the multiple range test of Duncan (14).

Chronic Anti-Inflammatory Evaluation—Nonfasted male rats were randomly caged in groups of 5 and allowed to equilibrate with their environment for 1 week prior to experimentation. Daily determinations of body weight, medial foot thickness (caliper method), and average food and water consumption were made. Commencing on day 0 animals were dosed daily for 2 weeks (day 0 through 13) with the test agents. These drugs were administered orally as suspensions in 0.25% agar (10 ml./Kg.). On day 1 all animals received an injection of a suspension of *Mycobacterium butyricum* (Difco Laboratories 0640-33) in light liquid petrolatum prepared according to the method of Nuss (15). Twenty milliliters of the petrolatum was added to 100 mg. of the desiccated organisms in a Kontes hand homogenizer. After fine subdivision the resultant suspension was autoclaved at 15 lb. pressure for 20 min., allowed to cool, and placed into a refrigerator overnight. Prior to injection the suspension was allowed to equilibrate to room temperature and resuspended by shaking by hand. Each rat was injected with 0.05 ml. into the right plantar aponeurosis using a 27 gauge 0.5 in. needle directed medially following its insertion under the skin in order to avoid back seepage and to minimize local vascular involvement. Medication was discontinued on days 14 through 30; however, body weight, foot thickness, and food and water consumption measurements were continued throughout the duration of the experiment. The percentage inhibition of increased foot thickness for the injected foot was calculated according to the methods of Newbould (16) on days 13 and 30. Gross skeletal specimens of the "arthritic" paws were prepared on day 40 using the antiformin method (17).

Tissue taken for histopathologic examination included sagittal sections of both femorotibial articulations and transverse sections of metatarsal areas, myocardium, liver, spleen, lung, kidney, and abdominal skin. Following euthanasia, the tissue was fixed in 10% formalin. Osseous sections were decalcified by means of a thermo-agitating bone decalcifier employing an 18% solution of formic and hydrochloric acids. After decalcification, sections were placed into a saturated solution of lithium carbonate for a period of 24 hr., rinsed, and replaced in formalin. Tissue blocks were embedded in paraffin, sectioned at 10 μ, and stained with hematoxylin and eosin.

Antipyretic Evaluation Using Peptone-Induced Fever—Male rats were acclimatized overnight in a temperature controlled room (23°). Control rectal temperatures of the animals were recorded using a thermistor-type thermometer with the flexible probe inserted to a depth of 5 cm. and held in position for 35 sec. The rats were rendered febrile by the injection of 0.6 ml. of a 5% aqueous solution of peptone tryptic digest of casein (N-Z amine type E tryptic digest, Sheffield Chemical Co. 02-537) which had been previously filtered clear and incubated in a Dubnof shaker at 37.5° for 12 hr. The site of the subcutaneous injection was in the dorsal region of the rat's neck. Four hours after injection of the pyrogen, core temperatures were recorded again. Any animal showing a temperature elevation of less than 1° was not considered adequately febile and was discarded from the test. At this time 5 rats were assigned randomly to each of the treatment groups and the drugs administered orally. At +1 and 2 hr. following drug administration, the rectal temperatures were determined. During the experiment food was withheld, although water was freely accessible.

Analgesic Evaluation—A modified conduction

dolorimeter (Metro Scientific, Inc. ME-5410) was utilized for measuring the rat tail flick response to a standard thermal stimulus. Only male rats possessing healthy nonkeratinized tails were employed. In order to record accurately the reaction times to the thermal stimuli, a photocell and an electric timer were adapted to the instrument so that as the rat's tail was placed over the photocell opening, a circuit was closed activating both the heater and the electric timer. Subsequent removal of the tail opened the circuit and stopped the timer. Control reaction times for each animal were determined just prior to drug administration and at +15, 30, 45, 60, and 120 min. after injection. The per cent analgesia was calculated by considering a reaction time of 20 sec. minus the control reaction time as the equivalent of 100% analgesia. Exposure to the thermal stimulus for longer than 20 sec. is generally regarded as undesirable since irreversible damage to the sensory receptors in the tail may occur.

Simultaneous Analgesic and Anti-Inflammatory Testing—The method employed was a modification of the technique initially developed by LaBelle and Tislow (18). Freshly prepared aqueous 1% silver nitrate was injected (0.2 ml.) into the right paw between the Achilles tendon, ankle, and heel bones *via* the plantar surface of the paw using a 27 gauge needle. This technique allows only insignificant amounts of silver nitrate to penetrate the foot areas subsequently measured for edema. It has been suggested that a local release of endogenous mediators acting in a humoral manner are responsible for the inflammation (19). Eighteen hours after injection of silver nitrate, the animals' reaction to pain in the injected paw was evaluated. This required the abrupt manual flexion of the ankle joint with scoring of the vocalization (squeal) and struggling using an arbitrary scale of 0 to +3 (0 = no squealing and struggling, +1 = audible squeals but no struggling, +2 = definite squealing and some struggling, +3 = loud squeals and violent struggling). Any animal either failing to produce a pain response of +2 or 3 or not showing a measurable edema (caliper technique) was eliminated from the experiment. Rats meeting criteria were subdivided randomly into the treatment groups and the drugs then administered orally as suspensions. Animals were scored for pain responses at hourly intervals for 5 hr. and at the end of this time foot widths were determined again. At the time of the silver nitrate injection, the animals were taken off food and at the time of the drug administration taken off water for the balance of the experiment.

Antiphlogistic Evaluation Using a Histamine-Induced Intradermal Wheal—Unanesthetized albino rabbits of either sex obtained from local suppliers were securely fastened to rabbit boards in a supine position. Abdominal hair was carefully removed 2 hr. prior to the experiment using electric clippers. Each shaved abdomen was then marked with 8–10 circular regions (approximately 30 mm. in diameter) using a felt marking pen. The test drugs were injected intraperitoneally 30 min. prior to intradermal injection of 0.3 ml. of 0.02 mg./ml. (as base) of histamine diphosphate into the center of each of the marked regions. Fifteen minutes after the intradermal injections, trypan blue was administered intravenously (1 ml./Kg. of a 1% aqueous solution) and the colors of the histamine-induced wheals rated at +30, 60, and 180 min. later using the scoring scale of Hoppe *et al.* (20).

Serotonin-, Bradykinin-, and Histamine-Induced Pedal Edema—Serotonin creatinine sulfate (0.01 mg. as the base), synthetic bradykinin (0.05 mg.), or histamine diphosphate (0.15 mg. as base) contained in an injection volume of 0.05 ml. were administered into the plantar aponeurosis of rats. Inflammation was measured (caliper technique) at 30-min. intervals for 180 min. following injection of the phlogiston. Cryogenine base was administered orally as a suspension 1 hr. prior to the pedal injections.

Antagonism Studies on Isolated Guinea Pig Ileum—Nonfasted virgin female guinea pigs obtained from local suppliers were sacrificed by cranio-vertebral dislocation and 2–3 cm. segments of ileum immediately excised and placed into calibrated 50 ml. baths containing fresh Tyrode's solution (37.5°) constantly oxygenated with a 95% O_2 and 5% CO_2 gas mixture. Isotonic frontal recording levers were used to record longitudinal contractions of the tissues. Bath concentrations of 1×10^{-6} *M* for serotonin (creatinine sulfate), histamine (diphosphate), and synthetic bradykinin were used to induce reproducible submaximal contractions of the tissue. After thoroughly washing out the agonists, various log molar concentrations of cryogenine (acetate) were incubated (1×10^{-9} to 1×10^{-7} *M*) with the ileum for 5 min. prior to addition of the agonists in order to ascertain its effects on the reference contractions. A fresh individual ileum segment was used to study the effects of cryogenine in regard to each agonist.

Fibrinolytic Evaluation—The method of von Kaulla (21) was employed with the reference clots formed from human plasma by the addition of 0.25 ml. of 5% calcium chloride to each 5.0 ml. of plasma used. The preformed clots were suspended in various molar concentrations of the drugs studied and then incubated at 37° for 24 hr. Evaluation of fibrinolytic activity was made at the end of this time and expressed as per cent lysis of clot. Any cloudiness of the solution, shrinking of the clot, or sediment formation was also noted.

RESULTS

Acute Anti-Inflammatory Evaluation Using Carrageenin-Induced Edema—The results as summarized in Table I show cryogenine to possess significant antiphlogistic activity at an oral dosage of 100 mg./Kg. when compared to the acetic acid vehicle

TABLE I—ANTI-INFLAMMATORY ACTIVITY OF 100 mg./Kg. OF CRYOGENINE ORALLY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Mean % Increase Paw Width	Paw Vol.	Level of Significance Observed *P*
Cryogenine	13.4 (2–24)[a]	...	0.01–0.05
Acetic acid control	26.8 (18–36)[b]	...	...
Cryogenine	...	27 (2–61)[b]	<0.001
Acetic acid control	...	118 (51–197)[b]	...

[a] Observed range of 5 observations 3 hr. after carrageenin injection. [b] Observed range of 6 observations 3 hr. after carrageenin injection.

controls. The inflammatory state induced by carrageenin did not elevate core temperature and treatment with either cryogenine alkaloid or the vehicle control did not cause significant changes in rectal temperature.

Assay of Cryogenine Versus Phenylbutazone—Calculation of the 2 × 2 assay revealed that cryogenine has 0.86 times the anti-inflammatory potency of phenylbutazone in this acute test procedure. The 95% confidence limits for the potency are 0.61–1.11. The λ of the assay (calculated as either s/b or as $s_M\sqrt{N/2}$) equaled 0.22. Cryogenine and phenylbutazone may be considered equipotent orally since there was no significant difference between drugs (P = 0.25–0.50) and no significant departure from parallelism (P = >0.50).

Multiple Drug Comparisons—The results of this one level experiment as treated by Duncan's multiple range test (14) are summarized in Table II

TABLE II—MULTIPLE DRUG COMPARISIONS OF ANTI-INFLAMMATORY ACTIVITY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Oral Dosage, mg./Kg.	Mean Increase in Paw Vol., ml.	Significant Subsets[a]
Control	...	0.45(0.37–0.56)[b]	A
Cinnamic acid	100	0.44(0.32–0.66)	A
Control	...	0.44(0.39–0.54)	A
Papain	400	0.35(0.29–0.40)	B
Sparteine	100	0.34(0.30–0.38)	B
Cryogenine	100	0.27(0.19–0.36)	B
Indomethacin	5	0.22(0.18–0.31)	B
Chloropromazine	100	0.21(0.05–0.33)	B

[a] The treatment effects represented by A and B differ significantly from each other (P = 0.05); however, the treatments within these subsets do not differ significantly (P = 0.05) from one another. [b] The figures within the parentheses represent the observed range of the 4 experimental values.

and show a division of the various treatment effects into two major subsets of means. The anti-inflammatory properties of cryogenine were confirmed. Although both cinnamic acid and sparteine have some structural chemical relationships to cryogenine, only sparteine could be considered as having some potential for anti-inflammatory activity. It should be noted here that in the two previous experiments cryogenine was administered as the soluble acetate salt, whereas in this experiment the base itself was administered orally as a suspension.

Chronic Anti-Inflammatory Evaluation—The effects of cryogenine and phenylbutazone therapy on the progression of the artificially-induced, chronic "arthritic" state in rats is presented graphically in Fig. 1 and 2. Table III also summarizes the results for several other selected reference compounds tested concurrently by this procedure. These compounds can categorically be classified as either well documented reference anti-inflammatory agents (phenylbutazone, indomethacin, DMSO, chloroquine, hydrocortisone), structurally related moieties of cryogenine (cytisine, sparteine, cinnamic acid), or pharmacologically related agents (chlorpromazine, papaverine, atropine) (7–9). The dosages employed are either those suggested in the literature or previously determined in these laboratories to be effective and apparently nontoxic for the rat after acute administration. The protection afforded by cryogenine and by phenylbutazone when compared with a nontreated control is very apparent in Fig. 3 which pictures representative gross specimens 40 days following the *M. butyricum* adjuvant administration. Secondary lesions in other tissues similar to those described in the literature by Newbould (16) were observed; however, the variability of lesions as to frequency, size, and site did not allow them to be used as a test metameter.

Two of the three control animals selected for histopathologic evaluation had no lesions of the

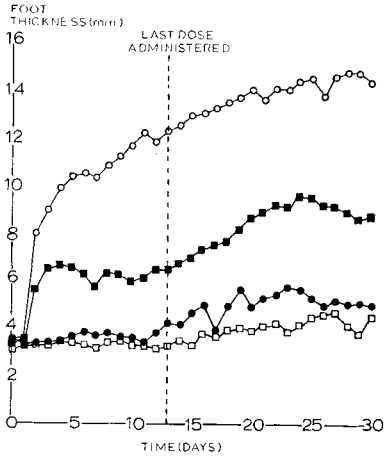

Fig. 1—Effect of 2 weeks of daily administration of 100 mg./Kg. of cryogenine orally on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); ■, mean width of injected foot (cryogenine);. □, mean width of contralateral foot (cryogenine).

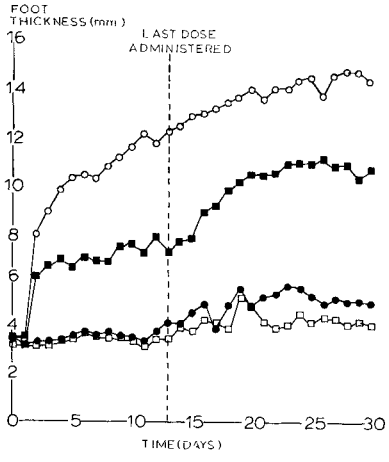

Fig. 2—Effect of 2 weeks of daily administration of 100 mg./Kg. of phenylbutazone orally on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); ■, mean width of injected foot (phenylbutazone); □, mean width of contralateral foot (phenylbutazone).

TABLE III—ANTI-INFLAMMATORY EVALUATION UTILIZING ADJUVANT-INDUCED CHRONIC POLYARTHRITIS IN RATS

Treatment	Daily Oral Dosage, mg./Kg.[a]	Quantal Survival Day 13	Quantal Survival Day 30	Inhibition of Foot Thickness[b] Day 13	Inhibition of Foot Thickness[b] Day 30	Mean Body Wt., Gm. Day 0	Mean Change in Body Wt., Gm. Day 13	Mean Change in Body Wt., Gm. Day 30	Mean Consumption: Food, Gm./Water, ml. Day 0	Mean Consumption: Food, Gm./Water, ml. Day 13	Mean Consumption: Food, Gm./Water, ml. Day 30
Cryogenine	100	5/5	5/5	63	55	166	+26	+71	14/24	17/27	16/30
Phenylbutazone	100	5/5	5/5	58	41	201	+32	+65	18/33	20/33	17/32
Indomethacin	0.5	5/5	5/5	50	30	190	+32	+74	15/23	20/24	18/35
Dimethylsulfoxide	...[c]	5/5	5/5	20	28	236	+22	+50	18/27	20/29	21/34
Chloroquine	100	4/5	3/5	76	35	246	−70	−6	17/26	5/12	36/20
Hydrocortisone	100	5/5	5/5	66	43	249	−20	+18	18/25	16/23	18/29
Aspirin	100	5/5	5/5	33	29	216	+15	+46	20/31	15/24	16/31
Sparteine	100	5/5	4/5	16	4	242	+24	+56	23/35	17/26	16/26
Cytisine	25	5/5	5/5	42	1	213	+13	+70	22/31	20/27	17/38
Cinnamic acid	100	5/5	5/5	8	2	209	+35	+83	20/32	20/29	16/39
Papaverine	100	5/5	5/5	−6	10	166	+37	+91	...[d]	...	...
Atropine	100	4/5	4/5	41	4	162	+35	+85	...[d]	...	...
Chlorpromazine	25	4/5	4/5	15	0	150	+26	+71	...[d]	...	...
Controls	...	30/30	28/30	(10)[e]	(8)[e]	191	+27	+81	19/29	16/22	17/28

[a] Administered as suspensions in 0.25% agar from day 0 through day 13, with the mycobacterium adjuvant injected into the right foot on day 1. [b] Percentage inhibition = $100[1 - (a - x)/(b - y)]$ where y = mean thickness right feet of controls before adjuvant, b = mean thickness right feet of controls on specified day, x = mean thickness right feet of treated rats before adjuvant, and a = mean thickness right feet of treated rats on specified day. [c] The adjuvant-injected paw was soaked for 1 min. daily. [d] Values not determined quantitatively. [e] Absolute differences between the larger and smaller values.

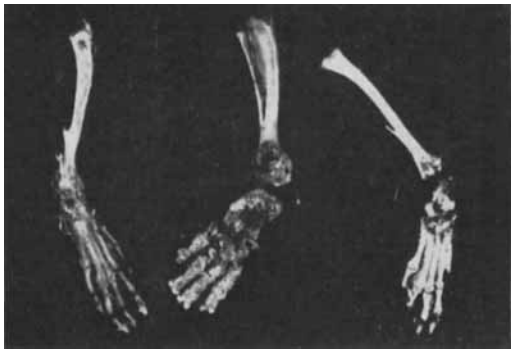

Fig. 3—Typical gross skeletal specimens of adjuvant-injected rat paws. Cryogenine-treated (left), non-treated controls (center), and phenylbutazone-treated (right).

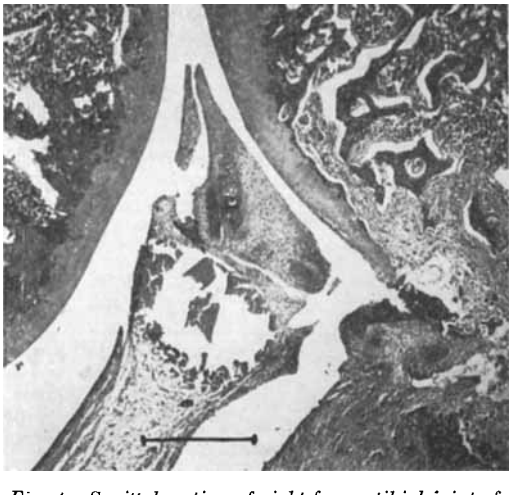

Fig. 4—Sagittal section of right femorotibial joint of a control animal with granulomatous reaction and destruction of the tibial cartilage seen at right. Marked distance = 0.5 mm.

femorotibial articulations, while all three revealed changes in the metatarsal areas. Lesions present in the most severely affected control animal were similar to those described by Glenn and Grey (22) with suppurative granulomas and fibrosis in zones of primary and secondary ossification bordering the femorotibial joint. Destruction of articular cartilage, an occasional finding, appeared secondary to the progressing subchondral granulomatous response. Within the joint proper, the synovial membrane was swollen, edematous, hyperplastic, and invaded by lymphocytes, mast cells, and mononuclear cells (Fig. 4). Progressive involvement resulted in fibrosis of the synovial membrane with an occasional pannus-like proliferation completely filling the joint cavity between the articular surfaces. Transverse sections of metatarsal areas had multiple subdermal granulomas and foci of mononuclear cells. A striking periosteal new bone proliferation was present about the periphery of all metatarsal bones. New bone was highly cellular, coarsely woven, and easily differentiated from mature cortical lamellar bone from which it was separated by a distinct basophilic cement line (Fig. 5). A decreased inflammatory response was observed in femorotibial sections of the cryogenine-treated rats (Fig. 6). Suppurative granulomatous reaction was seen in one instance in the tibial metaphysis and, with the exception of edema of the synovial membrane, pathologic changes were not present within the joint cavity. Metatarsal sections from cryogenine-treated animals revealed lesions as severe as those seen in the control rats (Fig. 7).

One rat received 100 mg./Kg. of cryogenine orally daily from day 0 through day 13 but did not receive an injection of the mycobacterium adjuvant. Examination of the soft tissues revealed no lesions of the myocardium, liver, lung, kidney, spleen, or abdominal skin.

The possibility of spurious anti-inflammatory activity can be detected by the adjuvant-induced polyarthritis procedure when chronic toxic mani-

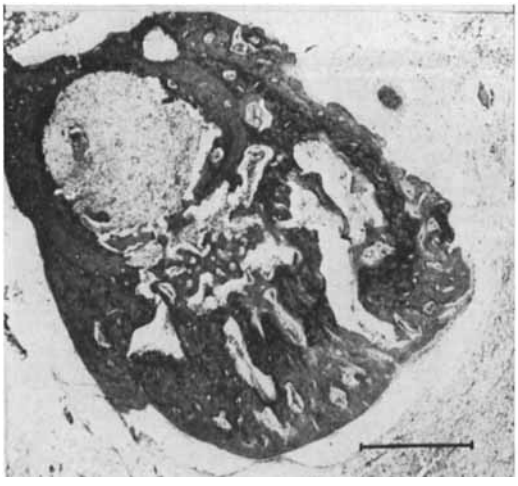

Fig. 5—Transverse section of right metatarsus of a control animal with extensive periosteal new bone proliferation. Marked distance = 0.5 mm.

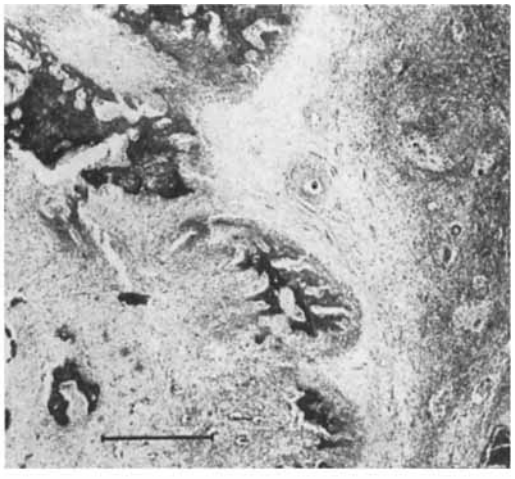

Fig. 7—Transverse section of right metatarsus of a cryogenine-treated rat with multiple granulomas and severe destruction of osseous tissue with periosteal new bone proliferation. Marked distance = 0.5 mm.

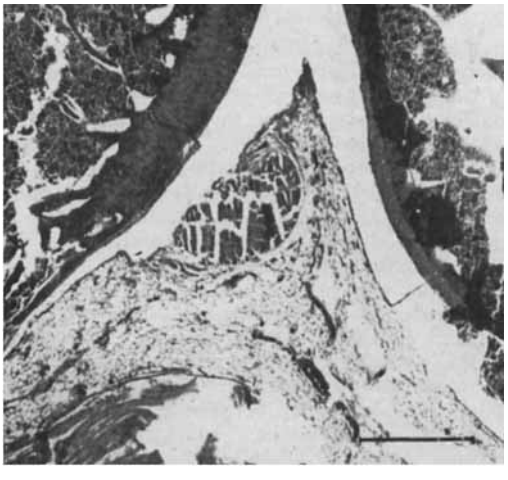

Fig. 6—Right femorotibial joint of a cryogenine-treated rat with absence of both articular cartilage destruction and inflammation of synovial membrane. Marked distance = 0.5 mm.

festations are noted. In Table III this is illustrated by decreased quantal survival rates (chloroquine, atropine, chlorpromazine), changes in the total body weight (chloroquine, hydrocortisone), reduced food and water consumption (chloroquine), and gross changes in general appearance and reactivity (chloroquine, atropine, chlorpromazine). Sparteine and cinnamic acid did not show any cryogenine-like activity. Cytisine may have some potential, although this activity appears to be short-lived and correlated with a decreased rate of body weight gain. In this experiment cryogenine, phenylbutazone, and indomethacin all appear as desirable anti-inflammatory agents, with indomethacin being considerably more potent than the other two.

Antipyretic Evaluation Using Peptone-Induced Fever—Figure 8 represents the results of the antipyretic evaluation. At +4 hr. and just prior to drug administration there was no significant difference between the respective means and variances of the three randomly assembled, experimental groups ($P > 0.50$). At +5 hr. there was no significant difference between the controls and the cryogenine-treated rats ($P = 0.25$–0.50), while there was a highly significant difference between the controls and aspirin-treated ($P = <0.001$) and the cryogenine-treated and aspirin-treated groups ($P = <0.001$). Subsequently, at +6 hr. a highly significant difference was still present between the untreated controls and the aspirin treated ($P = <0.001$) and the cryogenine-treated and aspirin-treated ($P = <0.001$). Moreover, there was a significant difference between the controls and the cryogenine-medicated animals ($P = 0.01$–0.025). Aspirin was most effective in that an oral dosage of 300 mg./Kg. almost totally abolished the pyretic response (69 and 91% reduction at 1 and 2 hr. after dosage, respectively). Cryogenine base at an oral dosage of 100 mg./Kg. was much less effective, although a 41% reduction of the peptone-induced fever was observed 2 hr. after administration.

Analgesic Evaluation—Table IV presents a summary of the analgesic evaluation using the rat tail flick technique which is considered generally to be most valid in the detection of narcotic analgesics. Cryogenine had an apparent low order of activity using this procedure; however, this may be

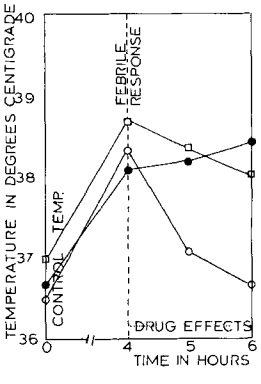

Fig. 8—Effects of orally administered cryogenine and aspirin on peptone-induced fever in the rat. Key: □, 100 mg./Kg. cryogenine; ○, 300 mg./Kg. aspirin; ●, untreated controls.

TABLE IV—ANALGESIC SCREENING OF CRYOGENINE AND REFERENCE AGENTS

Treatment	Dosage, mg./Kg.[a]	Mean % Analgesia[b] +15	+30	+45	+60	+120 min.
Control	...[c]	−2	−2	−6	−4	+2
Cryogenine	100	−10 (0)[d]	+17 (+)	+1 (±)	+3 (+)	−2 (±)
Phenylbutazone	100	+1 (±)	−2 (±)	−2 (±)	−2 (±)	−1 (±)
Morphine	10	+68 (+)	+86 (+)	+88 (+)	+80 (+)	+87 (+)

[a] Cryogenine was administered orally, continuing the mode of administration established in the earlier experiments where it was felt necessary to minimize possible counter-irritating influences of parenteral administration on the anti-inflammatory testing. However, all of the other agents and the control group were injected intraperitoneally as is usual with analgesic testing. Five rats per test group. [b] Per cent analgesia = 100 $[(R_x - R_0)/(20 - R_0)]$ where R_0 = pretreatment reaction time in seconds and R_x = post-treatment reaction time in seconds at the time specified. [c] The mean reaction times in seconds (observed range) for the 25 control animals are—preinjection: 4.58 (2.7–10.2); +15 min.: 4.46 (2.9–9.0); +30 min.; 4.41 (2.5–7.6); +45 min.: 3.94 (2.7–6.5); +60 min.: 4.22 (2.4–5.7); and +120 min.: 3.81 (2.7–6.0). [d] Key: 0, no significant analgesia in that the mean per cent analgesia is lower than −2 standard errors of controls; ±, mean per cent analgesia is included within ±2 standard errors of controls; +, significant analgesia in that the mean per cent analgesia is greater than +2 standard errors of controls.

due to its capacity to block conditioned responses (8) rather than to true analgesic ability. Without doubt cryogenine at effective anti-inflammatory dosage levels lacks morphine-like analgesic potency.

Simultaneous Analgesic and Anti-Inflammatory Testing—Both cryogenine and phenylbutazone demonstrated analgesic activity (Fig. 9), but it was significantly less both as to degree and duration than the response produced by indomethacin. None of the agents produced the degree of analgesia produced by morphine. By +5 hr. cryogenine, phenylbutazone, and indomethacin had caused reductions of the developed pedal edemas (10, 11, and 8%, respectively, 4 rats per treatment), whereas the control and morphine-treated animals demonstrated slight increases in the edematous response (0.4 and 2%, respectively). Using this technique it is quite apparent that the morphine-like narcotic analgesics can be distinguished readily from the nonsteroidal anti-inflammatory compounds which have an analgesic component.

Antiphlogistic Evaluation Using a Histamine-Induced Intradermal Wheal—Histamine diphosphate uniformly elicited the classic blue wheal in nontreated animals with the maximal score of 16 noted at all time intervals. Premedication with diphenhydramine HCl (20 mg./Kg. intraperitoneally) partially blocked the inflammatory response with mean scores of 0, 0.8, and 2.0 noted at +30, 60, and 180 min. after the histamine injection. Cryogenine (10 mg./Kg. intraperitoneally) completely blocked the action of histamine producing mean scores of 0, 0, and 0.2 at +30, 60, and 180 min., respectively. In addition, cryogenine produced a noticeable sedative action on the secured rabbit.

Serotonin-, Bradykinin-, and Histamine-Induced Pedal Edema—The progression of the edematous responses in the control and cryogenine-treated animals is illustrated in Fig. 10. The edemas produced by all of the phlogistic agents approached maximal response within 30 min. after injection. The histamine inflammation appeared much less pronounced in comparison to that produced by serotonin and synthetic bradykinin. The total areas under the respective curves were determined with the aid of a compensating polar planimeter. Cryogenine pretreatment afforded statistically significant protection ($P < 0.05$) against serotonin-induced edema. No significant protection ($P > 0.05$) was seen against bradykinin or histamine.

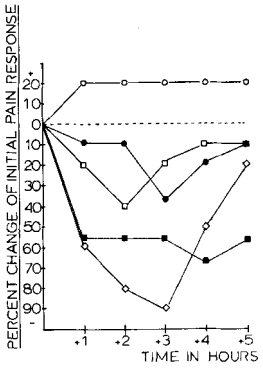

Fig. 9—Analgesic evaluation of orally administered cryogenine and other reference agents against silver nitrate-induced pain. Key: □, 100 mg./Kg. of cryogenine; ■, 100 mg./Kg. of indomethacin; ●, 100 mg./Kg. of phenylbutazone; ◇, 25 mg./Kg. of morphine; ○, untreated controls.

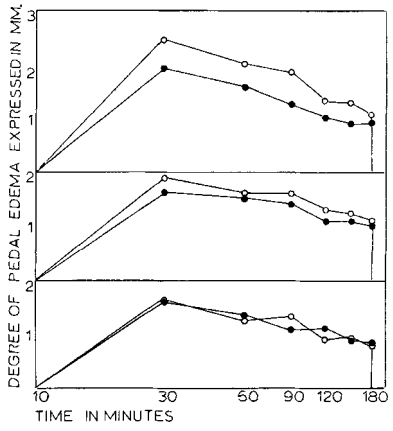

Fig. 10—Effect of oral cryogenine premedication on serotonin (top), bradykinin (middle), and histamine (bottom) induced pedal edema. Key: ●, 100 mg./Kg. of cryogenine; ○, nonmedicated controls. Five rats per each test group.

Antagonism Studies on Isolated Guinea Pig Ileum—At final bath concentrations of $10^{-7}M$, cryogenine almost totally blocked contractions induced by the reference agonists: serotonin, histamine, and bradykinin. In the cases of bradykinin and serotonin, the inhibition could not be reversed by increasing the concentration of the agonist; however, a partial reversal was possible in dealing with histamine.

Fibrinolytic Evaluation—A summary of the results is given in Table V. The various concentra-

TABLE V—FIBRINOLYTIC SCREENING OF CRYOGENINE AND REFERENCE AGENTS

	% Clot Lysis at *M* Concn.					
	0.04[a]	0.03	0.02	0.01	0.008	0.005
Phenylbutazone	10	100	>95	0	0	0
Indomethacin	10	90	...[b]	0	0	0
Cryogenine	...[b]	...[c]	...[c]	...[c]	...[c]	0
Sparteine sulfate	...[b]	...[d]	...[d]	...[d]	...[d]	...[d]
Chloroquine diphosphate	0	0	0	0	0	0

[a] Sufficient amount of the drug was weighed to produce 0.05 *M* stock solution upon dilution to 10 ml. Either NaOH or HCl was used to facilitate solution and/or adjust the final pH to 7.4. Cryogenine, phenylbutazone, and indomethacin were not totally soluble at a pH of 7.4; therefore, their saturated solutions (37°) were filtered through glass wool until clear. Appropriate dilutions were made with triple distilled water to make the final bath volume 2.5 ml. [b] Concentration was not tested. [c] No detectable lysis; some cloudiness of perfusion liquid noted. [d] No detectable lysis; some sediment formation noted.

tions of cryogenine, sparteine, and chloroquine did not produce visible lysis of the preformed clots. In the incubated test solutions of cryogenine, some degree of cloudiness was observed and in solutions of sparteine a white sediment was apparent. These phenomena may be expected considering the possible interactions between the soluble drug and the macromolecular matrix of the clot. Both phenylbutazone and indomethacin produced varying degrees of clot lysis.

DISCUSSION AND CONCLUSIONS

Cryogenine was shown to be partially effective in preventing the development of artificially induced inflammatory responses in rats using both acute (carrageenin) and chronic (*M. butyricum* adjuvant) experimental procedures. The possibility of interference due to "counter-irritation" was minimized by using the oral route of administration. In both acute and chronic evaluations, cryogenine appeared to be equipotent to phenylbutazone. Histopathological studies indicated that cryogenine may have some anti-inflammatory activity at the femorotibial joint. It is important to emphasize that the efficacy of cryogenine in both acute and chronic procedures appeared to be unrelated to toxic phenomena. After treatment(s) with cryogenine there were no indications of malaise and no deaths were recorded. The histopathological evaluation of a cryogenine-treated rat (100 mg./Kg. orally for 14 days) revealed no lesions of the organs examined.

Although cryogenine has been demonstrated to induce hypothermia in normothermic acclimatized animals upon intraperitoneal injection (7), it possessed only slight antipyretic activity in rats rendered febrile by peptone administration and then it was clearly inferior to aspirin in this respect. Cryogenine produced only equivocal analgesic activity utilizing the rat tail flick thermal technique. This low order of activity may be due to cryogenine's ability to alter conditioning phenomena. Untreated control animals usually show negative mean per cents of analgesia since they become conditioned to the heat stimuli and actually remove their tails from the area prior to actually receiving their threshold painful stimulus. Robichaud *et al.* (8) have shown cryogenine to be active after intraperitoneal administration in blocking conditioned responses in both nondiscriminated (continuous) avoidance and conditioned discrete avoidance escape situations. It is not uncommon to find anti-inflammatory, antipyretic, and analgesic activity for a single chemical entity. Rosenkilde (23) points out that the two central actions (antipyretic and analgesic) are apparently linked to a peripheral activity (anti-inflammatory). Since cryogenine did show some analgesic and antipyretic potential and was capable of partially reversing both silver nitrate-induced edema and pain responses, it may have both central and peripheral mechanisms involved in its anti-inflammatory activity.

Cryogenine inhibited responses both *in vivo* (histamine in the rabbit and serotonin in the rat) and *in vitro* (histamine, serotonin, and bradykinin in isolated guinea pig ileum) by certain suspected mediators of the inflammatory process. However, its action appeared to be relatively nonspecific since the results did not implicate any one mediator.

REFERENCES

(1) Blomster, R. N., Schwarting, A. E., and Bobbitt, J. M., *Lloydia*, **27**, 15(1964).
(2) Rother, A., Appel, H., Keily, J. M., Schwarting, A. E., and Bobbitt, J. M., *ibid.*, **28**, 90(1965).
(3) Ferris, J. P., Boyce, C. B., Briner, R. C., Douglas, B., Kirkpatrick, J. L., and Weisbach, J. A., *Tetrahedron Letters*, **30**, 3641(1966).
(4) Standley, P. C., *Contrib. U. S. Nat. Herb.*, **23**, 1025 (1924).
(5) Reko, V. A., *Pharm. Monatsh.*, **16**, 155(1935).
(6) Martinez, M., "Las Plantas Medicinales de Mexico," Editiones Botas, Mexico, 1959, p. 293.
(7) Robichaud, R. C., Malone, M. H., and Schwarting, A. E., *Arch. Intern. Pharmacodyn.*, **150**, 220(1964).
(8) Robichaud, R. C., Malone, M. H., and Kosersky, D. S., *ibid.*, **157**, 43(1965).
(9) Kaplan, H. R., and Malone, M. H., *Lloydia*, **29**, 348 (1966).
(10) Jiu, J., *ibid.*, **29**, 250(1966).
(11) Winter, C. A., Risley, E. A., and Nuss, G. W., *Proc. Soc. Exptl. Biol. Med.*, **111**, 544(1962).
(12) Harris, J. M., and Spencer, P. S. J., *J. Pharm. Pharmacol.*, **14**, 464(1962).
(13) Bliss, C. I., and Calhoun, D. W., "An Outline of Biometry," Yale Co-Operative Corp., New Haven, Conn., 1954.
(14) Duncan, D. B., *Biometrics*, **11**, 1(1955).
(15) Nuss, G. W., personal communication, 1965.
(16) Newbould, B. B., *Brit. J. Pharmacol.*, **21**, 127(1963).
(17) Farris, E. J., and Griffith, J. Q., Jr., "The Rat in Laboratory Investigation," 2nd ed., J. B. Lippincott Co., Philadelphia, Pa., 1949, p. 468.
(18) LaBelle, A., and Tislow, R., *J. Pharmacol. Exptl. Therap.*, **98**, 19(1950).
(19) Margolin, S., "International Symposium on Non-Steroidal Anti-Inflammatory Drugs," Garattini, S., and Dukes, M. N. G., eds., Excerpta Medica Foundation, Amsterdam, The Netherlands, 1964, pp. 214–217.
(20) Hoppe, J. O., Alexander, E. B., and Miller, L. C., *J. Am. Pharm. Assoc., Sci. Ed.*, **39**, 147(1950).
(21) von Kaulla, K. N., *J. Med. Chem.*, **8**, 164(1965).
(22) Glenn, E. M., and Grey, J., *Am. J. Vet. Res.*, **26**, 1180(1965).
(23) Rosenkilde, H., "Animal and Clinical Pharmacologic Techniques in Drug Evaluation," Nodine, J. H., and Siegler, P. E., eds., Year Book Medical Publishers, Inc., Chicago, Ill., 1964, pp. 492–501.

Tetracyclines I

Separation and Examination by Thin-Layer Chromatography

By PETER P. ASCIONE, JOHN B. ZAGAR, and GEORGE P. CHREKIAN

A thin-layer chromatographic (TLC) method for the examination of tetracyclines has been developed. This technique provides a rapid means for the separation and identification of tetracyclines and their derivatives in a wide variety of mixtures. Acid-washed diatomaceous earth served as the support. The support was treated with a buffer consisting of 0.1 *M* ethylenediaminetetraacetic acid disodium salt (EDTA) at pH 7.0, glycerin, and polyethylene glycol 400 (PEG 400). The developing solvent was ethyl acetate saturated with 0.1 *M* EDTA at pH 7.0. While the work reported was limited to demethylchlortetracycline (DMCTC), tetracycline (TC), and chlortetracycline (CTC), it has also been successfully used with other tetracyclines.

AT THIS TIME, a technique is needed for thin-layer chromatography for separating tetracyclines, whether in specifically prepared mixtures or in mixtures resulting from failure of the strains used to produce pure tetracylcines. Such a technique should be applicable not only to separating mixtures of tetracyclines, but also for studying their stability in pharmaceutical formulations.

The application of TLC for the separation of antibiotics was presented by Nicolaus, Coronelli, and Binaghi (1) in which several tetracyclines were examined in many solvents using various adsorption layers. Kapadia and Subba Rao (2) described a circular thin-layer separation employing sequestering agents in a silica gel layer for better resolution. Sonanini and Anker (3) studied the chromatography of tetracyclines using a support impregnated with glycerin, which maintained moisture in the support. A method for quantitation on thin-layer was reported by Simmons, Koorengevel, Kubelka, and Seers (4) using microcrystalline cellulose as a support. These TLC methods used in the separation of tetracyclines as described by the various workers (1–4) were examined in these laboratories. However, more than two tetracyclines could not be resolved by a single system on one chromatogram, and the resolution of the related compounds of each tetracycline could not be accomplished without a great deal of streaking and tailing.

This report presents the results of preliminary work in which various adsorbants, sequestering agents, PEG 400–glycerin mixture, and pH were directed toward obtaining clear separations. Satisfactory separation of the tetracyclines and their related compounds was achieved on diatomaceous earth, impregnated with EDTA at pH 7.0, glycerin–PEG 400, using ethyl acetate as the developing solvent.

Received May 22, 1967, from the Department of Analytical Development, Quality Control Section, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965

Accepted for publication August 11, 1967.

The authors thank Mrs. D. Budd, Literature Services Department, for valuable assistance with this report.

EXPERIMENTAL

Reagents—Disodium ethylenediaminetetraacetate A.C.S., glycerin A.C.S., polyethylene glycol 400 U.S.P., ammonium hydroxide A.C.S., ethyl acetate A.C.S.

Buffer—Mix 5 ml. of 20% v/v PEG 400 in glycerin with 95 ml. of 0.1 *M* EDTA previously adjusted to pH 7.0 with ammonium hydroxide.

Developing Solvent—Equilibrate 120 ml. of ethyl acetate with 20 ml. of 0.1 *M* EDTA previously adjusted to pH 7.0. Use the organic layer to develop the plates.

Preparation of Support—Wash diatomaceous earth[1] with hot 6 *N* hydrochloric acid until the washings no longer contain calcium or iron. Wash with water until a neutral pH is obtained and then dry at 105°.

Preparation of Plates—Prepare glass plates (20 × 20 cm.) according to the method of Lees and DeMuria (5). Place a double thickness of 1-cm. masking tape[2] on opposite edges of the plate. Triturate a slurry of 8 Gm. of acid-washed diatomaceous earth and 16 ml. of buffer in a glass mortar until smooth. Pour this slurry onto the plate and spread over the surface with a stout glass rod using the taped edges of the plate to control the thickness of the layer; press the glass rod against the taped edges at one end and slide to the opposite end in one motion. Air-dry the plate for 35–50 min. or until the layer surface appears whitish gray. Remove the tape from the edges of the plate and use immediately.

Preparation and Application of the Samples—Prepare a methanol solution of each of the tetracyclines to be chromatographed using a concentration of 1 mg./ml. At the same time, prepare standards of tetracyclines to be carried through the same procedure. Apply 1 μl. of each solution at 1-cm. intervals, 1.5 cm. from one edge of the plate. Inscribe a solvent-front line across the surface of the plate 12 cm. from the origin.

[1] Marketed by Desaga/Brinkmann Co. under the trademark of Kieselguhr G.

[2] Scotch brand tapes, cores No. 0300, 3M Co., St. Paul, Minn.

Development of Chromatogram—Line a rectangular glass jar (20 × 8 × 20 cm.) internal dimensions) with Whatman 3 MM chromatography paper. Pour the mobile phase into the glass jar. After the glass jar has equilibrated for 30 min., chromatograph the plates. When the mobile phase reaches the solvent-front line, remove the plate and air-dry for 2–3 min. Rechromatograph the plate for additional resolution. The plates may be developed for a third or fourth time if necessary.

After the plate has dried under ambient condition for 30 min., examine it under long wavelength ultraviolet light. The sample spots are identified by comparison with individually chromatographed tetracycline standards.

RESULTS AND DISCUSSION

The use of EDTA, glycerin, and pH buffer to improve separation in chromatographic procedures of tetracyclines has been reported (1–3). However, in this investigation these methods were unsuccessful in resolving more than two tetracyclines by a single solvent system on one chromatogram. Also, during the development of these chromatograms, tailing, streaking, and possible degradation occurred which was undesirable.

When Silica Gel G was used as a support on thin layer, the tetracyclines were strongly adsorbed and could not be eluted satisfactorily. On diatomaceous earth, the tetracycline could be developed with various solvent systems, but with pronounced tailing and little resolution of the various tetracyclines.

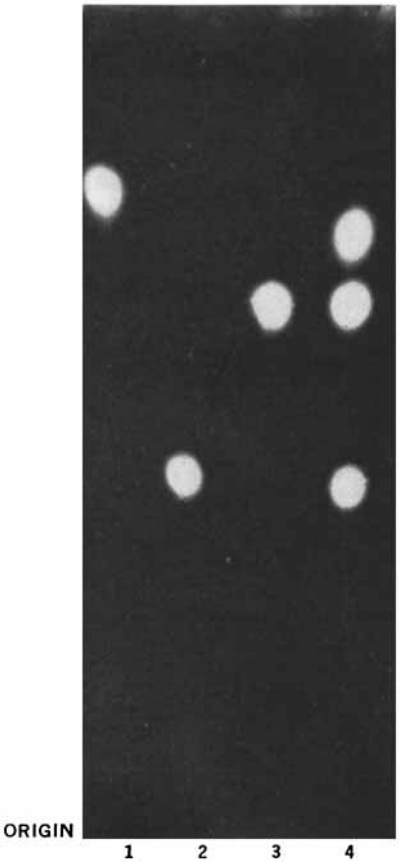

Fig. 1—Thin-layer chromatography of tetracyclines. Key: 1, CTC; 2, TC; 3, DMCTC; 4, mixture.

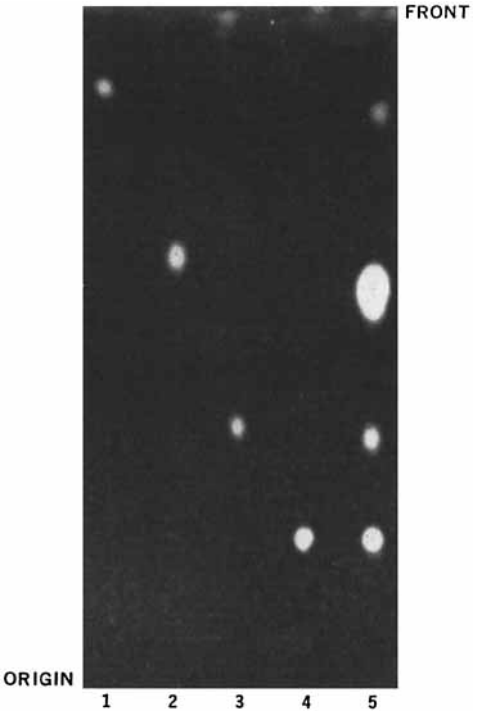

Fig. 2—Thin-layer chromatography of demethylchlortetracycline. Key: 1, ADMCTC; 2, DMCTC; 3, DMTC; 4, EDMCTC; 5, mixture.

Kelly and Buyske (6) found that paper impregnated with 0.1 *M* EDTA greatly improved paper chromatograms of tetracyclines. In our work, thin-layer chromatograms were developed without streaking and tailing by using diatomaceous earth which first had the binder removed and then EDTA added.

Sonanini and Anker (3) studied the chromatography of several tetracyclines by impregnating the support with glycerin to maintain a constant water concentration and thus obtain better resolution of the tetracycline. For this reason we used a mixture of glycerin and PEG 400 for the resolution of closely related tetracyclines.

Figures 1–4 show separations using these various techniques—acid-washed diatomaceous earth impregnated with EDTA–PEG 400–glycerin, and ethyl acetate as the solvent. Figure 1 shows the separations and resolution of three tetracyclines, CTC,[3] TC,[4] and DMCTC,[5] which were specifically prepared. Figures 2, 3, and 4 show the separation and resolution of each tetracycline, such as might result from failure of the strains to produce pure tetracycline, as well as possible degradation during storage. In Fig. 2, DMCTC is separated from

[3] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Aureomycin.
[4] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Achromycin.
[5] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Declomycin.

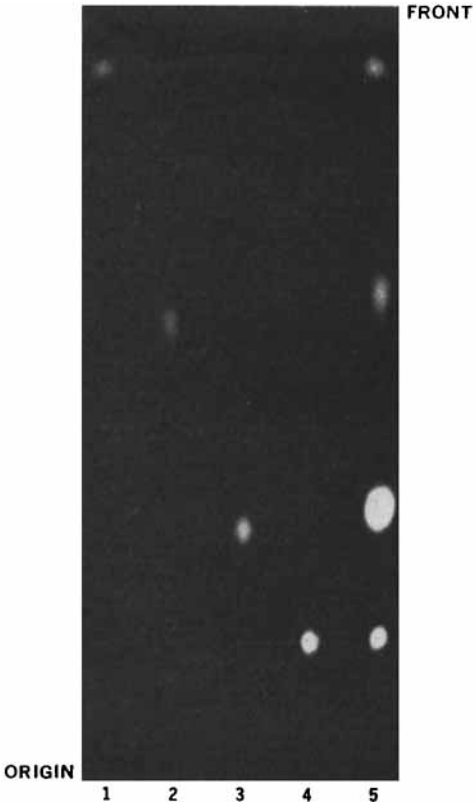

Fig. 3—Thin-layer chromatography of tetracycline. Key: 1, ATC; 2, CTC; 3, TC; 4, ETC; 5, mixture.

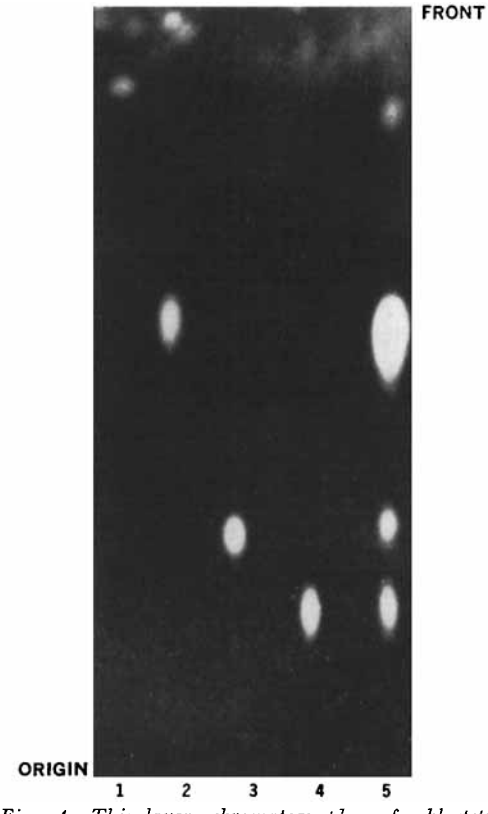

Fig. 4—Thin-layer chromatography of chlortetracycline. Key: 1, ACTC; 2, CTC; 3, TC; 4, ECTC; 5, mixture.

anhydrodemethylchlortetracycline (ADMCTC), demethyltetracycline (DMTC), and epidemethychlortetracycline (EDMCTC); Fig. 3, TC from anhydrotetracycline (ATC), CTC, and epitetracycline (ETC); and Fig. 4, CTC from anhydrochlortetracycline (ACTC), TC, and epichlortetracycline (ECTC).

The apparatus, reagents, developing solvents, plate preparation, and sample application were all evaluated to eliminate unnecessary steps and/or variables for obtaining useful chromatograms. All glassware was scrupulously clean, and only reagents of low native fluorescence were used. The support, diatomaceous earth, was acid-washed to remove the binder and other acid-soluble materials which might contribute to streaking and tailing. The glycerin-PEG 400 mixture maintained the necessary level of moisture that produced well-separated, sharply defined spots. The buffer at pH 7.0 minimized the danger of degradation of the tetracyclines.

Excess moisture due to insufficient drying of the plate reduced the rate of movement of the tetracycline and necessitated chromatographing three or four times. Too little moisture caused elongated spots and poor resolution. Standards were always run for comparative evaluation of samples.

By visual observation under long wavelength ultraviolet light, as minute a quantity as 50 nanograms was easily seen. Additional work is being done in the area of fluorescence analysis using a recording and scanning spectrophotofluorometer which will permit detection of even lower levels of materials, plus quantitation of these tetracyclines and their related compounds.

REFERENCES

(1) Nicolaus, B. J. R., Coronelli, C., and Binaghi, A., *Farmaco (Pavia) Ed. Prat.*, **16**, 349(1961).
(2) Kapadia, G. J., and Subba Rao, G., *J. Pharm. Sci.*, **53**, 223(1964).
(3) Sonanini, D., and Anker, L., *Pharm. Acta Helv.*, 39, 518(1964).
(4) Simmons, D. L., Koorengevel, C. K., Kubelka, R., and Seers, P., *J. Pharm. Sci.*, **55**, 219(1966).
(5) Lees, T. M., and DeMuria, P. J., *J. Chromatog.*, **8**, 108(1962).
(6) Kelly, R. G., and Buyske, D. A., *Antibiot. Chemotherapy*, **10**, 604(1960).

Tetracyclines II

Separation and Determination by Column Chromatography

By PETER P. ASCIONE, JOHN B. ZAGAR, and GEORGE P. CHREKIAN

A column chromatographic method for the determination of demethylchlortetracycline (DMCTC), tetracycline (TC), and chlortetracycline (CTC) was developed. The chromatogram involved the separation of these tetracyclines from their related compounds on a column of acid-washed diatomaceous earth treated with a buffer consisting of 0.1 *M* ethylenediaminetetraacetic acid disodium salt (EDTA) at pH 6.0 and pH 7.0, glycerin, and polyethylene glycol 400 (PEG 400), followe dby a modified spectral determination of column eluates. Although only DMCTC, TC, CTC, and their related compounds were studied in the work reported here, it appears reasonable to assume that the technique described could be extended to the assay of other tetracyclines.

THE MOST widely used and official method of analysis of tetracyclines is the microbiological assay which is based on the ability of tetracyclines to inhibit growth of certain organisms. Inhibition of growth is used as a direct measure of activity and/or potency of these tetracyclines.

However, the microbiological assay is subject to inherent sources of error which limits its precision to ±15%. Specificity is also limited by the fact that several tetracycline-related compounds exhibit activity toward the test organism similar to the parent compound.

The methods currently in use for the determination of tetracyclines and related compounds are based on spectral behavior in the ultraviolet. The procedure of Woolford and Chiccarelli (1), which determined TC in CTC, is based upon the change in spectrophotometric absorption in acid and basic aqueous media. A second paper by Chiccarelli *et al.* (2) described an additional assay method for TC and CTC based on the formation of anhydro compounds, and the fact that CTC heated at pH 7.5 is cleaved to isochlortetracycline, whereas TC under the same conditions is relatively stable. Doskocil and Vondracek (3) determined TC and CTC with a method based on the different rates at which tetracyclines are inactivated in alkaline medium. A differential spectrophotometric method has been used in these laboratories for determination of related compounds.

Various chromatographic methods—paper, column, and thin-layer—are being used for the quantitation and investigation of tetracyclines. Selzer and Wright (4) described paper chromatography of tetracyclines and epimers using complex solvent systems and buffered paper. Kelly and Buyske (5) used sequestering agents in paper chromatography. Whatman modified cellulose phosphate cation-exchange paper has been used also (6). Hrdy and Vesely (7) described column chromatography using powdered cellulose impregnated with tartrate buffer. A column chromatographic method was developed by Kelly (8) for the determination of both anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) in the presence of large quantities of TC. The use of Sephadex G25 polydextran in columns was reported by Griffiths (9). However, these procedures lack the necessary accuracy and/or specificity for proper evaluation of the true tetracycline content.

A thin-layer chromatographic (TLC) method was developed in these laboratories for the separation and examination of tetracyclines (10). The separation was carried out on acid-washed diatomaceous earth[1] impregnated with 0.1 *M* EDTA pH 7.0, glycerin, and PEG 400, with ethyl acetate as a solvent. With modification of both the thin-layer chromatography and spectrophotometric methods, a column method was developed.

Although the method, as described, is concerned only with crystalline samples, this technique has also made it possible to determine individual tetracyclines in fermentation-process control samples in which mixtures resulted from failure of the strain used to produce a pure tetracycline, in manufacturing processes in which potency was determined in finished products, and also in the study of stability of each individual tetracycline in pharmaceutical formulations, which will be reported later.

Received May 22, 1967, from the Department of Analytical Development, Quality Control Section, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965
Accepted for publication July 11, 1967.
The authors thank Mrs. D. Budd, Literature Services Department, for valuable assistance with this report.
Previous paper: Ascione, P. P., Zagar, J. B., and Chrekian, G. P., *J. Pharm. Sci.*, **56**, 1393(1967).

[1] Marketed by Desaga/Brinkmann Co. under the trademark Kieselguhr G.

EXPERIMENTAL

Materials and Methods

Apparatus—Chromatographic column, 1.3 cm. × 40 cm. (Bellco VIP 50-ml. pipet, graduated in 0.2 ml., with the delivery tip cut off at the 48-ml. graduation). Glass tamping rod, with a diameter 1 mm. less than the internal diameter of the chromatographic column.

Preparation of Acid-Washed Diatomaceous Earth[2]—Slurry 200 Gm. of diatomaceous earth with 750 ml. of 6 *N* HCl for 10–15 min. Filter off diatomaceous earth and wash with distilled water until pH is neutral. Slurry the diatomaceous earth in 500 ml. of a solution of equal volumes of ethyl acetate and methanol for 10–15 min. Filter and vacuum dry at 60°.

Reagents—EDTA, pH 6.0 and 7.0: dissolve 37.2 Gm. of ethylenediaminetetraacetic acid disodium salt in approximately 800 ml. distilled water. Adjust the pH to 6.0 or 7.0 with ammonium hydroxide and dilute to 1 L. with distilled water.

Twenty per cent v/v polyethylene glycol 400 (PEG 400) in glycerin: to 80 ml. of glycerin, add sufficient PEG 400 to make 100 ml. Mix well.

Buffer, pH 6.0 and 7.0: to 95 ml. of EDTA pH 6.0 or 7.0 add sufficient 20% v/v PEG 400 in glycerin to make 100 ml. Shake well.

Benzene, chloroform, *n*-butanol, and methanol, reagent grade.

Alkaline methanol solution: dilute 5 ml. concentrated ammonium hydroxide with 95 ml. of methanol.

Determination of Demethylchlortetracycline[3] and Related Compounds

Procedure—*Column Support*—Mix 100 Gm. acid-washed diatomaceous earth with 50 ml. of buffer, pH 7.0, in a plastic bag or in a glass jar on rollers, until the diatomaceous earth is uniformly moist.

Column Preparation—Place a disk of filter paper (Whatman 541, approximately 1.2-cm. diameter) into the bottom of the column, as a plug. Weigh 8 ± 0.1 Gm. of column support and add to the column in three portions. After each addition settle the diatomaceous earth by firmly tapping the lower end of the column on a padded bench top from a height of 2 to 4 in. while maintaining the column in a perpendicular position. Then lightly tamp the surface with the glass tamping rod. The column height should be about 10.5 to 11.5 cm.

Sample Preparation—*Demethylchlortetracycline HCl*—Weigh accurately about 20 mg. DMCTC HCl, transfer to a 25-ml. volumetric flask with 4 ml. distilled water, and dissolve. Dilute to mark with buffer, pH 7.0. Prepare not more than 30 min. before assay. Pipet 1.0 ml. of sample into a 50-ml. beaker. Add 2 ± 0.1 Gm. of acid-washed diatomaceous earth, stir well, and pack on the column. "Rinse" the beaker with 0.5 ± 0.1 Gm. of column support, add to the column, and tamp well.

Demethylchlortetracycline Neutral—Accurately weigh 18 mg. of DMCTC neutral, transfer to a 25-ml. volumetric flask using 3 ml. of 0.1 *N* HCl, and dissolve. Add 15 ml. of buffer, pH 7.0, followed by 3 ml. of 0.1 *N* NaOH, swirl, and dilute to 25 ml. with buffer, pH 7.0. Prepare this solution not more than 30 min. before assay. Pipet 1.0 ml. of this solution into a 50-ml. beaker, add 2 ± 0.1 Gm. of acid-washed diatomaceous earth and transfer to the column as described above.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column. Add 25 ml. of 40% benzene in chloroform to the column. When the solvent level reaches the top of the column packing, add 50 ml. of 30% benzene in chloroform. After collecting 22 ml. (cut *1*), remove the primary receiver and replace with 50-ml. volumetric flask. Collect the eluate in this flask until the level of the solvent in the column again reaches the top of the packing. Replace the 50-ml. volumetric flask (cut *2*) with a 10-ml. graduate, and add 30 ml. of chloroform to the column. When the volume of eluate in the graduate reaches 8 ml. (cut *3*), replace the graduate with a 50-ml. glass-stoppered graduate and continue collecting the eluate. When the chloroform reaches the top of the packing, add 40 ml. of 50% *n*-butanol in chloroform. When the 50-ml. glass-stoppered graduate contains 29.0 ml. (cut *4*), replace with another 50-ml. glass-stoppered graduate and collect all of the last solvent added to the column (cut *5*).

Assay of Column Eluates—To cut *2* (DMCTC), add cut *3*, and mix well. Add 2.0 ml. of alkaline methanol solution and dilute to volume with chloroform. Determine the absorbance of this solution in a 1-cm. cell against chloroform at a wavelength of 372 mμ on a suitable spectrophotometer within 10 min. after making the solution alkaline. To cut *4* demethyltetracycline (DMTC), add 1.0 ml. of alkaline methanol solution and mix. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 366 mμ within 10 min. after making the solution alkaline. To cut *5* epidemethylchlortetracycline (EDMCTC) add 2.0 ml. alkaline methanol, adjust the volume to the nearest milliliter graduation with chloroform, mix, and record the volume. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 372 mμ within 10 min. after making the solution alkaline.

Determination of Tetracycline[4] and Related Compounds

Procedure—The column support, column, and sample preparation are the same as for DMCTC.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column. Add 20 ml. of benzene to the column. When the solvent level reaches the top of the column packing, add 60 ml. of chloroform. After collecting 25 ml. (cut *1*), remove the primary receiver and replace with a 50-ml. volumetric flask. Collect the eluate in this flask until the level of the solvent in the column again reaches the top of the packing. Replace the 50-ml. volumetric flask (cut *2*) with a 10-ml. graduate and add 40 ml. of 50% *n*-butanol in chloroform to the column. When the volume of eluate in the graduate reaches 8 ml., replace the 10-ml. graduate (cut *3*) with a 50-ml. glass-stoppered graduate and collect all of the last solvent added to the column (cut *4*).

[2] Marketed as Celite 545 by Johns-Manville Co.

[3] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Declomycin.

[4] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Achromycin.

Assay of Column Cuts—To cut *2* (TC), add cut *3*, and mix well. Add 2.0 ml. of alkaline methanol solution and dilute to volume with chloroform. Determine the absorbance of this solution in a 1-cm. cell against chloroform at a wavelength of 366 mμ on a suitable spectrophotometer within 10 min. after making the solution alkaline. To cut *4* epitetracycline (ETC), add 2.0 ml. of alkaline methanol, adjust the volume to the nearest milliliter graduation with chloroform, mix, and record the volume. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 366 mμ within 10 min. after making the solution alkaline.

Determination of Chlortetracycline[5] and Related Compounds

Procedure—Column support, column, and sample preparation are the same as for DMCTC, but at pH 6.0.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column, add 25 ml. of 80% benzene in chloroform to the column. When the solvent level reaches the top of the column packing, add 50 ml. of 40% benzene in chloroform. The development of this column to completion is described under *Procedure* for DMCTC continuing from and including cut *1*.

Assay of Column Cuts—The cuts are treated as directed under *Procedure* for DMCTC with cut *2* (CTC) and cut *5* epichlortetracycline (ECTC) read at 377 mμ, and cut *4* (TC) read at 366 mμ.

RESULTS AND DISCUSSION

Earlier investigation into the separation of tetracyclines by thin-layer chromatography suggested the development of this column chromatographic technique. Thin-layer chromatograms of these tetracyclines (10) showed the possible occurrence of related compounds in each tetracycline. In developing the column, various modifications were made to adapt the basic TLC system. The mobile phase was varied on the column from low to high polarity, starting with benzene–chloroform and ending with *n*-butanol–chloroform. The pH was varied from 6.0 to 7.0, but the ratio of PEG 400 to glycerin was maintained at 1:5.

The effectiveness of the column for separating each tetracycline from its related compounds was demonstrated by the use of TLC (10). Figures 1–3 show the chromatography of subsequent column eluates. According to the results of TLC, the tetracyclines were separated on a column of acid-washed diatomaceous earth treated with EDTA, PEG 400–glycerin at pH 6.0 or 7.0, using benzene, chloroform, and butanol as the developing solvents. In Fig. 1, DMCTC is separated from anhydrodemethylchlortetracycline (ADMCTC), demethyltetracycline (DMTC), and epidemethylchlortetracycline (EDMCTC). The column eluates were collected in fractions of 5 ml. and examined by TLC. The TLC shows the distribution of the DMCTC components isolated on the column and having R_f values similar to those of standard test references. In Fig. 2, TC is separated from anhydrotetracycline (ATC), CTC, and epitetracycline (ETC) on a column as described previously; in Fig. 3, CTC is separated from anhydrochlortetracycline (ACTC), TC, and epichlortetracycline (ECTC) on a column as described above but at a pH 6.0.

[5] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Aureomycin.

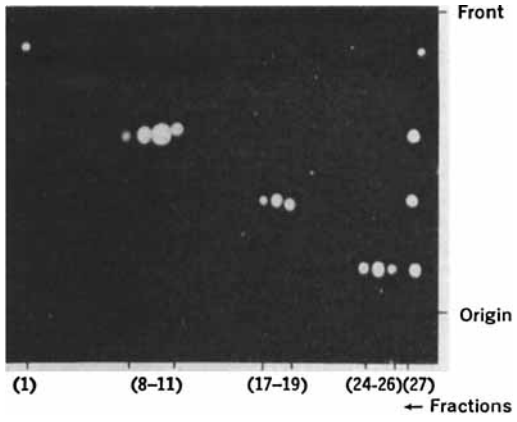

Fig. 1—Thin-layer chromatography of demethylchlortetracycline column fractions. Key: 1, ADMCTC; 8–11, DMCTC; 17–19, DMTC; 24–26, EDMCTC; 27, reference standard mixture.

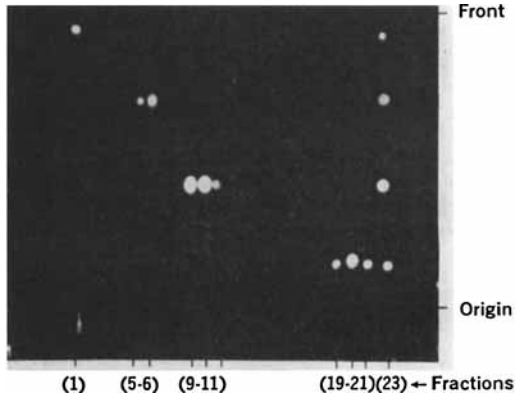

Fig. 2—Thin-layer chromatography of tetracycline column fractions. Key: 1, ATC; 5–6, CTC; 9–11, TC; 19–21, ETC; 23, reference standard mixture.

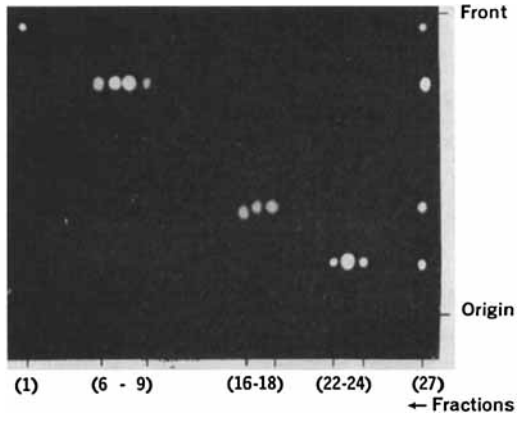

Fig. 3—Thin-layer chromatography of chlortetracycline column fractions. Key: 1, ACTC; 6–9, CTC; 16–18, TC; 22–24, ECTC; 27, reference standard mixture.

The effectiveness of the column and thin-layer chromatographic techniques for the determination of each tetracycline was corroborated by use of microbiological assays. The TLC-chromatogramed spots of tetracyclines were scraped and assayed by the pad-plate diffusion method while the column-chromatographed cuts were assayed by the turbidimetric method. In general, the microbiological results correlated well with the chromatographic data.

For convenience and ease of handling a 1.3-cm. × 40-cm. glass column was chosen. The pH of the buffer was adjusted to eliminate degradation during assay as well as to produce the desired chromatogram of the tetracycline. In the work reported, the sequestering agent (EDTA) was used in the development of TLC and the column. Kelly and Buyske (6) reported that EDTA showed strong chelating properties which improved the separation of tetracyclines. Benzene, chloroform, ethyl acetate, and *n*-butanol were tried as developing solvents, but none served well alone. The best separations were obtained when mixtures of the solvents were used.

Absorption Spectra—Absorption spectra in the region of 300 to 500 mμ were determined on a Cary model 11 recording spectrophotometer. Standard solutions of each tetracycline hydrochloride containing 0.8 mg./ml. were prepared by dissolving 80 mg. of standard in 10 ml. of methanol and then diluting to 100 ml. with chloroform. Two milliliters of the chloroform solution was transferred to a 50-ml. volumetric flask. Forty milliliters of chloroform and 2.0 ml. of alkaline methanol were added, and then diluted to 50 ml. with chloroform. Figures 4–6 show the effect of alkaline methanol, an auxochromic agent, on the absorption spectra of DMCTC, TC, and CTC. In each instance the alkaline methanol had a hypochromic as well as a hyperchromic effect. The wavelengths where maximum absorption occurred when alkaline methanol was introduced into the tetracycline chromaphoric system are as follows: DMCTC, 372 mμ; TC, 366 mμ; CTC, 377 mμ.

Standard calibration curves of DMCTC, TC, and CTC hydrochlorides were prepared with alkaline methanol reagent in chloroform solutions. Three standard curves are presented in Fig. 7. Typical linear Beer's law plot of absorbance *versus* tetracycline concentration range from 0.1 mg./50 ml. to 1.0 mg./50 ml. for all three tetracyclines.

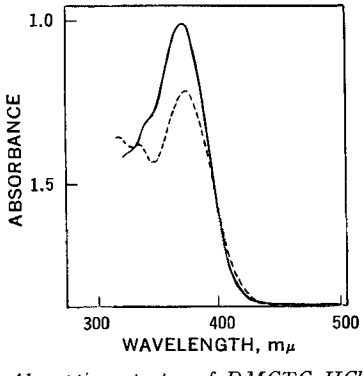

Fig. 4—Absorption spectra of DMCTC·HCl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

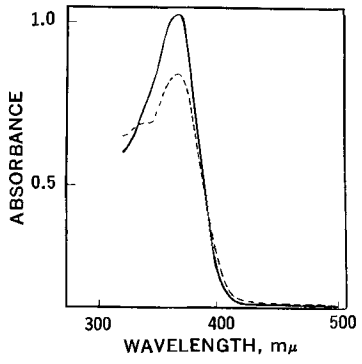

Fig. 5—Absorption spectra of TCH·Cl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

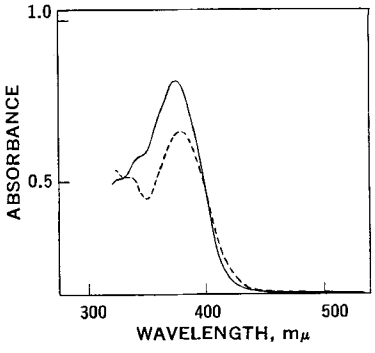

Fig. 6—Absorption spectra of CTC·HCl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

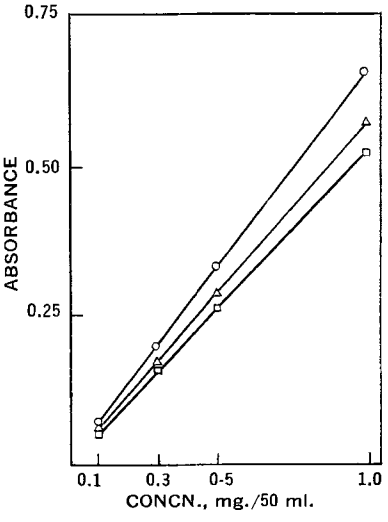

Fig. 7—Standard calibration curve. Key: ○, TC; △, DMCTC; □, CTC.

The absorptiometric system obtained a maximum absorbance value immediately upon the addition of the alkaline methanol reagent and this absorbance remained constant over a practical length of time. (Table I.) This should conservatively estimate the

span of time suitable for accurate quantitation. For example, data in Table I indicate at least 10 min. of stability in absorbance when CTC is being tested, and 30 min. stability for DMCTC and TC.

TABLE I—STABILITY OF TETRACYCLINES IN CHLOROFORM SOLUTION CONTAINING ALKALINE METHANOL

Time, min.	DMCTC, 372 mμ	TC, 366 mμ	CTC, 377 mμ
0	0.459	0.527	0.418
2	0.459	0.527	0.418
4	0.459	0.527	0.418
6	0.459	0.527	0.418
8	0.459	0.527	0.418
10	0.459	0.527	0.417
12	0.459	0.527	0.417
14	0.459	0.527	0.417
16	0.459	0.527	0.416
30	0.458	0.527	0.414

The concentrations of the three compounds tested were 0.80 mg./50 ml. for demethylchlortetracycline hydrochloride, tetracycline hydrochloride, and chlortetracycline hydrochloride, respectively.

The absorptivity for DMCTC, TC, and CTC was determined at the wavelength of maximum absorbance and found to be 2.89×10^{-2} for DMCTC, 3.071×10^{-2} for DMTC, 3.29×10^{-2} for TC, and 2.65×10^{-2} for CTC, as the hydrochlorides. The absorptivity is defined as the absorbance at the wavelength of each particular tetracycline for a solution containing 1 mcg. of tetracycline hydrochloride per milliliter, using a 1-cm. cell. Through examination of standards available in these laboratories, the absorptivity for each tetracycline and its epimer was found to be the same. The determination of absorbances of the column eluates after the addition of the auxochromic agent provided a quantitative spectrophotometric method for assaying tetracyclines in solvent solutions when compared with standards treated in like manner.

REFERENCES

(1) Woolford, M. H., Jr., and Chiccarelli, F. S., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 400(1956).
(2) Chiccarelli, F. S., Woolford, M. H., Jr., and Avery, M. E., *ibid.*, **48**, 263(1959).
(3) Doskocil, J., and Vondracek, M., *Chem. Listy*, **46**, 564(1952).
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(5) Kelly, R. G., and Buyske, D. A., *ibid.*, **10**, 604(1960).
(6) Addison, E., and Clark, R. G., *J. Pharm. Pharmacol.*, **15**, 268(1963).
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In Vivo Method for the Simultaneous Determination of Potassium and Sodium Depletion

By GORDON S. BORN, STANLEY M. SHAW, and JOHN E. CHRISTIAN

An *in vivo* radiotracer technique utilizing whole body liquid scintillation counting for the simultaneous detection of ^{42}K and ^{24}Na retention within the intact animal was developed. The technique allowed direct comparison of the retention of potassium and sodium in treated and control animals. The differences in retention indicated the kaliuretic and natriuretic effects of proven diuretic agents. Experimentation with standards was used to develop the simultaneous technique for ^{42}K and ^{24}Na determination. The method was tested *in vivo* in rats and swine, using furosemide and hydrochlorothiazide to show the effects of these diuretic agents in altering potassium and sodium metabolism, to illustrate the adaptability of the method in studies with various animal species, and to confirm the versatility of the method with a whole body liquid scintillation detector of the "human type." Results indicated the technique to be a sensitive method for measuring alterations in potassium and sodium metabolism in rats and swine caused by the diuretic agents.

RADIOTRACER techniques utilizing *in vivo* whole body liquid scintillation counting have been used in studying the effects of diuretics on either potassium or sodium metabolism. Using ^{22}Na, Rupe, Bousquet, and Christian (1) evaluated compounds for their natriuretic and diuretic activity in rats. Born *et al.* (2) and Seno (3), using ^{42}K, studied the kaliuretic properties of diuretics in the rat. Shaw, Kessler, and Christian (4) investigated the kaliuretic and natriuretic properties of a diuretic by using ^{42}K and ^{24}Na in separate studies in swine. The advantages of the whole body liquid scintillation counting technique over photometric methods of determining potassium and sodium elimination have been stated in the above references. However, an undesirable aspect of the whole body liquid scintillation counting technique was the inability to study the kaliuretic and natriuretic properties of a diuretic in the same group of animals in a single experiment.

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Accepted for publication July 21, 1967.
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RADIOTRACER techniques utilizing *in vivo* whole body liquid scintillation counting have been used in studying the effects of diuretics on either potassium or sodium metabolism. Using ^{22}Na, Rupe, Bousquet, and Christian (1) evaluated compounds for their natriuretic and diuretic activity in rats. Born *et al.* (2) and Seno (3), using ^{42}K, studied the kaliuretic properties of diuretics in the rat. Shaw, Kessler, and Christian (4) investigated the kaliuretic and natriuretic properties of a diuretic by using ^{42}K and ^{24}Na in separate studies in swine. The advantages of the whole body liquid scintillation counting technique over photometric methods of determining potassium and sodium elimination have been stated in the above references. However, an undesirable aspect of the whole body liquid scintillation counting technique was the inability to study the kaliuretic and natriuretic properties of a diuretic in the same group of animals in a single experiment.

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The purpose of the following research was to develop an *in vivo* technique for the simultaneous detection of both potassium and sodium retention, thereby achieving simultaneous determination of the kaliuretic and natriuretic properties of a diuretic. The technique would limit biological error, reduce investigational time, and allow greater economy resulting from the utilization of fewer animals.

Following the administration of radioactive potassium and sodium, and their equilibration with the body stores of the electrolytes, whole body radioactivity was determined by dual channel counting, to establish the amount of each radionuclide present in the body. Subsequent measurements were made during drug or control dosage. Direct comparisons between the radionuclide retentions of treated and control animals allowed the evaluation of drugs for kaliuretic and natriuretic properties.

EXPERIMENTAL

The investigation was divided into three parts which were: development of the technique, study of the natriuretic and kaliuretic effects of furosemide[1] and hydrochlorothiazide[2] in the laboratory rat, and a study of the saluretic effects of furosemide in swine. The first study, which was conducted using the PUSAC (5), was carried out to develop a whole body liquid scintillation counting technique which would allow the resolution of the component parts from a ^{42}K and ^{24}Na mixture. The PUSAC was modified to allow dual channel counting by splitting the signal from the preamplifier with a tee connector and directing the split signal through separate amplifiers, pulse height analyzers, and scalers. One pulse height analyzer was calibrated to monitor the 1.26 Mev. Compton peak of ^{42}K with a 9% window (5.0 Mev. full scale). The other was calibrated to monitor the 2.5 Mev. Compton peak from ^{24}Na with a 20% window (3.2 Mev. full scale). The base line discriminators on both analyzers were set to maximize the sample-squared-to-background ratios at the stated window widths. Three mixtures of ^{42}K and ^{24}Na, with varying but known amounts of each radionuclide, were prepared. Additional standards of the single radionuclides at the same concentration were prepared. Mixture *1* was a standard containing both ^{24}Na and ^{42}K in the same concentration as the ^{24}Na standard 1 and the ^{42}K standard 1; other mixed samples were similarly prepared. Counts were taken on the standards and the mixtures of known concentration at 0, 6, 18, 30, and 42 hr. after preparation to simulate an *in vivo* study. At each time interval, the net count rate was obtained in each channel. The counting procedure yielded a counting error for the net sample counts of 5% or less at the 95% confidence level.

[1] Furosemide is 4-chloro-*N*-(2-furyl-methyl)-5-sulfamoyl-anthranilic acid. This compound was supplied by Lloyd Brothers, Inc., Cincinnati, Ohio, and is marketed as Lasix.

[2] 6 - Chloro - 3,4 - dihydro - 7- sulfamoyl - 2*H* - 1,2,4-benzothiadiazine-1,1-dioxide. The compound was supplied by Merck Sharp and Dohme, West Point, Pa., as Hydrodiuril.

Observed net count rates for the mixtures (from both the potassium and sodium channels) were mathematically manipulated, resolving the relative amount of each radionuclide present. The simultaneous equation method was used to eliminate interference in each channel caused by spectral overlap of the other radionuclide. The ratio of any portion of the spectra to other portions was constant and independent of the activity.

The net count rate of a composite spectrum of the radionuclides ^{42}K and ^{24}Na, for the Compton peak channel of ^{42}K (N_K) may be written as:

$$N_K = K_K + f_{NaK}\, Na_{Na} \qquad \text{(Eq. 1)}$$

where K_K = count rate contribution by ^{42}K to the Compton-peak channel of ^{42}K, f_{NaK} = interference factor which expresses the fractional contribution of the Compton-peak channel of ^{24}Na to the Compton-peak channel of ^{42}K.

Similarly, the net count rate in the ^{24}Na channel (N_{Na}) may be written as:

$$N_{Na} = Na_{Na} + f_{KNa}\, K_K \qquad \text{(Eq. 2)}$$

where Na_{Na} = count rate contribution by ^{24}Na to the Compton-peak channel of ^{24}Na, f_{KNa} = interference factor which expresses the fractional contribution of the Compton-peak channel of ^{42}K to the Compton-peak channel of ^{24}Na.

Equations 1 and 2 are simultaneous linear independent equations containing two unknowns, K_K and Na_{Na}. The net count rates (N_K, N_{Na}) of the composite sample are obtained from the composite spectrum, and interference factors (f_{NaK}, f_{KNa}) obtained experimentally from standard spectra.

The solution to Eqs. 1 and 2 by substitution is:

$$K_K = N_K \cdot \frac{1}{1 - f_{NaK} f_{KNa}} - N_{Na} \cdot \frac{f_{NaK}}{1 - f_{NaK} f_{KNa}}$$

and

$$Na_{Na} = N_{Na} \cdot \frac{1}{1 - f_{KNa} f_{NaK}} - N_K \cdot \frac{f\mathrm{KNa}}{1 - f_{KNa} f_{NaK}}$$

Now let

$$C_1 = \frac{1}{1 - f_{NaK} f_{KNa}}$$

$$C_2 = \frac{f_{NaK}}{1 - f_{NaK} f_{KNa}}$$

$$C_3 = \frac{1}{1 - f_{KNa} f_{NaK}}$$

$$C_4 = \frac{f_{KNa}}{1 - f_{KNa} f_{NaK}}$$

The following new equations result:

$$K_K = C_3 N_K - C_4 N_{Na}$$
$$Na_{Na} = C_1 N_{Na} - C_2 N_K$$

where K_K = ^{42}K in the potassium channel, Na_{Na} = ^{24}Na in the sodium channel, N_K = observed count rate in the ^{42}K channel, N_{Na} = observed count rate in the ^{24}Na channel, and C_1, C_2, C_3, C_4 = constants whose value was derived from the ^{42}K and ^{24}Na standards.

The resolved potassium (K_K) and sodium (Na_{Na})

standard data at the first observation period were taken to represent 100% values. Both net count rates for later time intervals were corrected for counter efficiency changes, resolved, and then corrected for radioactive decay. Half-life values of 12.5 hr. for ^{42}K and 15.0 hr. for ^{24}Na were used. The per cent of potassium and sodium found in each of the three mixed samples was calculated and averaged for each observation period and the error (difference between observed value and 100%) calculated.

In using the simultaneous method to compare the effects of furosemide and hydrochlorothiazide upon potassium and sodium, two rat trials at different dosage levels were conducted. In addition to the simultaneous determination in trial *A*, an integral sodium study was conducted. In trial *B* an integral potassium determination was conducted as well as the simultaneous study, allowing comparison of the interference free (single radionuclide) integral and the simultaneous (dual radionuclide) detection methods. The PUSAC was utilized as previously described in this paper for the dual studies and as described by Born *et al.* (2) for the integral studies.

Female Sprague-Dawley albino rats (175–225 Gm.) were divided into groups consisting of 6 rats each. The animals were housed in individual metabolism cages. Twenty-four hours prior to the initiation of whole body counting, the animals were injected intraperitoneally with the isotope(s) in 0.5 ml. of neutral aqueous solution to allow equilibration of the radionuclides with body stores. For the simultaneous determinations, based upon supplier assay,[3] about 1 μc. of ^{42}K and 0.1 μc. of ^{24}Na were given each rat. In the case of the single radionuclide studies, approximately 3 μc. of ^{42}K or 0.3 μc. of ^{24}Na was administered. At the time of radionuclide administration, fasting was initiated and continued throughout the experiment. Distilled drinking water was allowed *ad libitum*. Immediately after initial determination of whole body radioactivity (time 0), the test drug (furosemide or hydrochlorothiazide) was administered. The animals were dosed again at each counting period (each animal receiving 220 mg./Kg./day in trial *A* and 250 mg./Kg./day in trial *B*). Whole body counts were made at 8, 16, 24, 32, and 40 hr. The net sample counts from each animal were manipulated as before to resolve the relative potassium and sodium counts. The resolved count rates for each radionuclide were treated separately, using a digital computer and Fortran programming for correcting data for radioactive decay, calculating the per cent ^{42}K and ^{24}Na retention in each animal, computing the average per cent retention of each electrolyte for the drug treated *versus* the control groups, and computing the Student *t* value for this difference in per cent retention.

To demonstrate the adaptability of the dual radionuclide detection method to other whole body counters, as well as to other test animals, domestic pigs were utilized in the Sinco-p (6) which was calibrated to allow dual channel counting of ^{24}Na and ^{42}K. A 20% window width (3.2 Mev. full scale) for each channel was used with this counter. The base line for each window was selected by maximizing the sample-squared-to-background ratio using ^{42}K and ^{24}Na standards.

Pigs of both sexes (25–30 lb.) were randomly divided into 2 groups of 3 animals. One group was designated the control group, and the other group selected for drug therapy. The animals were housed in metabolism cages throughout the experimental period. Forty-eight hours prior to the first drug administration, whole body ^{40}K was determined, the animals being immobilized in a steel container (4). Twenty-four hours prior to drug administration, ^{42}K (about 5 μc.) and ^{24}Na (about 1 μc.) were administered intraperitoneally to allow equilibration with body stores. Fasting, with water allowed *ad libitum*, was initiated and continued throughout the experiment. Following radionuclide equilibration, each animal was counted at 0, 6, 12, 24, 30, and 36 hr. Furosemide (100 mg./Kg./day) was administered in 2 equal aliquots at 12-hr. intervals starting at time zero.

The calculation of data was the same as previously described. However, natural ^{40}K radioactivity in each swine was subtracted from the net sample count of each channel before proceeding with further calculations.

RESULTS AND DISCUSSION

Table I shows the results of the study with standards which was undertaken to show the feasibility of a simultaneous method for detection of ^{42}K and ^{24}Na. The 100% values for ^{42}K and ^{24}Na at time zero were assigned, while the percentages at later time intervals were calculated. As can be seen, the greatest difference between known and observed standard activity was about 3% for ^{24}Na and less for ^{42}K. The sources used in this study had widely varied proportions of ^{42}K and ^{24}Na, representing extremes not often encountered in controlled experiments with animals. With observed count rates from ^{42}K and ^{24}Na approximating each other, as in the animal studies, the over-all error of simultaneous determinations should be reduced.

Table I—Per Cent of Isotope Found in Known Sources by the Simultaneous Method

Time[a] Elapsed	% [b] ^{42}K	% [b] ^{24}Na	% Error [c] ^{42}K	% Error [c] ^{24}Na
0	100.0	100.0	0.0	0.0
6	98.6	98.6	1.4	1.4
18	99.8	101.8	0.2	1.8
30	100.1	97.0	0.1	3.0
42	97.3	98.2	2.7	1.8

[a] Time elapsed in hours from first determination. [b] Average per cent for three samples. [c] One hundred per cent minus calculated per cent.

Table II shows the differences in per cent sodium retention between drug and control animals observed in trials *A* and *B*, and the calculated Student *t* values for these differences. As may be seen in Table II, both drug treatments caused a decrease in sodium retention in relation to control animals, and thereby, an increase in sodium excretion.

In trial *A*, in addition to the simultaneous study, an integral study of sodium retention was conducted allowing comparison of the data from both methods. In both studies in trial *A*, a significant difference

[3] New England Nuclear Corp., Boston, Mass.

TABLE II— EFFECTS OF FUROSEMIDE AND HYDROCHLOROTHIAZIDE UPON SODIUM RETENTION IN THE RAT

Time[a] Elapsed	Furosemide						Hydrochlorothiazide					
	Trial A[b]				Trial B[c]		Trial A				Trial B	
	Integral		Differential		Differential		Integral		Differential		Differential	
	D[d]	t[e]	D	t	D	t	D	t	D	t	D	t
8	13.6	9.72	9.4	4.19	13.0	9.26	9.7	7.02	6.5	4.01	9.9	11.07
16	13.2	12.10	14.7	9.33	15.3	8.15	6.8	6.33	8.8	5.66	10.8	6.38
24	14.5	8.76	16.9	12.60	18.5	9.39	7.0	4.18	8.4	5.36	10.7	6.27
32	14.5	13.15	12.6	12.03	17.2	8.82	6.4	6.75	5.1	2.80	7.9	5.16
40	11.2	7.82	12.7	7.51	15.5	8.60	4.6	3.55	3.7	2.36	7.2	4.83

[a] Time elapsed in hours from first drug administration. [b] Drug dosage 220 mg./Kg./day. [c] Drug dosage 250 mg./Kg./day. [d] Per cent retention controls minus per cent retention drug. [e] Student t value of 2.76 reflecting a confidence level of 99% for a "one tail" test.

(p = 0.01) in per cent retention was observed for sodium at all time intervals for both drugs. Figure 1 shows the average per cent retention of sodium,

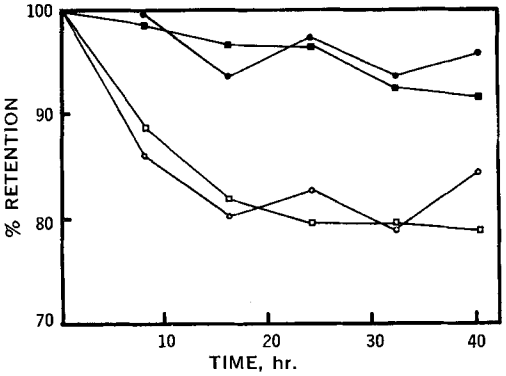

Fig. 1—Effect of furosemide on sodium retention. Key: ●, integral controls; ■, differential controls; ○, integral furosemide; □, differential furosemide.

TABLE III—DIFFERENCE IN EFFECT OF FUROSEMIDE AND HYDROCHLOROTHIAZIDE UPON SODIUM RETENTION (TRIAL A)

Time[a] Elapsed	Integral		Differential	
	D[b]	t[c]	D	t
8	3.8	3.29	3.0	1.51
16	6.4	8.23	5.9	3.72
24	7.5	8.27	8.6	5.49
32	8.0	8.30	7.5	4.05
40	6.6	5.77	9.1	5.65

[a] Time elapsed in hours from first drug administration. [b] Per cent retention hydrochlorothiazide treated minus furosemide treated. [c] Student t value of 2.76 reflecting a confidence level of 99% for a "one tail" test.

as affected by furosemide, for drug and control animals *versus* time for the simultaneous and integral studies. The observed retention values shown in Fig. 1 are representative of the average retention values used to calculate the differences in per cent retention. In Table III a comparison between furosemide and hydrochlorothiazide is made. In this case, the hydrochlorothiazide-treated group were considered as the controls, and activity of furosemide in relation to hydrochlorothiazide evaluated to show the differences in potassium and sodium elimination. In trial A of both integral and differential studies, furosemide was shown to cause significantly (p = 0.01) more sodium elimination than hydrochlorothiazide.

In Table IV the differences in per cent potassium retention between drug and control therapy are given for trials A and B, and their Student t values reported. In trial B, both integral and differential studies were conducted allowing comparison of the suitability of the two methods for potassium determination. From the data in Table IV, the integral method showed statistical differences (p = 0.01) in potassium retention between furosemide and control-treated animals at all time periods except the 16-hr. observation. In the differential study, the data were significantly different (p = 0.01) at the 8 and 24-hr. time period. With hydrochlorothiazide, only in the integral study was any difference in potassium retention between control and drug-treated animals observed, and then, only at two time intervals (8 and 32 hr.). The percentages of potassium eliminated in the integral and differential study are comparable. The statistical significance of potassium elimination in the integral study and nonsignificance in the differential study may indicate limitations to the reliability of a simultaneous method. The limitations are believed to be imposed by 3 combined factors: small number of animals utilized, counting error, and the inherently

TABLE IV—EFFECTS OF FUROSEMIDE AND HYDROCHLOROTHIAZIDE UPON POTASSIUM RETENTION IN THE RAT

Time[a] Elapsed	Furosemide						Hydrochlorothiazide					
	Trial A[b]		Trial B[c]				Trial A		Trial B			
	Differential		Integral		Differential		Differential		Integral		Differential	
	D[d]	t[e]	D	t	D	t	D	t	D	t	D	t
8	0.9	0.64	2.8	6.39	4.3	2.87	0.4	0.26	1.5	2.93	1.8	1.17
16	−0.0	−0.03	2.3	2.54	3.3	1.71	−2.2	−1.28	1.1	0.92	0.7	0.39
24	1.8	0.85	4.0	3.26	6.6	3.49	−0.3	−0.17	1.0	0.77	2.8	1.63
32	3.2	1.68	7.8	8.90	2.3	1.10	0.2	0.15	2.9	3.84	−1.9	−0.72
40	6.1	2.82	7.7	6.23	[f]	[f]	3.5	1.94	1.8	1.28	1.2	0.29

[a] Time elapsed in hours from first drug administration. [b] Drug dosage 220 mg./Kg./day. [c] Drug dosage 250 mg./Kg./day. [d] Per cent retention controls minus per cent retention drug. [e] Student t value of 2.76 reflecting a confidence level of 99% for a "one tail" test. [f] No observation made at this time interval.

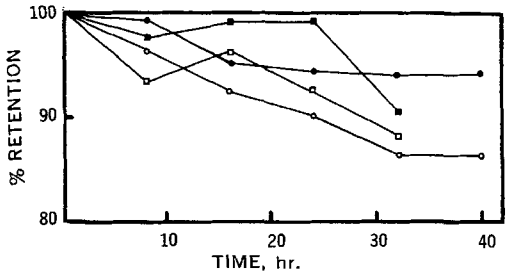

Fig. 2—Effect of furosemide on potassium retention. Key: ●, integral controls; ■, differential controls; ○, integral furosemide; □, differential furosemide.

TABLE V—DIFFERENCE IN EFFECT OF FUROSEMIDE AND HYDROCHLOROTHIAZIDE UPON POTASSIUM RETENTION (TRIAL *B*)

Time[a] Elapsed	Integral D[b]	Integral *t*[c]	Differential D	Differential *t*
8	1.3	2.21	2.5	2.13
16	1.3	1.56	2.6	1.28
24	3.0	3.89	3.8	1.94
32	4.9	6.14	4.2	1.77
40	6.0	6.04	...	...

[a] Time elapsed in hours from first day administration. [b] Per cent retention hydrochlorothiazide-treated minus furosemide-treated groups. [c] Student *t* value of 2.76 reflecting a confidence level of 99% for a "one tail" test.

small amounts of potassium actually excreted as a result of drug action. Figure 2 illustrates the average per cent potassium retention for furosemide and control therapy *versus* time for both the integral and differential study.

Table V shows differences in the effect of furosemide and hydrochlorothiazide on potassium metabolism, when the hydrochlorothiazide group was considered as control animals. The integral study shows that the two drugs affect potassium elimination to a different degree, furosemide causing a significant ($p = 0.01$) elimination of potassium as compared to hydrochlorothiazide. With the differential method, no statistically significant differences are observed, although the differences in per cent potassium elimination are comparable. Here again, any difference that may exist between the diuretics has been masked.

The effect of furosemide (100 mg./Kg./day) upon sodium and potassium retention in swine is shown in Table VI. The differences in per cent retention of ^{42}K and ^{24}Na between control and drug groups at different time intervals is indicated, as well as the Student *t* values for these differences. The furosemide treatment, when compared to control treatment, caused an insignificant excretion of potassium at all time periods. The results of the study show the natriuretic effect of furosemide upon sodium excretion, with significant differences being observed at 24, 30, and 36-hr. observation periods.

The results of the swine experiment are not in complete agreement with the integral study of the kaliuretic and natriuretic properties of furosemide by Shaw, Kessler, and Christian (4). While their investigation indicated a statistically significant

TABLE VI—EFFECTS OF FUROSEMIDE (100 mg./Kg./DAY) UPON POTASSIUM AND SODIUM RETENTION IN SWINE

Time[a] Elapsed	Potassium C−F[b]	Potassium *t*[c]	Sodium C−F	Sodium *t*
0[d]	0.0	0.000	0.0	0.000
6	3.0	2.229	13.3	3.237
12[d]	7.8	1.376	9.0	1.797
24[d]	0.2	0.087	17.9	7.732
30	2.0	0.376	18.9	12.903
36	11.7	1.907	23.7	4.358

[a] Time elapsed in hours from first drug administration. [b] Average per cent retention controls minus average per cent retention furosemide. [c] Student *t* value of 3.75 reflects a confidence level of 99% for a "one tail" test. [d] Drug or control dosage.

difference in potassium elimination between treated and control animals and this study indicates only a trend, as previously noted, the differential method lacks the sensitivity of the integral method in determining potassium retention. However, the sodium data are consistent with their study of furosemide.

SUMMARY AND CONCLUSIONS

Using standards, a suitable method was developed which would allow simultaneous detection of ^{42}K and ^{24}Na in a composite sample by large volume liquid scintillation counting. The standard data indicate that the determination of the amount of each radionuclide, in the presence of the other, can be accomplished with an error of about 3%. In rats, a comparison of the simultaneous and integral detection methods showed both methods capable of detecting potassium elimination as affected by furosemide or hydrochlorothiazide, though the integral was the more sensitive to small differences. The simultaneous method showed no statistical differences (between the 2 drugs) in potassium elimination. The simultaneous and integral methods were equally sensitive in the detection of sodium elimination. The effect of furosemide on the rat was shown, statistically, to be more pronounced than that of hydrochlorothiazide with respect to an increased elimination of sodium. Experimentation with swine illustrated the adaptability of the simultaneous method to other species and other whole body counters.

The results of the investigation indicate that the simultaneous method for determining the kaliuretic and natriuretic properties of diuretics would be applicable to animal studies and feasible for use in clinical evaluations. The high detection sensitivity of whole body counters, in combination with the short half-lived radionuclides ^{24}Na and ^{42}K, limits whole body exposure to ionizing radiation and allows clinical evaluation with a minimal health hazard.

REFERENCES

(1) Rupe, B. D., Bousquet, W. F., and Christian, J. E., *J. Pharm. Sci.*, **52**, 579(1963).
(2) Born, G. S., Shaw, S. M., Christian, J. E., and Kessler, W. V., *ibid.*, **54**, 1646(1965).
(3) Seno, S., unpublished data.
(4) Shaw, S. M., Kessler, W. V., and Christian, J. E., *J. Pharm. Sci.*, **54**, 1208(1965).
(5) Dunavant, B. G., and Christian, J. E., *Intern. J. Appl. Radiation Isotopes*, **8**, 223(1960).
(6) Christian, J. E., Kessler, W. V., and Ziemer, P. L., *ibid.*, **13**, 557(1962).

Phytochemical and Hypoglycemic Investigation of *Casearia esculenta*

By K. D. CHOUDHURY and N. K. BASU*

In the course of a systematic investigation of *Casearia esculenta*, the following components were isolated: a sterol, m.p. 132–134°; a substance resembling gutta-percha, m.p. 59–60°; a flavonoid compound, m.p. 255–258°; a colorless crystalline compound, neutral and water-soluble, m.p. 184°; and two resins. A reducing sugar present in the drug was identified by paper chromatography as arabinose. The hypoglycemic activity of the different fractions, both isolated and in alcoholic and aqueous extracts, has been studied.

THE ROOTS OF *Casearia esculenta* Roxb. (N. O. *Samydaceae*) are used as an antidiabetic drug in southern and southwestern India, particularly in Maharastra, by physicians who practice the Indian system of medicine known as Ayurvedha. The drug is considered to be a specific remedy for diabetes (1), and is widely used throughout Decan (2). A search of available literature (1, 3, 4) and abstracts revealed little or no scientific information concerning the evaluation of the medicinal properties of the plant. The present investigation was undertaken primarily on the basis of a preliminary report (5) which indicated the presence of hypoglycemic activity in a water-soluble crystalline body *S*, m.p. 182°, and also in two resin fractions, one of them water soluble but acid insoluble. From the available experimental data it is now evident that a water-soluble crystalline body, m.p. 184°, isolated from the roots of the plant, is responsible for the hypoglycemic activity.

EXPERIMENTAL

Source and Preparation of Material—Drug plant roots used in these investigations were received through the courtesy of Sri K. G. Gune, Ayurvedhashram Pharmacy, Ahmednagar, and authenticated by Sri Balwant Singh, Ayurvedic Garden, Banaras Hindu University, by comparison with the samples kept at the herbarium of the Ayurvedic Division, College of Medical Sciences, Banaras Hindu University. The roots were reduced to moderately fine powder.

Percentage of Extracts and Ash Determination—Suitable samples of the powdered drug were extracted successively with a series of selective solvents in a Soxhlet apparatus according to a modified Rosenthaler procedure (6). The solvents utilized and the per cent of extracts obtained with each solvent were: petroleum ether (b.p. 40°–60°), 2.55%; ether, 0.73%; benzene, 0.75%; chloroform, 0.15%; acetone, 2.66%; and alcohol, 5.34%. The determinations for ash content as recommended by the B.P. (7) afforded the following results: total ash, 3.25%; acid-insoluble ash, 0.243%; and water-soluble ash, 0.58%.

Extraction of the Drug with Benzene and Alcohol and Separation of Different Fractions (Scheme I)—Ten kilograms of the drug was completely extracted with benzene, and the extract was concentrated and ultimately dried. The residue obtained was labeled as fraction A.

The residue left after the extraction with benzene was further extracted with alcohol by percolation. The total alcoholic extract was concentrated under reduced pressure to a small volume and set aside. Some solid matter deposited from the concentrated extract and was separated from time to time. This process was continued until there was no further deposition on standing. The mixed solid was washed with a little cold alcohol and then with dry ether. It was labeled as fraction B. The ethereal washings gave positive tests for sterols and was labeled as fraction C. It was examined along with fraction A.

The alcoholic extract left after the removal of fraction B was poured in a thin stream, with constant stirring, into a large volume of water. The precipitated mass was separated, dried, and labeled as the resin. The supernatant liquid after acidification with dilute hydrochloric acid gave another precipitate which was collected and dried. This fraction was labeled as the acid-insoluble resin.

The filtrate gave positive tests for reducing sugars. A portion, labeled as fraction F, was used for the identification of the sugar.

Isolation of Sterol—*From Fraction A*—A suitable amount of fraction A was saponified with alcoholic potash and the unsaponifiable portion, after removal of alcohol, was taken up in ether. No crystalline matter separated from the ethereal extract even on prolonged storage at low temperature (10°). On addition of acetone to the ethereal extract, one yellowish body was precipitated. The precipitate gave faint tests for sterols. It was purified by repeatedly dissolving in ether and reprecipitating with the addition of acetone. After purification, this solid failed to respond to any of the tests for sterols. This was labeled as fraction K. After the removal of fraction K, only one sterol, m.p. 132–134°, could be isolated from the mother liquor.

From Fraction C—A sterol, m.p. 132–134°, separated during attempts at crystallization from

Received January 30, 1967, from the School of Pharmacy, Zaria, Northern Nigeria.
Accepted for publication May 10, 1967.
Abstracted in part from a thesis submitted by K. D. Choudhury to Banaras Hindu University in partial fulfillment of Doctor of Philosophy degree requirements.
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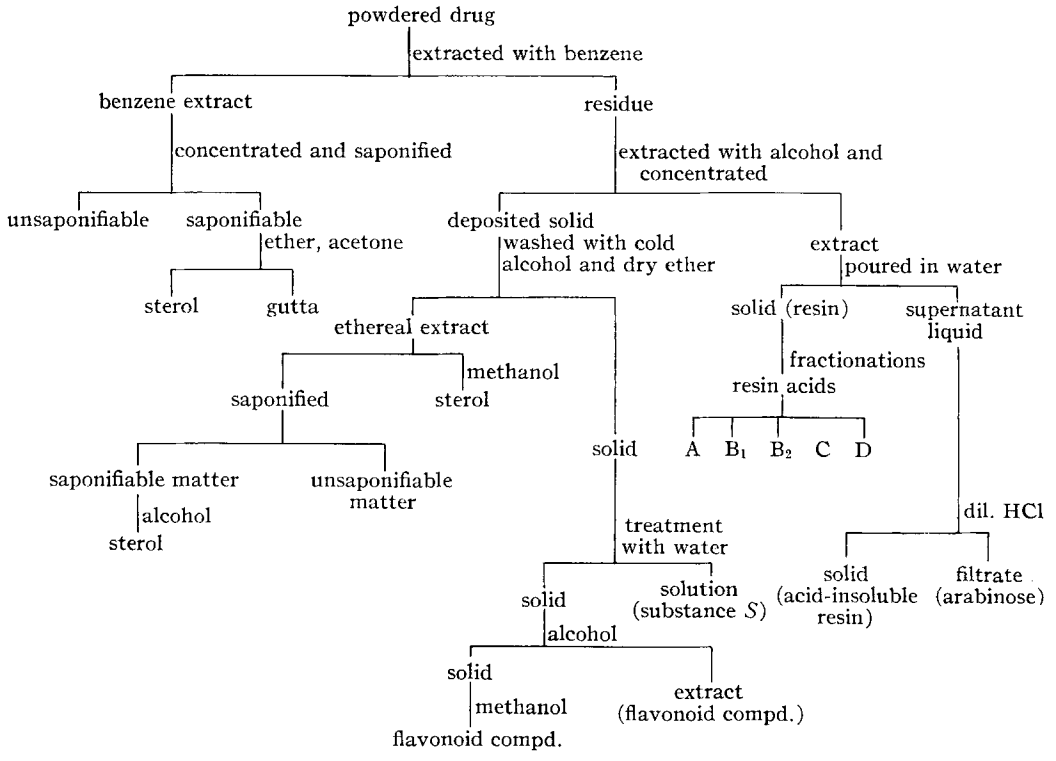

Scheme I

methanol and also by the process of saponification and taking up in ether and ultimate crystallization from ethanol. All the sterol fractions were found to be the same since there was no depression when mixed melting points were taken.

The following derivatives of the sterol were prepared and their melting points noted: digitonide, m.p. 202–203°; acetyl, m.p. 113–114°; and benzoyl, m.p. 113–116°.

Anal.—Calcd. for $C_{24}H_{38}O_2$: C, 80.44; H, 10.61. Found: C, 80.71; H, 10.47.

Molecular weight (Rast) using camphor as solvent.

Gutta-Like Substance—Fraction K, found during the isolation of sterol, was found to melt at 59°–60°. Elemental analysis (8) indicated the absence of nitrogen, sulfur, phosphorus, and halogens. It was classified [as per Tracey (9)] as gutta from its physical behavior and chemical tests. Since this substance did not show any hypoglycemic activity, it was not studied further.

Isolation of Substance S—Fraction B was treated with hot water. The suspension was filtered and the residue was repeatedly treated with warm water until a small volume of the filtrate did not leave anything on evaporation. The residue which did not go into solution was marked fraction P and set aside. A flavonic compound, isolated from fraction P, is described later. The mixed filtrate was concentrated to a small volume and kept for crystallization. A number of crops of crystals, m.p. 178–179°, were isolated. The crops were recrystallized separately from water and their melting points were found to lie between 182–184°. The mixed melting points of different crops did not show any depression. All the crops were, therefore, mixed together and dissolved in water and allowed to crystallize slowly. The crystals, as they appeared, were collected separately in several lots. The melting points of first and last fraction were taken and found to be 184°. When tested for hypoglycemic activity, this product was found to be active both on normal rats (Table VI) and rabbits (Table VII). The chemical constitution and antidiabetic activity of this compound, named substance *S*, (C, 39.6; H, 6.82) will be reported in a separate communication.

Isolation of Flavonoid Compound—Fraction P was found to have a silky appearance in water. It decolorized acid permanganate solution and gave positive tests for phenols with a solution of ferric chloride. Five grams of fraction P was refluxed several times with ethanol which took a small portion of it in solution. From the alcohol-soluble portion a granular body crystallized, m.p. 246–251° dec. This product turned green in a solution of ferric chloride. Light yellow needle-shaped crystals, m.p. 254–258°, with repeated crystallization were obtained from the granular body. This was labled fraction P/alcohol.

The mass left after refluxing with alcohol was refluxed several times with methanol. From the mixed methanolic extract a yellow crystalline product separated on cooling. This was separated and repeatedly crystallized from hot methanol until there was no rise in the melting point, 255–257°. This product was also found to turn green

TABLE I—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF ALCOHOLIC EXTRACT (1:10)[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	110.8	99.20	91.82	97.01	100.6	103.5
2	113.5	100.3	94.14	97.87	101.5	104.6
3	124.1	107.5	101.3	105.9	109.8	114.2
Mean % variation		−11.82	−17.52	−13.63	−10.42	−7.47
			Control			
1	118.4	117.1	116.6	114.7	114.2	113.1
2	115.9	114.8	114.4	112.6	112.1	111.6
3	112.2	111.1	110.7	108.8	108.2	107.2
Mean % variation		−1.00	−1.37	−3.00	−3.46	−4.21

[a] Dose, 25 ml./Kg.

TABLE II—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF AQUEOUS EXTRACT (1:10)[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	125.4	106.9	100.8	102.6	109.7	115.7
2	119.4	102.7	98.86	100.0	104.4	111.5
3	102.8	89.14	84.91	85.86	88.94	96.22
Mean % variation		−13.98	−18.08	−17.02	−12.86	−6.91
			Control			
1	116.3	115.6	114.5	113.1	112.8	111.9
2	114.6	113.3	111.7	108.8	109.6	108.5
3	105.7	104.8	103.9	102.8	102.6	100.2
Mean % variation		−0.86	−1.92	−3.52	−3.43	−4.67

[a] Dose, 25 ml./Kg.

TABLE III—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF CRUDE RESIN[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	105.7	98.80	93.60	93.10	95.40	99.60
2	113.7	103.3	97.45	98.23	103.5	105.5
3	110.2	101.5	94.63	97.82	99.70	101.6
Mean % variation		−7.73	−12.75	−11.63	−9.23	−6.81
			Control			
1	110.2	108.7	107.9	107.1	105.8	104.3
2	118.6	116.9	115.3	114.2	112.4	112.1
3	121.3	120.2	118.5	117.7	115.9	115.3
Mean % variation		−1.23	−2.39	−3.16	−4.55	−5.26

[a] Dose, 2 Gm./Kg.

TABLE IV—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF CRUDE ACID-INSOLUBLE RESIN[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	126.7	119.2	113.8	113.0	117.2	120.1
2	119.2	113.5	107.1	105.3	111.1	113.8
3	100.6	95.32	91.35	91.60	94.29	97.25
Mean % variation		−5.31	−9.50	−10.31	−6.85	−4.41
			Control			
1	115.6	114.8	114.6	112.3	110.7	109.4
2	123.3	122.5	121.8	119.8	118.6	118.1
3	121.7	120.4	120.1	118.6	117.8	117.3
Mean % variation		−0.80	−1.06	−2.71	−3.74	−4.39

[a] Dose, 2 Gm./Kg.

TABLE V—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF ETHER-INSOLUBLE PORTION OF RESIN[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	112.8	103.0	97.73	96.65	100.9	105.3
2	108.9	101.6	96.65	96.42	99.0	102.3
3	118.5	107.2	103.0	106.6	106.2	111.0
Mean % variation		−8.24	−12.56	−11.34	−9.84	−6.62
			Control			
1	119.6	118.4	117.8	116.9	116.6	114.8
2	128.3	126.9	126.1	124.3	122.8	122.5
3	107.5	106.1	105.4	104.7	103.9	103.1
Mean % variation		−1.13	−1.87	−2.65	−3.37	−4.20

[a] Dose, 2 Gm./Kg.

TABLE VI—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF SUBSTANCE *S* IN RATS[a]

Rat	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	123.9	106.2	95.66	99.27	104.1	111.4
2	105.3	90.48	82.93	84.85	87.54	93.63
3	119.8	104.2	93.28	98.43	101.8	108.9
Mean % variation		−13.79	−12.23	−19.06	−16.0	−10.07
			Control			
1	117.1	115.5	114.8	112.6	111.9	110.3
2	113.6	112.0	111.0	108.7	107.7	106.6
3	114.8	113.8	112.5	111.0	110.1	109.9
Mean % variation		−1.21	−2.08	−3.88	4.57	−5.55

[a] Dose, 0.5 Gm./Kg.

in a solution of ferric chloride and labeled as fraction P/methanol.

Both fraction P/alcohol and P/methanol exhibited the following colors: (*a*) ferric chloride solution, green; (*b*) magnesium and HCl, orange red; (*c*) boric acid and HCl, yellow; (*d*) sodium hydroxide solution, greenish yellow; (*e*) neutral lead acetate, yellow. As the above color reactions were characteristics of flavanoids (10), the method of Gupta and Seshadri (11) for the isolation of flavanoid compounds was adopted and one crystalline yellow product, m.p. 255–258°, was isolated. This fraction was also found to respond to all the color tests mentioned above. All the three fractions were then subjected to paper chromatography, and the chromatograms were developed with various reagents (10). The same color and R_f values were obtained with all the fractions. Mixed melting points of the different fractions did not show any depression. This indicated that they were identical. Microcombustion analysis gave the following results: C, 54.02; H, 3.88. These fractions were found to be inactive when tested for hypoglycemic activity and were not further investigated.

Studies with Resin Fractions—Both the resin fractions reported above were found to lower the blood sugar level of normal albino rats (Tables III and IV). But on purification this hypoglycemic property was found to have disappeared completely from both the fractions. However, the portion that did not go into solution in ether during fractionation of the resin was found to have hypoglycemic activity (Table V). This indicated that the hypoglycemic activity might be due to adsorption of substance *S* in the crude resin fractions. On fractionation by a chemical method (12), the following resin acids were isolated from the resin: (*a*) resin acid A, m.p. 147–164° (with frothing); (*b*) resin acid B_1, m.p. 85–91° (with frothing); (*c*) resin acid B_2, m.p. 81–132° (with frothing); (*d*) resin acid C, m.p. 144–150° (with frothing); (*e*) resin acid D, m.p. 130–144° (with frothing).

Identification of Reducing Sugar—The reducing sugar present in the drug was identified as arabinose by paper chromatography (13). This was done by making separate chromatograms of a pure sample of arabinose, fraction F, and a mixture of fraction F and added arabinose. After development, each showed only one spot at the same R_f value.

Hypoglycemic Studies—Healthy albino rats of the same breed and of nearly the same weight range were selected for carrying out the experiments. They were divided into two groups: one for control and the other for the drug. Food was withdrawn 18 hr. before starting the experiments, and no food was given during the experiment. The drug was given *per os*. Blood samples were taken from the tails of the animals and blood sugar was determined by the method of Folin and Wu (14). A color comparison was made in a Lumetron photoelectric colorimeter (Lumetron model 402EF). Of all the fractions tested, only the alcoholic and aqueous extracts, two crude resin fractions, and substance *S* were found to be active, and the results

TABLE VII—CONCENTRATION OF BLOOD SUGAR AFTER A SINGLE ORAL DOSE OF SUBSTANCE *S* IN RABBITS[a]

Rabbit	Initial Blood Sugar, mg./100 ml.	Blood Sugar, mg./100 ml. After 2 hr.	4 hr.	6 hr.	8 hr.	10 hr.
			Drug			
1	120.4	102.9	97.80	101.6	107.3	110.6
2	118.3	103.5	98.30	103.1	108.3	108.6
3	112.4	103.8	96.60	105.2	108.1	112.2
Mean % variation		−14.53	−19.12	−14.63	−10.68	−8.09
			Control			
1	113.8	112.7	112.3	111.5	110.4	109.9
2	116.5	115.0	114.4	113.3	112.8	112.4
3	121.1	119.0	118.3	117.9	117.3	116.3
Mean % variation		−1.32	−1.81	−2.44	−3.10	−3.64

[a] Dose, 0.5 Gm./Kg.

obtained with these fractions are given in the Tables I–VII.

From the observations recorded in the tables, it is quite evident that the hypoglycemic activity of the drug is due to substance *S* alone. Experiments to prove the antidiabetic activity of substance *S* are presently in progress.

SUMMARY

A number of fractions were isolated by a chemical method from the drug, *C. esculenta*. All of the isolated fractions were tested biologically for their hypoglycemic activity and only a few fractions, *viz.*, the two crude resin fractions and a water-soluble crystalline body *S*, showed positive results. On purification, both the resin fractions proved to be inactive, and this indicated that the activity might be due to adsorption of substance *S* in the crude resins. This was further confirmed by the fact that when the crude resin was extracted by ether the portion that did not go into solution in ether gave a positive response.

The chemical constitution and the antidiabetic activity of substance *S* will be presented in another paper.

REFERENCES

(1) Dymock, W., Warden, C. J. H., and Hooper, D., "Pharmacographia Indica," vol. II, Trubner and Co., London, England, 1891, p. 51.

(2) Kirtikar, K. R., and Basu, B. D., "Indian Medicinal Plants," vol. II, 2nd ed., Lalit Mohan Basu, Allahabad, India, 1935, p. 1092.

(3) Chopra, R. N., Nayar, S. L., and Chopra, I. C., "Glossary of Indian Medicinal Plants," 1st ed., Council of Scientific and Industrial Research, New Delhi, India, 1956, p. 53.

(4) Nadkarni, A. K., "Indian Materia Medica," vol. I, Popular Book Depot, Bombay, India, 1954, p. 281.

(5) Basu, N. K., and Choudhury, K. D., *Current Sci.*, **29**, 136(1960).

(6) Rosenthaler, L., "The Chemical Investigation of Plants," translated by Ghosh, S., G. Bell and Sons, Ltd., London, England, 1930, pp. 31–39.

(7) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1958, p. 884.

(8) Clarke, H. T., "A Hand Book of Organic Analysis," Edward Arnold Ltd., London, England, 1956, pp. 9, 91.

(9) Peack, K., and Tracey, M. V., "Moderne Methoden Der Pfanzenanalyse," vol. III, 1st ed., Springer Verlag, Berlin, Germany, 1955, pp. 312–315.

(10) *Ibid.*, pp. 467–474.

(11) Gupta, S. R., and Seshadri, T. R., *Proc. Indian Acad. Sci.*, 35A, 242(1952).

(12) "Allen's Commercial Organic Analysis," vol. IV, 5th ed., p. 220.

(13) Block, R. J., Emett, L. D., and Gunter, Z., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press Inc., New York, N. Y., 1958, pp. 170–214.

(14) Hawk, P. B., Oser, B. L., and Summerson, W. H., "Practical Physiological Chemistry," The Blackiston Co., Philadelphia and Toronto, 1946, p. 520.

Diffuse Reflectance Studies of Dye-Adjuvant Chemisorption

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A number of pharmaceutical adjuvants were screened by means of diffuse reflectance spectroscopy for possible solid–solid interaction with certified Red No. 3, Blue No. 1, and Yellow No. 5 dyes. Results indicate that a metallic or polyfunctional adsorbent molecule is necessary for these interactions. Furthermore, it was generally observed that the order of metallic adjuvant interaction with these dyes is $Mg^{2+} > Ca^{2+} > Zn^{2+} \backsim Al^{3+}$. Spectral and visual color changes, elution studies, and accelerated fading techniques support the reflectance data presented.

SEVERAL ARTICLES have recently appeared in the literature which point out drug–adjuvant interactions (1, 2) and the need for prescreening of the excipients used in the preparation of solid dosage forms. Since such interactions may be responsible for the various therapeutic discrepancies reported in related formulations, these interactions may also be of significant importance in color and drug stability.

EXPERIMENTAL

Reagents—FD&C Red No. 3 dye and aluminum lake (National Aniline), FD&C Blue No. 1 dye (National Aniline), FD&C Yellow No. 5 dye and aluminum lake (Warner-Jenkinson Mfg. Co.), acacia, aluminum hydroxide, aluminum stearate, calcium hydroxide, cellulose acetate phthalate, citric acid, lactose, magnesium citrate, magnesium hydroxide, pectin, polyethylene glycol 6000 (Carbide and Corton Chemical Corp.), starch U.S.P., titanium dioxide, zinc oxide, zinc sulfate.

Apparatus—Beckman DU spectrophotometer with a diffuse reflectance attachment, constant-temperature water bath set at 30 ± 0.5° with rotating spindles (1), assorted round amber bottles from 30 to 200 ml. with caps, Parafilm (Marathon Co.), aluminum foil (Reynolds), vacuum oven (National Appliance Co.), glass desiccator, lyophilizer (National Research Corp.) attached to a refrigeration system (Webber Refrigeration).

PROCEDURES

Equilibration Technique—The general method of studying these interactions involves equilibrating a weighed amount of adsorbent for 24 hr. with distilled water or an organic equilibration medium. After equilibration the dispersion medium is removed either by vacuum filtration followed by vacuum drying at 35° for 24 hr., by vacuum evaporation from a glass desiccator, or by freezing the aqueous media with the aid of a dry ice–methanol mixture and lyophilization for 24 hr. The particular drying method used is described under *Results and Discussion*.

Preparation of the Sample—A specified amount of pre-equilibrated dye and adsorbent (pharmaceutical adjuvant) are weighed. The powders are then placed in a suitable amber bottle and 20 ml. of the dispersion media is added. The bottle is covered with Parafilm or aluminum foil, capped, and equilibrated for 24 hr. at 30 ± 0.5° in order to effect interaction. After equilibration, one of the following three techniques is employed in order to dry the samples.

Vacuum Filtration and Drying—Used for samples with aqueous dispersion media reported in Tables I and II. The suspension is vacuum filtered after equilibration and the powder is dried in a vacuum oven at 35° for 24 hr. The resulting dry powder is then triturated, and its diffuse reflectance (DRS) measured using a magnesium carbonate reference standard.

Evaporation by Vacuum—The equilibrated solution or suspension is placed in an evaporation dish and placed in a vacuum desiccator. The organic solvent is removed at room temperature, and the resulting powder is triturated and its DRS measured.

Drying by Lyophilization—This process is used exclusively in all the samples reported in Table II. The equilibrated aqueous solution or suspension is frozen in a dry ice–methanol mixture and lyophilized for 24 hr. The resulting powder is triturated and its DRS measured.

Preparation of the Control—A specified weight of the previously equilibrated and dried adsorbent is physically mixed with an indicated amount of pre-equilibrated and dried adsorbate using a mortar and pestle. The DRS of this control is then measured using a magnesium carbonate reference standard.

SPECIAL TECHNIQUES

Washing the Sample—The dry powdered sample is placed in a 51-mm. Büchner funnel and 200 ml. of distilled water is added and removed by vacuum filtration. The resultant powder is dried and its DRS measured.

Accelerated Light Studies—The powder under study is packed into an inverted 4-cm. diameter bottle cap and placed into an Envira-Lite cabinet (Thermal Research, Inc.) which houses 10 Westinghouse F-40 CW/RFL fluorescent lamps and 4 GE lumiline No. L60 IF/T8 incandescent lamps and is

Received April 14, 1967, from the Research Center, Pitman-Moore Division of the Dow Chemical Co., Zionsville, IN 46077

Accepted for publication July 12, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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TABLE I—FD&C RED NO. 3 DYES OR LAKES: DYE OR LAKE REACTIONS WITH PHARMACEUTICAL ADJUVANTS USING AN EXCESS OF COLORING MATTER

Dye or Lake + Adjuvant	Concn., Dye:Adjuvant	Equilibration Media	Method of Solvent Removal	Relative Size of Spectral Changes	Visual Color Changes (Control to Sample)
Dye + TiO_2	200 mg.:4.00 Gm.	EtOH	Vacuum evaporation	No significant change	White to off white
Dye + TiO_2	200 mg.:4.00 Gm.	H_2O	Vacuum filter, wash and dry	Medium change	White to white
Dye + starch	100 mg.:4.00 Gm.	Dil. EtOH (1:1)	Vacuum evaporation	Small change	Light pink to pink
Lake + starch	100 mg.:4.00 Gm.	Dil. EtOH (1:1)	Vacuum evaporation	No significant change	Light pink to light pink
Dye + lactose	200 mg.:4.00 Gm.	EtOH	Vacuum evaporation	No significant change	Light pink to light pink
Dye + CAP	200 mg.:4.00 Gm.	H_2O	Vacuum filter, wash and dry	Small change	Lavender with white specks to fire engine red (heterogeneous)
Dye + CAP	200 mg.:4.00 Gm.	EtOH	Vacuum evaporation	Small change	Lavender with white specks to fire engine red, solid mass
Dye + PEG 6000	200 mg.:4.00 Gm.	Acetone	Vacuum evaporation	Small change	Light violet to deep, fluorescent red

TABLE II—FD&C YELLOW NO. 5 DYES OR LAKES: DYE OR LAKE REACTIONS WITH PHARMACEUTICAL ADJUVANTS USING AN EXCESS OF COLORING MATTER

Dye or Lake + Adjuvant	Concn., Dye:Adjuvant	Equilibration Media	Method of Solvent Removal	Relative Size of Spectral Changes	Visual Color Changes (Control to Sample)
Dye + Al stearate	200 mg.:2.00 Gm.	H_2O	Vacuum filter, wash and dry	No significant change	Heterogeneous whitish pink to light yellow
Dye + $ZnSO_4$	100 mg.:4.00 Gm.	EtOH	Vacuum evaporation	Small change	Orange to intense yellow
Dye + ZnO	200 mg.:4.00 Gm.	H_2O	Vacuum filter, wash and dry	No significant change	Off white to off white
Dye + Mg citrate	100 mg.:4.00 Gm.	$CHCl_3$	Vacuum evaporation	Large change	Light peach to intense yellow
Dye + citric acid	100 mg.:4.00 Gm.	$CHCl_3$	Vacuum evaporation	Small change	Orange to yellow-gold
Dye + TiO_2	200 mg.:4.00 Gm.	H_2O	Vacuum filter, wash and dry	No significant change	White with red specks to white
Dye + starch	100 mg.:4.00 Gm.	Dil. EtOH (1:1)	Vacuum evaporation	Medium change	Pink to bright yellow
Lake + starch	100 mg.:4.00 Gm.	Dil. EtOH (1:1)	Vacuum filtration	Small change	Pink to bright yellow
Dye + lactose	200 mg.:4.00 Gm.	EtOH	Vacuum evaporation	No significant change	Orange to heterogeneous orange with dark pink crystals
Lake + CAP	100 mg.:4.00 Gm.	H_2O	Vacuum filter, wash and dry	No significant change	Yellow to yellow
Dye + PEG 6000	200 mg.:4.00 Gm.	$CHCl_3$	Vacuum evaporation	Increased reflectance	Heterogeneous light orange to orange
Dye + PEG 6000	200 mg.:4.00 Gm.	Acetone	Vacuum evaporation	No significant change	Heterogeneous light orange to deep yellow
Dye + acacia	100 mg.:4.00 Gm.	EtOH	Vacuum evaporation	Medium change	Heterogeneous light orange to intense orange
Lake + acacia	50 mg.:4.00 Gm.	EtOH	Vacuum evaporation	No significant change	Yellow to yellow
Dye + pectin	100 mg.:4.00 Gm.	Dil. EtOH (1:1)	Vacuum evaporation	Increased reflectance	Golden yellow to golden yellow

equipped with a rheostat dimmer and fan. The light intensity used throughout this study was 2,000 foot candles (f.c.) and the temperature within the cabinet was 32 ± 3°.

RESULTS AND DISCUSSION

Although it is generally acknowledged that the most common type of dye instability in pharmaceutical and cosmetic systems is due in part to light and thermal rearrangements, it is possible that some of these color changes occurring in solid dosage formulations may be due to dye–excipient interactions (1). During the past 10 years some attention has been given to color stability in pharmaceutical dosage forms (3, 4). A number of reports have appeared in the literature dealing with studies to elucidate the nature of this interaction, the effect of excipients with respect to fading, and methods employed to retard such an interaction (5–8). Since most of the reported investigations dealing with this dye–adjuvant interaction have been studied in solution, very little information is available concerning the degree of this interaction observed with diffuse reflectance techniques.

Since diffuse reflectance spectroscopy (DRS) has been shown to be a useful tool for interaction studies in the solid state (1), this technique was employed to further study dye–adjuvant interactions.

In this present study, various certified dyes were screened with a large group of adjuvants for the existence of such interactions. The dyes, FD&C Red No. 3, Blue No. 1, and Yellow No. 5, were selected either on the basis of their chemical structure, light instability, or use in pharmaceuticals. The adjuvants were chosen on the basis of the metal fraction or polyfunctional groups they contain (8) or because of their use in medicinal dosage forms.

As has been pointed out in previous communications (9–11) the adsorbate/adsorbent ratio must be carefully considered in order to observe these solid–solid interactions; if an excess of adsorbate is used, the monomolecular chemisorption occurring at the surface of the adsorbent is masked by subsequent

physical layers of the adsorbate, and the DRS spectrum approaches that of the pure dye under investigation. In this preliminary dye–adjuvant interaction study, 50 to 200 mg. of dye was used with 4.00 Gm. adjuvant samples. The controls, reported in Tables I and II, were prepared by physically mixing the dye with pre-equilibrated and vacuum dried adjuvants; the samples represent the result of equilibrating dye–adjuvant combinations in the designated equilibration media, followed by either vacuum evaporation of the solvent at room temperature or removal of the solvent by vacuum filtration, washing of this sample, and vacuum drying at about 35° for 24 hr.

An examination of the data reported in Tables I and II suggests that these dyes either undergo little interaction or that the dye–adjuvant ratio was such as to completely mask any effects due to chemisorption (unimolecular layer). The absorbance behavior of multilayers of the dye on these excipient particles becomes similar to that of the pure dye (1).

The spectral changes observed with Red No. 3 dye after equilibration with various adjuvants (see Table I) were small and correlate well with the lack of visual color changes observed. It is interesting to point out, however, that the spectral changes observed as a result of equilibrating Yellow No. 5 dye with starch or magnesium citrate did produce significant spectral and visual color changes at these high dye–adjuvant concentration ratios, suggesting chemisorption. Figure 1 illustrates this Yellow No. 5 dye–starch interaction. It is clearly seen that the absorbance and visual color change of the sample (see Table II) along with a new band formation at 315 mμ is indicative of a strong interaction. It is also interesting to note here that the interaction of the corresponding Yellow No. 5 lake with starch is much smaller, suggesting that a number of the adsorption sites are not available in the aluminum lake.

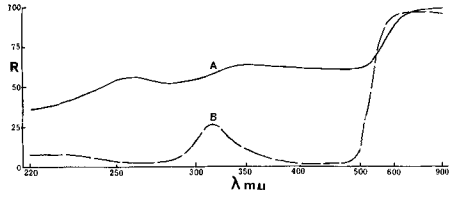

Fig. 1—DRS of FD&C Yellow No. 5 dye (100 mg.) and starch (4.00 Gm.). Key: A, *control;* B, *sample equilibrated in 30 ml. dilute ethanol (1:1).*

As has already been mentioned, spectral changes due to chemisorption are readily seen when a single layer of the dye is chemisorbed onto the surface of the adsorbent (8,9). Since the dye–adjuvant ratios employed in the initial phase of this investigation were high, these interactions were again studied, using lesser quantities of the dye. Dye concentrations of 30 mg./10.00 Gm. of adjuvant were found suitable in exhibiting this chemisorption effect.

In order that all experimental conditions be kept constant, the controls in this phase of study were prepared by separately equilibrating the dye and adjuvant in distilled water for a period of 24 hr., followed by lyophilization to remove the solvent media. The appropriate concentration of dye and adjuvant was then weighed and physically mixed by geometric dilution, and the diffuse reflectance spectrum (DRS) of this control was measured, using a magnesium carbonate reference standard. Interacted samples were prepared by placing a weighed amount of pre-lyophilized dye and adjuvant in a suitable container, adding distilled water, and equilibrating the mixture for 24 hr. This interacted sample was then lyophilized and triturated and its DRS was obtained. Although the degree of spectral change depends on both the dye and excipient under investigation, "no significant change," reported in the tables, will be defined as spectral intensity changes of 10 reflectance units (RU) or less. A "small change" will generally indicate intensity changes of 10–30 RU, whereas a "medium change" implies a spectral change of about 30–60 RU; a "large change" indicates hyperchromic changes of 60 or more reflectance units at the maximum wavelength. It is interesting to point out that the aqueous solvent serves as a dispersion medium in order to facilitate maximal interaction with the sites available. These interactions will also occur in the solid unequilibrated state in the presence of moisture (10).

Tables III, IV, and V summarize the data obtained in this study, using the structurally different certified Red No. 3, Blue No. 1, and Yellow No. 5 dyes. The spectral changes observed in these low dye concentration systems again correlate well with visual color changes observed. Typical interaction spectra of several Red No. 3–adjuvant systems are presented in Figs. 2–4. An examination of these figures certainly illustrates the high degree of interaction as evidenced by the large absorbance and other spectral differences of the equilibrated sample as compared with the control. The strength of these interactions with various adjuvants can also be demonstrated with the use of an elution technique. For example, the dye in the Red No. 3–$Al(OH)_3$ or Red No. 3–ZnO system exhibiting small or medium-sized spectral changes (Fig. 4) was readily eluted with water. In comparison, starch or $Mg(OH)_2$ interactions with this same dye, presented in Figs. 2 and 3, did not undergo significant spectral changes after the sample was washed with distilled water, indicating a stronger interaction.

It should be pointed out, however, that in spite of the fact that a dye can be readily desorbed from an excipient, indicating a relatively weaker adsorption, the interaction is nevertheless important since the color stability of the intact dosage form may be affected by this weaker interaction. In contrast, desorption of a medicinal agent from an adjuvant material is extremely important in that the therapeutic availability of this agent is directly related to this desorption phenomenon or chemical interaction.

The strength of these interactions was sometimes further correlated by subjecting the equilibrated sample to accelerated light fading conditions. For example it is seen that under these conditions, the color intensity of a Red No. 3–$Al(OH)_3$ equilibrated sample undergoes 61% increased reflectance. This change occurs at the $\lambda_{max.}$ after a 42-hr. exposure to 2,000 f.c. artificial light. On the other hand, the Red No. 3–$Mg(OH)_2$ equilibrated sample exhibits approximately 17% increased reflectance under the same conditions. This slower rate of fading or dye decomposition in the dye–$Mg(OH)_2$ system may be indicative of a stronger chemisorbed system. This is

TABLE III—FD&C RED NO. 3 DYE: DYE REACTIONS WITH PHARMACEUTICAL ADJUVANTS (CONCENTRATION: 30 mg. DYE/10.00 Gm. ADJUVANT)

Red No. 3 Dye + Adjuvant	Relative Size of Spectral Changes	Visual Color Changes (Control to Sample)	Effect of Washing Sample with 200 ml. Water	Sample Exposure to Artificial Light, 2,000 f.c.	
				42 hr.	184 hr.
$Al(OH)_3$	Small to medium change	Light pink to light pink	Eluted; spectrum approaches adjuvant	Increase of 21 RU or 61% fading[a]	Increase of 34 RU or 95% fading
$Ca(OH)_2$	Medium to large change	Light pink to pink	No elution	Increase of 16 RU or 22% fading	
$Mg(OH)_2$	Large change	Light pink to lavender	No elution	Increase of 12 RU or 17% fading	Increase of 29 RU or 41% fading
ZnO	Medium change	Faint pink to sharp pink	Eluted		
$ZnSO_4$	No significant change	Light pink to light pink			
Mg citrate	Small change	Pink to reddish-pink			
Citric acid	No significant change	Light pink to whitish pink			
Starch	Large change	Light pink to pink	No elution	Increase of 27 RU or 42% fading	Increase of 49 RU or 78% fading
Lactose	Medium change	Light pink to rosy red			Increase of 2 RU or 4% fading
Acacia	Small to medium change	Light pink to pink			

[a] % Sample fading at $\lambda_{max.} = \frac{R_{faded\ sample} - R_{sample}}{R_{control} - R_{sample}} \times 100.$

TABLE IV—FD&C BLUE NO. 1 DYE: DYE REACTIONS WITH PHARMACEUTICAL ADJUVANTS (CONCENTRATION: 30 mg. DYE/10.00 Gm. ADJUVANT)

Blue No. 1 Dye + Adjuvant	Relative Size of Spectral Changes	Visual Color Changes (Control to Sample)	Effect of Washing Sample with 200 ml. Water	Sample Exposure to 42 hr. Artificial Light at 2,000 f.c.
$Al(OH)_3$	Medium change	Powder blue to bluish-gray	Eluted	Increase of 14 RU or 31% fading
$Ca(OH)_2$	Medium change	Gray to light lavender	Eluted	Increase of 12 RU or 30% fading
$Mg(OH)_2$	Large change	Faint blue to deep blue	Little elution	Increase of 3 RU or 3% fading
ZnO	Medium to large change	Powder blue to blue	Eluted	
$ZnSO_4$	Large change	Light blue to sky blue		
Mg citrate	Large change	Light blue to dark blue-green		
Citric acid	No significant to small change	Light blue to powder blue		
Starch	Large change	Light blue to blue	Little elution	Increase of 1 RU or 1% fading
Lactose	Large change	Light blue to blue-green		
Acacia	Large change	Gray to deep blue		

TABLE V—FD&C YELLOW NO. 5 DYE: DYE REACTIONS WITH PHARMACEUTICAL ADJUVANTS (CONCENTRATION: 30 mg. DYE/10.00 Gm. ADJUVANT)

Yellow No. 5 Dye + Adjuvant	Relative Size of Spectral Changes	Visual Color Changes (Control to Sample)	Effect of Washing Sample with 200 ml. Water	Sample Exposure to 42 hr. Artificial Light at 2,000 f.c.
$Al(OH)_3$	Medium change	Off white to yellow	Eluted; spectrum approaches adjuvant	No significant change
$Ca(OH)_2$	Medium change	Off white to yellow	About $^2/_5$ eluted	Increase of 7 RU or 11% fading
$Mg(OH)_2$	Large change	Off white to golden yellow	No significant elution evident	No significant change
ZnO	Medium change	Off white to yellow	Eluted	
$ZnSO_4$	Medium change	Faint yellow to light orange-yellow		
Mg citrate	Large change	Faint yellow to canary yellow		
Citric acid	Small to medium change	Light pink to intense gold		
Starch	Large change	Faint yellow to darkish yellow	Little elution	No significant change
Lactose	Large change	White to deep yellow		
Acacia	Large change	Off white to bright yellow		

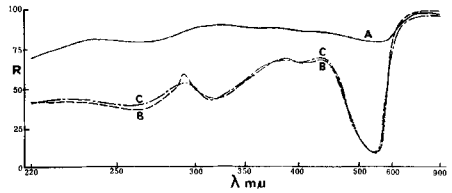

Fig. 2—DRS of FD&C Red No. 3 dye (30 mg.) and magnesium hydroxide (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample washed with 200 ml. distilled water.*

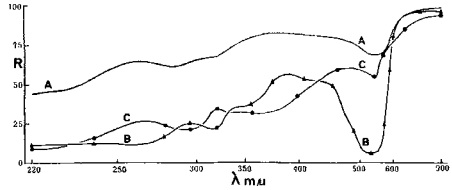

Fig. 3—DRS of FD&C Red No. 3 dye (30 mg.) and starch (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample exposed to 184 hr. artificial light at 2,000 f.c.*

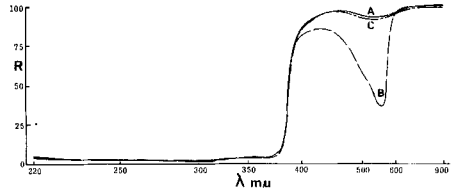

Fig. 4—DRS of FD&C Red No. 3 dye (30 mg.) and zinc oxide (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample washed with 200 ml. distilled water.*

clearly substantiated in the comparison of Blue No. 1 dye fading of starch and $Ca(OH)_2$ equilibrated samples presented in Figs. 5 and 6 and Table IV. These results indicate the Blue No. 1 fades about 1% in a dye–starch chemisorbed sample as compared to 30% fading in the dye–$Ca(OH)_2$ equilibrated sample, exposed to similar accelerated light conditions.

It is also interesting to note that although lactose shows comparable spectral interaction changes with the dyes to that of starch, its fading characteristics are somewhat different. For example, Fig. 7C, which represents an equilibrated Red No. 3–lactose system, shows little fading tendency even after 184-hr. exposure to artificial light at 2,000 f.c.; a Red No. 3–starch sample fades extensively under the same conditions, as illustrated in Fig. 3C. These differences show the complexity of these interactions and the need for individual dye–excipient study.

Although the structure of the dyes is quite different, suggesting different binding sites, it is nevertheless of interest to point out that the interaction obtained is dependent on both the dye and adjuvant employed. For example, Figs. 8 and 9 as well as Tables III, IV, and V illustrate that zinc sulfate reactions are markedly different with the dyes studied. Equilibration of this excipient with Red No. 3 dye, containing a sodium salt of a carboxyl group, but lacking phenylsulfonic acid functional groups,

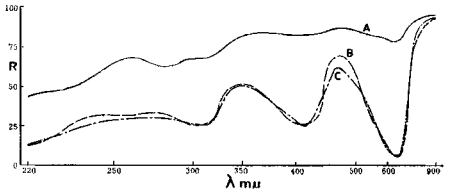

Fig. 5—DRS of FD&C Blue No. 1 (30 mg.) and starch (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample exposed to 42 hr. artificial light at 2,000 f.c.*

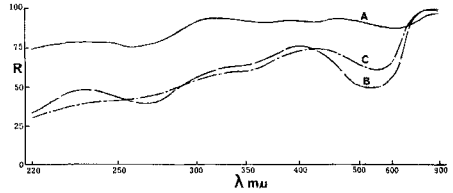

Fig. 6—DRS of FD&C Blue No. 1 dye (30 mg.) and calcium hydroxide (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample exposed to 42 hr. artificial light at 2,000 f.c.*

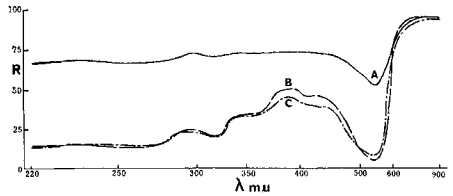

Fig. 7—DRS of FD&C Red No. 3 dye (30 mg.) and lactose (10.00 Gm.). Key: A, *control;* B, *sample;* C, *sample exposed to 184 hr. artificial light at 2,000 f.c.*

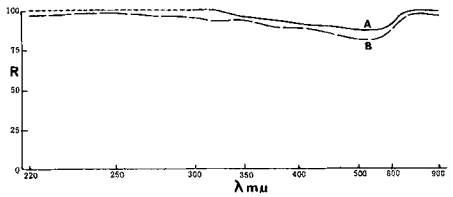

Fig. 8—DRS of FD&C Red No. 3 dye (30 mg.) and zinc sulfate (10.00 Gm.). Key: A, *control;* B, *sample.*

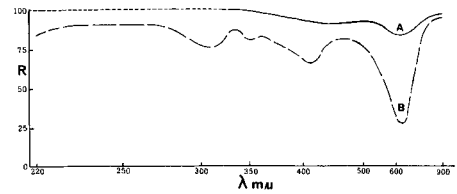

Fig. 9—DRS of FD&C Blue No. 1 dye (30 mg.) and zinc sulfate (10.00 Gm.). Key: A, *control;* B, *sample.*

found in the other two dyes under investigation, results in only very minor spectral changes, as seen in

Fig. 8. In contrast, $ZnSO_4$ does react to a large extent with Blue No. 1 dye, containing three phenylsulfonic acid groups, as well as with FD&C Yellow No. 5 which also contains a phenylsulfonic acid group, one carboxyl, and one hydroxyl functional group. These phenylsulfonic acid groups may therefore be involved in the observed chemisorption.

Furthermore, the same dye has been shown to react differently, depending on the excipient under investigation. For example, the strength of $Mg(OH)_2$, $Ca(OH)_2$, and $Al(OH)_3$ interaction with Yellow No. 5 dye is markedly different as observed in Figs. 10, 11, and 12, as well as Table V. An examination of these figures indicates that dye–$Mg(OH)_2$ equilibration facilitates a large decreased reflectance of about 74 units at the $\lambda_{max.}$ of 435 mμ as reported in Fig. 10B; the strength of this large interaction is further supported with the aid of elution studies presented in Fig. 10C. Although $Ca(OH)_2$ equilibration with this yellow dye does produce a 59 reflectance unit decrease at the $\lambda_{max.}$ of 410 mμ, seen in Fig. 11B and Table V, aqueous washing of this sample indicates that about 40% of the dye can be eluted. It is also interesting to point out that although Yellow No. 5 dye equilibration with $Al(OH)_3$ also produces a 47 reflectance unit decrease at the $\lambda_{max.}$ of 400 mμ, Fig. 12, this dye is eluted to a much greater extent from the surface of the adsorbent with aqueous washing of the sample. The above bathochromic and hyperchromic spectral changes again indicate that reactivity differences of the metallic ion present in these hydroxide adjuvants is $Mg^{2+} > Ca^{2+} > Al^{3+}$.

With regard to these interactions, although some systems may not exhibit visual color changes after equilibration, the spectral changes observed in the ultraviolet region may be significant in the photodecomposition of a particular dye.

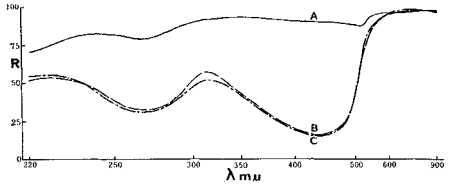

Fig. 10—DRS of FD&C Yellow No. 5 dye (30 mg.) and magnesium hydroxide (10.00 Gm.). Key: A, control; B, sample; C, sample washed with 200 ml. distilled water.

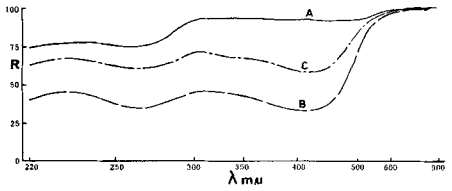

Fig. 11—DRS of FD&C Yellow No. 5 dye (30 mg.) and calcium hydroxide (10.00 Gm.). Key: A, control; B, sample; C, sample washed with 200 ml. distilled water.

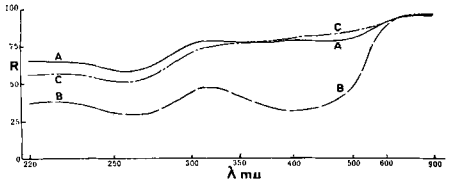

Fig. 12—DRS of FD&C Yellow No. 5 dye (30 mg.) and aluminum hydroxide (10.00 Gm.). Key: A, control; B, sample; C, sample washed with 200 ml. distilled water.

CONCLUSION

The data presented here indicate that the "inert" materials, including starch, lactose, and acacia, do undergo significant interactions with the dyes investigated. Furthermore, various metal-containing adjuvants also chemisorb with certified dyes; the order of chemisorption capacity of the metallic adjuvants with certified dyes was as follows: $Mg^{2+} > Ca^{2+} > Zn^{2+} \backsim Al^{3+}$. These dye–adjuvant interactions may be responsible for the various color problems encountered in solid pharmaceutical formulations.

It appears that the mechanism involved in these interactions is a combination of a chelation type of interaction and one of chemisorption. This is particularly true where the excipient material does not undergo ionization. It is apparent that these solid–solid interactions are of importance, not only in drug–adjuvant studies, but also in the investigation of color and drug stability.

These studies and those previously conducted with respect to drug–excipient interactions point out the need for incompatibility studies of drug or dye–adjuvant interactions in conjunction with other considerations in the formulation of pharmaceuticals.

REFERENCES

(1) Lach, J. L., and Bornstein, M., *J. Pharm. Sci.*, **54**, 1730(1965).
(2) Bornstein, M., and Lach, J. L., *ibid.*, **55**, 1033(1966).
(3) Lachman, L., Weinstein, S., Swartz, C. J., Urbanyi, T., and Cooper, J., *ibid.*, **50**, 141(1961).
(4) Goodhart, F. W., Everhard, M. E., and Dickcius, D. A., *ibid.*, **53**, 338(1964).
(5) Lachman, L., Kuramoto, R., and Cooper, J., *J. Am Pharm. Assoc., Sci. Ed.*, **47**, 871(1958).
(6) Scott, M. W., Goudie, A. J., and Huetteman, A. J., *ibid.*, **49**, 467(1960).
(7) Kuramoto, R., Lachman, L., and Cooper, J., *ibid.*, **47**, 175(1958).
(8) Brownley, C. A., Jr., and Lachman, L., *J. Pharm. Sci.*, **52**, 86(1963).
(9) Kortum, G., Braun, W., and Herzog, G., *Angew. Chem., Intern. Ed. Engl.*, **2**, 333(1963).
(10) Lach, J. L., and Bornstein, M., *J. Pharm. Sci.*, **55**, 1040(1966).
(11) Lieu, V. T., and Frodyma, M. M., *Talanta*, **13**, 1319 (1966).

Effect of Preservatives on the Heat Sterilization of Sesame Oil

By ROBERT L. ROBISON* and MELVIN H. WEINSWIG†

There is little information available concerning heat sterilization of oils containing antibacterial agents. Two species of thermoresistant bacterial spores, *B. subtilis* and *B. stearothermophilus,* were used as positive controls to determine requirements for the dry heat sterilization of sesame oil containing 0.5 per cent phenol and 2 per cent benzyl alcohol. Sesame oil, polyethylene glycol (PEG), and air controls were also studied. Spores were impregnated on filter disks, dried, and sterilized in oil. Data were collected in the range of 100–160° at 0.5-hr. intervals to 2 hr. Phenol significantly reduced the sterilization requirements for *B. subtilis;* benzyl alcohol gave variable results. Controls survived higher temperatures in air than in oils. The sterilization requirements for *B. stearothermophilus* were not altered by the presence of phenol or benzyl alcohol. Sterilization in oils required higher temperatures than in air. Spores of both organisms were killed at lower temperatures in PEG than in the oils.

THE VIEW is widely held that most antibacterial agents have limited activity in anhydrous systems. Under normal conditions and within the context of the usage of preservative agents in oil vehicle injectables, there is ample evidence to support the premise that preservative agents are, indeed, ineffective if water is absent (1, 2).

Injectable grade fixed vegetable oils are normally anhydrous or contain only traces of moisture and it is unlikely that any currently acceptable parenteral preservative is of value in the usual sense unless microbial contamination is introduced into an ampul in a moist condition. The effectiveness of the preservative agent would be dependent upon its transfer from the oil to the aqueous phase where it would exert an effect if time and concentration values were adequate (3).

In an aqueous medium, the activity of antibacterial agents generally increases with rises in temperature. This increased activity is utilized in the sterilization process known as "heating with a bactericide" (4).

There is little information relating to the activity of antibacterial agents in oils at higher temperatures. This is probably due to the reported ineffectiveness of preservatives in oils which have been tested at ambient temperatures.

In reference to the sterilization of nonaqueous injections, the U.S.P. (5) states "non-aqueous injections in sealed containers may fail to be sterilized if the customary temperature and time are used, since in the absence of water vapor within the container the processing is only the equivalent of dry heat sterilization. Processing of such solutions must be accomplished by heating at higher temperatures in a dry heat sterilizer, the cycle being dependent upon the stability of the particular solution. The process should be selected only on the basis of previously demonstrated ability to sterilize representative samples of such products which have been inoculated with thermoresistant spores." The U.S.P. indicates that 2 hr. at 160° is a suitable dry heat sterilization cycle.

The requirements for the dry heat sterilization of injectable oils have not been studied extensively. Pasquale *et al.* (6) determined the time and temperature requirements for the sterilization of unpreserved vegetable oils inoculated with the spores of 3 species of aerobic bacilli and found that the requirements varied both with the species and the oils used. The maximum survival time observed was for *B. subtilis* in sesame oil and in corn oil, being 2 hr. at 150°. Perkins (7) recommends that oils be sterilized by heating for 2 hr. at 160°, the timing to start after the oil has reached full sterilization temperature. The B. P. recommends oils be sterilized by holding for 1 hr. at 150° (4).

It was the purpose of this investigation to determine if two commonly used preservatives would lower the time and temperature requirements for the dry heat sterilization of a commonly used injectable oil containing thermoresistant spores.

Received April 14, 1967, from the †College of Pharmacy, Butler University, Indianapolis, IN 46207, and the *Lilly Research Laboratories, Indianapolis, IN 46206

Accepted for publication July 17, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted from a thesis prepared by Robert L. Robison for the Graduate School, Butler University, in partial fulfillment of Master of Science degree requirements.

The authors are indebted to the Lilly Research Laboratories for generously supplying materials and facilities for this investigation. The authors express their appreciation to Dr. H. Allisbaugh, Eli Lilly and Co., and to Mr. R. J. Russomanno, Hoffmann-LaRoche, Inc., for the spore cultures. Special gratitude is expressed to Mr. R. P. Roman and to Dr. H. Campbell, Jr., Eli Lilly and Co., for their valued advice and technical assistance.

EXPERIMENTAL

Equipment—A circulating hot air oven[1] was used for all dry heat sterilization tests. The temperature of the oil was monitored with a battery operated potentiometer[2] with copper-constantan thermocouples.

Materials—Sesame oil U.S.P.,[3] polyethylene glycol 300,[4] polyethylene glycol 400,[5] phenol reagent, and benzyl alcohol reagent.

Organisms—A preliminary series of experiments was conducted to obtain data on the dry heat resistance characteristics of five strains of nonpathogenic aerobic bacilli. Four strains of mesophilic spore formers and a thermophilic strain were evaluated.

Mesophiles—Three strains of *Bacillus subtilis* and one strain of *Bacillus cereus* were obtained from the following sources: a spore suspension of *B. subtilis* from Dr. H. Allisbaugh, Eli Lilly and Co. (HA), a lyophilized spore culture of *B. subtilis* from Mr. R. Russomanno, Hoffmann-LaRoche, Inc. (HL), a lyophilized spore culture of *B. subtilis* (ATCC 9372), and a spore suspension of *B. cereus* (ATCC 13061) from Mr. R. P. Roman, Eli Lilly and Co. The spore suspensions were used as received. The lyophilized cultures were inoculated into media to grow a suitable number of spores for the study.

Thermophile—*Bacillus stearothermophilus* was obtained by culturing a commercial autoclave control[6] which contained spores of this organism. The strain has been identified in the literature as NIH No. 7953 (8).

Growth and Harvest of Spores—*Mesophiles*—The *B. subtilis* (HL) lyophilized culture was inoculated into fluid thioglycollate medium U.S.P. (BBL) from which a 24-hr. culture was transferred to nutrient agar and held 7 days at 30–32°. The agar culture was then held at room temperature for 1 week during which sporulation became nearly complete. A spore suspension was prepared by washing off the growth with normal saline. The suspension was heated in a water bath at 80° for 15 min. to kill any vegetative cells remaining. A count by the most probable number (MPN) method (9) in fluid thioglycollate indicated 240 million viable spores per ml.

A 20-day-old fluid thioglycollate culture of *B. subtilis* (ATCC 9372) was harvested by centrifuging and resuspending three times in sterile distilled water. The suspension which contained approximately 60% spores was heated for 15 min. at 80° to kill the remaining vegetative cells. The MPN count in fluid thioglycollate was approximately 70 million viable spores per ml.

Thermophile—*B. stearothermophilus* spore strips were transferred into fluid thioglycollate and incubated for 48 hr. at 60°. The growth was harvested by centrifuging and washing three times with distilled water. The cell suspension which was comprised mostly of vegetative cells with a low percentage of spores was boiled 30 min. in a water bath. An inoculum from the heated suspension was then transferred two times in fluid thioglycollate followed each time by centrifuging, washing, and heating the cell suspension as before. The percentage of sporulation increased with each subculture and was approximately 50% on the third transfer. This procedure seemed to produce more synchronous sporulation and uniform morphology than other methods investigated and was perhaps a variant selective mechanism. Water replacement techniques (10) followed by agitation on a rotary shaker were unsuccessful as there was a marked tendency for this procedure to produce variable spore forms with low viable counts.

Preparation of Spore Disks—One-half inch filter paper disks[7] were sterilized by heating overnight at 105°. The sterile disks were then impregnated with appropriate dilutions of the spore suspensions using a 0.1-ml. capillary pipet. The disks were impregnated in a sterile enclosed stainless steel hood to prevent incidental air-borne contamination of the disks. They were then stored over silica gel in a desiccator for a minimum of 48 hr. prior to initial heat resistance studies.

Initial Resistance Studies—*Mesophiles*—Dried spore disks were placed in screw-cap bottles and heated in the preheated oven at 130° in 0.5-hr. increments to 2 hr. Disks were removed at the designated intervals and tested for growth in fluid thioglycollate medium U.S.P.

The results as shown in Table I indicated disks prepared from the *B. subtilis* (HL) and ATCC 9372 strains were the most resistant to dry heat.

Further evaluation of *B. subtilis* (HL) and *B. subtilis* ATCC 9372 indicated that the ATCC 9372 strain was somewhat more resistant. When drying was extended to 6 days the spores of ATCC 9372 survived 2 hr. at 130° while the spores of *B. subtilis* (HL) became less heat resistant. Results in tests described later show even greater resistance for the ATCC 9372 strain.

Initial Resistance Studies—*Thermophile*—The freshly harvested spore suspension of *B. stearothermophilus* was heated in a boiling water bath for 1 hr. to kill vegetative cells. The viable count by the MPN method was approximately 3,500,000 spores per ml. Dilutions of this stock suspension were made for the preparation of the disks.

A series of experiments were conducted with the spore disks to determine the proper load, the effect of drying on the resistance, and a suitable recovery medium for the spores of *B. stearothermophilus*. Since the spores of this organism are primarily used as an autoclave control, it was thought that it would also be of interest to compare its relative resistance to moist and dry heat.

Preliminary studies were conducted with disks containing approximately 50,000 spores each. In the initial experiment, the disks were dried for 2 days before testing for heat resistance and were cultured in fluid thioglycollate only. A second experiment was conducted with disks that had been dried for 6 days. Fluid thioglycollate with and without the addition of 0.1% soluble starch were used as recovery media.

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[1] Young Brothers circulating hot air oven with a Leeds and Northrup Micromax controller-recorder.
[2] Leeds and Northrup battery potentiometer, single range.
[3] Magnus, Maybee, and Reynard, Inc.
[4] Marketed as Carbowax 300 by the Union Carbide Corp. (PEG 300.)
[5] Marketed as Carbowax 400 by the Union Carbide Corp. (PEG 400.)
[6] Kilit Sporstrip 1, Baltimore Biological Laboratory, Inc.
[7] No. 740-E, Carl Schleicher & Schuell Co., Kenne, N. H.

TABLE I—DRY HEAT RESISTANCE OF MESOPHILIC SPORES[a]

Organism	Spores/Disk	Control, No Heat	130°C., hr. 0.5	1.0	1.5	2.0
B. subtilis (HA)	22,000	5/5[b]	5/5	0/5	0/5	0/5
B. subtilis (HL)	70,000	5/5	5/5	4/5	0/5	0/5
B. subtilis (ATCC 9372)	110,000	5/5	5/5	5/5	3/5	0/5
B. cereus (ATCC 13061)	100,000	5/5	0/5	0/5	0/5	0/5
	200,000	5/5	0/5	0/5	0/5	0/5
	400,000	5/5	0/5	0/5	0/5	0/5

[a] Disks were dried a minimum of 2 days over silica gel before use. [b] 5/5 = number of tubes showing growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 7 days at 30–32°.

TABLE II—EFFECT OF DRYING ON RESISTANCE IN MOIST HEAT
(*B. stearothermophilus*, 50,000 SPORES/DISK)

Days[a] Dried	Culture Medium	Time, min. at 121°C. Steam 0	1	2	3	4	5	6	7	8	9	10
2	FT[c]	2/2[b]	2/2	2/2	2/2	2/2	2/2	0/2	...	...	...	...
6	FT	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2
	FTS[d]	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2

[a] Dried over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. Incubated 4 days at 60°C.

TABLE III—EFFECT OF DRYING ON RESISTANCE IN DRY HEAT
(*B. stearothermophilus*, 50,000 SPORES/DISK)

Days[a] Dried	Culture Medium	Time, min. at 130°C. Dry Heat 0	15	30	45	60	75	90
2	FT[c]	2/2[b]	1/2	0/2	0/2	0/2	0/2	0/2
6	FT	2/2	2/2	1/2	0/2	0/2	0/2	0/2
	FTS[d]	2/2	2/2	2/2	0/2	0/2	0/2	0/2

[a] Dried over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. Incubated 4 days at 60°C.

The results as shown in Tables II and III indicated that additional drying had caused a significant increase in resistance, especially to dry heat. The slight increase in recovery of dry heated spores in fluid thioglycollate containing soluble starch was not considered significant because of the very limited number of disks tested at each temperature. The initial results demonstrated that the spore was inadequate and that a heavier load should be used in subsequent studies.

The composition of the recovery medium has an important influence on the germination and outgrowth of bacterial spores and thus will affect resistance values. This is particularly so if the spores have been damaged in some way such as by heat or chemicals since damaged cells may become fastidious and require specialized media (11). It was believed the reliability of the results would be improved if additional media were evaluated for the recovery of *B. stearothermophilus*. The U.S.P calls for the use of fluid thioglycollate medium in the official sterility test; however, the literature suggests that the inclusion of soluble starch or phytone in the medium might improve the recovery of heat damaged spores of *B. stearothermophilus* (8, 12–14). The three media evaluated to determine a suitable recovery medium were: fluid thioglycollate medium, fluid thioglycollate plus 0.1% soluble starch, formulated phytone–starch medium containing phytone peptone 1.5%, trypticase peptone 1.0%, dextrose 0.5%, soluble starch 0.1%, sodium chloride 0.15%, pH adjusted to 7.2.

Disks containing approximately 100,000 spores each were used to determine the effect of the increased spore load and to compare the three media. The results, as shown in Tables IV and V, indicated greater resistance from the heavier spore load and improved recovery with the fluid thioglycollate containing soluble starch. Growth by visual inspection was essentially the same with fluid thioglycollate medium with or without starch and was somewhat greater than with the phytone–starch medium. Visible growth occurred sooner, however, with the phytone–starch medium than with either of the thioglycollate media. The recovery after dry heat was slightly greater in fluid thioglycollate with starch than in the phytone–starch medium. Both media gave appreciably better recovery than the fluid thioglycollate without starch.

The fluid thioglycollate with soluble starch also gave the best recovery after steam heat. The phytone–starch medium showed a definite tendency toward incomplete recovery after sublethal time intervals in steam heat. These results tend to support the theory that moist and dry heat kill or damage bacteria by different mechanisms (15). It has been suggested that starch has an adsorption effect on substances in the medium that repress spore germination (16).

Oil Sterilization—Dried spore disks were inserted into 10 ml. size ampuls, filled with 5 ml. oil, and flame sealed. Oil sections included 10 ampuls each of sesame oil, sesame oil containing 0.5% phenol, and sesame oil containing 2% benzyl alcohol. Controls consisted of spore disks in PEG 300 and in air. The sealed ampuls were placed into the pre-

TABLE IV—EFFECT OF RECOVERY MEDIUM ON RESISTANCE IN MOIST HEAT (*B. stearothermophilus*, 100,000 SPORES/DISK[a])

Culture Medium	Time, min. at 121°C. Steam								
	0	5	6	7	8	9	10	11	12
FT[c]	2/2[b]	5/5	5/5	5/5	4/5	0/5	0/5	0/5	0/5
FTS[d]	2/2	5/5	5/5	5/5	5/5	1/5	0/5	0/5	0/5
PS[e]	2/2	5/5	2/5	4/5	3/5	0/5	0/5	0/5	0/5

[a] Dried 2 days over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. [e] Phytone–starch medium. Incubated 4 days at 60°C.

TABLE V—EFFECT OF RECOVERY MEDIUM ON RESISTANCE IN DRY HEAT (*B. stearothermophilus*, 100,000 SPORES/DISK[a])

Culture Medium	Time, min. at 130°C. Dry Heat						
	0	15	30	45	60	75	90
FT[c]	2/2[b]	5/5	5/5	2/5	0/5	0/5	0/5
FTS[d]	2/2	5/5	5/5	4/5	2/5	0/5	0/5
PS[e]	2/2	5/5	4/5	4/5	1/5	0/5	0/5

[a] Dried 2 days over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. [e] Phytone–starch medium. Incubated 4 days at 60°C.

heated oven at the designated temperature plus a 2° override to reduce the decrease in the rate of heat uptake that occurs as the temperature approaches final temperature. The controller was reset after the oil reached the designated temperature. The oil temperature was determined with a potentiometer which was connected to a thermocouple placed in an identical ampul containing 5 ml. sesame oil. The time required to reach the designated temperature (lag time) as well as the temperature during the run was monitored each time. The temperature range was from 100 to 160° in 10° increments. Ampuls were removed at 0.5, 1, 1.5, and 2 hr. after the oil reached temperature. The lag time of the oil varied only slightly from run to run, regardless of the sterilization temperature, ranging from a low of 45 min. to a high of 55 min.

Sterility Testing—Sterility tests were performed within 12 hr. of the time of sterilization. The ampuls were wiped with 5% phenol, flamed and broken open, and the oil allowed to drain. The disks were then aseptically removed with sterile-flamed tweezers, tapped lightly to remove excess oil, and transferred into fluid culture medium in 30 × 165 mm. tubes. The *B. subtilis* disks were tested in fluid thioglycollate medium U.S.P. Incubation was for 14 days at 30–32°. The *B. stearothermophilus* disks were tested in fluid thioglycollate medium containing 0.1% soluble starch. Incubation was for 4 days at 60–62°.

The tubes, which contained approximately 40 ml. of medium, were shaken vigorously by hand at the time the disks were placed on test and at periodic intervals throughout the incubation periods to disperse oil remaining on the disks and to effect transfer of the spores to the aqueous media.

RESULTS AND DISCUSSION

B. subtilis ATCC 9372—Interpretation of the results obtained with *B. subtilis* was complicated initially by a change in resistance that occurred during the approximately 5-week period in which the first series of oil tests was conducted. In the preliminary heat resistance studies, disks containing approximately 110,000 spores, which were dried for 2 days over silica gel prior to use, had a maximum resistance of 1.5 hr. at 130° in air. Disks that had been dried for 6 days were used for the initial oil sterilization at 130°. Ten disks per each time increment were included as air controls. The survival in air at this sterilization temperature was 2 hr., with nine of ten disks producing growth when cultured. Although this represented a change in resistance, the magnitude of the change was not recognized until later tests were conducted at 140°. With the first tests at 130° completed, it appeared that the preservatives had caused a decrease in the sterilization time and that the spores would survive less time in the oils or PEG 300 than in air.

As tests were conducted at the other temperatures designated for the study a rather irregular pattern of sterilization results emerged. Spores that initially survived only 1 hr. at 130° in sesame oil later survived 1 hr. at 140°. Spores that had been sterilized in 1.5 hr. at 120° in sesame oil with 2% benzyl alcohol later were not sterilized after 2 hr. at 130°. The 4 days additional drying perhaps accounted for the increased resistance first observed in air at 130°. The spore disks, however, continued to show changes in resistance which could not be related to drying of the disks as they had been stored in sealed jars after 6 days drying over silica gel.

It was noted that while sterilization in unpreserved sesame oil required 1.5 hr. at 130° and later 1.5 hr. at 140°, sterilization in sesame oil with benzyl alcohol had shown "skips" at 130° with minimal but definite survival at 140°. Repeat tests were then conducted in the range of the critical sterilization and sublethal temperatures and a quite different pattern of resistance was seen, especially as regarded the sections with benzyl alcohol in sesame oil.

The results obtained on spore disks less than 5 weeks of age are shown in Table VI. On the basis of these results alone, and relating the one survivor out of ten after 0.5 hr. at 140° to an extremely resistant spore, it would have been enticing to conclude that both the preservatives tested had a definite lowering effect on the sterilization requirements for sesame oil. Data acquired using spore disks which were aged more than 5 weeks showed that this was not the case with benzyl alcohol. The repeat tests indicated that the spores had an increased resistance to the action of benzyl alcohol but not to phenol.

The data in Table VII were extracted from all the *B. subtilis* test results without regard to the age of the spores, and represent the maximum resistance observed with each of the sterilization vehicles.

The results, although they do not help explain

TABLE VI—DRY HEAT STERILIZATION TESTS OF *B. subtilis* ATCC 9372 (110,000 SPORES/DISK[a])

Temp., °C.	Control, No Heat	Time, hr.[b] 0.5	1.0	1.5	2.0
		Air			
100	2/2[c]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	5/5	5/5	5/5	5/5
130	2/2	10/10	10/10	10/10	9/10
140	2/2	5/5	4/5	2/5	3/5
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
		PEG 300			
100	5/5	0/5	0/5	0/5	0/5
110	5/5	0/5	0/5	0/5	0/5
120	5/5	0/5	0/5	0/5	0/5
130	5/5	0/5	0/5	0/5	0/5
		Sesame Oil			
100	5/5	8/10	8/10	10/10	10/10
110	5/5	10/10	10/10	10/10	10/10
120	5/5	10/10	8/10	9/10	6/10
130	5/5	7/10	2/10	0/10	0/10
140	5/5	4/10	1/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
		Sesame Oil + 0.5% Phenol			
100	5/5	8/10	6/10	4/10	5/10
110	5/5	0/10	1/10	3/10	2/10
120	5/5	0/10	0/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	0/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
		Sesame Oil + 2% Benzyl Alcohol			
100	5/5	9/10	10/10	7/10	7/10
110	5/5	9/10	8/10	6/10	7/10
120	5/5	3/10	1/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	1/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10

[a] Aged less than 5 weeks. [b] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [c] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

why, point to a possible difference in mechanism of phenol and benzyl alcohol or to different mechanisms of spore resistance to the activity of the preservatives. It would also suggest that the resistance mechanism may be age dependent. Although there was no additional drying of the spore disks beyond 6 days, it is possible that a further loss in spore moisture content took place by equilibration with the disk material. If this hypothesis were accepted, then it could be suggested that the benzyl alcohol activity was dependent upon the moisture content of the spores whereas phenol activity was not dependent on moisture.

Additional tests were conducted at irregular intervals over a 8-month period with essentially the same findings, *i.e.*, aged spores continued to be resistant to benzyl alcohol but not to phenol.

Relation of Phenol Activity to Vehicle—Additional tests were run with another lot of sesame oil and mineral oil since earlier results indicated that phenol markedly reduced the sterilization requirements for sesame oil containing spores of *B. subtilis*. Although these tests were conducted at only limited time and temperature ranges, the results point out the significant effect of the vehicle on the resistance of the spores and the effect of the vehicle on the preservative action. The results as shown in Table VIII indicate that the resistance of the spores was about 10° greater in light mineral oil than in sesame oil and that phenol exerts less effect in mineral oil than in sesame oil. The results obtained with the second lot of sesame oil did not vary appreciably from those of the first lot.

Effect of Drying on Resistance of B. subtilis to Benzyl Alcohol—A second batch of spore disks was prepared from the original *B. subtilis* spore suspension. The MPN count on the suspension, which was

TABLE VII—DRY HEAT STERILIZATION TESTS OF *B. subtilis* ATCC 9372 AT MAXIMUM RESISTANCE LEVEL (110,000 SPORES/DISK)

Temp., °C.	Control, No Heat	Time, hr.[a] 0.5	1.0	1.5	2.0
		Air			
100	2/2[b]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	5/5	5/5	5/5	5/5
130	2/2	10/10	10/10	10/10	9/10
140	2/2	5/5	4/5	2/5	3/5
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
		PEG 300			
100	5/5	0/5	0/5	0/5	0/5
110	5/5	1/5	0/5	0/5	0/5
120	5/5	0/5	0/5	0/5	0/5
130	5/5	0/5	0/5	0/5	0/5
		Sesame Oil			
100	5/5	8/10	8/10	10/10	10/10
110	5/5	0/10	10/10	10/10	10/10
120	5/5	10/10	10/10	10/10	10/10
130	5/5	10/10	8/10	8/10	7/10
140	5/5	4/10	1/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
		Sesame Oil + 0.5% Phenol			
100	5/5	8/10	6/10	4/10	5/10
110	5/5	0/10	1/10	3/10	2/10
120	5/5	0/10	0/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	0/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
		Sesame Oil + 2% Benzyl Alcohol			
100	5/5	9/10	10/10	10/10	10/10
110	5/5	10/10	10/10	10/10	10/10
120	5/5	10/10	9/10	8/10	8/10
130	5/5	10/10	10/10	7/10	6/10
140	5/5	1/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10

[a] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

TABLE VIII—EFFECT OF STERILIZATION VEHICLE ON PHENOL ACTIVITY (*B. subtilis*, 110,000 SPORES/DISK)

Temp., °C.	Time, hr.[a] 0.5	1.0	1.5	2.0
	Sesame Oil[c]			
130	10/10[b]	8/10	8/10	7/10
140	4/10	1/10	0/10	0/10
150	0/10	0/10	0/10	0/10
	Sesame Oil[d]			
130	...	7/10	...	5/10
	Sesame Oil[d] + 0.5% Phenol			
110	1/5	1/5	0/5	0/5
120	0/5	0/5	0/5	0/5
	Mineral Oil			
140	3/3	...	...	...
150	4/5	0/5	0/5	0/5
	Mineral Oil + 0.5% Phenol			
130	5/5	5/5	...	...
140	1/5	0/5	...	...
150	0/5	0/5	...	...

[a] Time after reaching designated temperature, dry heat. [b] 10/10, number of tubes with growth/number of tubes inoculated with disks. [c] Original lot of sesame oil. [d] A second lot of sesame oil. Fluid thioglycollate medium incubated 14 days at 30–32°C.

3 months old, was approximately 80 million per ml. and was considered to have shown no significant change. The disks were prepared to contain 200,000 spores each. They were then stored in a tightly sealed can over silica gel for 10 days, after which disks were removed and heated in sesame oil with 2% benzyl alcohol at 130° for periods of 0.5 and 1 hr. This procedure was repeated again after 20, 30, and 90 days of drying. The results in Table IX showed a similar trend to those obtained with the earlier batch (Tables VI and VII). However, the degree of maximum resistance during the 3-month drying interval was not so great as when the disks were dried only 6 days. These results did not clarify the relationship of age and degree of desiccation to resistance but they did confirm the same general pattern of increased resistance developing with time.

TABLE IX—EFFECT OF DRYING ON RESISTANCE OF *B. subtilis* SPORES IN SESAME OIL WITH 2% BENZYL ALCOHOL (200,000 SPORES/DISK)

Days Dried[a]	Time, hr. at 130°C. Dry Heat 0.5	1.0
10	2/10[b]	1/10
20	5/10	1/10
30	3/10	3/10
90	6/10	5/10

[a] Dried over silica gel. [b] 2/10, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

B. stearothermophilus—Disks containing approximately 100,000 spores each were held over silica gel for 6 to 8 days and then used for the sterilization tests. As shown in Table X the maximum survival of *B. stearothermophilus* spores in air was 1.5 hr. at 130°. The maximum survival time in oil occurred in unpreserved sesame oil, being 1 hr. at 150° or 2 hr. at 140°. This increased resistance in sesame oil indicated the oil protected slightly against the effects of heat. Based on the maximum survival temperature alone, it would appear that benzyl alcohol and phenol caused a slight reduction in the sterilization requirements. Comparison of the results obtained with sublethal time intervals in the 130° and 140° temperature range, however, indicated neither 0.5% phenol nor 2% benzyl alcohol had any effect on the survival or death of the spores. In a repeat of the sterilization at 150°, there were no survivors in any of the oil sections and repeat tests at 140° did not give evidence of the preservatives having any effect. It would be tenuous, indeed, to relate any activity to the preservatives even though there was a slight increase in survival observed in the initial sterilization test of unpreserved oil at 150°. Sterilization of spores of *B. stearothermophilus* in PEG 300 occurred at significantly lower temperatures than in oil or air.

TABLE X—DRY HEAT STERILIZATION TESTS OF *B. stearothermophilus* (100,000 SPORES/DISK)

Temp., °C.	Control, No Heat	Time, hr.[a] 0.5	1.0	1.5	2.0
		Air			
100	2/2[b]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	9/10	10/10	10/10	10/10
130	2/2	8/10	4/10	1/10	0/10
140	2/2	0/10	0/10	0/10	0/10
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
		PEG 300			
100	2/2	3/5	2/5	1/5	1/5
110	2/2	0/5	1/5	0/5	1/5
120	2/2	0/5	0/5	0/5	0/5
130	2/2	0/5	0/5	0/5	0/5
140	2/2	0/5	0/5	0/5	0/5
150	2/2	0/5	0/5	0/5	0/5
		Sesame Oil			
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	10/10	8/10	2/10
140	2/2	6/10	1/10	0/10	1/10
150	2/2	2/10	1/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10
		Sesame Oil + 0.5% Phenol			
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	10/10	7/10	8/10
140	2/2	10/10	0/10	0/10	0/10
150	2/2	0/10	0/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10
		Sesame Oil + 2% Benzyl Alcohol			
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	9/10	10/10	8/10
140	2/2	9/10	1/10	0/10	0/10
150	2/2	0/10	0/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10

[a] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium with 0.1% soluble starch incubated 4 days at 60°C.

Minimum Inhibitory Level of Phenol in Aqueous Media—Phenol was added to fluid thioglycollate in concentrations ranging from 1:1200 to 1:600. The tubes were then inoculated with dried spore disks of *B. subtilis* and *B. stearothermophilus* containing 110,000 and 100,000 spores, respectively. Growth of *B. subtilis* occurred with 1:1000 but not with 1:900 phenol. Growth of *B. stearothermophilus* occurred with 1:800 but not with 1:700 phenol.

The *B. subtilis* spores used in the oil sterilization study were killed at lower temperatures in oil containing phenol than in oil alone while the *B. stearothermophilus* spores were not significantly affected by phenol under the conditions of the test. The *B. stearothermophilus* was more resistant to phenol than the *B. subtilis* in aqueous media. This suggests a possible reason why phenol in oil did not reduce the sterilization requirements for *B. stearothermophilus*.

Polyethylene Glycol for Oil Sterilization Control—It has been reported that oils containing only a few spores will frequently pass the sterility test by the U.S.P. method of direct inoculation because the spores may not transfer from the oil to the aqueous medium. It has been suggested that spores be placed in a water miscible nonaqueous liquid such as polyethylene glycol (PEG) and that containers of spores in this vehicle be placed in the oven when sterilizing oils (17). After sterilization, the spore controls in the PEG are tested for sterility by the regular U.S.P. method. Since the vehicle is miscible with water, it will mix readily with the culture medium and any spores not killed will be detected. The results in this series of experiments did not substantiate that this is a satisfactory means of gauging the adequacy of an oil sterilization cycle.

One lot of PEG 300 was used throughout the initial series of oil sterilizations. Two additional lots of PEG 300 and 2 lots of PEG 400 were subsequently used in sterilization tests at 110° and 120°. PEG 300 gave results similar to those obtained in the earlier tests. PEG 400 caused nearly as great a reduction in the sterilization requirements as the PEG 300. The results in these tests confirmed that the combined effect of heat and polyethylene glycol is a marked reduction in sterilization time when compared to that in either oil or air.

SUMMARY

The spores of two thermoresistant species of nonpathogenic aerobic bacilli were heated in preserved sesame oil to determine the effect of the preservatives on the time and temperature requirements for dry heat sterilization.

B. subtilis ATCC 9372 and *B. stearothermophilus*, mesophilic and thermophilic species, respectively, were selected as test organisms. Spores of these organisms were impregnated on filter disks and heated in (*a*) sesame oil, (*b*) sesame oil with 0.5% phenol, (*c*) sesame oil with 2% benzyl alcohol, (*d*) PEG 300, and (*e*) air. Additional comparisons were made with mineral oil and PEG 400. Data were collected in the range of 100–160° in 10° increments at 0.5 hr. intervals to 2 hr.

The *B. subtilis* spores survived 2 hr. at 140° in air. The requirements for sterilization in the sesame oil and PEG 300 were lower than in air. Maximum survival time in unpreserved sesame oil was 1 hr. at 140°. The maximum survival time in sesame oil with 0.5% phenol was 2 hr. at 110°. Benzyl alcohol gave variable results which were related to an increase in resistance of the spores. It had only minimal effect against aged spores. Mineral oil required higher temperatures for sterilization than sesame oil. Phenol had less effect in mineral oil than in sesame oil.

The *B. stearothermophilus* spores survived 1.5 hr. at 130° in air. Higher temperatures were required for sterilization of the oils. There was limited survival after 1 hr. at 150° in unpreserved sesame oil. Sesame oil containing phenol and benzyl alcohol showed no survivors at 150°. Review of the survival data at sublethal temperatures as well as repeat tests indicated no significant effect from phenol or benzyl alcohol.

The spores of both organisms were killed at lower temperatures in PEG 300 than in oil. PEG 400 gave similar results.

The results of this study do not lead to any overall generalizations regarding the effect of preservatives on the heat sterilization of oils. It would appear, however, that phenol would have a wider application and would be more effective than benzyl alcohol as an injectable oil preservative.

REFERENCES

(1) Bullock, K., and Keepe, W. G., *J. Pharm. Pharmacol.*, **3**, 717(1951).

(2) Eisman, P. C., Jaconia, D., and Mayer, R. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 659(1953).

(3) Bean, H. S., Heman-Ackah, S. M., and Thomas, J., *J. Soc. Cosmetic Chemists*, **16**, 15(1965).

(4) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 396.

(5) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 810–814.

(6) Pasquale, D., Jaconia, D., Eisman, P., and Lachman, L., *Bull. Parenteral Drug. Assoc.*, **18**, 1(1964).

(7) Perkins, J. J., in "Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization," 2nd ed., Reddish, G. F., ed., Lea & Febiger, Philadelphia, Pa., 1957, p. 766.

(8) Brewer, J. H., "The Becton, Dickinson Lectures on Sterilization," given at Seton Hall College of Medicine and Dentistry, Jersey City, N. J., April 10, 1959, p. 113.

(9) "Standard Methods for the Examination of Water, Sewage and Industrial Wastes," 10th ed., Waverly Press, Inc., Baltimore, Md., 1955, p. 383.

(10) Hardwick, W. A., and Foster, J. W., *J. Gen. Physiol.*, **35**, 907(1952).

(11) Wyss, O., in "Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization," 2nd ed., Reddish, G. F., ed., Lea & Febiger, Philadelphia, Pa., 1957, p. 205.

(12) Cook, A. M., and Brown, M. R. W., *J. Pharm. Pharmacol.*, **16**, 725(1964).

(13) Brewer, J. H., and McLaughlin, C. B., *J. Pharm. Sci.*, **50**, 171(1961).

(14) "Products for the Microbiological Laboratory," 4th ed., Baltimore Biological Laboratory, Inc., Baltimore, Md., reprinted 1963, p. 169.

(15) Rahn, O., *Bacteriol. Rev.*, **9**, 1(1945).

(16) Wynne, E. S., and Foster, J. W., *J. Bacteriol.*, **55**, 61 (1948a).

(17) Brewer, J. H., in "Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization," 2nd ed., Reddish, G. F., ed., Lea & Febiger, Philadelphia, Pa., 1957, p. 158.

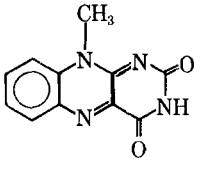
Hydrolytic Behavior of Isoalloxazines Related to Riboflavin III

Covalent Hydration of 1,2-Dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide

By DAVID E. GUTTMAN and THOMAS E. PLATEK

Previous studies concerned with the base-catalyzed degradation of 9-methylisoalloxazine led to the isolation of an intermediate which was reported to be an alloxan anil. The identification was based on comparing the properties of the isolated compound with those of what was thought to be authentic anil prepared by reacting *o*-methylaminoaniline with alloxan. Evidence has been obtained to indicate that an incorrect structure assignment was made and that the so-called anil is a compound which resulted from the covalent hydration of 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide. The hydrate is apparently formed by the addition of water across the double bond between N_4 and C_3 of the quinoxaline compound. It was found that in acid media, anhydrous and hydrated species coexisted in equilibrium while in neutral media, very little anhydrous form was detectable. The kinetics of attainment of equilibrium were studied at a number of different hydrogen-ion concentrations. Rate constants for forward and reverse reactions were determined. The influence of temperature on the rates of hydration and dehydration and on the equilibrium constant was also investigated.

RECENT PUBLICATIONS from this laboratory (1, 2) described the results of studies concerned with the base-catalyzed degradation of 9-methylisoalloxazine (I). It was reported that a white crystalline material was isolated from partially degraded solutions of the isoalloxazine and that the compound could undergo further reaction in basic solution to yield products which were identified as 1,2,3,4-tetrahydro-1-methyl-2,3-dioxoquinoxaline (II) and 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxylic acid (III). The properties and behaviors of the isolated material were shown to be identical to those exhibited by a product formed by the reaction in neutral medium of alloxan and *o*-methylaminoaniline and reported in the literature (3, 4) to be the alloxan anil (IV). This report will present evidence to suggest that the compound formed as an intermediate in the base-catalyzed decomposition of 9-methylisoalloxazine and which can be synthesized by the reaction of alloxan with *o*-methylaminoaniline is not the anil (IV), as previously suggested, but rather a product (VI) which is formed through the covalent hydration of the double bond between N_4 and C_3 of 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide (V). (Scheme I.)



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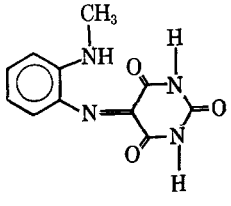
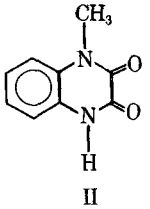


IV



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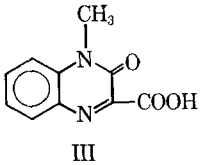

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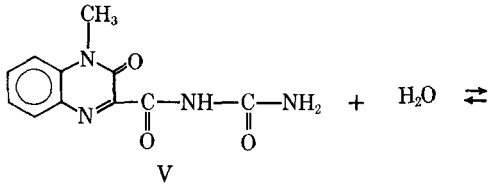

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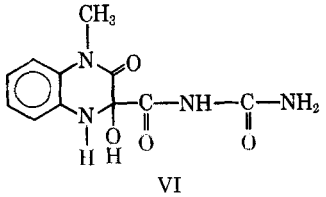


VI

Scheme I

Received April 14, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 28, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Previous paper: Wadke, D. A., and Guttman, D. E., *J. Pharm. Sci.*, **55**, 1363(1966).

King and Clark-Lewis (5, 6) and Clark-Lewis (7) have previously questioned the existence of alloxan anils such as IV. They reported that the condensation of *o*-methylaminoaniline with alloxan in neutral medium yielded a yellow crystalline compound with a characteristic spectrum which was identified as the ureide (V). That a white crystalline compound can also form under similar reaction conditions was not discussed by these authors. They did, however, refer to the work of Rudy and Majer (8) who obtained, from the reaction of 2-alkylamino-3-amino-pyridines with alloxan, two series of products, one yellow and unstable and the other colorless. The two different products were found to be interconvertible.

A number of considerations support the suggestion that the white compound in question is the covalent hydrate VI. These include: (*a*) the formation of products II and III can be readily explained on the basis of structure VI; (*b*) the compound on heating is converted to a yellow material with chromatographic and spectral characteristics of the ureide (V); (*c*) the equivalent weight of the compound, as determined by nonaqueous titration, corresponds to that of the hydrate (VI); (*d*) the ureide (V) in acidic and neutral media undergoes a rapid transformation to form a compound with spectral and chromatographic characteristics which are identical to those of the compound in question. Similarly, treatment of the latter compound with acid results in the formation of an equilibrium mixture composed of starting compound and ureide. Such facile transformations are readily explainable on the basis of a hydration–dehydration phenomenon.

The kinetics of the transformations described above were studied in some detail and, as will be seen, were found to be consistent with an acid-base catalyzed reversible covalent hydration.

RESULTS

Two different products were obtained when alloxan and *o*-methylaminoaniline were allowed to react in neutral alcoholic medium under slightly different conditions. One was the ureide (V) which is a yellow crystalline compound with the characteristic spectrum shown in Fig. 1. The other was a white crystalline compound which exhibited a much different spectrum, which is also shown in Fig. 1. This product, when heated above 100°, was converted to the ureide (V). The results of elemental analysis (C, 52.22%; H, 4.77%; N, 21.70%; O, 21.31%) were not consistent with structures IV (C, 43.6%; H, 4.10%; N, 22.8%; O, 19.5%) or VI (C, 50.0%; H, 4.58%; N, 21.20%; O, 24.2%) but were intermediate to the two possibilities. Equivalent weight determinations by nonaqueous titration yielded an average value of 265 which agreed with structure VI (theory 264). The white compound, in subsequent discussions, will be referred to as compound VI and the yellow compound as the ureide.

The spectrum of the ureide in essentially aqueous medium, buffered in the neutral range of pH, was found to rapidly change with time. The resulting spectrum appeared to be the composite of two spectra, that of the ureide and compound VI. Thin-layer chromatography showed the presence in such solutions of two components, a minor amount of ureide and a major amount of compound VI. The results suggested that an equilibrium was established between the two compounds. The differences in the spectral characteristics between the two were used to follow the kinetics of equilibration. A wavelength of 380 mμ was used for this purpose and a typical result is shown in Fig. 2. Two different data plots are contained in this figure. Decrease in absorbance as a function of time is shown by the open circles. The closed circles represent a plot of log $(A - A_\infty)$ *versus* time, where A is absorbance at time, t, and A_∞ is the constant absorbance value which was finally attained. The function $(A - A_\infty)$ reflects the concentration of ureide and the plot thus demonstrates that the rate of equilibration was first order with respect to ureide. The results of studies conducted at a number of different pH values are presented in Table I and Fig. 3. The A_∞ values shown in the table indicate that the equilibrium position was essentially independent of pH and that the equilibrium concentration of ureide was small. The plot of Fig. 3 yielded a slope of 1.03 and shows that the rate of attainment of equilibrium

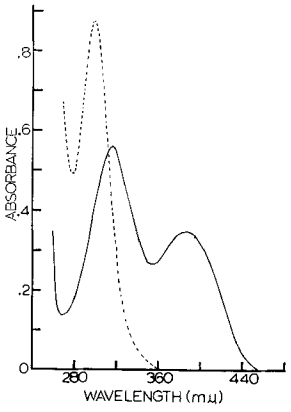


Fig. 1—Spectra exhibited by freshly prepared aqueous solutions of compounds V (——) and VI (– – –). The concentration of V was 6 × 10⁻⁵ M and that of VI was 1.97 × 10⁻⁴ M. The solutions contained 2% dimethylformamide.

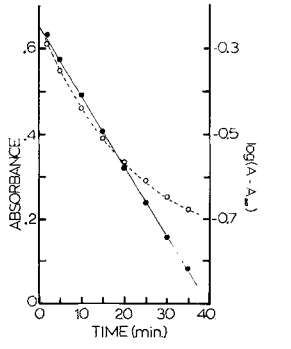


Fig. 2—A plot showing the rate of loss of absorbance at 380 mμ exhibited by a 1.22 × 10⁻⁴ M solution of the ureide (V) at pH 7.0 (μ = 1.0) at 35°. Key: – – –, absorbance vs. time plot; ——, obtained by plotting log (A − A∞) vs. time. The solutions contained 2% dimethylformamide.

TABLE I—OBSERVED RATE CONSTANTS AND A_∞ VALUES FOR THE TRANSFORMATION OF UREIDE IN BUFFERED AQUEOUS SOLUTION CONTAINING 2% DIMETHYLFORMAMIDE

pH	A_∞	$k_{obs.}$ (min.$^{-1}$)
6.20	0.05	0.0062
6.40	0.05	0.0151
6.86	0.05	0.0288
7.00	0.05	0.0388
7.40	0.05	0.0890
7.60	0.05	0.147
7.80	0.03	0.220
7.90	0.04	0.262
8.00	0.02	0.389

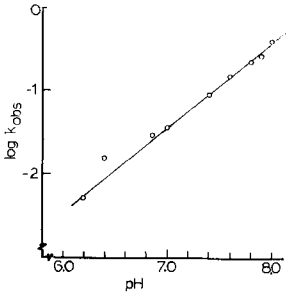


Fig. 3—A plot showing the relationship between observed rate constant and pH for the transformation of the ureide (compound V) at 35°.

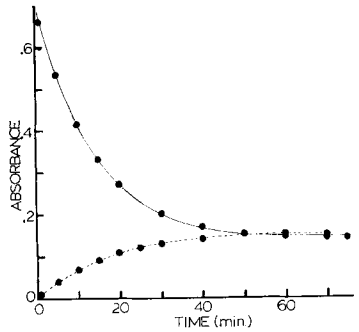


Fig. 4—A plot showing the time-dependent decrease in absorbance at 380 mμ for a 1.22 × 10^{-4} M solution of the ureide (V) (——) and the time-dependent increase in absorbance at 380 mμ for a 1.22 × 10^{-4} M solution of compound VI (– – –). The acid concentration was 0.58 N, μ = 1.0, T = 30°, and the solutions contained 2% dimethylformamide.

was first order with respect to hydroxide ion. The tabulated rate constants characterize rates of equilibration and are, therefore, sums of forward (k_f) and reverse (k_r) rate constants. The low equilibrium concentration of ureide precluded the accurate determination of an equilibrium constant and of the individual constants, k_f and k_r.

A similar transformation of the ureide, manifested by a loss of absorbance at 380 mμ, was observed in acidic media. Treatment of compound VI with acid, in contrast, resulted in a time-dependent generation of a 380 mμ absorbing species. A typical result is shown in Fig. 4 where absorbance loss of a ureide-containing system and absorbance gain of a compound VI-containing system are plotted as a function of time. It is seen that, within experimental error, the same asymptotic absorbance value was approached by the two systems. Thin-layer chromatography of equilibrated solutions confirmed the presence of two components, the ureide and compound VI. Both solutions on long standing exhibited a gradual increase in absorbance at 380 mμ. Spectrophotometric and chromatographic examination of aged solutions indicated the formation of the ketocarboxylic acid (III) which has significant absorbance at 380 mμ. The semilog plots of Fig. 5 show that the rate of equilibration was first order with respect to starting compound. The rate of attainment of equilibrium was studied at a number of different acid concentrations and from both directions. In all cases, a first-order approach to equilibrium was observed. Rate constants and A_∞ values are tabulated in Table II for systems in which the ureide was the starting material and in Table III for systems in which compound VI was the starting material. The results obtained with ureide systems are considered to be more accurate than those of the compound VI systems. A much lower concentration of ureide could be used to generate absorbance *versus* time plots. The compound VI systems were, by necessity, more concentrated and more susceptible to error because of the slow side-reaction which resulted in the formation of another compound which absorbed at 380 mμ.

It is seen from both tables that the rate of equilibration was first order with respect to the concentration of hydrogen ion, that the equilibrium absorbance value was independent of the concentration of hydrogen ion, and that at a particular concentration of hydrogen ion, essentially the same rate constant was obtained regardless of whether the starting material was ureide or compound VI.

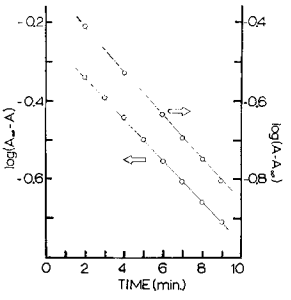


Fig. 5—A plot showing appearance (A$_\infty$ − A) of 380 mμ absorbing species in solutions of compound VI and disappearance (A − A$_\infty$) of 380 mμ absorbing species from solutions of the ureide (V). The acid concentration was 0.58 N, μ = 1.0, T = 35°. The initial concentration of VI was 6 × 10^{-4} M. The initial concentration of V was 1.22 × 10^{-4} M. The solutions contained 2% dimethylformamide.

TABLE II—OBSERVED FIRST-ORDER RATE CONSTANTS AND A_∞ VALUES OBTAINED IN FOLLOWING THE DECREASE IN ABSORBANCE AT 380 mμ EXHIBITED BY SOLUTIONS OF THE UREIDE IN ACID MEDIA[a]

Normality of Acid	A_∞	$k_{obs.}$ (min.$^{-1}$)
0.98	0.130	0.213
0.58	0.130	0.130
0.195	0.122	0.0452
0.097	0.125	0.0210

[a] Temperature, 35°; μ, 1. The initial concentration of ureide was 1.22 × 10^{-4} *M*. The solutions contained 2% dimethylformamide.

TABLE III—OBSERVED FIRST-ORDER RATE CONSTANTS AND A_∞ VALUES OBTAINED IN FOLLOWING THE INCREASE IN ABSORBANCE AT 380 mμ EXHIBITED BY SOLUTIONS OF COMPOUND VI IN ACID MEDIA[a]

Normality of Acid	A_∞	$k_{obs.}$ (min.$^{-1}$)
0.98	0.630	0.209
0.58	0.620	0.120
0.195	0.620	0.0403
0.097	0.600	0.0204

[a] Temperature, 35°; μ, 1. The initial concentration of compound VI was 6.00×10^{-4} *M*. The solutions contained 4% dimethylformamide.

Equilibrium constants describing the ureide–compound VI interconversion could be calculated with reasonable accuracy from the data obtained with systems in which the ureide was the starting material. Here, the molar absorptivity of the ureide was calculated from absorbance values which were obtained by extrapolating the semilog plots to zero time. The concentration of ureide at equilibrium was then calculated from the observed A_∞ value. The concentration of compound VI was obtained by difference between initial and final concentrations of ureide. The molar absorptivity was found to be independent of temperature and to have a value of 5750 (average of 11 determinations). The kinetics of ureide transformation was studied in 0.58 *N* hydrochloric acid at three different temperatures. Rate constants and equilibrium constants are presented in Table IV. As expected an increase in temperature resulted in an increase in the equilibrium concentration of ureide. The standard enthalpy change was estimated from these data to be approximately 4 Kcal. As can be seen, temperature had a much more pronounced influence on the rate of equilibration than on the equilibrium position.

DISCUSSION

Albert and Armarego (9) in their review of covalent hydration stated, "As chemists become more aware of the circumstances in which (covalent) hydration occurs and the means for detecting it, many new examples will probably be discovered and many puzzling discrepancies solved." A hydration–dehydration equilibrium such as depicted in Scheme I does provide a logical basis for explaining behaviors which previously had indeed been puzzling. Thus the spectral changes exhibited by the ureide in aqueous medium can be explained by the addition of water across the N_4—C_3 double bond to result in a reduction in the conjugation pathway of the molecule. Similarly, the conversion by heat of the white compound to yellow ureide is logically explained by a solid-state thermal dehydration. The anomalous elemental analysis could have resulted from partial dehydration by drying, to yield a mixture of hydrated and dehydrated product. In addition, Albert (9) reported that carbinolamines formed by the hydration of C=N bonds are readily oxidized by mild oxidizing agents to form oxo compounds and he suggested mild oxidation as a test for hydration and location of added water. That the dioxo compound (II) is formed by autoxidation of compound VI is added evidence to support hydration and the postulated structure. The kinetic data are consistent with those of other systems which were recently reviewed by Perrin (10).

Although the evidence strongly supports a hydration–dehydration phenomenon, the exact nature of the species undergoing reaction is not known. It is possible, for example, that in acid media both the ureide and the corresponding hydrate are protonated and that it was protonated species which underwent transformation. Similarly, at higher pH the reacting species could have been either neutral molecules or anions derived from the ionization of the imido hydrogen. Under all of the pH conditions studied, however, it was the hydrate that was the predominant species at equilibrium.

The identification of compound VI as a covalently hydrated ureide is strongly supported by the results of this investigation. The previously proposed scheme which summarized the degradative pathway of 9-methylisoalloxazine must then be revised and such a revision is presented in Scheme II.

It is also possible that the parent isoalloxazine might, in alkaline solution, undergo an equilibration to form a finite concentration of a hydrate resulting from the addition of water across the N_{10}—C_β double bond and that this species, by attack of hydroxide ion at C_α, directly formed the hydrated ureide. Although no confirmatory evidence of this possibility has yet been obtained, studies with 3,9-dimethylisoalloxazine, which will be reported in a future publication, do support such a possibility.

EXPERIMENTAL

Materials—1,2-Dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide (V) and the compound (VI) which is now thought to be the ureide formed by the covalent hydration of V were synthesized by methods which were previously described (1).

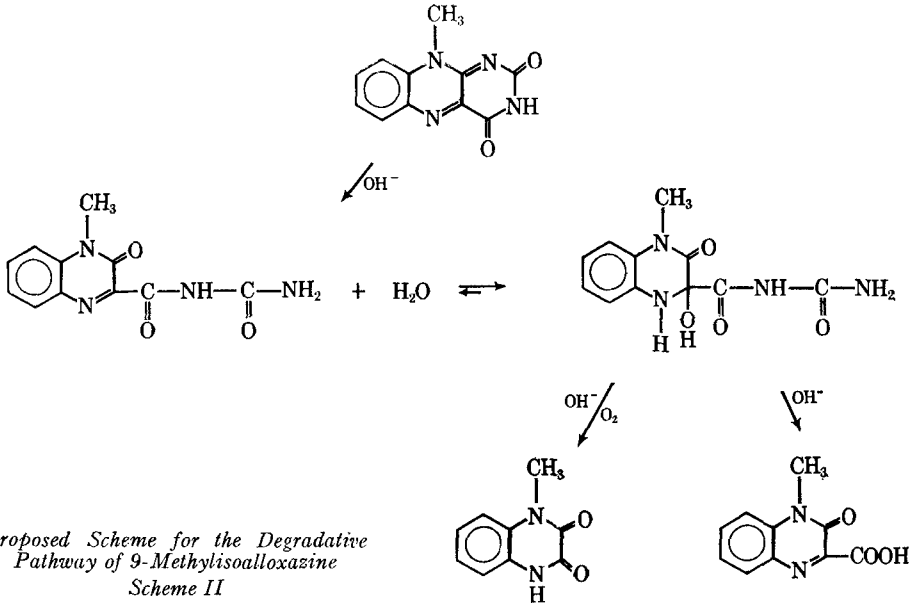
Thin-Layer Chromatography—Ten-microliter samples were applied on 300-μ silica gel plates and developed with a solvent system composed of chloroform–methanol–pyridine–water (180:15:1:1). After development, the plates were heated and examined under long wavelength ultraviolet light. The R_f value for the quinoxaline-3-carboxyureide was 0.90 and that for compound VI was 0.50.

Nonaqueous Titration—An accurately weighed

TABLE IV—INFLUENCE OF TEMPERATURE ON THE RATE OF EQUILIBRATION AND EQUILIBRIUM POSITION OF SYSTEMS INITIALLY CONTAINING 1.22×10^{-4} *M* UREIDE[a]

Temp., °C.	A_∞	$K_{equil.}$	$k_{obs.}$ (min.$^{-1}$)	k_r (min.$^{-1}$)	k_f (min.$^{-1}$)
10	0.080	7.75	0.00930	0.00106	0.00824
35	0.130	4.55	0.130	0.0234	0.107
50	0.172	3.07	0.413	0.101	0.312

[a] The acid concentration was 0.58 *N* and the ionic strength was 1.0. The solutions contained 2% dimethylformamide. The equilibrium constant was calculated as the ratio (compound VI)/(ureide).



Proposed Scheme for the Degradative Pathway of 9-Methylisoalloxazine
Scheme II

sample of compound VI was dissolved in 5 ml. of dimethylformamide and titrated with 0.1 *N* sodium methoxide in methanol to a thymol blue end point.

Spectra—All spectra were obtained with a Beckman model DB recording spectrophotometer.

Kinetic Studies—Stock solution of the ureide and of compound VI were prepared in dimethylformamide. Reaction mixtures were prepared by adding an aliquot of a stock solution to a volumetric flask containing a required volume of buffer or hydrochloric acid solution and at the desired temperature. The solution contained sufficient potassium chloride to make an ionic strength of 1.0. The solution was quickly mixed and a portion introduced to a cell which was placed in the cell compartment of a Beckman model DB spectrophotometer set at a wavelength of 380 mμ and equipped with a Sargeant SR recorder. The cell compartment of the spectrophotometer was maintained at the desired temperature by circulation of constant-temperature water from a P. M. Tamson N.V. circulating bath. Absorbance *versus* time plots were traced by the recorder. Zero time was taken to be the time at which the aliquot of stock solution of reactant was introduced to the volumetric flask. Limited studies showed that, in the concentration range encountered, changes in the dimethylformamide concentration of the reaction mixture had no effect on the observed rate.

REFERENCES

(1) Wadke, D. A., and Guttman, D. E., *J. Pharm. Sci.*, **55**, 1088(1966).
(2) *Ibid.*, **55**, 1363(1966).
(3) Kuhn, R., and Reinmund, K., *Chem. Ber.*, **67**, 1932 (1934).
(4) Tishler, M., Wellman, J. W., and Ladenburg, K. J., *J. Am. Chem. Soc.*, **67**, 2165(1945).
(5) King, F. E., and Clark-Lewis, J. W., *J. Chem. Soc.*, **1951**, 3080.
(6) *Ibid.*, **1951**, 3379.
(7) Clark-Lewis, J. W., *Chem. Ind.*, **1966**, 241.
(8) Rudy, H., and Majer, O., *Ber.*, **71B**, 1323(1938).
(9) Albert, A., and Armarego, W. L. F., in "Advances in Heterocyclic Chemistry," vol. IV, Katritzky, A. R., Boulton, A. J., and Lagowski, J. M., eds., Academic Press Inc., New York, N. Y., 1965, chap. 1.
(10) Perrjn, D. D., in *ibid.*, chap. 2.

Creaming of Phosphatide Stabilized Fat Emulsions by Electrolyte Solutions

By M. S. GRAY and W. S. SINGLETON

Creaming effects of electrolytes on emulsions of cottonseed and soybean oils have been investigated. The emulsions were stabilized with egg yolk phosphatides or with soybean phosphatides of different degrees of purity. The differences in creaming of the emulsions by the electrolyte solutions apparently were due to the differences in composition or purity of the phosphatide emulsifiers, and to the differences in properties of the respective oils. Emulsions of cottonseed or soybean oils stabilized with chromatographically homogeneous lecithin creamed to a greater extent than did emulsions of the same oils stabilized with impure phosphatides. In the latter, emulsions of cottonseed oil were slightly less creamed than emulsions of soybean oil.

IN A PREVIOUS STUDY (1), the electronically counted number of particles of specific diameters in fat emulsions developed for intravenous nutrition was found to be critically dependent upon the elapsed time of dilution of the emulsion with saline electrolyte. Because of the extreme dilution required by the electronic counting method, it could not be visually established whether creaming or coalescence occurred and was the cause of the change in particle count. Recently, Le Veen *et al.* (2) have shown that when certain sera are mixed *in vitro* with a fat emulsion, creaming or complete breaking of the emulsion may occur, and ascribe this effect to a globulin. It would appear that the electrolytes of serum or plasma may be equally significant factors in emulsion stability, based on the effect noted with saline in determining particle count. It must be assumed that the protein and other components of circulating plasma to some extent "buffer" the effect of plasma electrolytes on fat emulsions; however, the creaming and breaking effects of electrolytes on certain types of emulsions is well known. Van den Temple (3) reporting on o/w emulsions of monochlorobenzene and paraffin oil, found that the chlorides of sodium, potassium, and magnesium caused coagulation and finally breaking, the rate increasing with salt concentration. The cause of the breaking, according to Van den Temple (4) and to Martin and Hermann (5) involved discharge of the double layer potential. Electrolytes may affect the rate of creaming, and van Gils and Kraay (6) found that latex was creamed in the increasing order Li > Na > K among monovalent ions, and Ba = Ca > Sr among bivalent ions. The available reports do not include the effects of electrolytes on emulsions of vegetable oils, nor on emulsions stabilized with phosphatides.

In the phenomenon of creaming, droplets of the dispersed phase of an emulsion accumulate as a "cream" layer, and the continuous phase from which the dispersed phase is withdrawn becomes clearer, with less concentration of dispersed phase than originally. The continuous phase usually remains as an emulsion. Because turbidity of an emulsion decreases with creaming, measurements of transmitted light in the clearer phase seem to be a useful method of determining cream formation, as discussed by Griffin and Behrens (7).

The present investigation reports the creaming effect of solutions of some of the electrolytes found in human plasma on emulsions of two types of vegetable oils stabilized with egg phosphatides. The mechanism of creaming as a rate function, or correlation of light transmission with any of the parameters involved in rate functions, is beyond the scope of this study.

EXPERIMENTAL MATERIALS

Emulsions—Six emulsions were used, four prepared by high pressure homogenization as described previously (8), and two commercial products. The components of the various emulsions are shown in Table I.

The egg lecithin stabilizer in SR-151 and SR-183

TABLE I—COMPONENTS OF VARIOUS EMULSIONS

Emulsion	Lipid Phase	Stabilizer
SR-151	Soybean, 20%	Egg lecithin, 1%
SR-182	Soybean, 20%	Egg phosphatide, 1.2%
SR-183	Cottonseed, 20%	Egg lecithin, 1%
SR-181	Cottonseed, 20%	Egg phosphatide, 1.2%
Emulsion *A*[a]	Soybean, 20%	Egg phosphatide, 1.2%
Emulsion *B*[b]	Cottonseed, 10%	Soybean phosphatide, 2%

[a] Intralipid, a commercial emulsion; the composition is listed on the label. [b] Lipofundin, a commercial emulsion; the composition is listed on the label.

Received April 4, 1967, from the Southern Regional Research Laboratory, Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, New Orleans, LA 70119

Accepted for publication July 18, 1967.

This investigation was supported by funds from the Office of the Surgeon General, U. S. Department of the Army, Washington, D. C.

was chromatographically homogeneous (9); the egg phosphatide in SR-182 and SR-181 was not fractionated by column chromatography, and hence was less pure than the egg lecithin. The purity of the stabilizers of the commercial emulsion was not known, but when isolated from the emulsions, were nonhomogeneous, as shown by thin-layer chromatography. The isotonic aqueous phases were solutions of glycerol in all emulsions except emulsion *B* which contained sorbitol.

Electrolytes—All reagents were A. R. grade. Distilled water solutions of NaCl, KCl, $CaCl_2$, and $CaCl_2$–NaCl were made at salt concentrations of 0.25, 0.5, 1, 2, and 5%. The $CaCl_2$–NaCl solutions contained chemically equivalent weights of the two salts. A solution that simulated human plasma electrolytes was prepared with the following composition per liter: $CaCl_2$, 0.277 Gm.; KCl, 0.373 Gm.; $MgCl_2 \cdot 6H_2O$, 0.305 Gm.; $NaHCO_3$, 2.268 Gm.; $NaHPO_4 \cdot 12H_2O$, 0.358 Gm.; and NaCl, 6.546 Gm.; at pH 8.18. A fine precipitate which formed on standing was removed by filtration.

Method—Four milliliters of the respective salt solutions was mixed with 0.2 ml. of emulsion in a cell. After standing for 30 min., light transmission measurements of the solution–emulsion systems were made with a Lumetron photoelectric colorimeter, model No. 402E, operating through a constant voltage transformer and with galvanometer at direct reading. A monochromatic filter, 550 μ, was used. Under the conditions of operation, the light transmission of the freshly mixed emulsion–salt solution was 0%, after separation of the cream layer, 100%. The ratio of the per cent light transmission of a particular emulsion–salt solution after 30 min. of standing and the maximum per cent transmission of the clearer phase of a creamed emulsion was calculated. A ratio of 1.0 represents maximum creaming. Duplicate determinations of transmission were made, and the calculated results represent the average ±2%.

The transmission of the various emulsions, when diluted with distilled water, increased approximately 10–15% in 30 min. Only those increases above 15% were considered to be due to the salt solutions.

RESULTS AND DISCUSSION

The amount of creaming of the various emulsions was calculated as explained, and the calculated ratios are given in Table II. A ratio of 1.00 represents maximum creaming, and values less than 1.00 indicate some degree of creaming.

The increases in transmission of the four SR emulsions as creaming progressed, due to clearing of the dispersed phase, are plotted in Figs. 1–4. Where coincidence of data occurred, only one curve was plotted. The increases in transmission were due to decrease of dispersed phase, as electronic count of the dispersed particles (1) decreased by 400/μl./min.

Emulsions Stabilized with Homogeneous Lecithin—Emulsions SR-151 and SR-183, which were stabilized with pure lecithin but which differed in oil phase, had similar patterns of creaming with the various concentrations of salt solutions.

Upon dilution with each solution of NaCl, both emulsions were completely creamed. Results obtained with the KCl solutions were essentially similar to those obtained with NaCl.

Creaming was negligible at the lower concentrations of $CaCl_2$, with the exception of a slight amount observed in the soybean oil emulsion with the 2%

TABLE II—RATIO OF LIGHT TRANSMISSIONS OF EMULSIONS CREAMED BY VARIOUS CONCENTRATIONS OF ELECTROLYTES[a]

	Ratio of Light Transmission					
Salt and Concn., %	SR-151	SR-181	SR-183	SR-182	Emulsion *A*	Emulsion *B*
NaCl						
0.25	1.0	0.12	1.0	0.10	0.13	0.13
0.5	1.0	0.21	1.0	0.12	0.16	0.13
1.0	1.0	0.33	1.0	0.75	0.56	0.17
2.0	1.0	0.15	1.0	1.0	0.80	0.15
5.0	1.0	0.12	1.0	1.0	0.46	0.14
KCl						
0.25	0.80	0.13	1.0	0.10	0.13	0.11
0.5	1.0	0.14	1.0	0.10	0.12	0.11
1.0	1.0	0.28	1.0	0.12	0.15	0.11
2.0	1.0	0.18	1.0	0.65	0.39	0.13
5.0	1.0	0.12	0.97	0.92	0.30	0.16
$CaCl_2$						
0.25	0.10	0.25	0.13	0.14	0.12	0.17
0.5	0.10	0.24	0.14	0.14	0.19	0.12
1.0	0.10	0.30	0.13	0.12	0.13	0.12
2.0	0.40	0.20	0.14	0.25	0.21	0.13
5.0	1.0	0.20	1.0	1.0	1.0	0.13
$CaCl_2$–NaCl						
0.25	0.10	0.40	0.14	1.0	0.17	0.78
0.5	0.10	0.20	0.12	0.40	0.10	0.36
1.0	0.20	0.16	0.12	0.40	0.14	0.26
2.0	1.0	0.14	0.86	1.0	0.46	0.28
5.0	1.0	0.20	1.0	1.0	1.0	0.30
Plasma electrolytes	1.0	0.10	...	...	0.10	...

[a] Calculated as ratio of per cent light transmission 30 min. after mixing and maximum possible transmission of the continuous phase; a value of 1.0 represents maximum creaming.

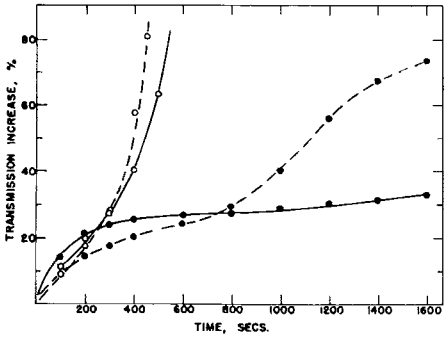


Fig. 1—Increase in transmission of SR emulsions with a 1% solution of NaCl. Key: ——, cottonseed oil emulsions; - - - -, soybean oil emulsions; ○, egg lecithin; ●, egg phosphatide.

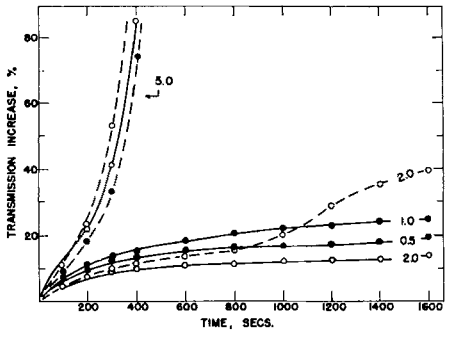


Fig. 3—Increase in transmission of SR emulsions with solutions of $CaCl_2$ of various concentrations. Key: ——, cottonseed oil emulsions; - - - -, soybean oil emulsions; ○, egg lecithin; ●, egg phosphatide.

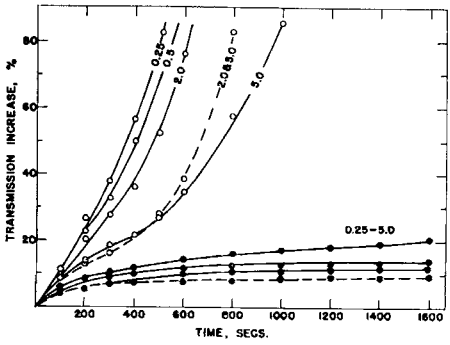


Fig. 2—Increase in transmission of SR emulsions with solutions of NaCl of various concentrations. Key: ——, cottonseed oil emulsions; - - - -, soybean oil emulsions; ○, egg lecithin; ●, egg phosphatide.

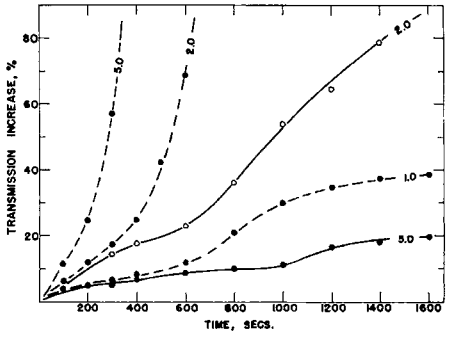


Fig. 4—Increase in transmission of SR emulsions with solutions of $CaCl_2$–NaCl of various concentrations. Key: ——, cottonseed oil emulsions; - - - -, soybean oil emulsions; ○, egg lecithin; ●, egg phosphatide.

solution; both emulsions were creamed by 5% solution of $CaCl_2$.

Creaming occurred in both emulsions with the 2% and 5% solutions of the mixed salts $CaCl_2$–NaCl.

Emulsions Stabilized with Crude Phosphatides—Emulsion SR-181, cottonseed oil, generally was less creamed than the soybean oil emulsion, SR-182, with all of the electrolytes.

Creaming in the two emulsions was negligible with the 0.25% and 0.5% concentrations of NaCl, but quite different with the higher concentrations. Creaming in the soybean oil emulsion increased to about the same extent as it did in the soybean oil emulsion stabilized with pure lecithin, whereas the cottonseed oil emulsion was relatively unaffected.

Similarly, the soybean oil emulsion was creamed by the higher concentrations of KCl, $CaCl_2$, and $CaCl_2$–NaCl, but the cottonseed oil emulsion was not.

Emulsions A and B—Emulsion *A* and emulsion SR-182, which are of similar composition, show similar patterns of creaming with the various concentrations of NaCl, KCl, and $CaCl_2$, except that emulsion SR-182 creamed to a greater extent with the higher concentrations of electrolytes. Emulsion SR-182 was more unstable than emulsion *A* with $CaCl_2$–NaCl.

There was little or no creaming of emulsion *B* at any of the concentrations of NaCl, KCl, and $CaCl_2$, and only a moderate amount with the concentrations of $CaCl_2$–NaCl. The oil content of emulsion *B* (10%) is less than that of the other emulsions and the phosphatide content larger, and the results may not be directly comparable with those of the other emulsions.

Plasma Electrolytes—Of the limited number of determinations with the mixture of plasma electrolytes, the soybean oil emulsion stabilized with pure lecithin was creamed, but the electrolyte had no effect on the other emulsions tested.

DISCUSSION

Creaming of the emulsions as a function of electrolyte concentration varied between emulsions of the same oil stabilized with pure lecithin and with crude phosphatide, and also between solutions of the various electrolytes. In some emulsions creaming was most complete with the 5% concentration of electrolyte; in others, with lower concentrations. In general, the amount of creaming was directly

proportional to electrolyte concentration. The inequality in creaming of the same emulsion with solutions of two different salts at identical concentrations apparently is explained by molarity of the cations (6).

The results obtained with the four SR emulsions indicated that the differences in stability of the emulsions to electrolytes apparently were due to the differences in properties of soybean and cottonseed oils, and to the differences in composition, or purity, of the stabilizers. Emulsions of cottonseed oil stabilized with chromatographically homogeneous egg lecithin or with egg phosphatides appeared to be more stable to electrolytes than were emulsions of soybean oil stabilized with the same emulsifiers. Also, emulsions of either oil stabilized with crude phosphatides were more stable to electrolytes than were emulsions stabilized with pure lecithin. The instability of the emulsions with NaCl, KCl, and $CaCl_2$ was marked with soybean oil–pure lecithin (SR-151), and was progressively less with cottonseed oil–pure lecithin (SR-183), soybean oil–phosphatide (SR-182), and cottonseed oil–phosphatide (SR-181).

Yeadon *et al.* (10) found that purified egg lecithin did not stabilize autoclaved fat emulsions in which a solution of dextrose was the aqueous phase. Zeringue *et al.* (8) produced a stable autoclaved fat emulsion stabilized with chromatographically homogeneous lecithin by substituting glycerol as the isotonic agent in the aqueous phase and adjusting the pH to 6.6–6.8. The marked instability of emulsions formulated with chromatographically homogeneous lecithin in the presence of electrolytes suggests that the electrolytes to which this type of emulsion is stable are quite limited and that salts known for their "salting out" effect bring about changes in the aqueous phase which are unfavorable to chromatographically homogeneous lecithin.

Although emulsion SR-182 and emulsion *A* were similar in composition, the egg phosphatide used in the emulsion *A* formula appeared to be nonhomogeneous, and could not be directly compared with the phosphatide in SR-182 which was quite highly purified. The greater amount of creaming of the SR-182 emulsion could be due to a difference in the composition of the two phosphatides, which lead to differences in the effects of the electrolyte solutions.

The creaming effects of the various salts with emulsion *B* were somewhat similar to those of emulsion SR-181, but differed considerably from those of the other emulsions. Emulsion *B*, although stabilized with phosphatide, differed in composition from the previously described emulsions. Soybean phosphatides were used as the stabilizer, sorbitol in place of glycerol as the isotonic phase, and a lower concentration in the oil phase, 10% cottonseed oil, as compared to 20%. The ratio of phosphatide to oil in the emulsion *B* formulation, 2.5% phosphatide to 10% oil, was considerably greater than the ratios of 1–1.2% phosphatide to 20% oil in the SR egg phosphatide-stabilized emulsions. These differences in composition may have contributed to the differences in creaming noted between emulsion *B* and SR emulsions.

It appeared that the electrolytes used caused agglomeration of the emulsions rather than coalescence. By visual inspection, cream formed in the cells and rose to the top surface. This cream could be redispersed, whereas coalescence would have resulted in free oil separation.

The relatively small amount of creaming that was observed in the phosphatide stabilized cottonseed oil emulsions essentially confirms the failure of dog blood sera to cream a similar emulsion (11). A conclusion of the latter study was that sera albumins did not complex with the fat component. It might be postulated from the present study that the sera electrolytes had but little effect on fat creaming because of the type of phosphatide used.

REFERENCES

(1) Singleton, W. S., and Brown, M. L., *J. Am. Oil Chemists' Soc.*, **42**, 312(1965).
(2) Le Veen, H. H., Giordano, P., and Johnson, A., *Am. J. Clin. Nutr.*, **16**, 129(1965).
(3) Van den Temple, M., *Rec. Trav. Chim.*, **72**, 442 (1953).
(4) *Ibid.*, **72**, 433(1953).
(5) Martin, A. R., and Hermann, R. N., *Trans. Faraday Soc.*, **37**, 30(1941).
(6) van Gils, G. E., and Kraay, G. M., "Advances in Colloid Science," vol. 1, Interscience Publishers, New York, N. Y., 1942, p. 259.
(7) Griffin, W. C., and Behrens, R. W., *Anal. Chem.*, **24**, 1076(1952).
(8) Zeringue, H. J., Brown, M. L., and Singleton, W. S., *J. Am. Oil Chemists' Soc.*, **41**, 688(1964).
(9) Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L., *ibid.*, **42**, 53(1965).
(10) Yeadon, D. A., Goldblatt, L. A., and Altschul, A. M., *ibid.*, **35**, 435(1958).
(11) Mann, G. E., Brown, M. L., Di Trapani, L. L., and Singleton, W. S., *Metabolism*, **10**, 859(1961).

Mechanisms of Interphase Transport I

Theoretical Considerations of Diffusion and Interfacial Barriers in Transport of Solubilized Systems

By A. H. GOLDBERG, W. I. HIGUCHI, N. F. H. HO, and G. ZOGRAFI

Based on first principles of diffusion, equations were derived to predict transport rates of micelle-solubilized drug from its aqueous environment to an oil phase. The basic equations also take into account the possible effects of an electrical barrier between the micelle and the charged oil–water interface. These processes may be considered as being analogous to absorption of water insoluble–bile soluble drugs, and may be used to evaluate the mechanism of transport in such situations. The physical model includes the effects of the free drug-solubilized drug equilibrium, the diffusion coefficients of both free and solubilized drug, ionic strength of the aqueous medium, and the micelle size. A novel experimental approach based on the use of emulsion droplets as the "sink" is described.

MUCH RESEARCH has been carried out, particularly in the last two decades, on the mechanisms of drug absorption. These studies have led to now well-established physicochemical concepts such as the importance of the oil–water partition coefficient, and the acid-base nature of the drug.

What appears to be conspicuously absent is an attempt to study the basic diffusional processes, both theoretically and experimentally with well-defined models. Such a study should provide a unified approach for explaining some of the discrepancies in the existing qualitative theories, and also suggest new rate-determining mechanisms for drug absorption. The authors are not so naive as to think that the simple laws of physical chemistry can generally describe so complex a subject as drug absorption. What is contended, however, is that where qualitative physicochemical relationships are established or expected, it is more than worthwhile to attempt to describe them on mechanistically quantitative bases.

DISCUSSION

The present communication is related to an aspect of drug absorption that has recently attracted some interest (1–4), *viz.*, the role of micellar solubilization. The theory governing the diffusion of both the solubilized and the free drug from an aqueous environment to a lipoidal interface is presented. The various interfacial boundary conditions influencing the rate of transport between phases have been considered. In order to study the transport rate theory, a novel experimental technique, which is based on the use of micron-sized oil emulsion droplets as a lipoidal "sink," has been developed.

Past Studies on Micellar Solubilization and Drug Absorption

The earliest studies involving micellar solubilization and biological availability were concerned with the activity of germicides in soap solutions (4–6). Allawala and Riegelman (7, 8) postulated that the thermodynamic activity of the free drug is the driving force behind the effectiveness of germicides dissolved in surfactant solutions.

Based on such studies, one would expect that if a surfactant is added to a solution of a drug in a concentration high enough to exceed its CMC, the drug's thermodynamic activity would be reduced. We would, therefore, expect a decrease in rate of transport because of a reduced driving force. This was shown by Levy *et al.* (1), who measured the absorption rates of secobarbital by goldfish in the presence of polysorbate 80 solutions.

To evaluate the effect of increasing surfactant concentration on the rate of transport of a solubilized drug, Matsumoto *et al.* (2, 3) used a cellulose membrane dialysis technique. These workers mathematically derived an equation for transport, based on the laws of diffusion and the concentration of free drug. Their experimental values matched their theoretical values only when no great interaction between the drug and the surfactant was observed. The degree of interaction was independently measured, and was classified as the ratio (r) of *total drug in solution* to *free drug*. When the values of r were four or greater,[1] the rates of transport exceeded the rates predicted. They postulated that this discrepancy was due to the transport of drug directly from the micelle. In these studies, it had been initially assumed that the micelles containing drug did not contribute to the transport phenomena. Matsumoto *et al.* (2, 3) eventually considered this a possibility, but only at high r values. According to their interpretation, however, it was not due to diffusion of the micelles, but rather to the coalescence of the micelles with the membrane. What was not considered was an enhanced flux that can be attributed to the diffusion of drug-containing micelles.

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication June 22, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by grant GM-13368-01 and fellowship A-F1-GM-34,237-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[1] In these experiments, the r value of the drug used is about 800, at a 1% polysorbate 80 concentration.

Present Considerations

In the transport of a drug from an aqueous solubilized system to an oil phase, the flux consists of the transport of the free drug and that of the solubilized drug (9). The solubilized drug may be considered as an interaction product between the drug and the surfactant. In order for the solubilized drug to pass from the aqueous phase to the oil phase, it must diffuse to some point close to the oil, and then leave the micelle, unless the micelle itself enters the oil. The greater the drug–surfactant interaction, the greater is the role played by the drug-containing micelle in the transport process.

There are several mechanisms by which a drug can leave a micelle. In the simplest case, where a nonionic drug is solubilized by a nonionic surfactant, we postulate that it is simply diffusion from the micelle, close to the oil "sink." In the case of drug in a charged micelle approaching a charged surface, there may be a strong electrical effect, either repulsive or attractive.

To study these ideas a procedure was conceived which involves the use of micron-sized oil emulsion droplets as a sink for the drug. There are a number of advantages, both theoretical and experimental, in employing such a system instead of one involving two or more bulk phases as has been done by some investigators (10). First, even when the rates of interphase transfer are purely diffusion controlled in the external (aqueous) phase, the results are relatively insensitive to the rate of agitation of the medium over a wide range of shear rates, and the infinite-sink, steady-state mathematics may be applied to the data. This is to be expected from hydrodynamic considerations and from the theory of diffusion involving small spheres. As a result, the data may be directly evaluated with diffusion theory without invoking the empirical "effective" diffusion layer thickness ideas which have been the major cause of difficulties in past studies (10). Another advantage is that, because droplet distortion is likely to be negligible for such small droplets, it is safe to assume in most instances that surface-active agents will not alter the effective surface area of the droplets as they frequently do in the case of large interfaces. Finally, it can be shown that rate experiments with small droplets are much more sensitive to interfacial resistances. Based on the estimated diffusion layer thickness of 200 to 400 μ from the studies of Rosano *et al.* (10), experiments with sinks consisting of micron-sized oil droplets should be more than a hundred times more sensitive to interfacial resistances.

Case A—*Simple Diffusion*—For the first case to be considered, that of diffusion of a nonionic drug from a nonionic surfactant, a planar model will be presented first, and then rederived for the case of spherically symmetric transport to an oil droplet. This derivation is based solely upon the principles of diffusion. A graphical representation for the planar diffusion case is shown in Fig. 1.

If the transport rate of the drug is diffusion controlled, then the rate of uptake by the oil is equal to the rate of transport through the aqueous phase. The steady-state rate of diffusion in the aqueous phase can be denoted by:

$$G = \left(A\, D_d \frac{dC_d}{dl} + A\, D_{dm} \frac{dC_{dm}}{dl} \right) \qquad \text{(Eq. 1)}$$

Fig. 1—Illustration of the planar model of diffusion. Key: C_{dm} *= concentration of drug in the micelles;* C_d *= concentration of free drug* l *= diffusion layer thickness.*

Where G = the steady-state rate of transport through the aqueous phase to the oil droplet, A = the area, D = the diffusion coefficients,[2] and dC/dl = the concentration gradients. If the area is maintained constant, Eq. 1 can be rearranged to:

$$\frac{G}{A}\, dl = D_d\, dC_d + D_{dm}\, dC_{dm} \qquad \text{(Eq. 2)}$$

Integration from $l = 1$ to $l = 0$ gives:

$$\frac{G}{A}\, l = D_d(\Delta C_d) + D_{dm}(\Delta C_{dm}) \qquad \text{(Eq. 3)}$$

Considering the transport from an aqueous phase to an oil phase which acts as a "perfect sink," (ΔC) can be denoted as (C^b-C^s), where C^b is the bulk aqueous concentration, and C^s is the concentration of the drug very close to the oil phase. The steady-state rate of diffusion is then finally denoted by:

$$G = \frac{A}{l} \left[D_d \left(C_d^b - C_d^s \right) + D_{dm} \left(C_{dm}^b - C_{dm}^s \right) \right] \qquad \text{(Eq. 4)}$$

The integral of this rate, with respect to time, should be equal to the uptake of the drug by the oil, and is expressed as:

$$\int G dt = C_{do} V \qquad \text{(Eq. 5)}$$

where C_{do} = the concentration of the drug in the oil, and V = the volume (ml./ml.) of the oil. Differentiation of both sides of Eq. 5 then expresses the rate of change of the concentration of the drug in the oil with respect to time, and is denoted by:

$$G = V \frac{dC_{do}}{dt} \qquad \text{(Eq. 6)}$$

Substitution for G from Eq. 4 gives:

$$\frac{A}{l} \left[D_d \left(C_d^b - C_d^s \right) + D_{dm} \left(C_{dm}^b - C_{dm}^s \right) \right] = V \frac{dC_{do}}{dt} \qquad \text{(Eq. 7)}$$

[2] Subscript d = free drug; subscript dm = drug in the micelles.

There are several relationships that can be used to reduce Eq. 7 to one equation with one unknown.

It has frequently been observed (11) that above the critical micelle concentration:

$$C_{dm} = KC_dC_{saa} \quad \text{(Eq. 8)}$$

where K = the pseudoequilibrium constant relating C_{dm} to C_d, and C_{saa} = the concentration of surfactant. If we assume that this relationship holds, then the substitution of Eq. 8 into Eq. 7 gives:

$$\frac{A}{l}\left[D_d\left(\frac{C_{dm}{}^b}{KC_{saa}} - \frac{C_{dm}{}^s}{KC_{saa}}\right) + D_{dm}(C_{dm}{}^b - C_{dm}{}^s)\right] = V\frac{dC_{do}}{dt} \quad \text{(Eq. 9)}$$

If the (KC_{saa}) terms in the denominator are factored, we can then factor $(C_{dm}{}^b\text{-}C_{dm}{}^s)$ to give:

$$\frac{A}{l}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)(C_{dm}{}^b - C_{dm}{}^s) = V\frac{dC_{do}}{dt} \quad \text{(Eq. 10)}$$

We now have only three concentration terms that are unknown.

We may base the analysis on a total emulsion volume of 1 ml. Then:

$$T = C_{do}V + C_{aq.}(1 - V) \quad \text{(Eq. 11)}$$

where T = the total amount of drug present and $C_{aq.}$ = the drug concentration in the aqueous phase. However, $C_{aq.}$ can be taken as $(C_{dm}{}^b + C_d{}^b)$. Inserting this into Eq. 11 and substituting for $C_d{}^b$ from Eq. 8, we arrive at:

$$T = C_{do}V + C_{dm}{}^b\left(1 + \frac{1}{KC_{saa}}\right)(1 - V) \quad \text{(Eq. 12)}$$

Rearrangement of Eq. 12 gives:

$$C_{dm}{}^b = \frac{T - C_{do}V}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} \quad \text{(Eq. 13)}$$

To relate $C_{dm}{}^s$ to C_{do}, we can use the apparent partition coefficient. The true partition coefficient for a drug between oil and water is given by:

$$PC_{o/w} = \frac{C_{do}}{C_d{}^s} \quad \text{(Eq. 14)}$$

The apparent partition coefficient may be expressed as:

$$PC_{app.} = \frac{C_{do}}{C_{aq.}} = \frac{C_{do}}{C_d{}^s + C_{dm}{}^s} \quad \text{(Eq. 15)}$$

Taking the reciprocal of Eq. 15 gives:

$$\frac{1}{PC_{app.}} = \frac{1}{PC_{o/w}} + \frac{C_{dm}{}^s}{C_{do}} \quad \text{(Eq. 16)}$$

Equation 16 rearranges to:

$$C_{dm}{}^s = C_{do}\left[\frac{1}{PC_{app.}} - \frac{1}{PC_{o/w}}\right] \quad \text{(Eq. 17)}$$

Substitution of Eqs. 13 and 17 into Eq. 10 yields:

$$\frac{A}{l}\left\{\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)\times\left(\frac{T - C_{do}V}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} - C_{do}\left[\frac{1}{PC_{app.}} - \frac{1}{PC_{o/w}}\right]\right)\right\} = V\frac{dC_{do}}{dt} \quad \text{(Eq. 18)}$$

The C_{do} terms can be factored to give:

$$\frac{A}{l}\left\{\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)\times\left[\frac{T}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} - C_{do}\left(\frac{V}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} + \left(\frac{1}{PC_{app.}} - \frac{1}{PC_{o/w}}\right)\right)\right]\right\} = V\frac{dC_{do}}{dt} \quad \text{(Eq. 19)}$$

We can collect constants, and let:

$$\alpha = \frac{T}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} \quad \text{(Eq. 20)}$$

and

$$\beta = \frac{V}{\left(1 + \frac{1}{KC_{saa}}\right)(1 - V)} + \left(\frac{1}{PC_{app.}} - \frac{1}{PC_{o/w}}\right) \quad \text{(Eq. 21)}$$

Insertion of Eqs. 20 and 21 into Eq. 19 and rearrangement yields:

$$\frac{dC_{do}}{\alpha - \beta C_{do}} = \left(\frac{D_d}{KC_{saa}} + D_{dm}\right)\frac{A}{lV}dt \quad \text{(Eq. 22)}$$

Integration of t from 0 to t and of C_{do} from 0 to C_{do} gives:

$$\ln\left(\frac{\alpha}{\alpha - \beta C_{do}}\right) = \left(\frac{\beta A}{lV}\right)\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)t \quad \text{(Eq. 23)}$$

A plot of the lefthand side of this function *versus* time should yield a straight line going through the origin, with a slope of $(\beta A/lV)\,(D_d/KC_{saa} + D_{dm})$.

If we now derive this equation for small spheres, we can graphically represent the model as in Fig. 2.

By rewriting Eq. 1 and replacing the area (A) by the area of a sphere, Eq. 1 becomes:

$$G = 4\pi r^2\left(D_d\frac{dC_d}{dr} + D_{dm}\frac{dC_{dm}}{dr}\right) \quad \text{(Eq. 24)}$$

where $\frac{dC}{dr}$ = the concentration gradient as a function of the radius. Equation 24 rearranges to:

$$\frac{dr}{r^2} = \frac{4\pi}{G}(D_d\,dC_d + D_{dm}\,dC_{dm}) \quad \text{(Eq. 25)}$$

Integration from $r = \infty$ to $r = a$, gives:

$$\frac{1}{a} = \frac{4\pi}{G}(D_d\Delta C_d + D_{dm}\Delta C_{dm}) \quad \text{(Eq. 26)}$$

or, with rearrangement and insertion of the same

concentration notations as in Eq. 4, it becomes:

$$G = 4\pi a[D_d (C_d^b - C_d^s) + D_{dm} (C_{dm}^b - C_{dm}^s)] \quad \text{(Eq. 27)}$$

The $4\pi a$ term has come from the derivation for a single sphere; however, for an emulsion that has a narrow distribution of oil droplet sizes, this can be replaced by $n4\pi a$, where n is the number of droplets. Since in a dilute, stable emulsion this term is constant with time, we can assign it the constant value A.

If we now make the same substitutions as were made for the planar case, the final equation becomes:

$$\ln\left(\frac{\alpha}{\alpha - \beta C_{do}}\right) = \frac{\beta A}{V}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right) t \quad \text{(Eq. 28)}$$

The only difference between this equation and Eq. 24 for the planar model is the disappearance of (l), the size of the diffusion layer.

Equation 28 may also be rewritten to give:

$$C_{do} = \frac{\alpha}{\beta}\left\{1 - \exp.\left[-\frac{\beta A}{V}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right) t\right]\right\} \quad \text{(Eq. 29)}$$

Every term, excluding C_{do}, can be independently determined. From such data, C_{do} values can be predicted for any value of time, and can be compared to the C_{do} values experimentally obtained.

There have been several assumptions made in the derivation of these equations. The first is that there exists a K between C_{dm} and C_d that holds below, as well as at saturation. It also has been assumed that the apparent partition coefficient expression correctly represents the relationship between C_{do} and C_{dm}^s. Both these assumptions can be shown to be valid with appropriate experiments.

Case B—*Electrical Effects*—The second case, that of a charged micelle diffusing to a charged lipid interface, will be presented for spheres directly. This is illustrated in Fig. 3. The steady-state rate of diffusion now has an additional term added that considers the force field of the electrical barrier. The expression for the rate becomes:

$$G = 4\pi r^2 \times \left[D_d \frac{dC_d}{dr} + D_{dm} \frac{dC_{dm}}{dr} + \frac{D_{dm}}{kT} C_{dm} \frac{dV^o}{dr}\right] \quad \text{(Eq. 30)}$$

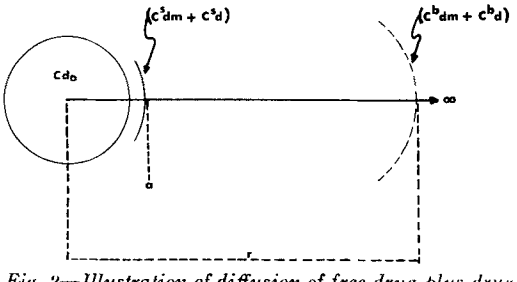


Fig. 2—Illustration of diffusion of free drug plus drug in the micelles from the aqueous phase to an oil droplet "sink." Key: C_{do} *= concentration of drug in the oil;* C^s_{dm} *= concentration of drug in the micelles, at some point* (a)*, arbitrarily close to the oil droplet;* C^b_{dm} *= concentration of drug in the micelles at an infinite distance (the bulk) from the oil droplet;* C_d *= concentration of free drug.*

The last term in the equation gives the influence of the electrical interaction upon the rate. Here V^o is the potential energy of interaction of the micelle with the charged oil–water interface. The factor, $(D_{dm}/kT)(dV^o/dr)$, is the average micellar velocity contribution due to the force. The k and T are the Boltzmann constant and the absolute temperature.

We may substitute for C_{dm} from Eq. 8 into Eq. 30. This gives:

$$G = 4\pi r^2 \left[D_d \frac{dC_d}{dr} + D_{dm}KC_d \frac{dC_{saa}}{dr} + D_{dm}KC_{saa} \frac{dC_d}{dr} + \frac{KC_dD_{dm}}{kT} C_{saa} \frac{dV^o}{dr}\right] \quad \text{(Eq. 31)}$$

Taking the case where the transport rate of the surface-active agent itself is zero, we may write:

$$C_{saa} = C^b_{saa} \exp.(-V^o/kT) \quad \text{(Eq. 32)}$$

where C^b_{saa} is the surfactant concentration at infinity. Equation 32 states that the micelle distribution obeys the usual Boltzmann distribution.

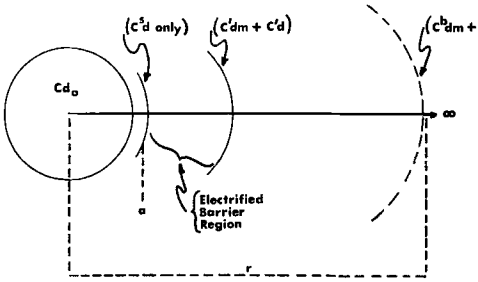


Fig. 3—Illustration of diffusion of free drug plus drug in the micelles freely diffusing to some distance from the oil droplet, where an electrical barrier permits only free drug to diffuse to some point (a)*, arbitrarily close to the oil droplet. Key:* C_{do} *= concentration of drug in the oil;* C_d *= concentration of free drug;* C_{dm} *= concentration of drug in the micelles; superscript* s *= surface,* b *= bulk, and the prime = the concentration at the start of the electrical barrier region.*

We may now substitute Eq. 32 into Eq. 31 and obtain:

$$G = 4\pi r^2 \times [D_d + D_{dm}KC^b_{saa} \exp.(-V^o/kT)] \frac{dC_d}{dr} \quad \text{(Eq. 33)}$$

This may be integrated as was done previously, C_d from C_d^s to C_d^b and r from a to ∞, to give:

$$G = \frac{4\pi (C_d^b - C_d^s)}{\int_a^\infty \frac{dr}{r^2 [D_d + D_{dm}KC^b_{saa} \exp.(-V^o/kT)]}} \quad \text{(Eq. 34)}$$

The integral in the denominator of Eq. 34 may be evaluated numerically. As it is constant with respect to time, it can be identified as the constant, γ.

As before, the drug transported from the aqueous phase to the oil phase can be represented by Eq. 5.

As was done for case A, we may substitute for G from Eq. 34 to give:

$$\int_0^t \frac{4\pi}{\gamma}(C_d^b - C_d^s)\,dt = C_{do}V \qquad \text{(Eq. 35)}$$

Differentiation of both sides gives:

$$\frac{4\pi}{\gamma}(C_d^b - C_d^s) = V\frac{dC_{do}}{dt} \qquad \text{(Eq. 36)}$$

By means of Eqs. 12, 15, and 36 we obtain the following equation:

$$\frac{4\pi}{\gamma}\left\{\frac{\text{tot.}}{(1 + KC_{saa}^b)(1 - V)} - C_{do}\times\left[\frac{V}{(1 + KC_{saa}^b)(1 - V)} + \left(\frac{1}{PC_{app.}(1 + KC_{saa}^b)}\right)\right]\right\} = V\frac{dC_{do}}{dt} \qquad \text{(Eq. 37)}$$

We can collect constants, and let:

$$\delta = \frac{\text{tot.}}{(1 + KC_{saa}^b)(1 - V)} \qquad \text{(Eq. 38)}$$

and also,

$$\epsilon = \left[\frac{V}{(1 + KC_{saa}^b)(1 - V)} + \frac{1}{PC_{app.}(1 + KC_{saa}^b)}\right] \qquad \text{(Eq. 39)}$$

If Eqs. 38 and 39 are substituted into Eq. 37, then rearranged and integrated from $t = 0$ to $t = t$, $C_{do} = 0$ to $C_{do} = C_{do}$, then we find that:

$$\ln\left(\frac{\delta}{\delta - \epsilon C_{do}}\right) = \frac{4\pi\epsilon}{\gamma V}t \qquad \text{(Eq. 40)}$$

This can be rewritten to:

$$C_{do} = \frac{\delta}{\epsilon}\left[1 - \exp.\left(-\frac{4\pi\epsilon}{\gamma V}t\right)\right] \qquad \text{(Eq. 41)}$$

Here, as in case A, all terms other than C_{do} can be independently determined and can be compared to experimentally obtained C_{do} values.

Discussion—A complete examination of models such as those discussed here requires extensive complementary studies of such parameters as K, $PC_{app.}$, D_d, D_{dm}, particle size, and a of the emulsion droplets, and the surface potential (actually the ζ-potential). Such studies are underway for several systems.

Preliminary results have been obtained on the transport rate of 2,3-bis(paramethoxyphenyl indole), a drug with very low water solubility. Isopropyl myristate was the emulsion oil phase with polysorbate 80 as the surfactant.

Figure 4 shows the comparison of experimental transport data for this system compared with theory (Eq. 29). The constants K, C_{saa}, α, and β were known from other experiments. However, only an estimate of the particle size of the emulsion and the diffusion coefficients, D_d and D_{dm}, were available. Therefore, the relatively good agreement is being regarded as tentative. A forthcoming communication (12) will present a full test.

Aside from the uncertainties in some of the parameters, the deviations between the experiments and theory in Fig. 4 are believed to be due to (*a*) neglect of the effects of the nonsteady-state diffusion, (*b*) the heterodispersity of the emulsion droplets, and (*c*) the effect of neighboring particles upon the particles in question. These factors are also being investigated, and it appears that all of these effects may be accounted for satisfactorily.

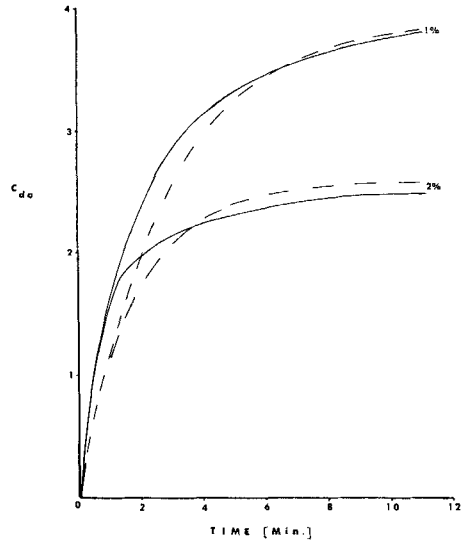


Fig. 4—Illustration of the correlation of predicted C_{do} *values to those experimentally found as a function of time. Key:* C_{do} *= concentration of drug in the oil; ——, experimental values; – – –, theoretical. The 1% and 2% refer to the concentration of polysorbate 80 present.*

Theoretical calculations of the integral in Eq. 34 have been carried out to estimate the expected electrical barrier effects upon G. The potential function, V^o, was taken to be:

$$V^o = V_A^o + V_R^o \qquad \text{(Eq. 42)}$$

where

$$V_A^o = -\frac{A}{6}\left[\frac{2a'(H + a')}{H(H + 2a')} - \ln\left(\frac{H - 2a'}{H}\right)\right] \qquad \text{(Eq. 43)}$$

is the Hamaker relation (13) for the attraction between a sphere and a plane. Here, a' is the radius of the micelle, and H is the closest distance of separation between the surface of the plane (oil droplet) and the surface of the sphere (micelle). A is the Hamaker constant, taken as 5×10^{-13} erg. V_R^o is given by:

$$V_R^o = \epsilon a'\psi_o^2 \ln\,[1 + \exp.\,(-KH)] \qquad \text{(Eq. 44)}$$

which is the electrical double layer repulsion between a sphere and a plane. ψ_o is the surface potential assumed to be the same for the micelle and the surface of the oil droplet, K is the Debye-Huckel κ, and ϵ is the dielectric constant of the medium. Equation 44 is strictly valid for the case in which $\psi_o \lesssim 25$ mv. and $a' \gg 1/K$. However, it is believed (14) to be a good approximation even for $a' \backsimeq 1/K$ and $\psi_o \backsimeq 50$ mv.

The calculations have shown that even for $\psi_o = 50$ mv., $a' = 50$ Å., and $1/K = 50$ Å., the interfacial electrical barriers may be great enough to significantly reduce G by a factor of two or more.

REFERENCES

(1) Levy, G., Miller, K. E., and Reuning, R. H., *J. Pharm. Sci.*, **55**, 394(1966).
(2) Matsumoto, H., Matsumura, H., and Iguchi, S., *Chem. Pharm. Bull.*, **14**, 398(1966).
(3) Matsumoto, H., *Yakagaku Zasshi*, **86**, 590(1966).
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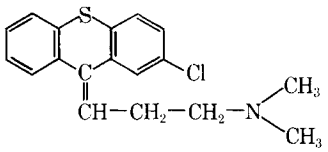
By JACK E. WALLACE

A rapid spectrophotometric method for determining chlorprothixene permits analysis of the drug in biologic specimens in the presence of its metabolites and other alkaline drugs without preliminary separation. The procedure is based upon the oxidation of chlorprothixene and its immediate metabolites to a mixture of reaction products by means of alkaline permanganate. In hexane the products have a characteristic ultraviolet absorption curve with a well-defined maximum at 233 mμ. The strong absorption at this wavelength is utilized as a basis for the analytical procedure. The method is sufficiently sensitive for the determination of chlorprothixene in urine 3 days after the ingestion of a single 50-mg. dose. Observations concerning the distribution of chlorprothixene in the rat and the excretion of the drug in man are presented.

CHLORPROTHIXENE,[1] *trans* isomer of 2-chloro-9-(13-dimethylaminopropylidene) thioxanthene, is a potent tranquilizing agent not only in acute and chronic schizophrenia but in severe psychoneuotic conditions. The chemical structure of chlorprothixene resembles that of the phenothiazines. Few methods for the determination of the drug are available in the scientific literature.

Ferrari and Toth (1) described a thin-layer chromatographic technique which identifies urinary metabolites of chlorpromazine, chlorprothixene, imipramine, and amitriptyline. Although the method is specific, it is time consuming and is not quantitative. Fluorometric procedures for determining chlorprothixene in blood and urine (2) are very sensitive, detecting 0.25-mcg. amounts of the drug, but they require the utilization of specialized analytical instruments.

Chlorprothixene strongly absorbs ultraviolet radiations, therefore, it can be determined spectrophotometrically in biologic extracts, but its strongest absorption is at 227 mμ. At that wavelength the spectral curve of the drug is often affected by background absorption and sulfoxide metabolites which induce a wide degree of variance in the ultraviolet absorbance curve, unless extensive purification of the drug extract is achieved. The method reported here is a specific spectrometric assay which requires no extensive purification procedure or no separation of unchanged drug and sulfoxides (3). The method permits a reliable evaluation of drug intake by patients treated with chlorprothixene.



Chlorprothixene

EXPERIMENTAL

Instrumentation—A Beckman DK-2A ratio recording spectrophotometer with linear presentation of the wavelength was used for the ultraviolet absorption measurements. The sample path was 10 mm. throughout. A Beckman IR-4 doublebeam infrared spectrophotometer was used for infrared spectral characterization of functional groups in the reaction products. Gas chromatographic analyses were performed by means of a Barber-Colman model 5000 gas chromatograph utilizing a 6-ft. U-shaped glass column containing 2% Carbowax 20 M on Gas-Chrom Q, 100–120 mesh. An F & M model

Received April 17, 1967, from the Forensic Toxicology Branch, U. S. Air Force Epidemiological Laboratory, Lackland Air Force Base, TX 78236
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REFERENCES

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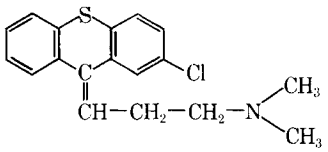

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185 carbon-hydrogen-nitrogen analyzer was used for elemental analysis of the chlorprothixene reaction products.

Method—Ten milliliters of whole oxalated blood, serum, or gastric contents are adjusted to pH 7.0–7.5 by the addition of a dilute aqueous solution of sodium hyroxide. Ten to 50 ml. of urine or 10 Gm. of homogenized tissue specimens are adjusted to a pH of 11.0 to 12.0 with 5 *N* sodium hydroxide. The resulting solution or homogenate is shaken vigorously for 3 min. with 200 ml. of *n*-hexane (spectroanalyzed) in a 500-ml. separator. The hexane layer is placed in a graduated cylinder by filtration through Whatman No. 541 filter paper. The volume of hexane recovered is recorded, and the lost hexane is considered in the final calculations. Five milliliters of 0.5 *N* HCl is added to the filtered hexane, and this mixture is shaken for 3 min. Four milliliters of the aqueous layer is placed in a 500-ml. round-bottom flask to which are added 20 ml. of 1% $KMnO_4$ buffered at pH 12.4 with 0.4 *M* sodium triphosphate–0.4 *M* sodium hydroxide buffer, 50 ml. of *n*-hexane (spectrophotometric grade), and a Teflon-coated magnetic stirring bar. The contents of the flask are refluxed for 30 min. with constant magnetic stirring. After cooling, the *n*-hexane is separated from the aqueous permanganate layer, washed with 5 ml. of 0.5 *N* HCl, and read in the spectrophotometer at 5 mμ intervals from 220 to 340 mμ against a blank of *n*-hexane. At least three maxima should be observed. If absorption at a single wavelength is desired, it may be determined at 233 mμ. Should the *n*-hexane contain less than 2.5 mcg./ml. of chlorprothixene as the reaction product, the following concentration technique should be performed. The recovered *n*-hexane, whose total volume is recorded, is placed in a 125-ml. flask which is then attached to a rotary vacuum evaporator. The solvent is evaporated by vacuum while the flask, partially submerged, rotates in a 50° water bath. Depending on the absorbance previously observed, the residue is dissolved in 2, 4, or 8 ml. of *n*-hexane. A standard curve is prepared from aqueous solutions of the drug which have been carried through the procedure described, including the concentration technique for solutions containing small amounts of chlorprothixene.

RESULTS AND DISCUSSION

At 233 mμ a linear relationship exists between the absorbance of the reaction products and the concentration of the drug in the original sample. This relationship is demonstrated in Table I. The ultraviolet absorption spectra of chlorprothixene

TABLE I—STANDARD CURVE DATA FOR CHLORPROTHIXENE REACTION PRODUCT

Chloroprothixene in Sample, mcg./ml.	Absorbance of Reaction Product in Hexane[a]	Absorbance/ Concn.
10.0	0.84	0.084
8.0	0.67	0.084
6.0	0.50	0.083
4.0	0.33	0.083
2.0	0.16	0.080

[a] Read at 233 mμ.

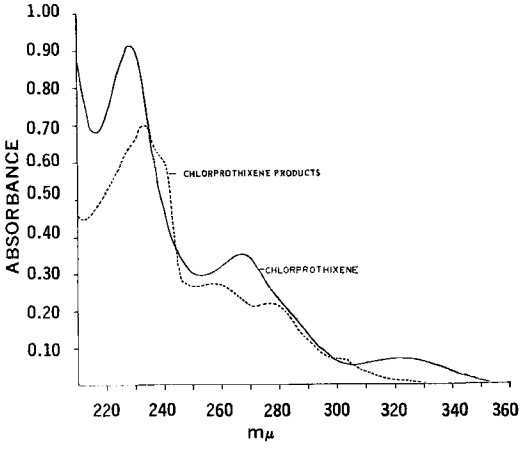

Fig. 1—Ultraviolet absorption spectra of chlorprothixene, 10 mcg./ml. in water, and of the chlorprothixene reaction products corresponding to a sample of equivalent concentration in hexane.

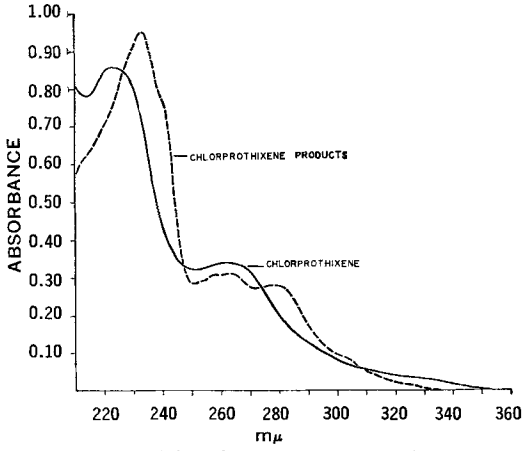

Fig. 2—Ultraviolet absorption spectra of chloroprothixene in water and of the chlorprothixene reaction products in hexane both obtained with chlorprothixene and its metabolites extracted from liver of rats.

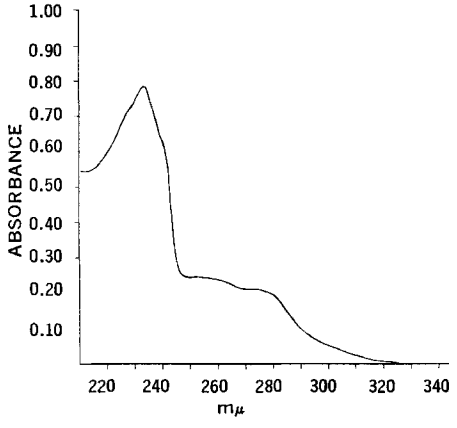

Fig. 3—Ultraviolet absorption spectrum of chlorprothixene reaction products obtained with chlorprothixene and its metabolites extracted from urine of humans.

and of its oxidation products are shown in Fig. 1. The absorption curve of the products is well defined, with absorbance maxima in hexane or ethanol at 278, 258, and 233 mμ and a shoulder at 302 mμ. The reaction products of chlorprothixene extracted from human urine and from the tissues of animals which have received the drug yield almost identical absorption curves to that observed for the reaction products of pure drug (Figs. 2 and 3). Direct analysis of chlorprothixene and its metabolic products from urine and animal tissues does not afford an ultraviolet absorption pattern from which accurate qualitative and quantitative data can be obtained (Fig. 2). The infrared spectrum of the reaction products of pure chlorprothixene is similar to the spectrum obtained from the reaction products of chlorprothixene extracted from human urine. Infrared spectra of chlorprothixene (Fig. 4) and of its reaction products (Fig. 5) show several distinct differences, of which the most significant is the appearance of a band at 1682 cm.$^{-1}$ in the spectrum of the reaction products. This band is thought to be produced by a carbonyl group (4) formed by mild permanganate oxidation of the olefinic carbon-to-carbon bond in the parent compound. Very strong absorption at 1150 and 1305 cm.$^{-1}$ in the spectrum of the chlorprothixene products suggests that a majority of the sulfur is oxidized to a sulfone group. Schrieber (5) examined the spectra of 13 sulfones and found two bands in the sulfones which were absent in the corresponding sulfides. These were at 1120–1160 cm.$^{-1}$ and at 1300–1350 cm.$^{-1}$. It seems likely that some sulfoxide is also present in the reaction products, since a strong band is obtained at 1020–1060 cm.$^{-1}$ (Fig. 5). Most normal sulfoxides will be found to give a strong band in the range 1020–1060 cm.$^{-1}$ in the solid state. The weak absorption

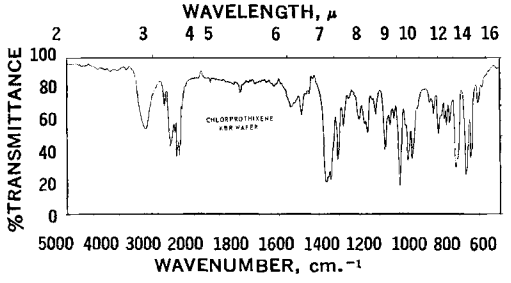

Fig. 4—Infrared absorption spectrum of chlorprothixene, 2 mg./400 mg. of potassium bromide.

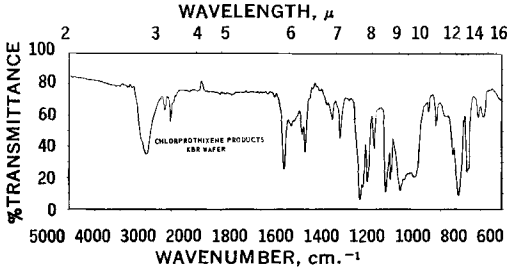

Fig. 5—Infrared absorption spectrum of chlorprothixene reaction products, 2 mg./400 mg. of potassium bromide.

TABLE II—COMPOUNDS INVESTIGATED FOR INTERFERENCE WITH THE DETERMINATION OF CHLORPROTHIXENE[a]

Compd.	Absorbance of Hexane Extract[b]
Chlorprothixene	1.61
Blank	0.02
Amitriptyline (250 mμ)	1.35
Amphetamine	0.02
Antazoline	0.06
Azacyclonol (247 mμ)	0.52
Caffeine	0.02
Captodiamine (247 mμ)	1.41
Carbinoxamine (270 mμ)	0.87
Chlordiazepoxide	0.05
Chlorothen	0.02
Chlorpromazine	0.02
Cyclizine (247 mμ)	1.24
Diazepam	0.18
Diphenhydramine (247 mμ)	1.34
Doxylamine	0.02
Ephedrine	0.02
Hydroxyzine (254 mμ)	0.85
Imipramine	0.02
Lidocaine	0.02
Methoxamine	0.02
Methylphenidate	0.02
Morphine	0.02
Nortriptyline (250 mμ)	1.08
Phenaglycodol	0.04
Phenindamine	0.02
Pheniramine	0.10
Phenmetrazine	0.02
Pilocarpine	0.02
Pipradrol (247 mμ)	0.85
Propoxyphene	0.09
Pyrilamine	0.05
Pyrrobutamine	0.20
Reserpine	0.02
Thenyldiamine	0.02
Tricyclamol	0.02
Triprolidine	0.15

[a] The absorbance in each case corresponds to a concentration of 20 mcg. of the compound per ml. of sample. [b] Read at 233 mμ unless otherwise indicated.

by the products in the 2800–3000 cm.$^{-1}$ band area to which carbon-hydrogen stretching vibrations have characteristic assignment (6) implies that the dimethylaminopropylidene group is removed during the oxidation process. Hydrogen, carbon, and nitrogen analysis of two fractions obtained by fractional crystallization of a saturated solution of the products in hexane supports the infrared data. One fraction appears to contain a carbonyl–sulfoxide product, the other a carbonyl–sulfone product.

Many organic bases were investigated for possible interference with quantitative determination of chlorprothixene. Several produced a reaction product(s) which gave considerable absorption at 233 mμ (Table II). However, none showed maximum absorption at 233 mμ, nor did any exhibit the absorption pattern characteristic of the chlorprothixene reaction products (Fig. 1). The reaction products of compounds listed in Table II which absorb maximally at 247 mμ, except that from captodiamine, are all benzophenone, and they can be identified as such by gas chromatography (7). The chlorprothixene reaction products chromatographed under the same conditions exhibit no chromatographic peak (Table III).

TABLE III—RELATIVE RETENTION TIMES OF REACTION PRODUCTS BY GAS CHROMATOGRAPHY[a]

Compd.	Max. Ultraviolet Absorption,[b] mμ	Reaction Ratio Reaction Product/Benzophenone Peak 1	Peak 2
Chlorprothixene	233	...[c]	...
Amitriptyline	250	4.5	5.5
Azacyclonol	247	1.0	...
Captodiamine	247	...	...
Carbinoxamine	270	3.0	...
Cyclizine	247	1.0	...
Diphenhydramine	247	1.0	...
Hydroxyzine	254	2.0	...
Nortriptyline	250	4.5	5.5
Pipradrol	247	1.0	...

[a] Chromatographic conditions: 6-ft. glass column packed with 100–120 mesh Gas-Chrom Q (Applied Science Laboratories) coated with 2% by weight Carbowax 20M (AnaLabs, Inc.). Column temperature 200°; injector temperature, 240°. Flow rate 50 ml. nitrogen/min. [b] In hexane. [c] No peak observed.

Recoveries of known amounts of chlorprothixene added to whole blood and urine are summarized in Table IV. Standard aqueous solutions of chlorprothixene hydrochloride were added to the biologic material to provide concentrations ranging from 0.50 to 10.0 mg. %. Ten milliliters of blood or 20 ml. of urine with added drug was analyzed. Recoveries of greater than 90% were obtained in each instance. It was observed very early in this work that the pH range 7.0–7.5 used in preliminary recovery studies was inadequate for maximum extraction of chlorprothixene and its metabolites from the urine of individuals who had taken the drug. Table V presents data from a healthy male who had taken a single 50-mg. oral dose, and whose urine was collected during the period 24–48 hr. after the dose. Samples of equivalent amounts were assayed for chlorprothixene, varying the pH for the initial extraction of the drug from 7.0 to greater than 12.5. Optimum recovery was obtained at pH 10.0 or greater.

TABLE IV—RECOVERY OF CHLORPROTHIXENE AFTER *In Vitro* ADDITION TO BLOOD AND URINE

Amt. Added, mcg./ml.	No. of Detn.	Recovery, Mean ± Std. (mcg./ml.) Whole Blood[a]	Urine[b]
50.0	10	47.1 ± 1.5	46.9 ± 1.7
25.0	12	23.7 ± 0.3	24.0 ± 0.5
10.0	11	9.2 ± 0.2	9.0 ± 0.3

[a] Extracted at pH 7.0–7.5. [b] Extracted at pH 11.0–12.0.

TABLE V—EFFECT OF pH ON EFFICIENCY OF EXTRACTION OF CHLORPROTHIXENE FROM HUMAN URINE[a]

pH of Extraction	Concn. Determined, mcg./ml.
7.0	0.29
8.0	0.53
9.0	0.75
10.0	0.92
11.0	0.96
12.0	0.93
12.5–13.0	0.94

[a] Urine obtained during the period 24–48 hr. after an oral dose of 50 mg.

To determine the optimum pH of extraction for biologic specimens other than urine, white albino rats were fed *via* stomach tube 70 mg. of chlorprothixene per kilogram of body weight. They were killed after 3, 4.5, 6, 12, and 24 hr. Tissues were assayed for chlorprothixene in duplicate, using a pH of either 7.0–7.5 or of 11.0–12.0 for the initial extraction (Table VI). In all tissues much more drug was recovered when the specimens were initially extracted at pH 11.0–12.0. Since chlorprothixene itself is efficiently recovered from biologic materials at pH 7.0–7.5 (Table IV) it seems reasonable to assume that certain metabolites in the tissues and urine necessitate the strongly alkaline extraction.

Table VII shows the distribution of the drug among various tissues of the rat 6 to 24 hr. after the animals had received the 70 mg./Kg. of body weight dose. The levels of drug and metabolites in various biologic fluids and organs in decreasing order are urine, liver, brain, fat, skeletal muscle, and blood. With the exception of the data for concentrations of the drug in muscle and fat tissues, the distribution ratios are in good agreement with those previously reported (8).

In three adult human males, each of whom received 50 mg. of chlorprothixene by mouth, the

TABLE VII—CHLORPROTHIXENE[a] DISTRIBUTION IN RAT

Tissue or Biologic Fluid[b]	Distribution Ratio[c] Concn. in Tissue or Fluid Concn. in Liver
Blood	0.02
Brain	0.37
Fat	0.23
Kidney	0.83
Liver	1.00
Muscle	0.16
Urine	2.50

[a] Chlorprothixene and metabolities. [b] Specimens obtained 6 to 24 hr. after rats had ingested 70 mg./Kg. of body weight. Blood initially extracted at pH 7.0–7.5. Remaining specimens initially extracted at pH 11.0–12.0. [c] Average of results from 16 rats.

TABLE VI—pH DEPENDENCE OF CHLORPROTHIXENE EXTRACTION[a]

Time, hr.	Extracted at pH	Chlorprothixene Recovered,[b] mcg./ml. or Gm. Blood	Brain	Fat	Kidney	Liver	Muscle
3	7.0–7.5	0.9	10.9	10.7	15.5	27.4	5.1
	11.0–12.0	...[c]	16.5	10.5	39.5	47.2	8.6
4.5	7.0–7.5	0.7	4.4	4.1	16.9	26.6	3.3
	11.0–12.0	...	12.5	10.0	30.7	37.1	4.9
6	7.0–7.5	0.8	6.4	6.3	16.3	16.0	3.3
	11.0–12.0	...	13.5	10.5	27.3	32.8	5.1

[a] Tissues from rats given chlorprothixene 70 mg./Kg. by stomach tube. [b] Pooled tissues from three rats. [c] Not determined.

TABLE VIII—CHLORPROTHIXENE RECOVERED FROM HUMAN URINE[a]

Time, hr.	Adult Male Subject I	II	III
	mcg./ml.		
4	0.14	0.55	3.33
8	1.46	3.76	7.59
12	1.48	4.45	3.99
24	4.19	2.32	2.26
48	2.21	0.88	1.14
72	1.19	0.20	0.35
Total recovered, mg.	3.6	3.4	3.6

[a] Each subject received 50 mg. chlorprothixene by mouth.

concentration of chlorprothixene measured in the urine over a 72-hr. period varied considerably from one subject to another (Table VIII). The total amount excreted in the urine by the three subjects during the 72 hr. was less variable: 3.4 to 3.6 mg.

Although the method for analysis which has been described does not distinguish between chlorprothixene and its sulfoxide metabolite, this lack of specificity is not likely to carry medical or legal importance. Sensitivity of the method is sufficient to permit the analyst to detect the drug and its metabolites in the urine 3 days after an individual has taken a single 50-mg. dose.

SUMMARY

A quantitative ultraviolet spectrophotometric procedure has been developed for the determination of chlorprothixene and its principal sulfoxide metabolite in biologic specimens. In the procedure, the drug is oxidized with buffered permanganate to a carbonyl derivative which has a well-defined ultraviolet absorption spectra. The method is sensitive and the combined ultraviolet spectrum of the oxidation products is sufficiently specific to establish qualitative identification of the drug. Results of a study to establish the excretion pattern of chlorprothixene and its metabolites in man, as well as their distribution in the tissues, and biologic fluids of the rat are presented.

REFERENCES

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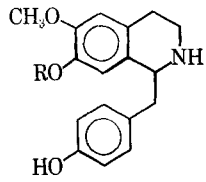
1-Benzyl-1,2,3,4-tetrahydroisoquinolines

By JOSEPH SAM and A. J. BEJ*

The syntheses of 1-benzyl-1,2,3,4-tetrahydroisoquinolines, particularly phenolic derivatives, are described. Preliminary pharmacological data also are reported.

NUMEROUS CHEMICAL and biological investigations have been conducted with synthetic as well as naturally occurring 1-benzylisoquinolines (1). Limited biological information is available, however, on phenolic 1-benzyl-1,2,3,4-tetrahydroisoquinolines. Kupchan and co-workers (2) noted weak analgesic activity with (−)-*N*-normarmepavine (I*a*). Colacurine (I*b*) was tested for curare-like activity in dogs but manifested no activity (3). Both of these alkaloids are secondary amines. Often the biological activity of secondary amines are lower than the corresponding *N*-methyl or tertiary amines (4). Accordingly, a program was initiated to synthesize and evaluate phenolic 1-benzyl-2-methyl-1,2,3,4-tetrahydroisoquinolines (II) for biological activity.



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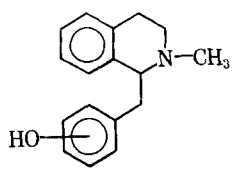
b, R = H



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The relationship of phenolic 1-benzyl-2-methyl-1,2,3,4-tetrahydroisoquinolines, both structurally (not sterically) and biogenetically (5), to morphine (III) gave additional impetus to the investigation. In this connection, Besendorf and co-workers (6) noted pronounced analgesic activity in 6,7-dimethoxy-1-(4-chlorophenethyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (IV).

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Accepted for publication July 5, 1967.

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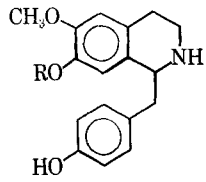

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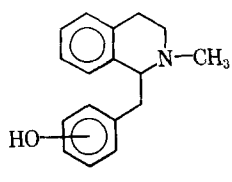

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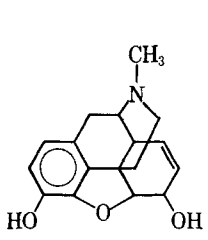
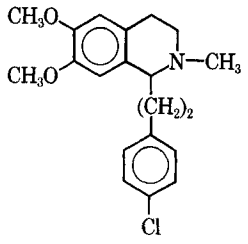


III



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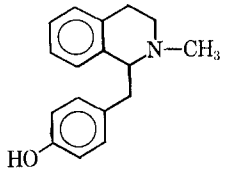


V

via (*a*) the Bischler-Napieralski reaction, (*b*) the Grignard reaction, (*c*) the Reissert compound, and (*d*) the reaction of pseudobases with 2- and 4-nitrotoluenes.

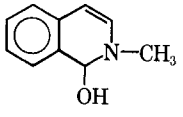
Kondo and Ishiwata (7) reported a 51% yield for the cyclization of VI to the corresponding 3,4-dihydroisoquinoline (VII) using phosphorus oxychloride. By using a mixture of phosphorus oxychloride and phosphorus pentoxide, a 70–80% yield of VII was obtained. Polyphosphoric acid on the other hand did not effect cyclization of VI at 190°. (Scheme I.)

The reduction of 1-(4-methoxybenzyl)-3,4-dihydroisoquinoline methiodide (VIII) with sodium borohydride gave the corresponding tetrahydroisoquinoline (IX). Demethylation of IX was effected with either 48% hydrobromic acid or 47% hydroiodic acid to yield V.

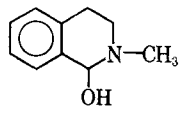
The treatment of isoquinoline methiodide (X) with 4-methoxybenzylmagnesium chloride, followed by reduction with sodium borohydride provided an alternate route to IX. The intermediate dihydro derivative (XI) was isolated but not identified. The reaction of 4-methoxybenzylmagnesium chloride with 3,4-dihydroisoquinoline methiodide (XII) also gave the tetrahydroisoquinoline (IX).

The reaction of pseudobases, XIII and XIV (obtained from isoquinoline methiodide and 3,4-dihydroisoquinoline methiodide, respectively), with 2- and 4-nitrotoluenes also was investigated. The condensation of isoquinoline methiodide (X) with 2-nitro-5-benzyloxytoluene (XV) according to the procedure described by Weisbach and co-workers (8) gave XVI. The platinum oxide reduction of XVI yielded XVII. The reaction of 4-nitrotoluene with either X or XIV, however, resulted only in the recovery of 4-nitrotoluene. (Scheme II.)

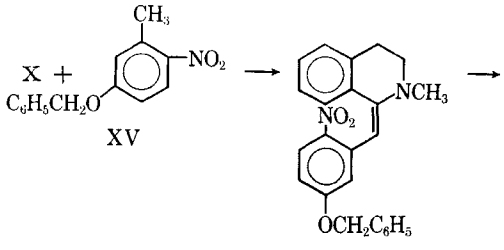
A final method of preparation of V involved initially the condensation of 1-cyano-2-benzoyl-1,2-dihydroisoquinoline (Reissert compound) (XVIII) with 4-benzyloxybenzyl chloride (XIX) or bromide



XIII

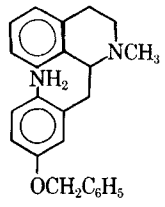


XIV

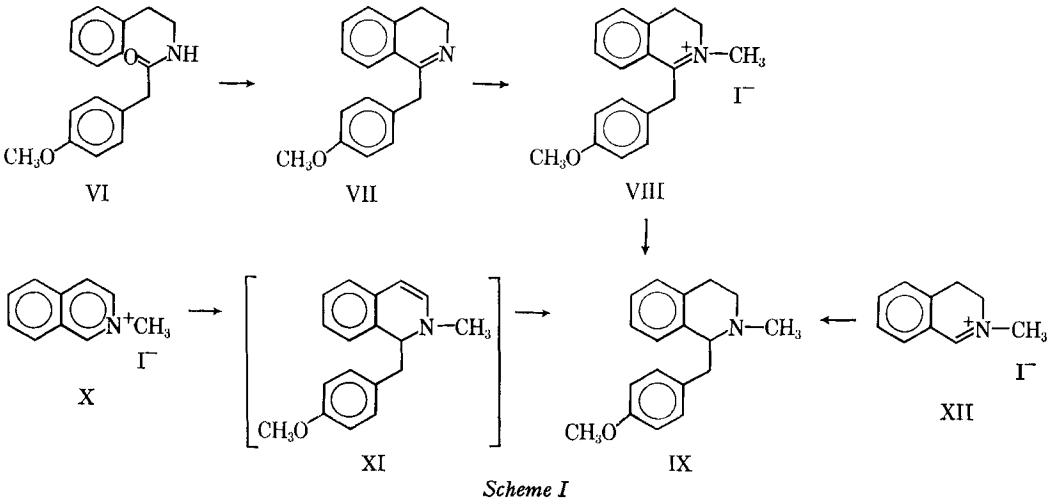


XV XVI

Scheme II



XVII



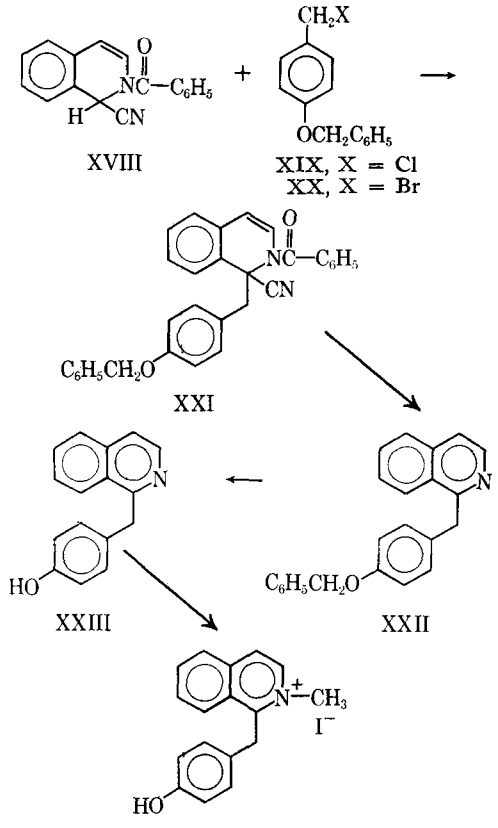
VI VII VIII X XI IX XII

Scheme I

(XX). Alkaline hydrolysis of XXI gave 1-(4-benzyloxybenzyl)isoquinoline (XXII). Heating XXII with 20% hydrochloric acid gave XXIII. The latter was converted to the methiodide (XXIV) and reduced to the desired product (V) with sodium borohydride. (Scheme III.)

Gibson and Popp (9) reported the preparation of XXII and XXIII from XVIII and 4-benzyloxybenzaldehyde. Ikehara (10) reported the synthesis of XXIII by the condensation of 1-chloroisoquinoline with 4-methoxyphenylacetonitrile, followed by demethylation.

Pharmacological Results[1]—Compounds V, XVI, and XVII have been investigated by a gross symptomological procedure in albino mice after intraperitoneal injection. There was little or no significant effect and no lethality with up to 680 mg./Kg. of XVI. However, compound XVII caused early deaths with excitation and clonic convulsions, and had an LD_{50} of 68 mg./Kg. Lower doses showed signs of CNS inhibitory actions; 2.0 and 20 mg./Kg. yielded significant prolongation of hexobarbital sleeping time. In the writhing test for analgesia, an ED_{50} of 11.5 mg./Kg. was noted. In a few albino rats, compound XVII appeared to produce drowsiness and partial analgesia at 10–20 mg/Kg., while at 46 mg./Kg. there was first sedation and later excitation and convulsions. An LD_{50} of 153 mg./Kg. was noted for V. In lethal dosage it produced multiple strong clonic convulsions and terminal tonic convulsion. In lower dosage symptoms of exophthalmos, immobility (catatonia-like), and loss of cutaneous pain reflexes were noted. At 100 mg./Kg., V reduced by 50% the number of writhing responses in the phenylquinone writhing test for analgesic action.



Scheme III

EXPERIMENTAL[2]

N-Phenethyl-4-methoxyphenylacetamide (VI)—The procedure utilized by Decker and Kropp (11) for the preparation of *N*-phenethylphenylacetamide was followed.

A mixture of 16.6 Gm. (1.0 mole) of 4-methoxyphenylacetic acid and 12.1 Gm. (1.0 mole) of β-phenethylamine was fused at 180° and the melt maintained at that temperature for 9 hr. The resulting homogeneous melt was poured into a mortar. The material, which solidified on cooling, was pulverized and recrystallized from ethanol. There was obtained 14.7 Gm. (55%) of a white crystalline solid, m.p. 96–97.5°. [Lit. (7) m.p. 95°.]

1-(4-Methoxybenzyl)-3,4-dihydroisoquinoline Methiodide (VIII)—A mixture of 26 Gm. (0.1 mole) of *N*-phenethyl-4-methoxyphenylacetamide (VI), 25 Gm. (0.22 mole) of phosphorus pentoxide, 23 Gm. (0.15 mole) of phosphorus oxychloride, and 200 ml. of dry toluene was refluxed at 130–135° for 3.5 hr. After 1 hr., 17 Gm. (0.11 mole) of phosphorus oxychloride was added to the reaction mixture. The reaction mixture was cooled to room temperature. The toluene layer was decanted from the brownish semisolid. The latter was treated with crushed ice and then extracted with 10 L. of boiling water. The water extract was divided into five portions; each portion was basified with 20% sodium hydroxide solution, and each portion was extracted with three 300-ml. portions of ether. The combined ether extract was washed with water and dried over anhydrous potassium carbonate. Distillation of the ether under reduced pressure gave 16 Gm. (70%) of the 3,4-dihydro free base. The latter was transferred to a pressure bomb (glass) and treated with 20 ml. of methyl iodide and then heated on an oil bath maintained at 50–55° for 6 hr. The crude solid was recrystallized from ethanol to yield 19 Gm. (50%) of product, m.p. 198–200°. Recrystallization twice from ethanol gave a pale yellow crystalline solid, m.p. 207–209°.

Anal.—Calcd. for $C_{18}H_{20}INO$: C, 54.98; H, 5.12; I, 32.38; N, 3.56. Found: C, 55.04; H, 5.34; I, 32.40; N, 3.34.

1-(4-Methoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (IX)—*Method A*—A stirred solution of 6.25 Gm. (0.017 mole) of 1-(4-methoxybenzyl)-3,4-dihydroisoquinoline methiodide (VIII) in 150 ml. of methanol was treated with 7 Gm. of sodium

[1] The authors are grateful to Dr. Marvin Davis, Department of Pharmacology, School of Pharmacy, University of Mississippi, for the pharmacological data.

[2] Melting points were taken in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 G Infracord spectrophotometer using potassium bromide pellets unless otherwise indicated. The NMR spectrum of V was obtained by means of the Varian A-60A spectrometer using tetramethylsilane (TMS) as the internal standard and deuterochloroform as the solvent. The shifts were measured on the τ-scale relative to the internal standard TMS (τ 10.0). Assignment of protons in a certain area is based on correct integral information from the NMR spectrum.

borohydride in small portions. The resultant mixture was stirred at room temperature for 45 min. and then concentrated under reduced pressure. The residue was treated with 500 ml. of 2% sodium hydroxide and extracted with four 100-ml. portions of ether. The ether extract was washed with 200 ml. of water and dried over anhydrous potassium carbonate. The ether was concentrated under reduced pressure to yield 3.2 Gm. (45%) of pale yellow oil. A picrate was prepared in the usual manner and recrystallized from ethanol, m.p. 159–160.5°.

Anal.—Calcd. for $C_{24}H_{24}N_4O_8$: C, 58.07; H, 4.87; N, 11.30. Found: C, 58.08; H, 4.84; N, 11.32.

Method B—The procedure utilized by Schnider and Grüssner (12) for the preparation of 1-(4-methoxybenzyl)-2-methyl-1,2,5,6,7,8-hexahydroisoquinoline was followed. A stirred ice-cooled suspension of 41 Gm. (0.15 mole) of isoquinoline methiodide (X) in 200 ml. of dry ether was treated dropwise over a 30-min. period with 4-methoxybenzylmagnesium chloride [prepared from 23.4 Gm. (0.15 mole) of 4-methoxybenzyl chloride according to the general method of Van Campen (13) and coworkers]. The resultant mixture was stirred at room temperature for 1 hr. and then poured into 1 Kg. of crushed ice and water containing 30 Gm. of ammonium chloride. The mixture was made alkaline with ammonium hydroxide. The ether layer was separated and the aqueous layer further extracted with ether. The combined ether solution was extracted with seven 100-ml. portions of cold 10% hydrochloric acid. The acid extract was made alkaline with cold ammonium hydroxide and extracted with four 200-ml. portions of ether. The ether extract, after drying over anhydrous sodium sulfate, was concentrated under reduced pressure. Trituration of the residual semisolid with ethanol gave 7 Gm. (17%) of the dihydro compound (XI) as an amorphous solid, m.p. 68–73°. This compound changed to reddish brown when exposed to air, and attempts to purify the substance proved abortive.

A solution of 4 Gm. (0.014 mole) of the above solid in 150 ml. of methanol was treated in portions with 5 Gm. of sodium borohydride and then stirred at room temperature for 2.5 hr. The product was isolated as in method *A* to give 2.3 Gm. (57%) of pale yellow oil. A picrate, m.p. 159–160.5°, showed no depression on admixture with a sample obtained by method *A*. The infrared spectra were identical.

Method C—The procedure employed in method *B* was followed using a mixture of 10 Gm. (0.037 mole) of 3,4-dihydroisoquinoline methiodide (XII) (14), 50 ml. of absolute ether, and 4-methoxybenzoylmagnesium chloride [prepared from 5.8 Gm. (0.037 mole) of 4-methoxybenzyl chloride]. The mixture was treated as in method *B* to yield 1.8 Gm. (17%) of product. The picrate was prepared in the usual manner, m.p. 159–160°. A mixed melting point with the picrate obtained from either method *A* or *B* was undepressed. The infrared spectra were identical.

1-(2-Nitro-5-benzyloxybenzal)-2-methyl-1,2,3,4-tetrahydroisoquinoline (XVI)—The procedure described by Weisbach and co-workers (8) for the preparation of 1-(2-nitrobenzal)-2-methyl-1,2,3,4-tetrahydroisoquinoline was followed. To a warm stirred solution of 1.86 Gm. (0.08 mole) of sodium in 70 ml. of absolute ethanol (commercial ethanol refluxed with calcium oxide and distilled) were added 7.2 Gm. (0.26 mole) of isoquinoline methiodide and 13 Gm. (0.52 mole) of 2-nitro-5-benzyloxytoluene (XV). The resultant solution was kept at room temperature for 24 hr. and then in a refrigerator for 48 hr. The solid that separated was removed by filtration and recrystallized from ethanol to give 8 Gm. (80%) of product, m.p. 102.5–103.5°.

Anal.—Calcd. for $C_{24}H_{22}N_2O_3$: C, 74.59; H, 5.73; N, 7.25. Found: C, 74.63; H, 5.64; N, 7.18.

1-(2-Amino-5-benzyloxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline Dihydrochloride (XVII)—A modified procedure of Weisbach and co-workers (8) was followed. A mixture of 10.5 Gm. (0.025 mole) of 1-(2-nitro-5-benzyloxybenzal)-2-methyl-1,2,3,4-tetrahydroisoquinoline (XVI), 1 Gm. of platinum oxide, and 250 ml. of absolute ethanol was shaken with hydrogen (initial pressure 40 p.s.i.) in a low pressure Parr hydrogenator for 2 hr. The uptake of hydrogen was essentially complete after the first 15 min. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure. Ethanolic hydrogen chloride was added to the residual oil; the resulting solution was kept in a refrigerator for 48 hr. The ethanol was distilled under reduced pressure and the residue treated with 40 ml. of ethanol. The ethanol was again distilled under reduced pressure. The residue was successively treated with three 40-ml. portions of ethyl acetate and finally with 40 ml. of dry benzene; each time the solvent was distilled under reduced pressure. The crude solid was recrystallized from a mixture of methanol and ethyl acetate with the aid of charcoal to give 10.2 Gm. (97%) of product, m.p. 213.5–214.5° dec.

Anal.—Calcd. for $C_{24}H_{28}Cl_2N_2O$: C, 66.80; H, 6.54; Cl, 6.43; N, 6.49. Found: C, 66.79; H, 6.70; Cl, 16.36; N, 6.41.

4-Benzyloxybenzyl Alcohol—To a suspension of 21 Gm. (0.1 mole) of 4-benzyloxybenzaldehyde in 150 ml. of methanol was added dropwise with stirring a solution of 2 Gm. of sodium borohydride in 2 ml. of 2 *N* sodium hydroxide and 18 ml. of water at 18–25°. The reaction mixture was stirred at room temperature for 3 hr. The ethanol was distilled under reduced pressure and the residue diluted with 100 ml. of water and extracted with 600 ml. of ether. The ether solution, after drying over anhydrous magnesium sulfate, was concentrated under reduced pressure. The residue was recrystallized from petroleum ether (30–60°) to give 20 Gm. (95%) of product, m.p. 85–86°. [Lit. (15) m.p. 85–86°.]

1-(4-Benzyloxybenzyl)-2-benzoyl-1,2-dihydroisoquinoaldonitrile (XXI)—*Method A*—The procedure utilized by Kershaw and Uff (16) for the preparation of 1-(4-methoxybenzyl)-2-benzoyl-1,2-dihydroisoquinoaldonitrile was followed. To a cooled (0°) and stirred solution of 2.76 Gm. (0.01 mole) of 1-cyano-2-benzoyl-1,2-dihydroisoquinoline (Reissert compound) (17) and 2.3 Gm. (0.01 mole) of 4-benzyloxybenzyl chloride (XIX) (15) in 50 ml. of *N,N*-dimethylformamide maintained in a nitrogen atmosphere was added 0.24 Gm. (0.01 mole) of a 50% dispersion of sodium hydride in mineral oil. The mixture was stirred at 0° for 1 hr. and at room temperature for 2 hr. The reaction mixture was poured onto 500 Gm. of crushed ice. The product was removed by filtration and recrystallized twice

from absolute ethanol; 3.1 Gm. (70%) of product was obtained, m.p. 144–146°. The infrared spectrum in Nujol possessed a rather weak band for the amide carbonyl group at 6.05 μ, possibly due to an interaction of the oxygen atom of the amide with the carbon atom of the cyano group. This was supported by a very weak absorption at 4.16–4.54 μ, the frequency range in which absorption due to a cyano group is observed.

Anal.—Calcd. for $C_{31}H_{24}N_2O_2$: C, 81.56; H, 5.34; N, 6.14. Found: C, 81.31; H, 5.47; N, 6.21.

Method B—The procedure described in method *A* was followed using 4-benzyloxybenzyl bromide (XX) instead of 4-benzyloxybenzyl chloride (XIX), m.p. 144–146°.

1-(4-Benzyloxybenzyl)isoquinoline (XXII)—The procedure utilized by Boekelheide and Weinstock (18) for the preparation of 1-methylisoquinoline was followed. A solution of 4.5 Gm. (0.01 mole) of 1-(4-benzyloxybenzyl)-2-benzoyl-1,2-dihydroisoquinaldonitrile (XXI) in 70 ml. of methanol was treated with 1.12 Gm. (0.02 mole) of sodium hydroxide in 8 ml. of water and then refluxed for 3.5 hr. The cooled mixture was diluted with 75 ml. of water and extracted with three 200-ml. portions of ether. The ether extract was dried over anhydrous magnesium sulfate. The ether was concentrated under reduced pressure; the residue was recrystallized from ethanol to yield 2.3 Gm. (69%) of product, m.p. 122–123°. [Lit. (9) m.p. 121–122°.]

1-(4-Hydroxybenzyl)isoquinoline (XXIII)—A mixture of 3.25 Gm. (0.01 mole) of 1-(4-benzyloxybenzyl)isoquinoline (XXII) and 100 ml. of 20% hydrochloric acid was heated on a steam bath for 1.5 hr. The reaction mixture was washed with two 30-ml. portions of chloroform and then basified with 28% ammonium hydroxide. The solid was removed by filtration, washed with water, and recrystallized from ethyl acetate to yield 1.5 Gm. (65%) of product, m.p. 180–182°. [Lit. (9) m.p. 177–179°.]

1-(4-Hydroxybenzyl)isoquinoline Methiodide (XXIV)—A solution of 0.9 Gm. (0.004 mole) of 1-(4-hydroxybenzyl)isoquinoline (XXVIII) and 15 ml. of methyl iodide in 10 ml. of methanol was refluxed for 3 hr. and then poured into ether. The solid was removed by filtration and recrystallized from methanol to yield 0.95 Gm. (82%) of product, m.p. 224–227°.

1-(4-Hydroxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (V)—*Method A*—The method utilized by Craig and associates (19) for the preparation of 1-alkyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines was followed. A mixture of 3.5 Gm. (0.013 mole) of 1-(4-methoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (IX) and 40 ml. of 48% hydrobromic acid (redistilled over a trace of 50% hypophosphorous acid and mixed with the latter in the proportion of 1 Gm. of 50% hypophosphorous acid to 100 Gm. of 48% hydrobromic acid) was gently refluxed at 135–140° in a current of nitrogen for 6 hr. The reaction mixture was basified with 28% ammonium hydroxide and extracted with four 100-ml. portions of benzene. The benzene extract was dried over anhydrous sodium sulfate and then distilled under reduced pressure to yield a pale yellow solid, m.p. 72–82°. The crude product was chromatographed on 25 Gm. of neutral Woelm alumina (activity I) prepared in benzene and eluted with 240 ml. of benzene, 210 ml. of acetone, 180 ml. of ethyl acetate, and finally with 180 ml. of methanol. The evaporation of the methanol eluents gave 0.420 Gm. (33%) of product, m.p. 130–132°. Recrystallization from carbon tetrachloride with the aid of charcoal gave product, m.p. 133.5–134.5°.

Anal.—Calcd. for $C_{17}H_{19}NO$: C, 80.6; H, 7.56; N, 5.59. Found: C, 80.8; H, 7.54; N, 5.82.

Method B—The procedure described in method *A* was followed using 47% hydriodic acid instead of 48% hydrobromic acid, m.p. 133.5–134.5°.

Method C—The procedure utilized by Gibson and co-workers (20) for the preparation of 1-(4-hydroxybenzyl)-2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was followed. A mixture of 0.7 Gm. (0.0019 mole) of 1-(4-hydroxybenzyl)isoquinoline methiodide (XXIV), 2 Gm. of sodium borohydride, 2 ml. of water, and 80 ml. of methanol was refluxed with stirring for 2 hr. Most of the methanol was distilled under reduced pressure, and the residue poured onto ice. The mixture was adjusted to pH 8 and extracted with two 50-ml. portions of ether. The ether extract was dried over anhydrous magnesium sulfate and then concentrated under reduced pressure. The residue was recrystallized from carbon tetrachloride to yield 0.5 Gm. (100%) of product, m.p. 132.5–134°. A mixed melting point with the sample obtained *via* the Bischler-Napieralski reaction was undepressed.

The nuclear magnetic spectrum (NMR) of V in deuterochloroform showed three *N*-methyl protons (singlet τ = 7.49), six ring methylene protons (multiplet centered at τ 7.1), one tertiary proton (triplet τ = 6.1), and eight aromatic protons (τ 2.75–3.5), which is consistent with the structure.

1-(4-Benzoyloxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline—To a solution of 0.25 Gm. (0.001 mole) of 1-(4-hydroxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (V) in 10 ml. of 1 *N* sodium hydroxide was added 0.2 Gm. (0.001 mole) of benzoyl chloride. The mixture was shaken vigorously for 10 min. and then extracted with two 20-ml. portions of chloroform. The chloroform extract was dried over anhydrous sodium sulfate and then concentrated under reduced pressure. The residual oil (0.25 Gm., 70%) was converted to a picrate in the usual manner and recrystallized from methanol, m.p. 201.5–203°.

Anal.—Calcd. for $C_{30}H_{26}N_4O_9$: C, 61.43; H, 4.47; N, 9.55. Found: C, 61.60; H, 4.57; N, 9.62.

REFERENCES

(1) Burger, A., "The Alkaloids," vol. IV, Manske, R. H F., and Holmes, H. L., eds., Academic Press Inc., New York, N. Y., 1954, p. 29.
(2) Kupchan, S. M., Dasgupta, B., Fujita, E., and King, M. L., *Tetrahedron*, **19**, 227(1963).
(3) Finkelstein, J., *J. Am. Chem. Soc.*, **73**, 550(1951).
(4) Schaumann, O., *Pharmazie*, **4**, 364(1949); through *Chem. Abstr.*, **43**, 9268(1949).
(5) Mothes, K., and Schutte, H. R., *Angew. Chem. Intern. Ed. Engl.*, **2**, 441(1963).
(6) Besendorf, H., Pellmont, B., Bachtold, H. P., Reber, K., and Studer, A., *Experientia*, **18**, 446(1962); through *Chem. Abstr.*, **57**, 17326(1962).
(7) Kondo, H., and Ishiwata, S., *Chem. Ber.*, **64**, 1533 (1931).
(8) Weisbach, J. A., Burns, C., Macko, E., and Douglas, B., *J. Med. Chem.*, **6**, 91(1963).
(9) Gibson, H. W., and Popp, F. D., *J. Chem. Soc.*, **1966**, 1860.
(10) Ikehara, M., *Pharm. Bull. (Japan)*, **3**, 294(1955); through *Chem. Abstr.*, **50**, 13056(1956).
(11) Decker, H., and Kropp, W., *Chem. Ber.*, **42**, 2077 (1909).

(12) Schnider, O. S., and Grüssner, A., *Helv. Chim. Acta*, **32**, 821(1949); through *Chem. Abstr.*, **43**, 6635(1949).
(13) Van Campen, M. G., Meisner, D. F., and Parmerter, S. M., *J. Am. Chem. Soc.*, **70**, 2296(1948).
(14) Leonard, N. J., and Leubner, G. W., *ibid.*, **71**, 3408 (1949).
(15) Shelton, R. S., Van Campen, M. G., Jr., Meisner, D. F., Parmerter, S. M., Andrews, E. R., Allen, R. E., and Wyckoff, K. K., *ibid.*, **75**, 5391(1953).
(16) Kershaw, J. R., and Uff, B. C., *Chem. Commun.*, **1966**, 331.
(17) Weinstock, J., and Boekelheide, V., "Organic Synthesis," coll. vol. IV, Rabjohn, N., ed., John Wiley & Sons, Inc., New York, N. Y., 1963, p. 641.
(18) Boekelheide, V., and Weinstock, J., *J. Am. Chem. Soc.*, **74**, 660(1952).
(19) Craig, P. N., Nabenhauer, F. P., Williams, P. M., Macko, E., and Toner, J., *ibid.*, **74**, 1316(1952).
(20) Gibson, H. W., Popp, F. D., and Noble, A. C., *J. Heterocyclic Chem.*, **3**, 99(1966).

Plasticizers in Medical Application I

Analysis and Toxicity Evaluation of Dialkyl Benzenedicarboxylates

By JAY NEMATOLLAHI, WALLACE L. GUESS, and JOHN AUTIAN

A series of dialkyl and dicycloalkyl benzenedicarboxylates (phthalates, isophthalates, terephthalates), of which some have been employed as plasticizers, were synthesized and purified. Thin-layer chromatography and infrared spectrometry were employed as methods of detection and identification of these compounds. Toxicity tests were conducted using mammalian cell cultures and limited mouse studies. The low molecular weight esters and their isomers manifested toxic responses while the esters containing alkyl groups C_6 to C_9 were devoid of toxic effect. The di-*n*-decyl phthalate and terephthalate also elicite toxic actions to cells. Results from this study indicate the need for good quality control procedures to prevent the access of potentially toxic plasticizers in devices for medical and paramedical applications.

A NUMBER of polymeric materials require the presence of one or more plasticizers to impart a degree of softness and flexibility not inherent in the parent polymer. The concentration of the plasticizer may vary from a rather small percentage to as high as 60%, depending upon the desired final properties. Selection of a plasticizer (or plasticizers) for a specific plastic is dependent upon a number of considerations; the initial consideration is that it will impart to the final plastic the optimum properties desired at a minimum cost consistent with quality production. If the plastic material is to be used as a food wrap or a container, the degree of migration of the plasticizer to the food and the toxicity profile of the migrated ingredient must be evaluated under the Food Additive Amendment. Development and use of the plasticized polymeric materials for various medical, pharmaceutical, and dental applications such as tubing, catheters, containers, and protective films raises the question as to the toxic potential of migrated plasticizer which may enter the patient by routes other than oral. In this regard, consideration must also be given to the exact composition of the "migrated ingredient" since it may actually be composed of the intact plasticizer, degraded products of the plasticizer, impurities in the plasticizer, and other additives such as stabilizing agents, antioxidants, degraded products of the additives, and impurities in the additives.

Within the past 5 years, a number of published reports (1–3) have demonstrated that certain types of polyvinyl chloride (PVC) devices could produce toxic effects if one or more ingredients migrated from the device into tissue or into an injectable solution.

Recent investigations (4) on a group of PVC blood bags revealed that certain ingredients were migrating from the plastic into acid citrate dextrose solutions. The combined ingredients were found to be toxic to mammalian cell cultures. Further investigations on the "leached" ingredients showed the presence of several chemicals, presumably plasticizers together with their degradation products. A frequently employed plasticizer in the PVC plastic used for the manufacture of the blood bags was found to be di(2-ethylhexyl)phthalate. Others such as dialkyl sebacates and epoxidized soybean oil were also detected in the PVC.

When a combined chemical and spectrophotometric method was employed for the detection and identification of these additives, it became obvious that a mixture of homologs of a few di-

Received May 18, 1967, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication July 12, 1967.
This investigation was supported in part by grant CA 06120-06 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors thank Mrs. Nawal Shafiq for her technical assistance.

(12) Schnider, O. S., and Grüssner, A., *Helv. Chim. Acta*, **32**, 821(1949); through *Chem. Abstr.*, **43**, 6635(1949).
(13) Van Campen, M. G., Meisner, D. F., and Parmerter, S. M., *J. Am. Chem. Soc.*, **70**, 2296(1948).
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alkyl phthalates could not be separated easily and under these experimental conditions their infrared spectra were indistinguishable. Likewise, when a commercial medical grade di(2-ethylhexyl) phthalate (DOP) was examined by thin-layer chromatography (TLC), the presence of several spots was observed. Again it was not possible by tedious column chromatography to separate some of the proximately located spots observed on the thin-layer plate, and the I.R. spectra of the fractions did not provide sufficient help for structural elucidation. This, and the fact that the commercial synthesis of plasticizing esters does not rule out the presence of isophthalates and terephthalates in phthalate plasticizers, necessitated the extension of this investigation to the synthesis and analysis of a series of authentic esters of the three isomers of benzenedicarboxylic acids. The detected TLC spots for the medical grade DOP, mentioned above, could be ascribed to the homologs of the phthalic acid esters as well as to its positional isomers (see Table I). One of the industrial methods of preparation of benzenedicarboxylic acids is *via* oxidation of xylenes. Quite often one isomer of xylene contains the other two, whose oxidation would give rise to a mixture of the three corresponding acids.

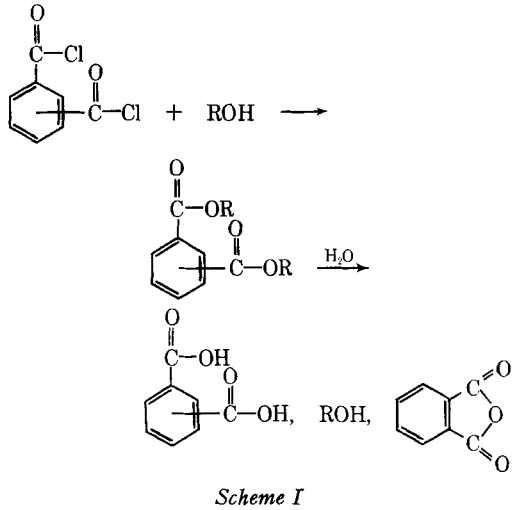
TABLE I—R_f VALUES FOR DIALKYL BENZENEDICARBOXYLIC ACIDS. ADSORBENT, SILICA GEL, DEVELOPER, ETHYL ACETATE–*n*-HEXANE (10:90)

R	Phthalate	Iso-phthalate	Tere-phthalate
CH_3	.27	.41	.56
C_2H_5	.42	.54	.79
n-C_3H_7	.55	.66	.82
iso-C_3H_7	.59	.67	.82
n-C_4H_9	.72	.76	.93
iso-C_4H_9	.76	.77	.92
n-C_5H_{11}	.83	.84	.95
iso-C_5H_{11}	.85	.86	.94
Cyclo-C_5H_9	.73	.77	.85
n-C_6H_{13}	.89	.86	.96
Cyclo-C_6H_{11}	.81	.81	.88
n-C_7H_{15}	.90	.92	.97
Cyclo-C_7H_{13}	.79	.92	.95
n-C_8H_{17}	.90	.93	.98
Cyclo-C_8H_{15}	.83	.85	.97
n-C_9H_{19}	.93	.94	.98
n-$C_{10}H_{21}$	.93	.95	.99
n-$C_{11}H_{23}$	.94	.96	.99
n-$C_{12}H_{25}$	.95	.97	.99

The main objectives of this investigation were: (*a*) to synthesize a homologous series of the authentic dialkyl and dicycloalkyl benzenedicarboxylates in order to acquire information on certain physical properties and spectroscopic data for the series, requisite both for present and further investigations in plastics; (*b*) to search for the existence of a correlation among chemical structures of the esters and their I.R. spectral patterns; (*c*) to correlate the chemical structures of the esters together with their products of hydrolysis to their toxic potential on the mammalian cell cultures and mice; and (*d*) to determine the rate of hydrolysis of the esters and relate this rate to chemical structure and toxic effect. The latter part will be discussed in another paper.

CHEMISTRY

The rapid rate of reaction of phthalyl, isophthalyl, and terephthalyl chloride with alcohol suggested the adoption of this route for the preparation of dialkyl and dicycloalkyl benzenedicarboxylates. The acid chlorides were employed slightly in excess of the stoichiometric quantity of the alcohol in order to facilitate the purification of the final product which consists of the removal of the acid from the ester by ether–dilute aqueous base extraction. In addition to *n*-alkyl and some iso-alkyl esters, four cycloalkyl esters were prepared: cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl benzenedicarboxylate. The general method of synthesis and the degradation by hydrolysis of the esters is shown in Scheme I.



Scheme I

Due to cell culture toxicity manifested by some esters and the products of hydrolysis of the esters of benzenedicarboxylic acids, the establishment of a detection method for esters mixed with such products became essential. This purpose was served well by the application of thin-layer chromatography. The esters, acids, and anhydride (the anhydride formed as one of the products of degradation of phthalic acid esters) could be detected visually on the thin-layer plate by the aid of a short wave ultraviolet light. Both the acid and anhydride remained near the origin whereas the esters showed a noticeable R_f value. The position of the alcohol on the TLC plate usually was in between the ester and acid. Since the presence of the alcohol could not be visualized by the aid of a U.V. light, its location was fixed by spraying the plate with a 10% solution of phosphomolybdic acid in 95% ethanol.

TABLE II—RESULTS OF THE TOXICITY EVALUATION OF THE DIALKYL BENZENEDICARBOXYLATE ON THE MAMMALIAN CELL CULTURES AND MICE

R	Phthalates			Isophthalate			Terephthalates		
	Chick Embryo Cells	L-Cells	Mice	Chick Embryo Cells	L-Cells	Mice	Chick Embryo Cells	L-Cells	Mice
CH_3	+	+	+	+	+	+	+	+	+
C_2H_5	+	+	+	+	+	+	+	+	+
n-C_3H_7	+	+	+	+	+	+	+	+	+
iso-C_3H_7	+	+	+	+	+	−	+	+	+
n-C_4H_9	±	+	+	+	+	+	+	+	+
iso-C_4H_9	+	+	±	+	+	+	−	−	±
n-C_5H_{11}	−	±	−	+	+	−	+	+	+
iso-C_5H_{11}	+	+	+	+	+	−	±	+	−
Cyclo-C_5H_9	+	+	±	+	+	+	±	±	±
n-C_6H_{13}	−	−	−	−	−	−	−	−	−
Cyclo-C_6H_{11}	−	−	−	−	−	−	−	−	−
n-C_7H_{15}	−	−	−	−	−	−	+	+	−
Cyclo-C_7H_{13}	+	+	−	+	+	−	−	−	−
n-C_8H_{17}	−	−	−	−	−	−	−	−	−
Cyclo-C_8H_{15}	−	−	...	−	−	...	±	±	...
n-C_9H_{19}	−	−	−	±	+	−	−	−	−
n-$C_{10}H_{21}$	±	+	±	−	−	−	+	+	−
n-$C_{11}H_{23}$	−	−	−	±	±	−	−	−	−
n-$C_{12}H_{25}$	±	+	−	−	−	−	−	−	±

The R_f values for the esters are listed in Table I. A possible occurrence of variation in the position of spots, due to change in the atmosphere of the chamber or the proportion of the solvent, was eliminated by spotting DOP as a reference on the plate along with the esters.

TOXICITY EVALUATION

The toxicity of the esters was evaluated by two methods: (*a*) a cell culture method and (*b*) an animal method. The details of the evaluation of a chemical by cell culture methods have been described previously (5).

The results of both animal and cell culture tests are listed in Table II. It is noteworthy that the results from the two tests coincide within the experimental error. A detailed assessment of this is described further under results and discussion.

EXPERIMENTAL

The infrared spectra were determined with a Perkin-Elmer 337 spectrophotometer. The instrument was calibrated with a polystyrene film and the "normal" slit program was employed throughout the experiments. A KBr disk method was used for solid samples and neat (between NaCl windows) for liquid samples. The recorded wave number values are within ±2 cm.$^{-1}$ of the actual values. The plates for thin-layer chromatography (TLC) were prepared using Brinkmann Silica Gel HF 254 + 366 and were activated at 180° for 30 min. A mixture of hexane–ethyl acetate (90:10) was employed at room temperature as a developing solvent. The solvent front height was maintained at 12 cm. ± 1. Spots were located both by visual examination under a short wave ultraviolet light and by the aid of an indicator, 10% alcoholic solution of phosphomolybdic acid. Elemental analyses[1] were conducted by the Microanalytical Laboratory, Department of Chemistry, University of Texas, Austin, Tex.

General Method of Synthesis of Dialkyl and Dicycloalkyl Benzenedicarboxylate—To 0.2 mole of a dry alcohol was added 0.11 mole of benzene dicarboxylic acid chloride. With the provision for exclusion of water, the mixture was heated under reflux for 4–6 hr. The reaction mixture was examined from time to time by the aid of TLC in order to evaluate the progress of the reaction. Upon completion of the reaction, the content of the flask was poured into a separator containing 100 ml. of diethyl ether. The ether solution was extracted with 20-ml. portions of 1 *N* NaOH solution until the aqueous portion became basic to an indicator paper. The ether layer was washed with water to neutrality, and then was placed in a dry flask containing anhydrous sodium sulfate. After 12 to 24 hr. the ether was filtered and evaporated under dry nitrogen. The residue dialkyl or dicycloalkyl benzenedicarboxylate was obtained in 55 to 75% yield. The purity of the product was verified by TLC (Table I). In a few cases, where the presence of the trace quantity of acid or alcohol was observed, the ester was purified by means of a column packed with a mixture of equal quantities of silica gel and diatomaceous earth,[2] and developed with 200 ml. of *n*-hexane, followed by 300–400 ml. of benzene. The collected fractions, 10–15 ml., were examined by TLC for the presence of the pure ester. In general, the benzene fraction contained the pure ester, which was obtained by the evaporation of benzene *in vacuo*. The main peaks of the infrared spectra of the esters are listed in Tables III, IV, and V.

Toxicity Test—*Mammalian Cell Culture Method*—Monolayers of L-cells and chick embryo cells overlaid with agar were used in this evaluation according to a method reported by Guess *et al.* (5). Several modifications, however, were necessary in

[1] Elemental analysis was done only on those dicycloalkyl benzenedicarboxylates whose structures were not well characterized previously. The results were found to coincide with the calculated values. None was reported in this manuscript.

[2] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

TABLE III—POSITION OF PEAKS OF THE INFARED SPECTRA OF DIALKYL AND DICYCLOALKYL PHTHALATE[a]

R	νC—H	νC=O	Aromatic Ring Skeletal Vibration	δC—H in Plane	O ‖ νC$_6$H$_5$C—OR Stretching	C—H Out of Plane, Aromatic o-di	Unassigned Peaks
CH_3	3000(w), 2950	1740	1590–1570	1480, 1440	1290–1265(b)	745	1190, 1125(b), 1075, 1045, 960(b), 840(w), 820, 700, 650
C_2H_5	2980	1740	1600, 1570	1450, 1360	1290, 1260(b)	747	1175(w), 1125, 1115–1114(b), 1075, 1045(w), 1015(w), 860(wb), 700
n-C_3H_7	2970, 2880	1740	1600, 1570	1460, 1390, 1350	1290–1265(b)	747	1125, 1075, 1040(w), 965(w), 940, 915(w), 700, 650
iso-C_3H_7	2970, 2910	1725	1590, 1570	1480, 1460–1450(d), 1370, 1350	1290–1275(b)	747	1185, 1145, 1110, 1075, 1040(w), 920, 850(w), 707(w)
n-C_4H_9	2960, 2880	1740	1600, 1580	1470, 1390	1290(b)	748	1135(sh), 1125, 1075, 1040, 945–965(d), 845, 708, 650
iso-C_4H_9	2960, 2880	1740	1600, 1580	1470, 1390, 1370	1290–1260(b)	747	1135(sh), 1120, 1075, 1040, 980, 945(w), 705, 650(w)
n-C_5H_{11}	2955, 2870	1740	1600, 1580	1470, 1385	1290–1260(b)	745	1140–1120(b), 1075, 1040, 965, 875, 705, 650
iso-C_5H_{11}	2955, 2860(w)	1740	1600, 1580	1470, 1390–1370(d)	1285(b)	740	1125(b), 1075, 1040(w), 960–940(d), 700
Cyclo-C_5H_9	2955, 2860	1725	1590, 1570	1480(w), 1440, 1355	1280(b)	740	1170(w), 1140–1125(d), 1070, 1040, 960, 900(w), 850(wb), 708
n-C_6H_{13}	2960–2940(d), 2860	1740	1600, 1585	1480, 1390	1290–1250(b)	745	1140–1120(d), 1075, 1040, 980(w), 910(w), 705, 650
Cyclo-C_6H_{11}	2940, 2860	1725	1600, 1580	1450, 1320(w)	1290–1250(b)	745	1160(w), 1125, 1075, 1040(w), 1015(w), 940(w), 920(w), 920(w), 890(w), 840(w), 708
n-C_7H_{15}	2960–2930(d), 2850	1735	1600, 1580	1470, 1385	1290–1250(b)	745	1140–1120(b), 1075, 1040, 960(wb), 704(w), 650(w)
Cyclo-C_7H_{13}	2930, 2850	1725	1600	1450	1285, 1245	752	1300, 1135–1120(d), 1075, 1035, 970, 880(wb), 840(w), 790, 700, 650, 600, 500
C_8H_{17}	2960–2925(d), 2850	1740	1595, 1580	1485(w), 1460, 1380, 1350	1290–1260(b)	745	1195(w), 1140–1170, 1075, 1040(w), 960(wb), 915(w), 845(w), 700, 650
n-C_8H_{17}	2950–2915(d), 2850	1725	1590, 1570	1460, 1380, 1350	1275(b)	739	1140–1115(d), 1075, 1045, 1035(w), 1000(w), 970(w), 945, 920, 895(w), 875(w), 825(w), 810(w), 795, 700, 650(w), 590(w)
Cyclo-C_8H_{15}	2960(sh), 2920, 2850	1720	1600	1475, 1450	1280, 1245	750	1140–1115(d), 1080, 1035, 1000(w), 970(w), 945, 920, 875(w), 825(w), 810(w), 795(w), 700, 590(w)
C_9H_{19}	2950, 2860	1740	1600, 1580	1470, 1380(w), 1360	1290–1250(b)	742	1200(wb), 1140–1115(b), 1075, 1040(w), 960, 705(w)
$C_{10}H_{21}$	2950(sh), 2915, 2850	1735	1600, 1580	1470, 1375(w)	1290–1270(b)	742	1135(sh), 1125, 1075, 1040(w), 965(wb), 705(w)
$C_{11}H_{23}$	2950(sh), 2915, 2850	1735	1600, 1580	1465, 1375(w)	1290–1270(b)	742	1140–1120(b), 1075, 1040(w), 960(wb), 705(w)
$C_{12}H_{25}$	2950(sh), 2920, 2850	1740	1600, 1580	1470, 1380(w)	1290–1270(b)	742(w)	1135(sh), 1120, 1075, 1040(w), 950(wb)

[a] Key: w, weak; d, doublet; b, broad; sh, shoulder; wb, weak broad. Solvent: the spectra of all the esters were determined neat except R = CH_3 and cyclo C_8.

the method to make it suitable for the samples tested in this investigation. If the esters were crystalline, approximately 20 mg. of each was placed directly on the agar overlay. For liquid esters, 35 mg. of each was applied directly to a paper disk, 12.7 mm. diameter, which had previously been placed on the agar overlay. The culture plates were then incubated for a period of 24 hr., at which time the cells adjacent to the test samples were observed for possible toxicity. If the compound affected the cells by creation of zone of dead cells (inhibition zone) it was assumed to be toxic [designated by a positive (+) sign]. If the cells remained intact, the compound was assumed to be nontoxic [negative (−) sign]. In those cases where the results appeared in doubt, pending further tests, the results were identified with a positive–negative sign (±). These results are shown in Table II. Each of the compounds were tested a minimum of 3 times.

Intraperitoneal Mice Method—Each of the compounds was administered i.p. in groups of 3 mice at a concentration level of 5 mmoles/Kg. in either cottonseed oil or in castor oil, depending upon the solubility of the specific compound. The animals were kept under observation for a period of 4 days or until death. In general, the esters exerted a depressant effect in mice and induced observable ptosis. Gross toxicity results based upon death are included in

TABLE IV—THE POSITION OF PEAKS OF THE INFRARED SPECTRA OF DIALKYL AND DICYCLOALKYL ISOPHTHALATES[a]

R	νC—H	νC=O	Aromatic Ring Skeletal Vibration	δC—H in Plane	O ‖ νC$_6$H$_5$C—OR Stretching	δC—H out of Plane Aromatic *m*-di	Unassigned Peaks
CH_3	3120(d), 2950	1730	1600, 1580	1480, 1460, 1445–1430(d)	1290, 1250(b)	825(w), 808(w), 725	1315, 1190, 1130, 1095, 1075, 1000(w), 980, 955(d), 930, 925, 865, 670, 655
C_2H_5	2980, 2900(w)	1735	1605(w)	1475, 1450, 1390(w), 1370	1285(w), 1240(b)	825(w), 735(d)	1310, 1170, 1135, 1100, 1080, 1025, 930(d,b), 865(w), 658(w)
n-C_3H_9	2970, 2880	1735	1605(w)	1460, 1440, 1390, 1350(w)	1240	735(d)	1305, 1165, 1135, 1100, 1080, 985(w), 960(b)
iso-C_3H_7	2985, 2940(w)	1735	1610(w)	1475, 1440, 1380–1360(d)	1285(w), 1245(b)	830, 730(d)	1305, 1180, 1165(w), 1145, 1110, 1075, 940, 915(w), 890(w), 650
n-C_4H_9	2960, 2940(w), 2870	1730	1600(w)	1460, 1430(w), 1380	1245(b)	730(d)	1300, 1185(w), 1165(w), 1135, 1095, 1080, 970–950(d,b)
iso-C_4H_9	2960, 2870(w)	1730	1600(w)	1460, 1430(w), 1390–1370(d)	1240(b)	730(d)	1300(d), 1170, 1130, 1092, 1075, 990, 940(w), 820(w)
n-C_5H_{11}	2950, 2920, 2850	1725	1600(w)	1465, 1435, 1380	1235	730(d)	1300, 1165(w), 1135, 1095, 1075, 975
iso-C_5H_{11}	2960, 2870	1730	1605(w)	1465, 1435(w), 1385–1370(d)	1235(b)	830(w), 730(d)	1300, 1170, 1135, 1098, 1080, 975(b)
Cyclo-C_5H_9	2960, 2870	1730	1605(w)	1440, 1360, 1325	1240	835(w,b), 1730(d)	1300, 1190(w), 1165, 1135, 1098, 1075, 1030(w), 960, 690
n-C_6H_{13}	2960, 2935, 2865	1740	1605(w)	1470, 1435(w), 1390	1240	830(w), 735(d)	1300, 1165(w), 1140, 1100, 1080, 900
Cyclo-C_6H_{11}	2940, 2860	1725	1605(w)	1450	1240	830(w), 730	1300, 1160(w), 1140, 1120, 1098, 1080, 1040, 1015, 960(w), 890(w)
n-C_7H_{15}	2960–2930(d), 2850	1725	1605(w)	1465, 1435(w), 1380	1235(b)	830(w), 735	1300, 1165(w), 1135, 1098, 1080
Cyclo-C_7H_{13}	2940, 2860	1725	1605(w)	1460	1280(w), 1240	820(w), 730	1300, 1160(w), 1135, 1095, 1075, 1015(w), 1000(w), 975, 885(w)
n-C_8H_{17}	2960(sh), 2935, 2860	1735	1605(w)	1470, 1440(w), 1380(w)	1235(b)	730	1305, 1165(w), 1135, 1095, 1075, 960(w,b)
Cyclo-C_8H_{15}	2960(sh), 2920, 2840(w)	1720	1600(w)	1475, 1450, 1375(w)	1275, 1240	750	1140, 1120, 1080, 1035, 1000(w), 970(w), 945, 920, 875(w), 700
n-C_9H_{19}	2960(sh), 2940, 2860	1740	1605(w)	1470, 1440(w), 1380(w)	1235	730	1305, 1135, 1098, 1075, 970(w,b)
n-$C_{10}H_{21}$	2950(sh), 2925, 2850	1740	1605(w)	1470, 1380(w)	1235	730	1305, 1130, 1095, 1075, 970(w,b)
n-$C_{11}H_{23}$	2940(sh), 2915, 2840	1735	1605(w)	1465, 1385(w)	1240	730	1305, 1160(w), 1135, 1095, 1075, 970(w,b)
n-$C_{12}H_{25}$	2950(sh), 2920, 2850	1735	1605(w)	1465, 1385(w)	1235	730	1305, 1160(w), 1130, 1095, 1075

[a] Key: same as in Table III.

Table II. A negative sign (−) indicates no deaths, a positive sign (+) 2 or 3 deaths, and a positive-negative sign (±) only one death.

DISCUSSION

Infrared—The major peaks of the I.R. spectra of dialkyl and dicycloalkyl benzenedicarboxylates, together with the spectra of phthalic acid, phthalic anhydride, and isophthalic and terephthalic acid, are listed in Tables III–VI. The peaks for stretching frequencies of C=O and of C—H for acids and their appropriate esters occur in different regions of the infrared spectrum. The presence of phthalic acid, a hydrolysis product of a dialkyl phthalate, can be verified by the presence of C=O band at 1700 cm.$^{-1}$ next to a conjugated ester C=O band at 1740 cm.$^{-1}$. This distinction, however, cannot be made if the quantity of acid to ester is less than 5%—only the peak for ester C=O will be observed. The presence of phthalic anhydride can be identified in a like manner by the presence of C=O bands at 1760 and 1855 cm.$^{-1}$. The bands in the fingerprint region, which are characteristic of a given compound, are further means of identifying an unknown molecule, providing the spectrum of the authentic sample is available. The latter statement, however, is not quite applicable in those cases where the compound ought to be differentiated from its closely related homologs.

The esters of the three isomeric benzenedicarboxylic acids can be differentiated (*a*) on the basis of peak positions for carbonyl frequency, 1710–1745 cm.$^{-1}$; (*b*) aromatic ring skeletal vibration, 1500–1605 cm.$^{-1}$; (*c*) in-plane C—H bending, 1350–1490 cm.$^{-1}$; (*d*) C_6H_5—$\overset{\overset{O}{\|}}{C}$—OR stretching vibration, 1230–1290 cm.$^{-1}$; and (*e*) out-of-plane aromatic C—H bending vibration, 720–840 cm.$^{-1}$. In general, the stretching frequency for C=O of

TABLE V—THE POSITION OF PEAKS OF THE INFRARED SPECTRA OF DIALKYL AND DICYCLOALKYL TEREPHTHALATE[a]

R	νC—H	νC=O	Aromatic Ring Skeletal Vibration	δC—H in Plane	O ‖ νC$_6$H$_5$C—OR Stretching	δC—H out of Plane Aromatic *p*-di	Unassigned Peaks
CH_3	3000(w), 2950(w)	1725, 1700(sh)	1575, 1505	1440, 1410, 1380(w)	1280	815, 790, 735(w)	1192, 1130, 1110, 1020, 955, 880, 800(w), 695(w), 550(w), 500(w)
C_2H_5	2990, 2940, 2905(w)	1730	1570(w), 1500	1480, 1450, 1410, 1370	1275	840, 810(w), 725	1130, 1100, 1020, 870, 500, 450
n-C_3H_7	2970	1725	1570(w), 1500(w)	1475, 1410, 1390(w), 1350(w)	1270, 1250(sh)	732	1195, 1120–1100(d), 1020, 940, 875, 700(w)
iso-C_3H_7	2980, 2920(w)	1720	1500(w)	1470, 1410, 1380(d), 1350(d)	1275	845, 740	1185, 1130, 1100, 1015, 920(w), 880(w)
n-C_4H_9	2960, 2930, 2875	1735	1575(w), 1500(w)	1470, 1410, 1380	1280, 1270(b)	835(w), 790(w), 725	1115–1100(d), 1018, 960–940(d), 870
iso-C_4H_9	2960, 2860	1720	1500	1475, 1400, 1375	1270, 1250	800, 730	1135, 1120, 1105, 1015, 980, 948, 900, 870, 500, 430
n-C_5H_{11}	2960, 2935, 2865	1735	1580, 1500	1460, 1410, 1380	1270, 1250(sh)	735	1120–1105(d), 1020, 970(wb), 875(w)
iso-C_5H_{11}	2950, 2870	1725	1580(w), 1500	1470, 1410, 1390, 1370(d)	1270, 1250	820(w), 730	1120–1105(d), 1020, 965(b), 875
Cyclo-C_5H_9	2960, 2870(w)	1715	1505	1440, 1410, 1365, 1330(w)	1265, 1250(sh)	735	1330(w), 1165, 1120, 1105, 1015, 960(w), 880(w)
n-C_6H_{13}	2935(d), 2850(d)	1715	1500(w)	1460, 1400, 1370(w)	1270	840, 730	1135(w), 1120, 1105, 1020, 955(b), 875(w)
Cyclo-C_6H_{11}	2930, 2850	1730, 1710	1500	1450, 1400, 1370	1270, 1250	835, 735	1155(w), 1120, 1105, 1040, 1015, 940, 910(w), 895, 880, 510, 450
C_7H_{15}	2945(sh), 2915, 2845	1730	1500	1470, 1410, 1390	1270, 1245(sh)	839, 725	1195(w), 1120, 1105, 1040(w), 1018, 985(w), 920(w), 870, 500
Cyclo-C_7H_{13}	2945(sh), 2920, 2855	1715	1500	1470, 1450, 1410, 1375(w)	1270	839, 820(w), 760(w), 730	1325, 1125, 1100, 1015, 975, 905(w), 872, 500
C_8H_{17}	2960, 2920, 2850	1720	1675, 1505	1480, 1470, 1410, 1380, 1350(w)	1280	840, 730	1320, 1135, 1125, 1105, 1040(w), 1020, 875, 500(w), 470(w), 435(w)
Cyclo-C_8H_{15}	2925, 2850	1710	1500(w)	1470, 1450, 1410, 1375(w)	1270, 1255, 1220	845, 820, 810, 740(d)	1330, 1140, 1125, 1105, 1035, 1015, 945(w), 920(w), 880(w), 870(w)
n-C_9H_{19}	2950(w), 2910, 2850	1725	1500	1470, 1400, 1380	1275	830(w), 728	1125(d), 1100, 1020, 950(w), 900(w), 870(w), 500(w)
$C_{10}H_{21}$	2900, 2840	1710	1500(w)	1475–1460(d), 1400, 1375(w)	1285, 1270	840, 740	1135, 1125, 1110, 1020(w), 1000(w), 960(w), 875
$C_{11}H_{23}$	2915, 2850	1730	1500(w)	1475, 1450(w), 1405, 1390	1275, 1250	825(w), 730	1130, 1105(w), 1070(w), 1020, 975(w), 930(w), 875, 500(w)
$C_{12}H_{25}$	2950, 2915, 2850	1720	1500(w)	1475(d), 1400(w)	1290, 1280, 1255	845(w), 730	1320(w), 1140, 1130, 1110, 1025, 960(w), 875(w), 500(w)

[a] Key: same as in Table III.

TABLE VI—THE POSITION OF PEAKS OF THE INFARED SPECTRA OF BENZENEDICARBOXYLIC ACIDS AND PHTHALIC ANHYDRIDE[a]

	νCH	νC=O	Aromatic Ring Skeletal Vibration	δCH in Plane	O ‖ νC$_6$H$_5$C—OH	δC—H out of Plane, Aromatic	Unassigned Peaks
Phthalic acid	3100–2850(b)	1695	1590	1490(w), 1410	1280	740	1150(w), 1075, 1005(w), 975(w), 910(wb), 830(w), 800, 675, 555
Isophthalic acid	3070–3000(b)	1695	1610–1585	1430	1290–1280(b)	730	1160(w), 1095(w), 1075(w), 930–920(b), 830(w), 690
Terephthalic acid	3100–2900(b)	1690	1570(w), 1500	1420	1275	730	1200, 1130, 1110, 1070, 930(b), 880, 780, 695
Phthalic anhydride	3075–3050	1850, 1765	1600, 1515(w)	1475, 1390, 1360	1255	715	1165, 1105, 1070(w), 1005, 905, 840, 800, 675, 640, 535

[a] Key: same as in Table III.

phthalates are slightly higher than isophthalates and terephthalates. The field effect of the proximately located C=O group may be a reason for observing a higher C=O stretching frequency for phthalates.

The doublet for aromatic ring skeletal vibration at 1580–1600 cm.$^{-1}$ observed in the spectra of a phthalate is absent in the spectra of iso- and terephthalates. Instead there is a single band at 1605 cm.$^{-1}$ for iso- and two bands at 1570 (weak) and 1500 cm.$^{-1}$ for terephthalates.

The bands due to the in-plane bending vibration, 1350–1490 cm.$^{-1}$, quite often vary somewhat among the esters of the three benzenedicarboxylic acids. The variation in this region, however, is not sufficient to lead one to an unequivocal identification of an unknown ester; a reference to the spectrum of an authentic sample is necessary.

Out-of-plane bending bands, 700–900 cm.$^{-1}$, are characteristic of the esters of each of the three isomeric acids. Positional isomers of disubstituted benzene usually contain I.R. bands which are at 690–840 cm.$^{-1}$.

$$C_6H_5—\overset{\overset{O}{\|}}{C}—OR$$

The stretching band for C_6H_5—COR for phthalates is quite close to terephthalates at 1275–1280 cm.$^{-1}$. This band for isophthalate comes at 1235–1240 cm.$^{-1}$ and can serve for identification purposes.

Finally, the position of the unassigned peaks in the tables could function as fingerprints for detection and differentiation of the esters of the three isomeric acids. Various esters of a given acid, such as phthalic acid, can also be discerned if the molecular weight of the homologs are not too close.

Thin-Layer Chromatography—TLC proved to be an excellent and rapid method for the detection of the product of hydrolysis of an ester or the presence of other esters and the parent acid and alcohol mixed with a given ester. The R_f values for the esters of the three isomeric benzenedicarboxylic acids are listed in Table I. In general, the R_f value in this series increases from *ortho* to *meta* to *para* isomer. Apparently, the R_f value in the hexane–ethyl acetate developer for a given molecular weight of these esters is inversely proportional to their dipole moment value. Cyclic compounds show lower R_f values as compared to their straight chain counterpart which, in a sense, indicates the influence of polarity of a compound on its R_f value. Since only a fraction of a mg. is necessary to identify a compound by TLC, this technique in conjunction with I.R. manifests a great potential in the detection and identification of plasticizers leached out of a plastic. Such identification, however, may become laborious if some esters unrelated to benzenedicarboxylate are in the mixture.

Toxicity—As is depicted in Table II, toxicity results obtained by the cell culture method quite frequently coincide with the toxicity evaluation conducted on mice.

In general, low molecular weight dialkyl benzenedicarboxylates (alkyl group C1 to C5) manifest toxic effect. This toxic effect could be ascribed to the higher degree of solubility of the low molecular weight esters, as compared with their higher homologs. However, since both di-*n*-decyl phthalate and di-*n*-decyl terephthalate show toxicity to cell cultures, the conclusion based merely on relating the toxicity of a compound in the series to its solubility or molecular weight becomes subject to some doubts. It is noteworthy that, unlike a freshly synthesized pure di-*n*-decyl phthalate, the commercial sample of this compound was found to be extremely toxic to mice. Although determining the toxicity of a commercial plasticizer is beyond the scope of this investigation, the importance of purity and stability of a compound should not be ignored. Apparently identical plasticizers obtained from various sources or subjected to various conditions during the process of incorporation into a polymer may manifest quite different toxic effects.

The question of whether toxicity effects of the esters are due only to their degradation products rather than to the intact ester molecule has been examined in the past with citrate plasticizers (6), where toxicity proved to be due to the ester, despite the fact that citric acid *per se* was also toxic. In the dialkyl benzenedicarboxylates, although their products of hydrolysis were found to be toxic, evaluation of toxicity data suggested that these esters, as citrates, exert their toxic effects as the intact ester molecule. The molecules, di-*n*-propyl, di-iso-propyl, di-*n*-butyl, di-iso-butyl, di-*n*-pentyl, and di-iso-pentyl, all show similar ratings in the toxicity evaluation, whereas the rate of hydrolysis of the iso derivatives would be expected to be lower than the *n* derivatives. Likewise, no great difference, if any, would be expected in the rate of hydrolysis of di-*n*-pentyl and its homolog di-*n*-hexyl phthalates, but biologically these two behave differently. As indicated above, phthalates with alkyl groups C_6 to C_9 do not show a toxic effect on the cell.

The reason for the toxic manifestation of di-*n*-decyl phthalate and terephthalate is not yet clear. Such behavior, however, could be postulated to be due to the inhibition of the function of certain enzyme systems, whose metabolites possess a structure analogous to that of the ester.

The mice tests were included only as another screening method to observe if the results noted in the cell tests could be manifested in animals. Keeping in mind the limitation in the mouse test, it is still interesting to note that a good correlation exists between the results shown for the mammalian cell method and for the i.p. mouse test. Further animal studies would, of course, be needed to establish true toxicity patterns based upon definite dose levels of the compounds. It is, however, important to state that the close correlation between the animal test and cell test result renders the latter an appealing method due to its inherent convenience, rapidity, and economy.

In conclusion, perhaps the important point to be gained from this study is that greater attention should be paid to the actual composition of the plasticizer which is to be used for a medical application. A pure plasticizer may have no demonstrable toxic response, but the presence of related homologs and isomers could add a toxic potential to the plasticizer. To this possibility one must also include the potential toxic effects from degradation products present or forming in the plasticizer during manufacture of a plastic device, or occurring after the device is stored under various conditions for undetermined periods of time. Good quality control procedures in the manufacture of a medical device should prevent the entrance of potentially toxic

plasticizers to the medical and para-medical professions.

REFERENCES

(1) Lawrence, W. H., Mitchell, J. L., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **52**, 958(1963).
(2) Rosenbluth, S. A., Weddington, G. R., Guess, W. L., and Autian, J., *ibid.*, **54**, 156(1965).
(3) Meyers, D. B., Autian, J., and Guess, W. L., *ibid.* **53**, 774(1964).
(4) Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, Tex., unpublished data.
(5) Guess, W. L., Rosenbluth, S. A., Schmidt, B. C., and Autian, J., *J. Pharm. Sci.*, **54**, 1545(1965).
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Synthesis of Tropine-Labeled Atropine II

Prototype Synthesis for the Preparation of Tropine-^{14}C and Atropine-^{14}C from Arabinose-^{14}C

By GILBERT C. SCHMIDT*, THOMAS E. ELING, JOHN M. McOWEN, and JOHN C. DRACH†

Prototype methods for the synthesis of tropines-^{14}C and tropine-labeled atropines are described. These compounds and the requisite labeled intermediates may be synthesized from arabinose-5-^{14}C or arabinose-UL-^{14}C. Yields for each step in the conversion of arabinose to succindialdehyde *via* 2,5-diethoxytetrahydrofuran have been determined. Predicted yields of 27 per cent atropine, based on starting pentose, were confirmed by synthesis of the alkaloid from 2 mmole quantities of arabinose. This procedure is the first published method for labeling atropine in the carbon skeleton of the tropine moiety.

FEW METHODS for selectively labeling atropine have been published. Labeled tropic acid has been synthesized by adaptations of Blicke's procedure (1), then converted to correspondingly labeled atropine by modifications of the Wolffenstein esterification (2). Using these methods, atropine has been labled with ^{14}C in the carboxyl position (3) and in the α-position (4) of the tropic acid moiety. Subsequent studies with the labeled alkaloids (5–9) have contributed much to an understanding of atropine metabolism, but the metabolic fate of the tropine moiety is not known.

The virtual absence of studies concerned with the metabolic fate of tropine indicates that suitably labeled compounds are not readily available for study. Randomly ^{14}C-labeled atropine has been biosynthesized (10) and atropine has been randomly labeled with tritium (11), but these compounds lack the desired specificity of labeling. Selective tritiation of tropine has been accomplished recently (12), but the heterocycle was converted to deptropine rather than atropine (13). *Datura metel* is known to incorporate 80–85% of the radioactivity from acetate-2-^{14}C into positions 2,3,4 of tropine (14, 15), but the exact position or positions of labeling are unknown. When *D. metel* is grown on acetate-1-^{14}C (14, 15) or *Datura stramonium* is grown on ornithine-2-^{14}C (16, 17), radioactivity is incorporated with stereochemical specificity into position 1 or position 5 of the tropine moiety. These biosynthetic compounds have not been available for metabolic studies and problems inherent in biosynthetic methods limit feasibility of this approach to tropine labeling. Standard synthetic methods for labeling the tropine moiety of atropine are needed, but few have been reported. Werner *et al.* (3), Fodor *et al.* (18), and Eling *et al.* (19) have used classical organic syntheses to obtain *N*-methyl-^{14}C-tropine and *N*-methyl-^{14}C-atropine, but these compounds are of limited value because the methyl carbon attached to the endo nitrogen bridge, rather than the carbon skeleton of tropine, is labeled. The need of practical methods for labeling the carbon skeleton of tropine is acute and the problem remains unsolved.

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REFERENCES

(1) Lawrence, W. H., Mitchell, J. L., Guess, W. L., and Autian, J., *J. Pharm. Sci.*, **52**, 958(1963).
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key compounds in the Robinson condensation. The problems of condensation and esterification have been studied recently (20) and reproducible microsynthetic methods now are available. The present needs are for suitably labeled intermediates and microsynthetic methods for converting them to labeled succindialdehyde or labeled acetone dicarboxylic acid.

This communication presents, in detail, a prototype method for the synthesis of tropine-labeled atropine from arabinose.[1] The starting material is available in labeled forms suitable for the synthesis of tropine-1,5,6,7-^{14}C or tropine-1-^{14}C (or tropine-5-^{14}C), from which the correspondingly labeled atropines can be synthesized. Except for biosynthetic procedures, it is the first microsynthesis suitable for selectively labeling the carbon skeleton of tropine.

EXPERIMENTAL

Synthesis of Furaldehyde from Arabinose—Furaldehyde was synthesized from arabinose by a modification of Pervier and Gortner's method (22). In a 500-ml. round-bottom flask fitted for steam distillation, 300 ml. of 12% HCl was heated to 100° and 300 mg. arabinose[2] dissolved in the hot solution.[3] While heating with a heating mantle at a rate to maintain the vapor phase at 103.5–105.5°, steam was passed into the distilling flask and distillation continued until 1000 ml. of distillate had been collected.[4] At this point, single drops of distillate were tested with 2,4-dinitrophenylhydrazine solution[5] until the red precipitate characteristic of furaldehyde no longer appeared.[6] The entire solution was used, without concentration, for conversion of furaldehyde to furoic acid.

Conversion of Furaldehyde to Furoic Acid—Furoic acid was prepared from furaldehyde by a modification of Wagner and Simons' procedure (25). Furaldehyde from the previous step,[7] contained in a 2-L. beaker, was adjusted to pH 12 with saturated NaOH, cooled to 10°, and continuously stirred with a mechanical stirrer during the gradual addition of 0.3 Gm. $KMnO_4$ dissolved in 25 ml. water. The reaction mixture was boiled gently for 15 min.,[8] filtered, and the filtrate acidified with HCl. The acidified filtrate was concentrated *in vacuo* at 70° to a volume of 30 ml., then five times extracted with 50-ml. portions of diethyl ether. The ether solution of furoic acid was transferred to

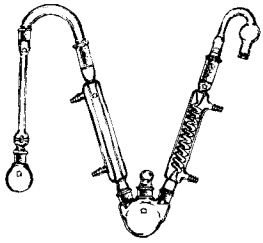


Fig. 1—Simplified decarboxylation apparatus.

a sublimation flask,[9] the solvent removed *in vacuo*, and the sublimation flask, containing the light tan product, dried for 2 hr. in a vacuum desiccator. After drying the flask was fitted with a cold finger containing dry ice–acetone and the furoic acid sublimed onto the cold finger by heating the flask for 30 min. at 15 mm. and 100°. The sublimates[10] were removed from the cold fingers with a small amount of absolute ether, the solvent was evaporated, and a residue of pure, white furoic acid obtained.[11]

Synthesis of Furan from Furoic Acid—Furoic acid was decarboxylated to furan by adapting for microsynthesis the method of Wagner and Simons (25). A simplified decarboxylation apparatus,[12] shown in Fig. 1 and constructed entirely of standard-taper microglassware,[13] was used. A 50-ml. three-neck flask was used as a receiving vessel. One opening of the receiver was fitted with a spiral water-cooled condenser, protected by a soda and lime tube. The second opening was fitted with a water-cooled straight condenser. The middle opening was fitted with a glass plug to permit access to contents of the receiver. The reaction vessel, a 5-ml. round-bottom flask, was fitted to the delivery end of an air-cooled condenser. Receiver and reaction assemblies were connected by a modified U-tube constructed of two adapters bent to the proper angles.

The receiver, containing a mixture of 5 ml. absolute ethanol and 20 ml. absolute diethyl ether,[14] was cooled to −40° and the temperature maintained at this level throughout subsequent steps. Furoic acid from the previous step,[15] 8.5 mg. cupric oxide, and 0.5 ml. water-free quinolin[16] were introduced into the reaction vessel and all joints sealed. The reaction vessel was heated on an oil bath until gas evolution became vigorous (about 200°) and the temperature gradually raised to 275°, at which point it was maintained for 15 min.[17] The resulting furan,

[1] A preliminary report was presented to the American Association for the Advancement of Science (21).

[2] Matheson, Coleman and Bell, Cincinnati, Ohio, m.p. 157–160°, used without further purification.

[3] In preliminary experiments to determine yields, 100–500 mg. arabinose was used. For conversion to tropanone, a minimum of 300 mg. arabinose is recommended.

[4] Complete distillation usually required 6–8 hr. and produced 1000–1250 ml. distillate. It is essential that the stated distillation range be maintained. At lower temperatures furaldehyde does not distil properly, and at higher temperatures decomposition of the product decreases yields.

[5] A standard solution of 2,4-dinitrophenylhydrazine, as described by Shriner *et al.* (23), was used in qualitative and quantitative precipitations.

[6] In quantitative studies to determine the yield for this step, the dinitrophenylhydrazone was isolated and weighed, m.p. 230°, after recrystallization from ethanol (24).

[7] In experiments to determine yields for this step, 0.2 ml. freshly distilled furaldehyde was dissolved in 1000 ml. water and treated as described.

[8] To insure complete precipitation of MnO_2.

[9] Of the design described by Werner (3), having a capacity of 250 ml.

[10] To obtain the last traces of furoic acid after the first sublimation, the flask was washed with ether to dissolve selectively any residual furoic acid. The solution was decanted into another sublimation flask, the solvent evaporated, and the residue sublimed as described previously.

[11] Melting point 130–132° without recrystallization.

[12] In the original apparatus there was a soda and lime trap between the two condensers, to remove CO_2 and water. In microsyntheses, the small quantities of furan produced were adsorbed on the soda and lime. The trap was, therefore, omitted from the reaction train.

[13] Microware, Kontes Glass Co., Vineland, N. J.

[14] Anhydrous conditions must be maintained.

[15] In experiments to determine yields for the conversion of furoic acid to furan, 190 mg. furoic acid was used routinely with the stated quantities of reagents. In other experiments, 5-Gm. quantities of furoic acid were used with corresponding increases in reagents.

[16] Quinolin must be water free and was redistilled immediately before use.

[17] At the stated temperature, quinolin refluxes about halfway up the air condenser. While heating from 200°–275°, furan began to distil into the receiving vessel. When larger quantities of furoic acid were used (5 Gm.), the resulting furan was purified by fractionally distilling at 31–32° and atmospheric pressure.

representing an 88% yield from furoic acid and a 70% yield from arabinose, was immediately converted to tetrahydrofuran, as described below.[18] The bromine number of furan was calculated from the difference in amounts of thiosulfate required to titrate blank and standard solutions.[19] Replicate determinations with known furan solutions gave bromine values of 460–470 mg. bromine per 100 mg. furan. To determine the furan yield in a particular synthesis, an aliquot of the diluted, glacial acetic acid solution of the product was assayed as just described. Furan production was then calculated on the basis of the previously determined bromine number for furan.[20]

Synthesis of 2,5-Diethoxytetrahydrofuran—Furan was converted to diethoxydihydrofuran and diethoxytetrahydrofuran by a micro modification of Fakstorp's (27) procedure. The solution of furan in ethanol and ether,[21] from the preceding step, was introduced into the reaction vessel,[22] cooled to −40° in a dry ice–acetone bath, and a solution of 0.08 ml. bromine in absolute ethanol[23] added, while stirring continually, at a rate to prevent a rise in temperature. Stirring was continued for 15 min. after adding all the bromine, the dropping funnel was replaced with an inlet tube and, while stirring, dry ammonia was bubbled into the reaction mixture until pH 6 was attained.[24] Stirring was continued until the color had disappeared, ammonia was again bubbled in to obtain a pH of 8, precipitated ammonium bromide removed by centrifugation, and the solution of 2,5-diethoxydihydrofuran concentrated to 10 ml.[25] The pale yellow solution of diethoxytetrahydrofuran was transferred to a micro hydrogenation apparatus,[26] using small volumes of absolute ethanol to transfer the last traces, and the volume finally adjusted to 50 ml. with absolute ethanol. With the magnetic stirrer in motion, three "micro spatelafuls" of freshly prepared Raney nickel[27] were added and a very slow[28] stream of hydrogen passed through the reaction mixture until reduction was complete.[29] Hydrogenation usually was complete in about 2 hr., at which time the catalyst was removed by centrifugation, leaving a colorless solution of diethoxytetrahydrofuran in ethanol.[30] The catalyst was washed with several 1-ml. portions of absolute ethanol and the washings combined with the previously obtained alcoholic solution of product.

Synthesis of Tropanone—Tropanone was prepared by the "standard" micro Robinson condensation previously described (20). Succindialdehyde was obtained *in situ* by removal of the solvent,[31] and was then condensed *in situ* with equimolar quantities of methylamine HCl and freshly prepared acetone dicarboxylic acid. The product was purified by micro sublimation and dried in a desiccator[32] before reduction to tropine.

Reduction to Tropine and Esterification with Tropic Acid—Tropanone was reduced to tropine,[33] converted to the hydrochloride and esterified with tropic acid,[34] as described previously (20).[35]

RESULTS AND DISCUSSION

The development of micro methods for the synthesis of tropine and for esterification of this heterocycle with tropic acid has made feasible the synthesis of tropine-labeled atropine. Atropine has been labeled in the bridge position of the tropine moiety by use of methylamine-^{14}C in the micro Robinson condensation, but methods for labeling the carbon skeleton of the tropine moiety have not been published. The feasibility of such labeling depends on on the availability of labeled Robinson intermediates

[18] The apparatus was allowed to cool to room temperature, then the last traces of product were washed from the condenser into the receiver. Receiver and contents were maintained at −40° until used in subsequent steps. In experiments to determine the yield of furan from furoic acid, 25 ml. of glacial acetic acid replaced the ether–alcohol mixture in the receiving vessel. After washing the condenser with a little glacial acetic acid, the solidified solution of furan in glacial acetic acid was allowed to liquefy and was then assayed as described.

[19] The bromine number of furan, defined as mg. bromine reacting with 100 mg. furan, was calculated as follows:

$$\frac{(\text{blank} - \text{sample})(N\ Na_2S_2O_3)(\text{mol. wt. } Br_2)(100)}{(\text{mg. furan in the known aliquot assayed})} =$$

bromine No. of furan = 460

[20] Using the previously determined bromine value of 460 mg. bromine/100 mg. furan, the furan contents of unknown samples were calculated as follows:

$$\frac{(\text{blank} - \text{unknown})(N\ Na_2S_2O_3) \times (\text{mol. wt. } Br_2)(100)\ (\text{dilution factor})}{460} = \text{mg. furan in total distillate}$$

[21] To determine yields of diethoxydihydrofuran and diethoxytetrahydrofuran from furan, 100 mg. pure furan was dissolved in a mixture of 20 ml. absolute diethyl ether and 5 ml. absolute ethanol.

[22] The reaction vessel was a 50-ml. three-neck flask that had been fitted with a thermometer, dropping funnel, and mechanical stirrer. Prior to use, the apparatus was dried by warming with a flame from a Bunsen burner.

[23] Bromine was dissolved in commercial, absolute ethanol, and a 1–2-ml. aliquot containing the stated amount of bromine was used. The alcoholic solution of bromine was cooled to −40° before addition to the cold furan solution.

[24] On bubbling in ammonia, ammonium bromide separated immediately. At pH 6 (Hydrion paper), the temperature had risen to −5° and was not permitted to rise above this level.

[25] To determine the yield of diethoxydihydrofuran from furan, the product from 500 mg. 5-Gm. furan was purified by fractional distillation at 69–72° and 5 mm. Fakstorp (27) reported b.p. 50–53° at 1 mm. Yield was determined by weighing the purified product.

[26] Available from Microware, Inc., Vineland, N. J. The apparatus was immersed in ice water and ice water was used to cool the "cold finger," to prevent evaporation of both product and reactant. Unless extreme care is exercised, losses occur by volatilization on passage of hydrogen.

[27] Raney nickel was prepared as described previously (20).

[28] It is essential that the flow of hydrogen be very slow to prevent losses of reactant and product by volatilization. It is probable that hydrogenation in a closed system, used in syntheses to be reported subsequently, would be equally effective and more convenient. This method was not, however, used in syntheses from arabinose.

[29] To test for completeness of reduction, a 1-ml. aliquot was removed and treated with 2,4-dinitrophenylhydrazine solution. A yellow precipitate of the succindialdehyde derivative indicated complete reduction, while an orange-red precipitate of the malaldehyde derivative indicated incomplete reduction.

[30] In experiments to determine the yield of 2,4-diethoxytetrahydrofuran, the product was acid-hydrolyzed to succindialdehyde, from which the bis-dinitrophenylhydrazone was prepared. The melting point (28) in semimicro and macro experiments was 260° without recrystallization and 272° on recrystallization from ethanol. In micro syntheses m.p. 272° was obtained without recrystallization.

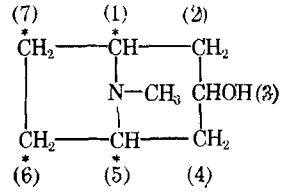
[31] Solvent was removed on the flash evaporator; succindialdehyde distilling at 78–80° and 20 mm. In experiments to determine succindialdehyde yields from diethoxytetrahydrofuran, the furan derivative was purchased from Beacon Chemicals, Cambridge, Mass., and was used without purification. In experiments to determine the dialdehyde yields from succinaldoxime, the oxime was prepared as described by Keagle and Hartung (28) and was recrystallized from water before use in the condensation.

[32] Tropanone yields were determined as the free base, after purification by micro sublimation, and as the picrate, m.p. tropanone 41–43°, m.p. tropanone picrate 220°.

[33] Because Raney nickel is extremely susceptible to poisoning, it was necessary to use highly purified hydrogen available in small cylinders, or to purify by washing the hydrogen obtained commercially in large cylinders.

[34] Nutritional Biochemicals, Cleveland, Ohio, m.p. 157–160°, used without further purification.

[35] Atropine determined gravimetrically and an aliquot converted to the picrate, m.p. free base 114–116°, m.p. picrate 174–175°. All melting points reported in this communication were determined with a Fisher-Johns melting point apparatus.

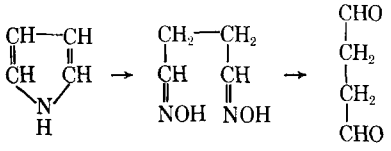


Tropine

other than methylamine. Starting with succindialdehyde, any or all of the indicated positions of tropine could be labeled, depending upon the labeling in succindialdehyde.

Succindialdehyde, the key intermediate for labeling positions 1,5,6,7 of tropine, has been prepared in a number of ways, many of which are not suitable for ^{14}C-labeling because of experimental conditions or because suitable starting materials are are not available. Among the potentially applicable procedures are preparation by ozonolysis of cyclooctadiene (29), oxidation and subsequent reduction of furan (30), preparation from pyrrole (28, 31), and synthesis by hydrolysis of 2,5-diethoxytetrahydrofuran (20, 32).

The authors initially prepared succinaldoxime from pyrrole, and the dialdehyde from this intermediate, as described by Keagle and Hartung (28).



Pyrrole Succinaldoxime Succindialdehyde

Using micro quantities of pyrrole, the expected 45% conversion to the intermediary dioxime was obtained. The purified dioxime was then converted to succindialdehyde, which was determined as the bis-dinitrophenylhydrazone. That the dioxime undergoes 90% conversion to the dialdehyde, as reported by Keagle and Hartung, was confirmed. When used in the Robinson condensation, the dioxime did not react to the same extent, as is shown in Table I. This difference in reactivity appears to account for the different yields reported by Keagle and Hartung (28) and by Werner *et al.* (3).

Because they obtained 90% conversion of succinaldoxime to the bis-dinitrophenylhydrazone of succindialdehyde, Keagle and Hartung assumed the same conversion to dialdehyde in the Robinson condensation.

Based on the calculated amount of aldehyde, 60% of theoretical tropanone yields were reported. Using the same method of calculation, the authors repeatedly confirmed the reported yields. In contrast, Werner *et al.* used a relatively large excess of dioxime and calculated tropanone yields on the basis of methylamine. Using the method of Werner and co-workers, the authors also obtained 70–75% tropanone yields from methylamine. Using 2,5-diethoxytetrahydrofuran as a source of succindialdehyde, and no excess of either reactant, 75% of theoretical tropanone yields were obtained. From these observations, it was concluded that an unpredictable excess of succinaldoxime is required in the Robinson condensation. Determination of the succindialdehyde as the bis-dinitrophenylhydrazone is not a reliable measure of the extent to which the oxime will react in the condensation.

TABLE I—COMPARISON OF TROPANONE AND SUCCINDIALDEHYDE YIELDS FROM PURIFIED SUCCINALDOXIME AND 2,5-DIETHOXYTETRAHYDROFURAN

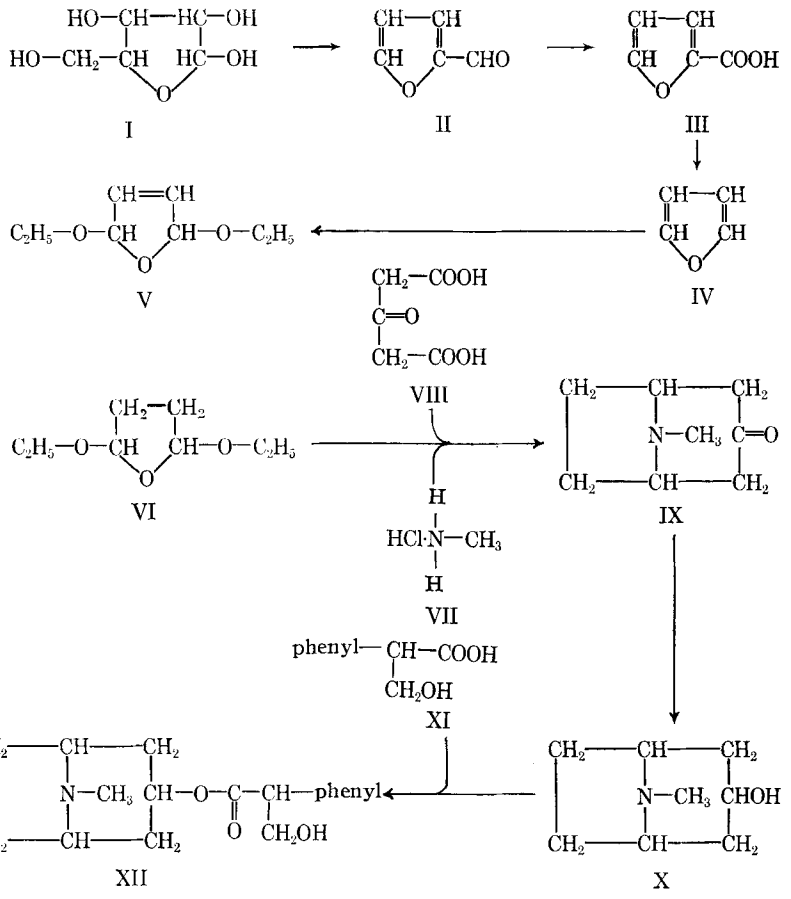
Source of Succindialdehyde	mmoles of Source Employed	Succindialdehyde[a] Yield, %	Tropanone[b] Yield, %
Succinaldoxime[c]	1.12	90 (89–91)	60 (58–62)
Succinaldoxime[d]	1.50	90 (89–92)	72 (70–75)
2,5-Diethoxytetrahydrofuran[e]	1.00	99.5 (99–100)	73 (70–75)
2,5-Diethoxytetrahydrofuran[e]	1.50	99.5 (99–100)	71 (68–74)
2,5-Diethoxytetrahydrofuran[e]	2.00	99.5 (99–100)	75 (74–77)

[a] Succindialdehyde was isolated and determined as the bis-dinitrophenylhydrazone, m.p. 278–279°. [b] Yields are tropanone, purified by microsublimation and melting at 41–43°; yields as the picrate, m.p. 220°, were 1–2% higher. [c] Tropanone yields based on calculated aldehyde production, as described by Keagle and Hartung (19); no excess of succinaldoxime. [d] Tropanone yields based on methylamine, using an excess of succinaldoxime, as described by Werner *et al.* (3). [e] Yields based on methylamine; equimolar quantities of methylamine, acetone dicarboxylic acid, and diethoxytetrahydrofuran were used in the Robinson condensation.

When the *N*-methyl carbon is to be labeled, poor conversion of pyrrole to the dioxime, the need to recrystallize this intermediate, and the requirement for an unpredictable excess of the purified oxime are disadvantages of little consequence. Circumstances are quite different if the resulting succindialdehyde is to be utilized as the instrument for ring labeling. The stated disadvantages then become a major concern and practically exclude pyrrole as a labeled precursor to radioactive succindialdehyde. As a result, 2,5-diethoxytetrahydrofuran was considered for use in the synthesis of labeled succindialdehyde.

Compared to pyrrole, the furan derivative proved to be a far superior source of succindialdehyde. Its use for this purpose has been reported in both chemical and patent literature, but it has not been used previously in the Robinson condensation, nor has the aldehyde been produced so simply as described in this communication. Most authors have hydrolyzed diethoxytetrahydrofuran with acid, in an atmosphere of nitrogen, and have removed the acid as the barium salt. Neither of these steps was necessary for use of this compound in the Robinson condensation. Direct conversion of the intermediate to succindialdehyde was effected by a very simple, rapid, *in situ* acid hydrolysis at room temperature. Derivatives of the resulting dialdehyde melted sharply and correctly without recrystallization. When used in the Robinson condensation, *in situ* hydrolysis obviated the need for isolation of an intermediate. No excess of diethoxytetrahydrofuran was required, the condensation proceeded smoothly, and reproducible 70–75% tropanone yields were obtained with 1 mmole or less of the aldehyde source.

The superiority of 2,5-diethoxytetrahydrofuran as a source of succindialdehyde led to development of the reaction sequence shown in Scheme I for its synthesis from commercially available labeled compounds.



Scheme I

For the proposed sequence, the steps from diethoxytetrahydrofuran (VI) to atropine (X) have been established by microsynthetic studies of the individual reactions (20). Diethoxytetrahydrofuran (VI) yields tropanone (IX) in 75% of theoretical quantities, conversion of tropanone to tropine (X) is essentially quantitative, and esterification of tropine with tropic acid (XI) produces atropine (XII) in 70–75% of theoretical yield. The predicted 49–53% yield of atropine from diethoxytetrahydrofuran has been confirmed repeatedly by microsynthesis of the alkaloid. Usefulness of the proposed method for labeling atropine therefore depends on the efficient conversion of arabinose to 2,5-diethoxytetrahydrofuran.

That arabinose (I) can be converted quantitatively to furaldehyde (II) was shown by Pervier and Gortner (22) during the development of a titrimetric assay for pentosans. Large volumes of distillate were required, but furaldehyde yields from either arabinose or xylose were quantitative. Bonner and Roth (33) prepared furaldehyde from xylose by essentially the same procedure, but did not isolate the intermediate furaldehyde. Direct oxidation with silver oxide gave 30% furoic acid (III), based on starting pentose. In contrast, Wagner and Simons (25) obtained "over 80%" furoic acid by permanganate oxidation of furaldehyde. Using the micro adaptations described under *Experimental*, the authors obtained 98% furaldehyde from arabinose and 83% furoic acid from furaldehyde. Observed and previously reported data are compared in Table II. These findings were confirmed by the direct conversion of micro quantities of arabinose to furoic acid. As shown in Table III, experimental yields were slightly below levels predicted from the individual steps. Based on these observations, the microsynthesis of furaldehyde (II) and furoic acid (III) from arabinose (I) was assumed to be practical. The efficiency of subsequent steps in the proposed sequence therefore became the key to tropine labeling.

For the conversion of furoic acid (III) to furan (IV), the catalytic decarboxylation of Wagner and Simons (25) was modified as described under *Experimental*. To determine efficiency of the conversion, micro quantities of furoic acid were decarboxylated, the product was trapped in glacial acetic acid, and the resulting solution assayed by a modification of Angelli's method (26). In parallel experiments, the acetic acid solution of furan was prepared directly from arabinose, then assayed. The data, shown in Tables II and III, confirm the findings of Wagner and Simons and establish the practicality of converting arabinose (I) to furan (IV).

The subsequent conversion of furan (IV) to the intermediate diethoxydihydrofuran (V) and to diethoxytetrahydrofuran (VI) was equally feasible.

TABLE II—COMPARISON OF OBSERVED AND REPORTED YIELDS FOR INDIVIDUAL STEPS IN THE CONVERSION OF ARABINOSE TO SUCCINDIALDEHYDE

Reaction	Ref.	Wt. of Starting Material	Yield for the Step Shown, %
Arabinose	[a]	500 mg.	98
	[a]	300 mg.	95
	[a]	100 mg.	98
	(22)	200 mg.	100
Furaldehyde	(33)	500 mg.	...
Furaldehyde	[a]	5.0 Gm.	83
	[a]	200 mg.	80
	(25)	25 Gm.	80
Furoic acid	(33)	...	30
Furoic acid	[a]	5.0 Gm.	88
	[a]	190 mg.	88
Furan	(25)	25 Gm.	90
Furan	[a]	5.0 Gm.	79
	[a]	500 mg.	76
	[a]	100 mg.	77
Diethoxydihydrofuran	(27)	36 Gm.	70
Diethoxydihydrofuran	[a]	5.0 Gm.	81
	[a]	100 mg.	79
	[a]	70 mg.	75
Diethoxytetrahydrofuran	(27)	79 Gm.	85
Diethoxytetrahydrofuran	[a]	1.0 Gm.	100
	[a]	150 mg.	99
	[a]	100 mg.	99
	[a]	75 mg.	99
	[a]	50 mg.	98
	(32)		100
Succindialdehyde	(27)	16 Gm.	30

[a] This report. [b] Yield is for conversion of xylose to furoic acid; the intermediate, furaldehyde, was not isolated.

TABLE III—PREDICTED AND OBSERVED YIELDS OF SUCCINDIALDEHYDE AND ITS PRECURSORS AND OF ATROPINE AND ITS PRECURSORS WHEN ARABINOSE IS USED AS A STARTING MATERIAL

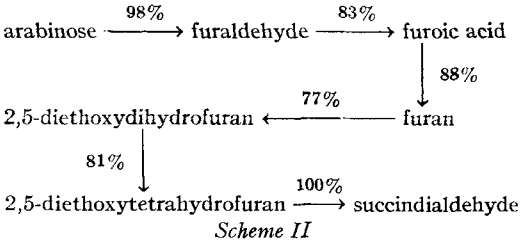
Compd. Synthesized	Observed Yields from Immediate Precursor, %	Yields Predicted from Individual Steps, %	Observed Yields from Arabinose, %
Furaldehyde	98	...	98
Furoic acid	83	81	70–73
Furan	88	71	70
2,5-Diethoxydihydrofuran	77	55	Not isolated
2,5-Diethoxytetrahydrofuran	81	45	50
Succindialdehyde	99.8	45	50
Tropanone	75	34	38
Tropine	98	33	34
Atropine	71–75	22–25	24

Yields for the individual steps were first determined with 5-Gm. quantities of furan and diethoxydihydrofuran. On isolation and purification of the dihydro compound, 77% of theoretical yields were obtained from furan. Conversion of the dihydro compound to the tetrahydro compound which was assayed by hydrolysis to succindialdehyde and isolation of the bis-dinitrophenylhydrazone, gave 81% of theoretical yields. In confirmatory, micro experiments furan was converted to diethoxytetrahydrofuran, without isolation of the intermediate. Yields for the direct conversion (70%) were higher than predicted from the individual steps (63%), a discrepancy that probably arose from losses during isolation and purification of the intermediate. Data for the conversion are in agreement with the findings of Fakstorp (27) and were confirmed by synthesis of 2,5-diethoxytetrahydrofuran from micro quantities of arabinose. The data, shown in Tables II and III, establish feasibility of these conversions and of the entire sequence from arabinose (I) to succindialdehyde. Establishment of this sequence simultaneously establishes the feasiblity of using labeled arabinose as an instrument for labeling tropine and the tropine moiety of atropine.

SUMMARY AND CONCLUSIONS

Previous work (20) has shown that the micro Robinson condensation is practical, that the reduction of tropanone to tropine is essentially quantitative, and that esterification of tropine with tropic acid gives 70–75% of theoretical atropine yields. In the present report, it has been shown that 2,5-diethoxytetrahydrofuran yields 75% tropanone when hydrolyzed *in situ*, employing a very simple procedure. Predicted yields of 49–53% atropine from 2,5-diethoxytetrahydrofuran have been confirmed by microsynthesis.

A reaction sequence for the production of diethoxytetrahydrofuran from arabinose has been proposed. Quantitative studies of each step in the sequence have yields for the individual steps shown in Scheme II.



Scheme II

Cumulative yields at each point in the sequence have confirmed yields for the individual steps. Validity of the sequence was finally established by the synthesis of 50% succindialdehyde and 37% tropine from arabinose. Additional verification was obtained by the synthesis of 26% atropine sulfate from 300-mg. quantities of arabinose.

Since arabinose-5-^{14}C and arabinose-UL-^{14}C are available commercially, the proposed sequence is a practical approach to the synthesis of tropine-1-^{14}C, tropine-1,5,6,7-^{14}C, and of atropines correspondingly labeled in the tropine moiety. Based on ^{14}C, 37% tropine and 26% atropine yields may be expected from arabinose-5-^{14}C, 29.6% labeled tropine and 20.8% labeled atropine from the uniformly labeled pentose. Expected yields of labeled furaldehydes, furoic acids, furans, and diethoxytetrahydrofurans can be calculated from the data presented and will vary, depending on which of the available pentoses is used and on whether one carbon of the uniformly labeled compound is lost during synthesis of the desired product.

Results of this investigation make available, for

the first time, a synthetic method for labeling the carbon skeleton of tropine, for synthesizing tropine-labeled atropine, and for synthesizing labeled intermediates between arabinose and tropine. Further studies will be published in the third paper of this series.

REFERENCES

(1) Blicke, F. F., Raffelson, H., and Barna, B., *J. Am. Chem. Soc.*, **74**, 253(1956).
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(4) Murray, A., and Williams, D. L., "Organic Synthesis with Isotopes," part I, Interscience Publishers, Inc., New York, N. Y., 1958, p. 550.
(5) Gosselin, R. E., Gabourel, J. D., Kalser, S. C., and Wills, J. H., *J. Pharmacol. Exptl. Therap.*, **115**, 217(1955).
(6) Kalser, S. C., Wills, J. H., Gabourel, J. D., Gosselin, R. E., and Epes, C. F., *ibid.*, **121**, 449(1957).
(7) Werner, G., and Schmidt, H., *Naturwiss.*, **46**, 626 (1959).
(8) Gabourel, J. D., and Gosselin, R. E., *Arch. Intern. Pharmacodyn.*, **115**, 416(1958).
(9) Werner, G., and Schmidt, H. L., *Abhandl. Deut. Akad. Berlin Kl. Chem. Geol. Biol.*, **4**, 207(1963).
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(11) Schmidt, H. L., and Werner, G., *Ann.*, **656**, 149(1962).
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(14) Kaczkowski, J., Schutte, H. H., and Mothes, K., *Naturwiss.*, **47**, 304(1960).
(15) Kaczkowski, J., Schutte, H. H., and Mothes, K., *Biochim. Biophys. Acta*, **46**, 588(1961).
(16) Bothner-By, A. A., Schutz, R. S., Dawson, R. F., and Salt, M. L., *J. Am. Chem. Soc.*, **84**, 55(1962).
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(18) Fodor, G., Janszo, G., Otvos, L., and Benfi, D., *Ber.*, **93**, 2681(1960).
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(22) Pervier, N. G., and Gortner, R. A., *Ind. Eng. Chem.*, **15**, 1167(1923).
(23) Shriner, R. L., Fuson, R. L., and Curtou, D. Y., "The Systematic Identification of Organic Compounds," 5th ed., John Wiley & Sons, Inc., New York, N.Y., 1964, p. 253.
(24) Blankama, K. L., and Walker, M. C., *Rec. Trav. Chim.*, **55**, 655(1936).
(25) Wagner, E. C., and Simons, J. K., *J. Chem. Educ.*, **13**, 265(1936).
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(27) Fakstorp, J., Raleigh, D., and Schnupp, L., *J. Am. Chem. Soc.*, **72**, 859(1950).
(28) Keagle, A. C., and Hartung, W. H., *ibid.*, **68**, 1608 (1946).
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(31) Wilstatter, R., and Huebner, W., *Ber.*, **40**, 3871 (1907).
(32) Findlay, S. P., *J. Org. Chem.*, **22**, 1385(1957).
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Model Catalysts Which Simulate Penicillinase II

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IT WAS PREVIOUSLY reported that the compound 3,6-bis(dimethylaminomethyl)catechol (CDM) rapidly catalyzed the hydrolysis of penicillin to penicilloic acid (1). Pyrocatechol itself, as the monoanion, was also shown to be catalytic but at a much lower rate than CDM. Studies of the relationship between structure of the catalyst and its reactivity revealed that the catalytic efficiency of CDM was at least partly due to an ionic interaction between catalyst and substrate, similar to the type of interaction which might be expected in formation of an enzyme–substrate complex. It was speculated at that time that this reaction proceeded by covalent catalysis (2) to form a penicilloate ester of the catecholamine which would then be rapidly hydrolyzed to product, releasing free catalyst.

$$\text{penicillin} + \text{CDM} \rightarrow \text{ester} \rightarrow \text{penicilloic acid} + \text{CDM}$$

The above-mentioned studies were conducted by measuring the rate of acid production by means of a pH-stat. The presence of an ester intermediate might be demonstrated, however, by also following loss of penicillin. If the latter were more rapid than acid formation there would be no doubt as to the presence of an intermediate in the reaction pathway. The present report concerns the results of such studies in which pyrocatechol was utilized as the catalyst rather than CDM because it was felt that ester hydrolysis would be slower with pyrocatechol than with

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Accepted for publication July 5, 1967.
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REFERENCES

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CDM where the amino groups might accelerate the reaction by intramolecular general acid catalysis. As will be seen, the data provide evidence not only for an ester intermediate but also for metastable intermediates in the reaction pathway prior to ester formation

EXPERIMENTAL

Materials and Apparatus—Potassium benzylpenicillin and sodium methicillin were supplied by Bristol Laboratories. Pyrocatechol, mercuric chloride, and buffer ingredients were all reagent grade. pH measurements were made with the Radiometer TTT-1 meter in a glass cell thermostated at the temperature of the kinetic studies. The meter was standardized with both pH 6.83 and 9.18 buffers. The Hitachi Perkin-Elmer model 139 spectrophotometer was used in the penicillin assays and the Radiometer TTT-1 pH-stat with SBR-2C recorder used for measurement of acid formation rates.

Kinetic Studies—All the rate measurements were carried out at 31.5° at ionic strength 0.2 maintained by addition of sufficient potassium chloride to the reaction mixture. All solutions were prepared with water which had been freshly boiled and cooled under nitrogen and a nitrogen atmosphere was maintained throughout the rate measurements to minimize oxidation of catechol.

Rates of acid formation from penicillin in the presence of catechol were measured on the pH-stat as previously described (1).

Rates of penicillin loss were measured as follows. Catechol solution containing sufficient potassium chloride was placed in the reaction cell of the Radiometer pH-stat and allowed to come to bath temperature. The pH was adjusted to the desired value by addition of NaOH solution and the reaction was initiated by addition of a solution of the penicillin which had been kept at bath temperature. Samples were removed at appropriate intervals and transferred to a 0.2 *M* pH 2.7 glycine buffer containing 0.01 *M* mercuric chloride. After standing a specified time (80 min. for benzylpenicillin, 130 min. for methicillin), the absorbance was read at 322 mμ for benzylpenicillin or 328 mμ for methicillin. This assay for residual penicillin in solution has been described previously (3).

The rates of alkaline hydrolysis of the penicillins in absence of catechol were determined by following acid production rates on the pH-stat.

RESULTS

Figure 1 shows the typical first-order dependence of rate of penicillin loss upon concentration of benzylpenicillin at constant pH. Similar linear plots were obtained for methicillin. The rate constants ($k_{obs.}$) obtained from the slopes of these plots were corrected for alkaline hydrolysis of the penicillin and converted to specific rate constants for monocatecholate ion (k_c) by Eq. 1:

$$k_c = \frac{k_{obs.} - k_{OH}[OH^-]}{\frac{K_a[C]_t}{K_a + [H^+]}} \qquad \text{(Eq. 1)}$$

where $[C]_t$ is the total of all catechol species in the system and K_a is the dissociation constant of catechol. The data are tabulated in Table I. The pKa of catechol under the conditions of these experiments was 9.40. The alkaline hydrolysis rate constants (k_{OH}) were: benzylpenicillin 12.5 and methicillin 6.0 L. mole^{-1} min.$^{-1}$.

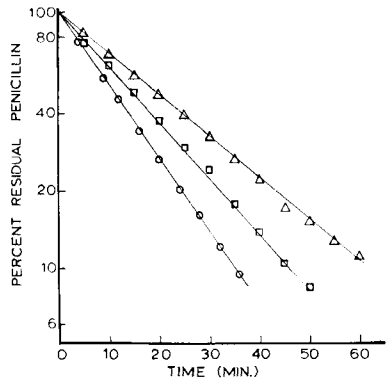


Fig. 1—First-order rate of loss of benzylpenicillin at various pH's. Catechol concentrations: O, *0.0435* M, *pH 9.15;* □, *0.0174* M, *pH 9.50;* △, *0.0087* M, *pH 10.0.*

At a single pH the observed first-order rate constants were found to be directly proportional to catechol concentration as shown in Fig. 2.

TABLE I—RATES OF PENICILLIN LOSS FROM SOLUTION IN PRESENCE OF CATECHOL

pH	Initial Penicillin Concn., $M \times 10^4$	Total Catechol Concn., M	$k_{obs.}$, min.$^{-1}$	k_c, L. mole^{-1} min.$^{-1}$
		Benzyl Penicillin		
8.35	2.0	0.067	0.0210	3.82
8.50	2.0	0.131	0.0577	3.95
9.00	2.0	0.044	0.0507	4.02
	20.0	0.044	0.0507	4.02
9.15	2.0	0.0435	0.0666	4.22
	20.0	0.0435	0.0666	4.22
	20.0	0.022	0.0333	4.22
	2.0	0.083	0.126	4.19
9.35	2.0	0.022	0.0478	4.56
9.42	2.0	0.0174	0.0436	4.83
9.50	2.0	0.0174	0.0477	4.85
9.60	2.0	0.0176	0.0546	4.98
10.00	2.0	0.0174	0.0745	5.22
	2.0	0.0087	0.0380	5.24
	2.0	0.0129	0.0555	5.20
10.25	2.0	0.0172	0.0912	5.81
10.50	2.0	0.0083	0.0564	6.55
		Methicillin		
8.50	2.0	0.129	0.0495	3.41
9.00	2.0	0.044	0.0468	3.71
	2.0	0.085	0.0912	3.74
	2.0	0.065	0.0673	3.65
9.15	2.0	0.043	0.0602	3.88
9.35	2.0	0.022	0.0420	4.02
9.50	2.0	0.022	0.0533	4.34
9.75	2.0	0.0174	0.0533	4.40
10.00	2.0	0.0086	0.0321	4.51
	2.0	0.0172	0.0641	4.51
	2.0	0.0129	0.0471	4.48
10.25	2.0	0.0085	0.0365	4.67
10.35	2.0	0.0081	0.0369	4.75
10.50	2.0	0.0086	0.0408	4.73

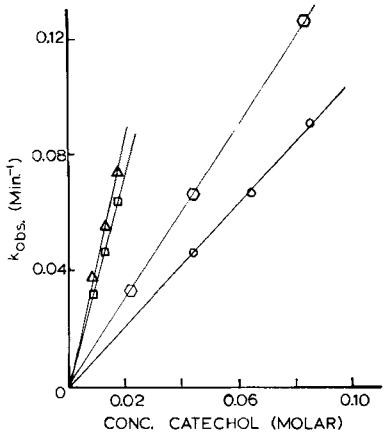


Fig. 2—Plot showing first-order dependence of rate of penicillin loss on catechol concentration at constant pH. Key: △, benzylpenicillin, pH 10.0; ○, benzylpenicillin, pH 9.15; □, methicillin, pH 10.0; ○, methicillin, pH 9.0.

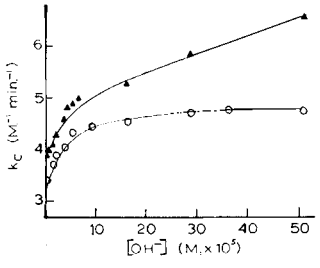


Fig. 3—Dependence of k_C *upon hydroxyl-ion concentration. Curves were fitted to Eqs. 2 and 3 for benzylpenicillin and methicillin, respectively. The points are the values determined experimentally. Key: ▲, benzylpenicillin; ○, methicillin.*

It was found that the values of k_c determined from rate of penicillin loss were not constant over the pH range studied, but varied as shown in Fig. 3.

In the case of benzylpenicillin the data were fitted to a curve of the form of Eq. 2:

$$k_c = \frac{a + b\,[OH^-] + c\,[OH^-]^2}{d + [OH^-]} \qquad \text{(Eq. 2)}$$

where a, b, c, and d are constants. For methicillin the data were fitted to Eq. 3:

$$k_c = \frac{a + b(OH^-)}{d + (OH^-)} \qquad \text{(Eq. 3)}$$

The curves in Fig. 3 were constructed from the values of the constants given in Table II, while the points are the actual values obtained experimentally. In both cases when (OH^-) is zero, $k_c = a/d$, whereas at high (OH^-):

benzylpenicillin: $k_c = b + c(OH^-)$
methicillin: $k_c = b$

The rates of acid formation were apparently first order and rate constants $k_{obs.}$ were determined from Guggenheim plots of the data. These values are given in Table III.

DISCUSSION

The specific rate constants (k_c) for loss of benzylpenicillin from solution, catalyzed by monocatecholate ion, are not independent of hydroxyl-ion concentration. This variation from a high degree of dependence at pH below 9.5 to only slight dependence at higher alkalinity is indicative of a change in mechanism of the reaction with increasing pH.

When (OH^-) = 0 the rate expression is:

$$\text{rate} = \frac{a\,(P)(C^-)}{d} \qquad \text{(Eq. 4)}$$

which is the typical expression for nucleophilic catalysis by (C^-).

At high [OH^-] the rate expression becomes:

$$\text{rate} = b\,[P][C^-] + c\,[P][C^-][OH^-] \qquad \text{(Eq. 5)}$$

Equation 5 contains terms showing both nucleophilic catalysis by monocatecholate ion and by dicatecholate ion. The second term could alternatively be attributed to specific base-catalyzed

TABLE II—CONSTANTS USED TO FIT EQS. 2 AND 3

	a	b	c	d
Benzylpenicillin	18.2×10^{-5}	5.35	3.0×10^{3}	4.74×10^{-5}
Methicillin	9.30×10^{-5}	4.90	...	3.00×10^{-5}

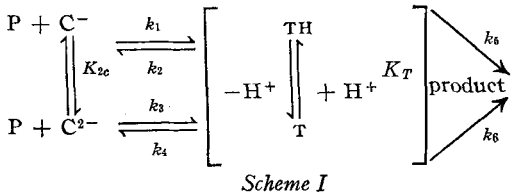
TABLE III—RATES OF ACID FORMATION[a]

Benzylpenicillin			Methicillin		
pH	Catechol Concn., M	$k_{obs.}$, min.$^{-1}$	pH	Catechol Concn., M	$k_{obs.}$, min.$^{-1}$
8.50	0.129	0.051	8.50	0.089	0.0344
9.00	0.124	0.130	8.75	0.088	0.0550
9.50	0.053	0.105	9.00	0.088	0.0912
10.00	0.049	0.139	9.25	0.087	0.0937
10.25	0.048	0.151	9.50	0.044	0.0667
10.50	0.049	0.169	9.75	0.0178	0.0377
			10.0	0.0178	0.0423
			10.5	0.0089	0.0289

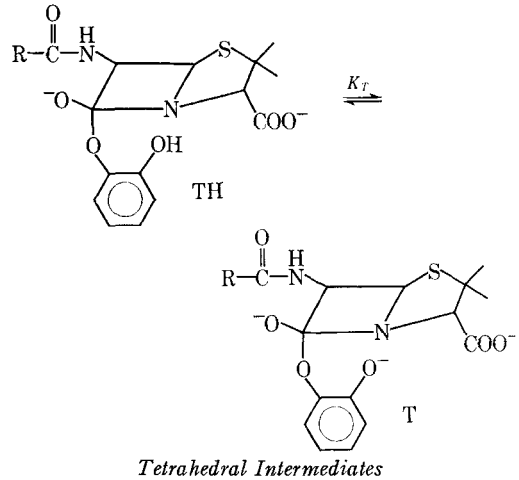
[a] Initial penicillin concentration was 0.02 M.

nucleophilic attack by monocatecholate ion, which is kinetically equivalent. At intermediate hydroxyl-ion concentrations a complex expression holds. This situation can only occur with the involvement of intermediates which are in acid-base equilibrium (4).

Such a mechanism is shown in Scheme I. C^{2-} represents dicatecholate ion, K_{2c} the second dissociation constant of catechol, and TH and T represent tetrahedral addition intermediates formed by nucleophilic attack of the catecholate ions on the penicillin.[1] These intermediates are depicted in Scheme II. The dissociation constant for TH is represented by K_T.



Scheme I



Tetrahedral Intermediates
Scheme II

Taking a steady state in both TH and T, since these would be metastable intermediates, gives the following expression for k_c:[2]

$$\frac{\text{rate}}{[P][C^-]} = k_c = \frac{\frac{k_1k_5K_w}{(k_4 + k_6)K_T} + \frac{k_1k_6K_T + k_3k_5K_{2c}}{(k_4 + k_6)K_T}[OH^-] + \frac{k_3k_6K_{2c}}{(k_4 + k_6)K_w}[OH^-]^2}{\frac{(k_2 + k_5)K_w}{(k_4 + k_6)K_T} + [OH^-]} \quad \text{(Eq. 6)}$$

This equation is the same form as Eq. 2 showing that this mechanism fits the experimental data.

In the case of methicillin there is a plateau reached above pH 9.5 where k_c becomes independent of hydroxyl-ion concentration. This would occur if the pathway through dicatecholate ion were not contributing significantly to the over-all reaction. If k_3 and k_4 are both set equal to zero, Eq. 6 becomes:

$$k_c = \frac{\frac{k_1k_5K_w}{k_6K_T} + k_1[OH^-]}{\frac{(k_2 + k_5)K_w}{k_6K_T} + [OH^-]} \quad \text{(Eq. 7)}$$

which is of the form of Eq. 3 to which the data for methicillin were fitted. From Eq. 7 it can be seen that when $[OH^-] = 0$:

$$k_c = \frac{k_1k_5}{k_2 + k_5}$$

and when $[OH^-]$ is high:

$$k_c = k_1$$

For methicillin k_1 equals 4.9 L. mole^{-1} min.$^{-1}$ and therefore:

$$\frac{k_5}{k_2 + k_5} = 0.7 \text{ or } k_5 = 2.3\ k_2$$

It is difficult at this time to explain the differences in reactivity toward dicatecholate ion shown by benzylpenicillin and methicillin. The latter, judging from its lower rate of reaction with hydroxyl ion in the absence of catechol, is generally less susceptible to nucleophilic attack and this difference may be sufficient to obscure any contribution made by dicatecholate ion in the pH range studied.

For both penicillins the apparent first-order rate constants for acid formation at the higher pH values are lower than those for penicillin loss at the same catechol concentration. This is a clear indication of a buildup of a covalent intermediate in the reaction pathway.

This intermediate is apparently the monopenicilloate ester of catechol. Thus, the over-all reaction scheme at one pH may be depicted as:

$$P + C^- \xrightarrow{k_1'} \text{ester} \xrightarrow{k_2'} \text{product} + C^-$$

It is quite difficult to determine the values of k_2' from the present data because the concentration of intermediate ester must be determined by difference. Thus, small errors in the amount of acid formed and residual penicillin could lead to large errors in ester concentration. Approximate values of k_2' were determined nevertheless, from the maxima in plots of ester concentration *versus* time. At the time when the concentration of intermediate ester is maximal:

$$k_1'[P] = k_2'[E]$$

The values of k_2' thus calculated were found to be fairly constant for both penicillins in the pH range 9.5–10.5. For methicillin the values were about 0.2 min.$^{-1}$ and for benzylpenicillin about 0.4 min.$^{-1}$.

It has been shown that the rates of hydrolysis of catechol monobenzoate (5) and catechol monoacetate (6) depend on the concentration of anion, and the rapid rates of these hydrolyses are attributable to intramolecular general base catalysis by the *ortho*-hydroxyl ion.

In the two reactions cited the rates were almost constant at pH values where all of the *ortho*-hydroxyl group was ionized. The pKa of the acetate and

[1] Attempts were made to find other mechanisms which would fit the experimental data, but none could be found.
[2] See *Appendix* for derivation of Eq. 6.

benzoate esters were 8.56 and 8.70, respectively, and the pKa of the penicilloate ester is therefore probably in the range 8.5–9.0. Thus, it would be expected that its rate of hydrolysis above pH 9.5 would become almost independent of pH as has been observed experimentally.

The ratio of k_2' for benzylpenicillin to that for methicillin is about 2 which is the same as the ratio of the k_{OH} values. The difference probably reflects the effect of the side chain on the susceptibility to nucleophilic attack of the β-lactam carbonyl. A similar mechanism has been proposed for the catechol-catalyzed hydrolysis of phenyl chloroacetate (7). Here it was shown that catechol monochloroacetate hydrolyzed much faster than phenyl chloroacetate, implicating the catechol monoester as a reaction intermediate.

In the catalysis of hydrolysis of benzylpenicillin by CDM it was shown (1) that one of the charged amino groups was responsible for binding the penicillin through ionic interaction with the carboxylate ion on the latter. The role of the other amino group, which apparently participated in the reaction, was not as clear. If the mechanism of hydrolysis catalyzed by catechol may be extrapolated to the situation with CDM, then there are revealed several possible functions for this second amino group. It may act to polarize the carbonyl group of the β-lactam increasing the susceptibility of the latter to nucleophilic attack by the adjacent hydroxyl ion. The amino function may also interact with the negatively charged oxygen of the tetrahedral intermediate thus stabilizing the latter relative to the reactants. Each of these actions would increase the rate of formation of ester relative to that observed with catechol. The amino group in all probability would also enhance the rate of hydrolysis of the ester by intramolecular hydrogen bonding to the ester carbonyl as has been shown in the hydrolysis of tertiary aminoalkyl acetates (8).

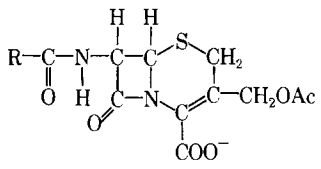
ANALOGY TO PENICILLINASE

The mechanism of action of the enzyme penicillinase and the nature of the functional groups involved in its active site are not known (9). Some speculation on these problems may, however, be based on consideration of the mechanism of hydrolysis of penicillins catalyzed by catechol and the aminocatechol derivatives previously reported (1).

While it would not be expected that catecholamines or even catechol groups will be found in the active site of the enzyme it would seem likely that a combination of nucleophilic and electrophilic (general acid) functions, located so as to act in concerted fashion could be responsible for its catalytic action. The binding of substrate to the enzyme is probably partly due to an ionic interaction between the carboxylate ion of the penicillin and a positively charged group in the active site of the enzyme. It has been shown that conversion of the carboxyl group to carboxamide decreases its susceptibility to enzymic hydrolysis (10, 11). Also, evidence for the presence of a positively charged group on the enzyme active site has been presented (12).

It has been shown that some penicillins resistant to the enzyme (such as methicillin) are competitive inhibitors of benzylpenicillin (13, 14) and activity is recovered only very slowly. In the light of the mechanism of catalysis of benzylpenicillin by catechol it may be that the competitive inhibition is due to a covalent compound formed by acylation of the enzyme by methicillin, analogous to the catechol ester. If the rate of hydrolysis of the "methicilloyl-enzyme" were slow, then the slow recovery of enzymic activity would be explained. In the catechol ester the adjacent groups which assist hydrolysis are fixed in position. In the penicilloyl-enzyme this need not be so and the side chain may cause conformational changes as a result of which the groups catalyzing deacylation may be shifted to a position in which they can no longer influence hydrolysis rate.

One further point meriting attention is the difference in enzymic activity toward penicillins and cephalosporins (15). The cephalosporins are generally resistant to hydrolysis by penicillinase and are also relatively resistant to hydrolysis catalyzed by CDM as shown by preliminary studies in this laboratory. It can be readily seen with molecular models that in the cephalosporins the steric relationship between the carboxylate ion and the β-lactam carbonyl is quite different from that of the pencillins. Thus, even though CDM may associate with the cephalosporins by ionic interaction, the nucleophilic hydroxyl ion would not be in an optimum position to exert its catalytic action. This argument may account for the simultaneous lack of catalytic activity on cephalosporins by penicillinase and the observed competitive inhibition of the enzyme by these compounds (15).



Cephalosporins

APPENDIX

Derivation of Eq. 6

$$\text{rate} = \frac{d\,(\text{product})}{dt} = k_5\,[\text{TH}] + k_6[\text{T}]$$

$$\text{TH} = \frac{[\text{H}^+]}{K_T + [\text{H}^+]}\,[\text{T}]_T;\quad \text{T} = \frac{K_T}{K_T + [\text{H}^+]}\,[\text{T}]_T$$

where $[\text{T}]_T$ represents [TH] + [T]

$$\text{rate} = \frac{k_5\,[\text{H}^+] + k_6K_T}{K_T + [\text{H}^+]}\,[\text{T}]_T$$

$$\frac{d\,[\text{T}]_T}{d_t} = k_1\,[\text{P}][\text{C}^-] + k_3\,[\text{P}][\text{C}^{2-}] -$$

$$[k_2 + k_5]\,[\text{TH}] - [k_4 + k_6][\text{T}] = 0 =$$

$$k_1\,[\text{P}][\text{C}^-] + \frac{k_3K_{2C}\,[\text{P}][\text{C}^-]}{\text{H}^+} -$$

$$\frac{(k_2 + k_5)\,[\text{H}^+] - [k_4 + k_6]\,K_T}{K_T + [\text{H}^+]}\,[\text{T}]_T$$

$$\text{T}_T = \frac{\left[k_1 + \frac{k_3K_{2C}}{[\text{H}^+]}\right][K_T + \text{H}^+]\,[\text{P}][\text{C}^-]}{(k_2 + k_5)\,[\text{H}^+] + (k_4 + k_6)\,K_T}$$

$$\text{rate} = \left\{\frac{k_1k_5\,[\text{H}^+] + k_1k_6K_T + k_3k_5K_{2c} + \frac{k_3k_6K_TK_{2c}}{[\text{H}^+]}}{(k_2 + k_5)\,[\text{H}^+] + (k_4 + k_6)\,K_T}\right\} \times [\text{P}][\text{C}^-]$$

Substituting $K_w/[OH^-]$ for $[H^+]$ and dividing numerator and denominator by $(k_4 + k_6)K_T$:

$$\frac{\text{rate}}{[P][C^-]} = \frac{\frac{k_1k_5K_w}{(k_4 + k_6)K_T} + \frac{k_1k_6K_T + k_3k_5K_{2c}}{(k_4 + k_6)K_T}[OH^-] + \frac{k_3k_6K_{2c}}{(k_4 + k_6)K_w}[OH^-]^2}{\frac{(k_2 + k_5)K_w}{(k_4 + k_6)K_T} + [OH^-]}$$

REFERENCES

(1) Schwartz, M. A., *J. Pharm. Sci.*, **54**, 1308(1965).
(2) Jencks, W. P., *Ann. Rev. Biochem.*, **32**, 645(1966).
(3) Brandriss, M. W., Denny, E. L., Huber, M. A., and Steinman, H. G., "Antimicrobial Agents and Chemotherapy—1962," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1963, p. 626.
(4) Bruice, T. C., and Fedor, L. R., *J. Am. Chem. Soc.*, **86**, 4886(1964).
(5) Capon, B., and Ghosh, B. C., *J. Chem. Soc., B*, **1966**, 492.
(6) Hansen, B., *Acta Chem. Scand.*, **17**, 1375(1963).
(7) Fuller, E. J., *J. Am. Chem. Soc.*, **85**, 1777(1963).
(8) Hansen, B., *Acta Chem. Scand.*, **12**, 324(1958).
(9) Citri, N., and Pollock, M. R., *Advan. Enzymol.*, **28**, 237(1966).
(10) Cooper, D. E., and Binkley, S. B., *J. Am. Chem. Soc.*, **70**, 3966(1948).
(11) Huang, H. T., Seto, T. A., Weaver, J. M., English, A. R., McBride, T. J., and Schull, G. H., "Antimicrobial Agents and Chemotherapy—1963," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1964, p. 493.
(12) Depue, R. H., Moat, A. G., and Bondi, A., *Arch. Biochem. Biophys.*, **107**, 374(1964).
(13) Gourevitch, A., Pursiano, T. A., and Lein, J., *Nature*, **195**, 496(1962).
(14) Citri, N., and Garber, N., *J. Pharm. Pharmacol.*, **14**, 784(1962).
(15) Crompton, B., Jago, M., Crawford, K., Newton, G. G. F., and Abraham, E. P., *Biochem. J.*, **83**, 52(1962).

Bactericidal Properties of Straight-Chained Alkyltrimethylammonium Bromides in a Simple Emulsion System

By THOMAS L. WELSH, GEORGE C. HOSS, BLAZE T. PALERMO, and ALLEN I. DINES*

The individual constituents of the potent bactericidal agent cetrimide B.P. are among those alkyltrimethylammonium bromides (C_8 through C_{18}). which have been synthesized by classical methods. Each of these quaternary compounds was then emulsified with six (C_8 through C_{18}) fatty alcohols. The bactericidal properties of these emulsions are compared by the critical killing dilution method. When emulsified, only the C_{12} and C_{14} quaternary compounds exhibited any appreciable bactericidal action. All of the quaternary compounds in the series are inactivated when emulsified with myristyl alcohol.

THE OFFICIAL COMPOSITION of the quaternary ammonium bactericidal agent cetrimide B.P. has gradually changed over the last decade (1–3). Other investigators (4, 5) have recorded significant effects on their studies brought about by these changes.

Similar effects were noted in this laboratory during *in vitro* bactericidal testing of an experimental product. This product, a medicated cream, was an oil-in-water emulsion which utilized cetrimide B.P. as its bactericidal agent. The disperse phase was comprised mainly of stearyl alcohol.

The erratic bactericidal results were discovered to have been caused by a shift in the predominant constituent in the cetrimide utilized. Cetrimide conforming to the 1963 B.P. monograph, *i.e.*, "comprised mainly of tetradecyltrimethylammonium bromide . . ." afforded excellent bactericidal activity. Cetrimide which conformed to the 1953 B.P. monograph, *i.e.*, "comprised mainly of hexadecyltrimethylammonium bromide . . ." was completely inactive in the product. Some anomalous bactericidal results were also obtained when alcohols other than stearyl were introduced into the formula.

The present study was initiated to determine: (*a*) the bactericidal properties of the component compounds which comprise cetrimide when they are incorporated into a simple emulsion system, and (*b*) if a pattern of relationship can be deter-

Received April 11, 1967, from the Product Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication July 12, 1967.
Presented to the Industrial Pharmaceutical Technology Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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The authors express their gratitude to Mr. R. A. Zehr, Mr. W. A. Beppler, and Miss Janet Shripka, Microbiology Department, Operating Services Division, Miles Laboratories, Inc., for their excellent cooperation in this project. We also thank Mr. John Devlin and Mr. Darrell Waterman for their technical assistance with this work.

Substituting $K_w/[OH^-]$ for $[H^+]$ and dividing numerator and denominator by $(k_4 + k_6)K_T$:

$$\frac{\text{rate}}{[P][C^-]} = \frac{\frac{k_1k_5K_w}{(k_4 + k_6)K_T} + \frac{k_1k_6K_T + k_3k_5K_{2c}}{(k_4 + k_6)K_T}[OH^-] + \frac{k_3k_6K_{2c}}{(k_4 + k_6)K_w}[OH^-]^2}{\frac{(k_2 + k_5)K_w}{(k_4 + k_6)K_T} + [OH^-]}$$

REFERENCES

(1) Schwartz, M. A., *J. Pharm. Sci.*, **54**, 1308(1965).
(2) Jencks, W. P., *Ann. Rev. Biochem.*, **32**, 645(1966).
(3) Brandriss, M. W., Denny, E. L., Huber, M. A., and Steinman, H. G., "Antimicrobial Agents and Chemotherapy—1962," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1963, p. 626.
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(5) Capon, B., and Ghosh, B. C., *J. Chem. Soc., B*, **1966**, 492.
(6) Hansen, B., *Acta Chem. Scand.*, **17**, 1375(1963).
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(9) Citri, N., and Pollock, M. R., *Advan. Enzymol.*, **28**, 237(1966).
(10) Cooper, D. E., and Binkley, S. B., *J. Am. Chem. Soc.*, **70**, 3966(1948).
(11) Huang, H. T., Seto, T. A., Weaver, J. M., English, A. R., McBride, T. J., and Schull, G. H., "Antimicrobial Agents and Chemotherapy—1963," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1964, p. 493.
(12) Depue, R. H., Moat, A. G., and Bondi, A., *Arch. Biochem. Biophys.*, **107**, 374(1964).
(13) Gourevitch, A., Pursiano, T. A., and Lein, J., *Nature*, **195**, 496(1962).
(14) Citri, N., and Garber, N., *J. Pharm. Pharmacol.*, **14**, 784(1962).
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Bactericidal Properties of Straight-Chained Alkyltrimethylammonium Bromides in a Simple Emulsion System

By THOMAS L. WELSH, GEORGE C. HOSS, BLAZE T. PALERMO, and ALLEN I. DINES*

The individual constituents of the potent bactericidal agent cetrimide B.P. are among those alkyltrimethylammonium bromides (C_8 through C_{18}). which have been synthesized by classical methods. Each of these quaternary compounds was then emulsified with six (C_8 through C_{18}) fatty alcohols. The bactericidal properties of these emulsions are compared by the critical killing dilution method. When emulsified, only the C_{12} and C_{14} quaternary compounds exhibited any appreciable bactericidal action. All of the quaternary compounds in the series are inactivated when emulsified with myristyl alcohol.

THE OFFICIAL COMPOSITION of the quaternary ammonium bactericidal agent cetrimide B.P. has gradually changed over the last decade (1–3). Other investigators (4, 5) have recorded significant effects on their studies brought about by these changes.

Similar effects were noted in this laboratory during *in vitro* bactericidal testing of an experimental product. This product, a medicated cream, was an oil-in-water emulsion which utilized cetrimide B.P. as its bactericidal agent. The disperse phase was comprised mainly of stearyl alcohol.

The erratic bactericidal results were discovered to have been caused by a shift in the predominant constituent in the cetrimide utilized. Cetrimide conforming to the 1963 B.P. monograph, *i.e.*, "comprised mainly of tetradecyltrimethylammonium bromide . . ." afforded excellent bactericidal activity. Cetrimide which conformed to the 1953 B.P. monograph, *i.e.*, "comprised mainly of hexadecyltrimethylammonium bromide . . ." was completely inactive in the product. Some anomalous bactericidal results were also obtained when alcohols other than stearyl were introduced into the formula.

The present study was initiated to determine: (*a*) the bactericidal properties of the component compounds which comprise cetrimide when they are incorporated into a simple emulsion system, and (*b*) if a pattern of relationship can be deter-

Received April 11, 1967, from the Product Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication July 12, 1967.
Presented to the Industrial Pharmaceutical Technology Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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The authors express their gratitude to Mr. R. A. Zehr, Mr. W. A. Beppler, and Miss Janet Shripka, Microbiology Department, Operating Services Division, Miles Laboratories, Inc., for their excellent cooperation in this project. We also thank Mr. John Devlin and Mr. Darrell Waterman for their technical assistance with this work.

TABLE I—ALKYLTRIMETHYLAMMONIUM BROMIDES, $R(CH_3)_3NBr$

R	Critical Killing Dilution Data[a]				Physical Constants and Analyses[b]				
	CKD *S. aureus*	Lit. CKD[c] *S. aureus*	CKD *S. typhosa*	Lit. CKD[c] *S. typhosa*	M.p.,[d] °C.	Lit. M.p., °C.	Formula	Br, % Calcd.	Br, % Found
n-Octyl	1/100	1/30	1/150	1/75	212–220°	215°[e]	$C_{11}H_{26}BrN$	31.68	31.46
n-Decyl	1/1,000	...	1/1,200	...	238–242°	239–242°[e]	$C_{13}H_{30}BrN$	28.51	28.31
n-Dodecyl (lauryl)	1/6,500	1/4,000	1/9,000	1/9,000	243–247°	243°[e]	$C_{15}H_{34}BrN$	25.91	25.47
n-Tetradecyl (myristyl)	1/50,000	1/38,000	1/90,000	1/36,000	243–245°	244–255°[c]	$C_{17}H_{38}BrN$	23.75	23.44
n-Hexadecyl (cetyl)	1/90,000	1/80,000	1/130,000	1/40,000	248–253°	237–243°[c]	$C_{19}H_{42}BrN$	21.92	21.43
n-Octadecyl (stearyl)	1/55,000	1/64,000	1/80,000	1/8,000	238–245° dec.	230–240°[c] dec.	$C_{21}H_{46}BrN$	20.36	20.01

[a] Critical killing dilution data were obtained by the Microbiology Laboratory, Operating Services Division of Miles Laboratories, Inc. [b] Analyses were performed by the Corporate Pharmacy Research Laboratory, Miles Laboratories, Inc. [c] See *Reference 9*. [d] Melting points are uncorrected. [e] See *Reference 15*.

mined among these constituents when fatty alcohols of varying chain lengths are used as the oil phase in the emulsion.

EXPERIMENTAL

Preparation of the Alkyltrimethylammonium Bromides—Straight-chained alkyltrimethylammonium bromides from C_8 to C_{18} were synthesized by classical methods (6). High purity synthetic fatty alcohols were used as starting materials.[1] The preparation of *n*-dodecyltrimethylammonium bromide is cited below for illustrative purposes.

In a 1-L. three-neck flask containing 102.3 Gm. (0.55 mole) of *n*-dodecyl alcohol, 62 Gm. (34 ml.) of concentrated sulfuric acid was slowly added along with 240 Gm. (1.4 moles) of 48% hydrobromic acid. This mixture was refluxed 7 hr. and allowed to cool to room temperature overnight. The mixture was diluted with 400 ml. water, then filtered through a sintered-glass funnel. The organic layer was separated mechanically and then was successively washed with 75-ml. portions of concentrated sulfuric acid, water, and 10% sodium carbonate solution. It was then dried over calcium chloride. The yield was 81 Gm.

The *n*-dodecylbromide, 81 Gm. (0.32 mole), was dissolved in 150 ml. dry acetone and 100 ml. of a 20% w/w solution of trimethylamine (0.33 mole) in dry acetone was added. After about 20 min. the crystalline *n*-dodecyltrimethylammonium bromide began to form. The crystals were collected after 5 hr., washed with dry acetone, dried, and weighed. The yield was 60 Gm. (60% of theory).

Recrystallization of the quaternary compounds with acetone A.R. resulted in products of excellent purity. The physical constants of these compounds are listed in Table I.

Establishment of the Critical Killing Dilution of the Quaternary Compounds in Solution—The complete technique for establishment of the critical killing dilution is outlined in the Association of Official Agricultural Chemists, Official Methods of Analysis (7). An excellent review of the method is offered by Reddish (8).

[1] These ethylene condensation compounds were generously supplied by the Continental Oil Co., Petroleum Chemicals Department, Ponca City, Okla., as Alfols 8 through 18.

Preparation of the 15% o/w Emulsion System Containing 0.5% Quaternary Compound—Each of the six quaternary compounds (C_8 through C_{18}) was emulsified with each of the six corresponding fatty alcohols (C_8 through C_{18}) in the following manner. The alkyltrimethylammonium bromide (0.5 Gm.) was dissolved in water (84.5 ml.) and heated on a water bath to 65°. The fatty alcohol (15.0 Gm.) was heated in a 250-ml. conical flask to 65°. The aqueous solution was then added to the alcohol and the flask was agitated for 5 min. on a wrist shaker. The emulsion was allowed to return to room temperature (about 1 hr.) and its physical appearance noted. These emulsions were left at ambient laboratory conditions and were examined after 24 hr. and again after 30 days for physical appearance.

Establishment of the Critical Killing Dilution for the Emulsions—To test the emulsions, the following modifications from the AOAC method (as cited above) are introduced. A 3.33% dilution of the emulsion is made in a glass-stoppered cylinder. Care is taken to insure that there is adequate uniform dispersion of the emulsion droplets throughout this system. Further dilutions are made from this stock solution. Five milliliters of the desired dilution is inoculated with the test organism. At time intervals of 5, 10, 15, and 20 min. a loopful of the seeded emulsion solution is withdrawn and subcultured in a nutrient broth. The subcultures are incubated at 37° for 48 hr. and examined macroscopically for growth. A standardized 5% phenol solution is used as a control to validate the results.

RESULTS AND DISCUSSION

The bactericidal properties, in solution, of the alkyltrimethylammonium bromides which were prepared from synthetic alcohols as well as those made from naturally occurring alcohols (9, 10) are summarized in Table I. In general, it can be seen that the same over-all pattern of CKD's has been manifested. Because strains of bacteria exhibit varying resistance in tests such as these, it is not possible to say if there has been a significant improvement in bactericidal action due to the purity of the starting materials. The values obtained, particularly those for the C_{18} homolog, are somewhat higher than the literature values against the Gram-negative *S. typhosa*.

References to the behavior of cetrimide or its con-

stituent compounds in emulsion systems are somewhat limited. Christenson and Shelton (11) reported inactivation of cetyltrimethylammonium bromide (CTAB) by various grades of lanolin in some pectin lotions. They also reported that any additions to the oil phase, such as glyceryl monostearate, glyceryl monooleate, or vegetable oils, increased the killing time of the lotion. Prusak and Mattocks (12) reported inactivation of CTAB by hydrophilic ointment U.S.P. and hydrophilic petrolatum U.S.P. Of the 6 ointment bases which they studied, only a Carbowax ointment and pectin paste N.F. appeared suitable to obtain bactericidal action with CTAB. Davies (13) noted that a fatty glycol ethylene oxide condensate neutralized the bacteriostatic effect of CTAB. Frank and Stark (14) found cetrimide to be completely inactivated in hydrous emulsifying ointment B.P.

Thirty-six 15% o/w emulsions with the quaternary compound present at 0.5% were prepared and were tested for bactericidal action by a modification of the AOAC phenol coefficient test. This test establishes the critical killing dilution (CKD) (that is, the maximum dilution which will kill a specified organism in 10 min. but not in 5). Any emulsion which achieved complete kill against *S. aureus* at a dilution of 1 to 30 or greater was then tested against the Gram-negative *Ps. aeruginosa*. Emulsions which failed to kill *S. aureus* at 1 to 30 were assessed as having no appreciable bactericidal activity. The critical killing dilutions for this series of emulsions are shown in Table II. Also shown in this table are the pharmaceutical properties of these emulsions based on their physical appearance.

None of the emulsions made with the C_8 and C_{10} quaternary compounds met the 1 to 30 criterion for bactericidal activity. This is not surprising in view of their relatively weak bactericidal action in aqueous solution. Because of poor physical stability, none of these emulsions were acceptable from a pharmaceutical standpoint. However, all of the emulsions made with the C_{12} and C_{14} quaternary compounds had CKD's of at least 1 to 30 with the exception of those made with myristyl alcohol. The C_{12} emulsions exhibited surprising strength against the very resistant Gram-negative *Ps. aeruginosa*.

The inactivation of these compounds by myristyl alcohol is, as yet, unexplained. Emulsions have been made with three other commercial sources of this alcohol, all of which inactivate these quaternary compounds.

Table II also shows that cetyltrimethylammonium bromide, by far the most active of the series in aqueous solution, was completely inactivated in this system; except when combined with the C_8 alcohol. This activity is attributed to the fact that more of the quaternary compound is in the aqueous phase be-

TABLE II—EMULSION BACTERICIDAL AND PHARMACEUTICAL PROPERTIES

Emulsion No. Quaternary Chain Length	Emulsion No. Alcohol Chain Length	Emulsion Physical Appearance[a]	Critical Killing Dilutions Against *S. aureus*	Critical Killing Dilutions Against *Ps. aeruginosa*[b]
8	8	*A*	$<1/30$	...
8	10	*A*	$<1/30$	...
8	12	*A*	$<1/30$	...
8	14	*A*	$<1/30$	...
8	16	*A*	$<1/30$	...
8	18	*A*	$<1/30$	...
10	8	*A*	$<1/30$	...
10	10	*A*	$<1/30$	...
10	12	*A*	$<1/30$	...
10	14	*A*	$<1/30$	...
10	16	*A*	$<1/30$	...
10	18	*A*	$<1/30$	...
12	8	*A*	$1/75$	$1/90$
12	10	*B*	$1/90$	$1/30$
12	12	*B*	$1/60$	$1/30$
12	14	*B*	$<1/30$	...
12	16	*B*	$1/60$	$1/75$
12	18	*C*	$1/45$	$1/30$
14	8	*A*	$1/400$	$1/120$
14	10	*A*	$1/500$	$<1/10$
14	12	*B*	$1/30$	$<1/10$
14	14	*C*	$<1/30$	...
14	16	*C*	$1/45$	$<1/10$
14	18	*C*	$1/60$	$<1/10$
16	8	*B*	$1/120$	$1/120$
16	10	*B*	$<1/30$	...
16	12	*B*	$<1/30$	...
16	14	*C*	$<1/30$	...
16	16	*C*	$<1/30$	...
16	18	*C*	$<1/30$	...
18	8	*A*	$1/30$	$1/250$
18	10	*A*	$<1/30$	...
18	12	*B*	$<1/30$	...
18	14	*C*	$<1/30$	...
18	16	*C*	$<1/30$	...
18	18	*C*	$<1/30$	...

[a] Key: *A*, very poor; creamed or broken within 24 hr.; *B*, initially good but creamed or broken within 30 days; *C*, pharmaceutically elegant; stable for more than 30 days.
[b] Emulsions whose CKD was less than $1/30$ against *S. aureus* were not tested against the Gram-negative *Ps. aeruginosa*.

TABLE III—MIXED ALCOHOL EMULSIONS

Emulsion System	Emulsion Physical Appearance	Critical Killing Dilution Against *S. aureus*	Critical Killing Dilution Against *Ps. aeruginosa*
0.5% C_{12} Quaternary 7.5% C_8 Alcohol 7.5% C_{16} Alcohol	Good initially but begins to separate within 30 days	$1/100$	$1/70$
0.5% C_{14} Quaternary 7.5% C_8 Alcohol 7.5% C_{16} Alcohol	Pharmaceutically elegant; stable for more than 30 days	$1/130$	$1/10$
0.5% C_{16} Quaternary 7.5% C_8 Alcohol 7.5% C_{16} Alcohol	Pharmaceutically elegant; stable for more than 30 days	$<1/30$	$1/10$
0.5% C_{18} Quaternary 7.5% C_8 Alcohol 7.5% C_{16} Alcohol	Pharmaceutically elegant; stable for more than 30 days	$<1/30$	$1/10$

cause of the relatively poor emulsion. The C_{18} analog parallels the C_{16} closely. Emulsions in these series have superior pharmaceutical properties and yet are inactive from a bactericidal standpoint.

Because emulsions made wih the C_8 alcohol did exhibit satisfactory bactericidal activity, although they were not pharmaceutically stable, a series of emulsions was made utilizing 7.5% of the C_8 alcohol and 7.5% of the C_{16} alcohol. It was postulated that they could have an acceptable bactericidal level as well as pharmaceutical stability. The CKD's for this series of emulsions are shown in Table III. Bactericidal activity is again found to be restricted to the C_{12} and C_{14} quaternary compounds. The C_{12} compound again shows its effectiveness against *Pseudomonas*. The C_{16} and C_{18} compounds again are inactivated in this system.

SUMMARY

Six straight-chained (C_8 to C_{18}) alkyltrimethylammonium bromides have been synthesized by classical methods, utilizing high purity synthetic alcohols as starting materials. Among these compounds are the individual constituents which comprise the bactericidal agent cetrimide B.P. The critical killing dilutions of these compounds, in solution, are comparable to those obtained from compounds prepared from naturally occurring alcohols. These quaternary compounds were then used as the emulsifying agent in a model oil-in-water system. Thirty-six emulsions were prepared and evaluated for bactericidal properties by the critical killing dilution method against *S. aureus* and *Ps. aeruginosa*. The results indicate that in addition to their poor bactericidal activity, the C_8 and C_{10} quaternary compounds lack the power to emulsify any of the alcohols in the selected system. The C_{12} and C_{14} quaternary compounds exhibit the greatest bactericidal activity. The C_{16} and C_{18} quaternary compounds are virtually inactivated by incorporation into this system. Emulsification with myristyl alcohol inactivates all of the quaternary compounds in this series.

REFERENCES

(1) "British Pharmacopoeia," General Medical Council, The Pharmaceutical Press, London, England, 1953, p. 131.
(2) *Ibid.*, 1958, p. 141.
(3) *Ibid.*, 1963, p. 148.
(4) Jones, A. S., *Nature*, **199**, 280(1963).
(5) Hart, S. L., and Nessim, J. A., *J. Pharm. Pharmacol.*, **18**, 288(1966).
(6) Gilman, H., and Blatt, A. H., "Organic Syntheses," coll. vol. I, John Wiley & Sons, Inc., New York, N.Y., 1941, p. 29.
(7) Horwitz, W., "Official Methods of Analysis of the AOAC," 10th ed., Association of Official Agricultural Chemists, Washington, D.C., 1965, pp. 80–94.
(8) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization," 2nd ed., Lea & Febiger, Philadelphia, Pa., 1957, pp. 107–126.
(9) Shelton, R. S., Van Campen, M. G., Tilford, C. H., Lang, H. C., Nisonger, L., Bandelin, F. J., and Rubenkoenig, H. L., *J. Am. Chem. Soc.*, **68**, 753(1946).
(10) Hoogerheide, J. C., *J. Bacteriol.*, **49**, 277(1945).
(11) Christenson, G. L., and Shelton, R. S., *J. Am. Pharm. Assoc., Sci. Ed.*, **37**, 354(1948).
(12) Prusak, L. P., and Mattocks, A. M., *ibid.*, **38**, 67 (1949).
(13) Davies, G. E., *J. Hyg.*, **47**, 271(1949).
(14) Frank, R., and Stark, G., *Pharm. Acta Helv.*, **29**, 81 (1954).
(15) McDowell, M. J., and Kraus, C. A., *J. Am. Chem. Soc.*, **73**, 2170(1951).

Solvolysis of 3,4-Dialkyl Sydnones

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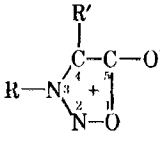
The kinetics of solvolysis of various dialkyl sydnones: 3-*tert*-butyl-4-methyl, 3-*n*-propyl-4-ethyl, 3-ethyl-4-methyl, 3-isopropyl-4-ethyl, 3-*n*-propyl-4-methyl, and 3-*n*-propyl-4-*n*-butyl sydnone, have been studied spectrophotometrically as a function of HCl and NaOH concentrations, pH, and temperature. The reactivity on acid-catalyzed solvolysis decreased in the order cited with only the first compound exhibiting pH-independent solvolysis and high reactivity, about 600 times that of the others. The reactivities of the remainder were only over a threefold range. The order of reactivity tended to reverse on alkaline solvolysis within a similar range, where 3-ethyl-4-methylsydnone was 1.7 times more reactive than the equally reactive 3-*n*-propyl-4-ethyl, 3-*n*-propyl-4-methyl, and 3-isopropyl-4-ethyl sydnones. The 3-*n*-propyl-4-*n*-butylsydnone was half as reactive as this group, whereas the 3-*tert*-butyl-4-methylsydnone was one quarter as reactive. The presence of α-hydrogens on the alkyl substituted sydnones tends to stabilize such structures against hydrogen ion and water attack. This can be explained by postulating extended equilibria among tautomeric structures which decrease the effective concentration of the reactive intermediate. The 3-benzylsydnone demonstrates a reactivity to hydroxyl ion attack equivalent to the more reactive furfuryl sydnone where the electron-attracting powers of the rings are not completely inhibited by the intervening methylene groups. The acid-catalyzed solvolysis of 3-benzylsydnone is of the order of magnitude of other 3-alkyl sydnones with α-hydrogens. The observed second-order dependency of reactivity on hydrogen ion concentration may be assigned to the delocalization of the charge of the singly protonated species, due to the probability of many mesomeric forms with the benzyl substituent.

THE MESOIONIC SYDNONES are of interest because of their unique structure and properties (1–3). In common with other mesoionic systems they may be potential medicinal agents (2–7). Some sydnones possess fungicidal (4) and ascaricidal (5) properties. Others have effects on the central nervous system (6) and show antitumor activity (7–9).

The sydnones have a small, planar, heterocyclic ring system and an aromatic character. They are highly polarized with relatively high dipole moments. Yet they are electrically neutral and have no apparent potentiometric or spectrophotometric pKa values (1–3). The sydnones cannot be represented by a single covalent structure. Suitable symbolism is as a hybrid of a large number of mesomeric structures such as I (1).



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They also possess a relatively high lipid solubility for such polar molecules (3). The sydnones are thus interesting model compounds to gain insight into the solvolytic mechanisms of mesoionic compounds or the transport of dipolar molecules across biological membranes.

The sydnones are prepared by cyclodehydration of a properly substituted *N*-nitrosoglycine (1–3, 10, 11). Alkaline hydrolysis results in formation of the starting *N*-nitrosoglycine. Acid hydrolysis gives a substituted hydrazine, a carboxylic acid, and carbon dioxide (1–3, 10, 11). The solvolytic stability of the sydnone ring has been shown to depend significantly on the nature of the substituents in the 3-position in the ring (12) in contrast to previous statements (11). Electron donating 4-substituents are said to cause an instability in the sydnone ring, but the need for further work has been stated (2).

The present work was undertaken to obtain further evidence to improve understanding of the solvolytic processes in the sydnone series. In particular, the effects of alkyl 4-substituents which should have greater electron-donating properties than hydrogen are investigated to compare the results with those obtained for relevant 3-alkyl sydnones. The hydrolysis of 3-benzylsydnone was also studied to gain insight as to the possible reasons for the anomalous solvolytic behavior of 3-furfurylsydnone (12) which is similar in structure.

EXPERIMENTAL

Materials and Apparatus—The synthesis and characterization of the sydnones studied here have been reported previously by Kier and Dhawan (13), who also prepared the 3-benzylsydnone by methods in the literature (14). The compounds were stored

Received May 25, 1967, from the College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication August 2, 1967.

Presented in part to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported by grant GM-09864-03,04,05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors thank Drs. L. B. Kier and D. Dhawan for the supplies of sydnones studied.

in a vacuum desiccator in a refrigerator. All other chemicals used were of analytical reagent grade. Nitrogen-purged, glass-distilled water was used to make up all solutions. Spectrophotometric readings were taken on the Beckman model DU spectrophotometer. Spectra were recorded on the Cary model 15 recording spectrophotometer. Matched quartz cells (1.00 cm.) were used for all measurements and a slit width of 0.10 mm. employed. Acid and alkali solutions were standardized using the Sargent recording titrator model D. The pH values at the temperatures of study were calculated from activity coefficient data available in the literature (15) or extrapolated from those values. Ionic strengths of all other buffer solutions were adjusted to 0.10. The pH of these buffers was measured at the temperature of study by a Beckman model G pH meter. Constant-temperature baths were maintained to within ± 0.1° of the temperature of study. The sydnones studied and the absorptivities at the wavelengths of their maximum spectrophotometric absorbances are listed in Table I.

Kinetic Procedures—The general procedure was to dissolve a sydnone, 2.0×10^{-4} moles, in a few drops of ethanol and to make up the volume to 200 ml. with distilled water. This 10^{-3} *M* master solution was used for kinetic runs in acidic and alkaline media. In a typical case, 10 ml. of the master solution was pipeted into a 100-ml. volumetric flask containing 80 ml. of 1.0 *M* hydrochloric acid solution maintained at 80°. The volume was brought to mark with distilled water maintained at the same temperature to give a 10^{-4} *M* sydnone in 0.80 *M* hydrochloric acid at 80°.

In the acetate and phosphate buffers, a weighed amount of sydnone to obtain a 10^{-4} *M* solution was dissolved with the aid of a few drops of ethanol and the volume brought to 100 ml. with the appropriate buffer solution maintained at the temperature of study.

The wavelengths of maximum absorbance of these sydnones in aqueous solution were between 284 and 292 mμ (ε 6,000 to 8,000) in acidic, neutral, and alkaline solutions (Table I). The wavelengths of maximum absorbance and the magnitude of absorbance did not show any significant change with pH.

The absorbance at the wavelength of maximum absorbance decreased with time in each case. The decrease was followed by withdrawal of samples at intervals of time, cooling rapidly to room temperature, and reading against an appropriate blank until the residual absorbance, A_∞, remained constant or asymptotically approached zero.

In all cases, except with the 3-*tert*-butyl-4-methylsydnone, a spectrophotometric absorbance assignable to the corresponding *N*-nitrosoglycine was found to increase concomitantly at a shorter wavelength (about 240 mμ) and strongly indicated a 1:1 transformation.

No such band assignable to an alkyl substituted *N*-nitrosoglycine appeared during the hydrolysis of 3-*tert*-butyl-4-methylsydnone in sodium hydroxide, or for all the sydnones in hydrochloric acid, acetate, and phosphate buffers. Typical spectral curves are given in Fig. 1 for the alkali-catalyzed hydrolysis of 3-benzylsydnone.

Thin-Layer Chromatography—The degradation in 0.8 *M* HCl of 10^{-2} *M* solutions of 3-benzylsydnone at 80° was followed by thin-layer chromatography. A small volume of reaction mixture was brought to pH 6–7 and 20 mm.[3] of the neutralized solution was applied on a thin-layer chromatography (TLC) plate. The TLC plates were prepared to obtain 0.4-mm. thick layers of Silica Gel GF_{254} containing phosphor indicator (catalog No. 7730, Brinkmann Institute, Westbury, N. Y.). The chromatograms were developed in *n*-butanol–acetic acid–water (4:1:5) system until the solvent front reached a height of 10 cm. They were then dried and the resulting separation observed under a short wavelength ultraviolet lamp. The relative positions of the spots were noted. For control purposes, 10^{-2} *M* solutions of the benzylsydnone and benzyl hydrazine (Aldrich Chemical Co., Milwaukee, Wis.) were also spotted on the TLC plate.

TABLE I—3R, 4R′ DIALKYLSYDNONES USED IN THE PRESENT INVESTIGATION

R	R′	λ_{max}, mμ	ε
tert-Butyl	Methyl	292	7,200
n-Propyl	Methyl	284	6,700
n-Propyl	Ethyl	284	6,700
n-Propyl	*n*-Butyl	291	6,800
i-Propyl	Ethyl	284	6,600
Ethyl	Methyl	284	6,400
Benzyl	H	292	7,000

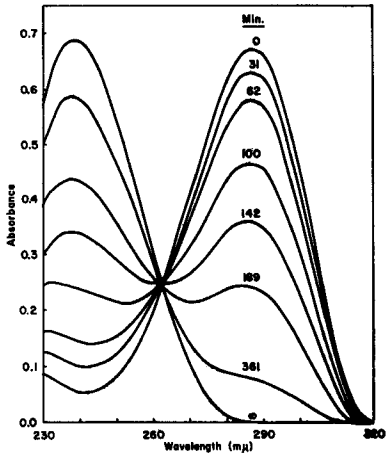


Fig. 1—Spectrophotometric absorbance curves for the solvolysis in 0.25 N *NaOH of 3-benzylsydnone* (10^{-4} M) *at 70.0°.*

RESULTS

Rate Constants—The apparent first-order rate constants, *k*, were calculated from the slopes of the plots of the logarithm of the difference in the absorbance *A* at time *t* and the final absorbance A_∞ against time according to:

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

where A_0 is the absorbance at zero time. Typical first-order fits obtained are shown for the acid-catalyzed hydrolysis of 3-*n*-propyl-4-methylsydnone in Fig. 2.

The conditions and rate constants for the apparent

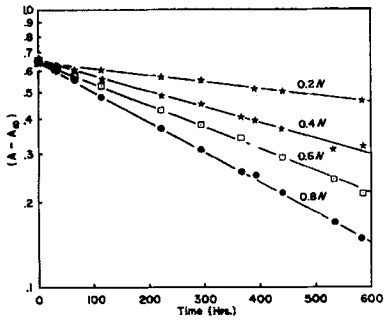


Fig. 2—Typical first-order plots at several [HCl] for the loss of the 284 mμ absorbance (A) *by acid-catalyzed solvolysis of 3-n-propyl-4-methylsydnone at 80.0°. The A_∞ value is the residual absorbance at infinite time.*

first-order acid- and alkali-catalyzed degradations of the sydnones studied are given in Tables II and III. The only compound showing pH-independent hydrolysis was 3-*tert*-butyl-4-methylsydnone (75°; pH 4.53, acetate buffer, $k_0 = 2.03 \times 10^{-5}$ sec.$^{-1}$; pH 5.83, phosphate buffer, $k_0 = 2.10 \times 10^{-5}$ sec.$^{-1}$; pH 7.82, phosphate buffer, $k_0 = 2.12 \times 10^{-5}$ sec.$^{-1}$). Catalysis by the buffer species was found to be absent (75°; pH 6.75, phosphate buffer in buffer strength 25% to 100% with a maintained ionic strength of 0.1, $k_0 = 1.95$ to 2.02×10^{-5} sec.$^{-1}$).

Dependence of Rate on the Catalytic Species—The dependence of the apparent first-order rate constants on the catalytic species is defined for specific alkali catalysis by:

$$k = k_{NaOH}[NaOH] + k_0 \qquad \text{(Eq. 2)}$$

where k is the apparent first-order rate constant, and k_0 is the apparent first-order rate constant for pH-independent hydrolysis. Plots of k against [NaOH] should give straight lines with slopes k_{NaOH}, the bimolecular rate constant for the NaOH-catalyzed hydrolysis where the k_0 values are the intercepts of such plots. The k_{NaOH} and k_0 values are given in Table IV.

The dependence of k on hydrochloric acid is defined by:

$$k = k_{HCl}[HCl] + k_0 \qquad \text{(Eq. 3)}$$

where k_{HCl}, the bimolecular rate constant for the HCl catalyzed hydrolysis is obtained from the slopes of the plots of k against [HCl] and k_0, the pH-independent apparent first-order rate constant, is obtained from the intercepts. The k_{HCl} and k_0 values are given in Table V. Examples of these plots are given in Figs. 3 and 4 where nonzero intercepts of Fig. 3 demonstrate the presence of pH-independent hydrolysis in 3-*tert*-butyl-4-methylsydnone.

The log k-pH profiles for the hydrolysis of the 3-*tert*-butyl-4-methylsydnone at several temperatures exhibit a pH-independent hydrolysis (Fig. 5). The slopes of the log k-pH graphs related to acid- and alkali-catalyzed hydrolysis for all sydnones that do not exhibit pH-independent hydrolysis are −1 and +1, respectively, and are characteristic of specific hydrogen and specific hydroxyl ion-catalyzed hydrolysis.

In the case of the 3-benzylsydnone, good first-order plots were obtained for the degradation in the

TABLE II—CONDITIONS AND RATE CONSTANTS (k sec.$^{-1}$) FOR THE APPARENT FIRST-ORDER ACID-CATALYZED DEGRADATION OF SYDNONES (10^{-4} M) AT 75°

Sydnone	[HCl]	10^6k (sec.$^{-1}$)
3-*tert*-Butyl-methyl	0.395	142.8
	0.592	205.0
	0.790	294.4
3-*n*-Propyl-4-methyl	0.208	0.093
	0.413	0.167
	0.627	0.380
3-*n*-Propyl-4-ethyl	0.208	0.095
	0.413	0.199
	0.627	0.342
	0.848	0.494
3-*n*-Propyl-4-*n*-butyl	0.220	0.046
	0.425	0.081
	0.637	0.122
	0.853	0.174
3-Ethyl-4-methyl	0.198	0.034
	0.395	0.192
	0.593	0.340
	0.790	0.505
3-Isopropyl-4-ethyl	0.220	0.162
	0.425	0.330
	0.637	0.475
	0.853	0.645
3-Benzyl	0.208	0.070
	0.413	0.210
	0.627	0.370
	0.848	0.560

TABLE III—CONDITIONS AND RATE CONSTANTS (k sec.$^{-1}$) FOR THE APPARENT FIRST-ORDER ALKALI-CATALYZED DEGRADATION OF SYDNONES ($10^{-4}M$) AT 75°

Sydnone	[NaOH]	10^6k (sec.$^{-1}$)
3-*tert*-Butyl-4-methyl	0.05	19.5
	0.10	20.5
	0.25	24.1
	0.50	30.6
3-*n*-Propyl-4-methyl	0.20	36.0
	0.40	86.7
	0.60	108.0
	0.80	154.0
3-*n*-Propyl-4-ethyl	0.20	32.1
	0.40	66.5
	0.60	112.0
	0.80	152.0
3-*n*-Propyl-4-*n*-butyl	0.20	21.6
	0.40	43.8
	0.60	68.1
	0.80	89.4
3-Ethyl-4-methyl	0.05	15.1
	0.10	29.2
	0.25	72.2
	0.50	156.6
3-Isopropyl-4-ethyl	0.20	4.87
	0.40	13.8
	0.60	25.6
	0.80	39.3
3-Benzyl	0.05	14.7
	0.10	28.4
	0.25	71.6
	0.50	154.3

TABLE IV—CATALYTIC CONSTANTS FOR THE ALKALI AND NEUTRAL SOLVOLYSIS OF SYDNONES

Sydnone	Temp., °C.	$10^5 k_{NaOH}$[a] (L. mole^{-1} sec.$^{-1}$)	$10^5 k_4$[a] (sec.$^{-1}$)
3-*tert*-Butyl-4-methyl	80.0	7.45	2.90
	75.0	3.05	1.75
	70.0	1.77	1.03
	60.0	1.02	0.32
3-*n*-Propyl-4-methyl	80.0	25.8	
	75.0	18.6	
	70.0	12.0	
	60.0	4.5	
3-*n*-Propyl-4-ethyl	80.0	25.9	
	75.0	17.1	
	70.0	12.0	
	60.0	4.7	
3-*n*-Propyl-4-*n*-butyl	80.0	13.9	
	75.0	10.2	
	70.0	7.62	
	60.0	3.65	
3-Ethyl-4-methyl	80.0	43.5	
	75.0	29.5	
	70.0	18.8	
	60.0	7.65	
3-Isopropyl-4-ethyl	80.0	27.8	
	75.0	16.5	
	70.0	10.1	
	60.0	3.75	
3-Benzyl	80.0	43.0	
	75.0	29.2	
	70.0	19.1	
	60.0	8.60	

[a] Determined as slopes and intercepts of plots of the apparent first-order rate constants (k sec.$^{-1}$) against [NaOH] according to the expression $k = k_{NaOH}$ [NaOH] + k_0.

TABLE V—CATALYTIC CONSTANTS FOR THE ACID AND NEUTRAL SOLVOLYSIS OF SYDNONES

Sydnone	Temp., °C.	$10^6 k^a_{HCl}$ (L. mole^{-1} sec.$^{-1}$)	$10^6 k_0$[a] (sec.$^{-1}$)
3-*tert*-Butyl-4-methyl	80.0	622.0	30.0
	75.0	322.0	20.0
	70.0	142.0	15.0
	60.0	34.0	5.0
3-*n*-Propyl-4-methyl	80.0	0.915	0.00
	75.0	0.475	
	70.0	0.295	
	60.0	0.120	
3-*n*-Propyl-4-ethyl	80.0	1.53	0.00
	75.0	0.670	
	70.0	0.340	
	60.0	0.120	
3-*n*-Propyl-4-*n*-butyl	80.0	0.470	0.00
	75.0	0.220	
	70.0	0.150	
	60.0	0.040	
3-Ethyl-4-methyl	80.0	1.10	0.00
	75.0	0.575	
	70.0	0.305	
3-Isopropyl-4-ethyl	80.0	1.01	0.00
	75.0	0.752	
	70.0	0.403	
	60.0	0.145	
		$10^6 k_1$[b]	$10^6 k_2$[b]
3-Benzyl	80.0	0.500	1.75
	75.0	0.280	0.830
	70.0	0.161	0.337
	60.0	0.050	0.069

[a] Determined as slopes and intercepts of the plots of the apparent first-order rate constants (k sec.$^{-1}$) against [HCl] according to the expression $k = k_{HCl}$ [HCl] + k_0. [b] Determined as intercepts, k_1, and slopes, k_2, of the plot of k/[HCl] against [HCl] according to the expression k/[HCl] = k_1 + k_2[HCl].

acid medium. Plots of the apparent first-order rate constants, k against [HCl], however, did not yield the expected straight lines but showed an apparent exponential increase in such constants with [HCl]. This was most readily observed at the higher temperatures. The plots were reasonably linear when k was plotted against [HCl] . In solutions of the

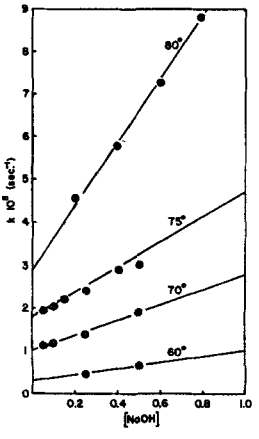


Fig. 3—Dependence of the apparent first-order rate constant, k, on [NaOH] for the base-catalyzed solvolysis of 3-tert-4-methylsydnone at several temperatures.

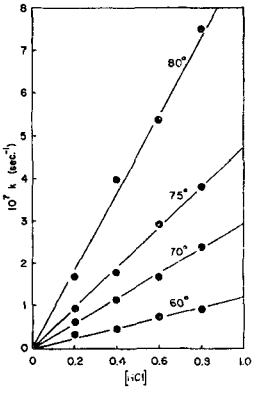


Fig. 4—Dependence of the apparent first-order rate constant, k, on [HCl] for the acid-catalyzed solvolysis of 3-n-propyl-4-methylsydnone.

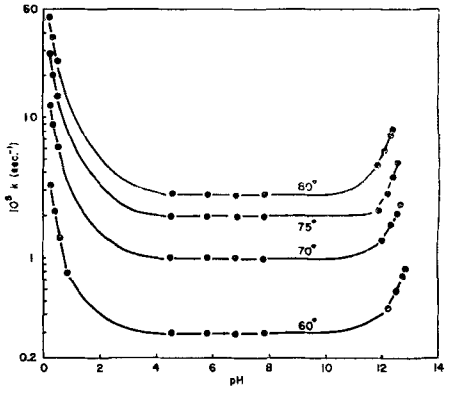


Fig. 5—Log k-pH profile of the dependence of the rate constant, k, on pH for the solvolysis of 3-tert-butyl-4-methylsydnone at several temperatures with a demonstrated region of pH-independent solvolysis.

TABLE VI—APPARENT FIRST-ORDER RATE CONSTANTS FOR THE ACID-CATALYZED SOLVOLYSIS (0.80 *M* HCl) AS A FUNCTION OF IONIC STRENGTH[a] AT 80°

Ionic Strength	$10^7 k$ sec.$^{-1}$ 3-Benzylsydnone	3-Isopropylsydnone
0.80	12.07	10.10
1.00	14.10	11.10
1.20	17.00	12.50

[a] Ionic strength adjusted with KCl.

TABLE VII—APPARENT THERMODYNAMIC QUANTITIES[a] FOR THE SOLVOLYSIS OF SYDNONES

Sydnone	HCl ΔH_a Kcal./mole	HCl log P	NaOH ΔH_a Kcal./mole	NaOH log P
3-*tert*-Butyl-4-methyl	32.8	16.9	33.1	17.0
3-*n*-Propyl-4-methyl	23.6	8.5	23.1	10.8
3-*n*-Propyl-4-ethyl	23.6	8.6	19.2	9.3
3-*n*-Propyl-4-*n*-butyl	30.0	12.2	24.2	10.2
3-Ethyl-4-methyl	31.8	13.2	20.0	9.0
3-Isopropyl-4-ethyl	24.1	8.9	23.6	8.3
3-Benzyl	27.5[b] 3.20[c]	10.8[b] 14.1[c]	18.6	8.1

[a] These quantities are obtained from the logarithmic form of the Arrhenius equation: $\log k = \log P - \Delta H_a/2.303RT$, where k is in sec.$^{-1}$ for the hydrolysis in 1.0 *M* HCl and 1.0 *M* NaOH, the ΔH_a is the experimental activation energy in Kcal./mole, R is the gas constant in cal. deg.$^{-1}$ mole^{-1}, T is the absolute temperature, and log P is a constant associated with the entropy of activation. [b] Determined from intercepts, k_1, of the plot of k/[HCl] against [HCl] according to the expression $k/[HCl] = k_1 + k_2$ [HCl]. [c] Determined from the slope, k_2, of such plots.

same hydrochloric acid concentration but differing ionic strengths, the apparent k increased as would be expected for a reaction between two like-charged species (16), according to the following equation:

$$\log k = \log k' + z_A z_B \sqrt{\mu} \qquad \text{(Eq. 4)}$$

where z_A and z_B are the respective signed charges on the reacting species.

The hydrolysis of 3-isopropylsydnone was also studied under similar conditions as a model compound for the other 3-alkyl sydnones. A smaller salt effect was observed in this case. The apparent first-order rate constants for these two sydnones as a function of the ionic strength are given in Table VI.

Plot of log k against pH for the acid-catalyzed hydrolysis of the 3-benzylsydnone gave slopes ranging from −1.4 at 60° to −1.8 at 80°, rather than the normally expected slopes of −1 for the single proton catalyzed hydrolysis.

Dependence of Rate on Temperature—The Arrhenius expression $k = Pe^{-\Delta H_a/RT}$ can be written in the logarithmic form:

$$\log k = \log P - \frac{\Delta H_a}{2.303\,RT} \qquad \text{(Eq. 5)}$$

Plots of the logarithms of the various catalytic rate constants against the reciprocal of the absolute temperature permit calculation of the ΔH_a and log P values of Eq. 5. These values are listed in Table VII.

Thin-Layer Chromatography—The acid hydrolysis of 3-benzylsydnone was monitored by thin-layer chromatography (*n*-butanol–acetic acid–water, 4:1:5) and observed under ultraviolet light. The spot, $R_f = 0.65$, due to the benzylsydnone became smaller and fainter with time and finally disappeared completely. The only other spot was due to appearance of benzyl hydrazine, $R_f = 0.48$, which increased in size and intensity with time. This showed that benzyl hydrazine was the major product of acid hydrolysis.

DISCUSSION

Comparative Reactivities of Sydnones—The reactivities of the 3,4-dialkyl sydnones can be readily compared with respect to their solvolytic rate constants (Tables II–V) when one substituent is kept constant and the other is varied. This is shown in Table VIII for alkali- and acid-catalyzed hydrolysis at 75°. The comparison for 4-H sydnones was based on data given previously (12).

The sequences (Table VIII) when the 4-substituent is methyl and the 3-substituent is varied, and when the 3-substituent is *n*-propyl and the 4-substituent is varied in the acid-catalyzed region, are in accordance with the expected induction effects of the alkyl groups. It is expected that the greater the electron-releasing capacity of a substituent, the less positive the sydnone ring, the less the reactivity to hydroxyl ion attack, and the greater the reactivity to hydrogen ion attack. However, the sequence is not rigorous in those cases (Table VIII) when the 4-substituent is ethyl, the 3-substituent is *n*-propyl, and the other substituent is varied. In fact, substitution of an ethyl or a methyl for a hydrogen in the 4-position does not significantly change the reactivity of 3-*n*-propyl sydnones to alkaline solvolysis. It does have an effect on acid solvolysis, however.

Kier and Roche (3) have cited unpublished data to support their contention that the 2-nitrogen is predicted to be energetically the least likely point of attack of hydroxide ion as based on molecular orbital calculations of anion localization energies. They preferred hydroxyl ion attack on the 5-carbon in I, the atom adjacent to the oxygen assigned a high formal and localized negative charge (3). Since the presence of a formal charge in all known molecules inhibits the attack of a like charged species (16), it is difficult to expect that a highly polar anion such as hydroxyl would attack an atom

TABLE VIII—RELATIVE REACTIVITIES OF THE 3R, 4R′-DIALKYL SYDNONES AS COMPARED AT 75°

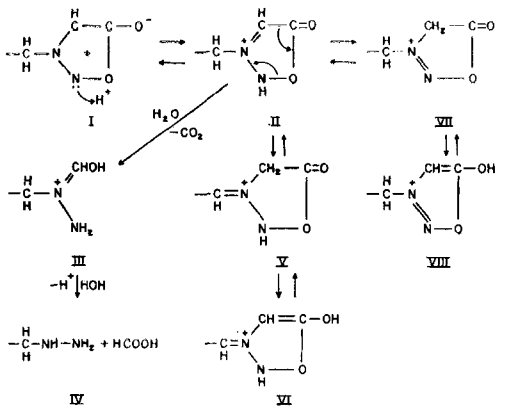
	k_{NaOH}	k_{HCl} (L./mole/sec.)
R = *n*-Propyl	R′: H ∼ Me ∼ Et > *n*-Bu	R′: H > Et > Me > *n* − Bu
R′ = Ethyl	R: *n* − Pr ∼ *i* − Pr	R: *n* − Pr ∼ i − Pr
R′ = Methyl	R: Et > *n* − Pr > *t* − Bu	R: *t* − Bu ≫ *n*-Pr ∼ Et

adjacent to one with a highly localized negative charge. It was predicted (12) that if this were so, 3,4-dialkylated sydnones should demonstrate significantly less susceptibility to hydroxyl ion attack than solely 3-alkylated sydnones. It is apparent (Table VIII) that the more electron-donating methyl and ethyl substitution for hydrogen in the 4-position, which is adjacent to the postulated attack on the 5-carbon site, had no effect on diminishing reactivity to hydroxyl ion attack. Substitution of a methyl for a hydrogen in the 4-position did decrease the rate of hydroxyl ion attack on the 3-*n*-propyl and 3 *tert*-butylsydnones by 40–60%, but substitution of an ethyl had no significant effect on 3-*n*-propylsydnone reactivity and actually increased the reactivity of 3-*i*-propylsydnone to hydroxyl ion attack by 40%.

It is just as probable that hydroxyl ion attack occurs elsewhere in the sydnone ring than on the 5-position adjacent to the strong anionic charge. Electronic redistributions can be readily induced on the approach of the hydroxyl ion through the bonded *p*-orbital of the atoms of the aromatic sydnone ring, and this sensitizes other sites for reaction. Orbital calculations are based on approximations for the ground state of a molecule, and the results reflect these approximations and exclude the perturbations induced by attacking species. A definitive experiment is needed to resolve the question of site and mechanism of hydroxyl ion attack on sydnones in aqueous solutions.

Still unresolved are the reasons for the enhancement of comparative acid-catalyzed solvolytic rates when the 3-substituent in 3-alkyl sydnones is highly branched, tertiary, and possesses no α-hydrogens, *e.g.*, 1,1,3,3-tetramethylbutyl, 3-*tert*-butyl (12), and 3-*tert*-butyl-4-methyl sydnones (Table V). In addition, these are the only alkyl sydnones that show significant water attack.

We may accept as likely the mechanism of acid hydrolysis based on that of Baker and Ollis (3, 17), *i.e.*, the sequence I–IV to the hydrazine in Scheme I. The net mechanism is similar whether we add water to II and then form III (12) or have the decomposition products of II react with the components of water (3). It is possible that the availability of hydrogens on the α-carbon of the 3-alkyl substituent permits the equilibria to V and VI *via* acid-catalyzed enolization-like mechanisms. This distribution among other species diminishes the concentration of a reactive intermediate, such as II, necessary for the production of the hydrazine product in acid solution. The conjugations of VI and VIII should be stabilizing influences. When no α-hydrogens on a 3-alkyl are available, such as in the *tert*-alkyl sydnone cases cited, the equilibria with VI and VII are not possible. Thus, the increased concentration of the reactive intermediate (II) could lead to a higher over-all rate of acid catalyzed solvolysis.



Scheme I

There is a great enhancement of hydrogen ion-catalyzed solvolysis when a methyl is substituted for a hydrogen in the 4-position in the case of *tert*-butylsydnone. The second-order rate constant for the acid catalyzed solvolysis of 3-*tert*-butyl-4-methylsydnone at 75° (Table V) is 16 times greater than that of 3-*tert*-butylsydnone (12). Admittedly, it could be argued that the electron-releasing properties of the 4-methyl substituent promoted the hydrogen ion attack on a more negative site in the sydnone ring. Its magnitude is difficult to accept on this premise, however, since the introduction of a 4-alkyl for the hydrogen has only minor enhancing effects on acid-catalyzed solvolysis of 3-alkyl sydnones with α-hydrogens. (Compare Table V and the data in *Reference 12.*) A possible explanation may be based on consideration of the equilibria in Scheme I of II ↔ VII ↔ VIII remaining available to *tert*-butylsydnone. Substitution of a methyl for a hydrogen in the 4-position should lower the electron density on the 4-carbon and not favor the net transfer of hydrogen from the 2-nitrogen in this equilibria. This could lead to a net increase in the concentration of the reactive species II.

The fact that of all the 3-alkyl sydnones only these tertiary alkyl sydnones demonstrate pH-independent solvolysis is significant. This pH-independent solvolysis implicates water attack since it drastically decreases in alcohol–water mixtures of lessened water content (12). An activated state similar to one or several of the postulated hybrids of the numerous dipolar and tetrapolar forms (3, 17) may undergo attack by water in a mechanism similar to that of Scheme I with an activated state similar to II (12). It can be argued that the concentration of this reactive intermediate is reduced by the formation of equilibrating structures similar to V and VI when α-hydrogens on the 3-alkyl are present. Only in the cases of 3-*tert*-alkyl sydnones may there be a sufficient concentration of the reactive intermediate for a pH-independent water attack to be observed. An argument similar to that made previously for increased concentration of the reactive species may be made for the enhancement of water attack on 3-*tert*-butyl-4-methylsydnone over 3-*tert*-butylsydnone, about 10 times more reactive at 75°. (Compare Table V and the data in *Reference 12.*)

Reactivity of 3-Benzylsydnone—The results obtained for the alkali-catalyzed hydrolysis of 3-benzylsydnone agree with expectations for an apparent first-order dependence on hydroxide ion concentration (Table III). However, the apparent first-order rate constant, k, is not strictly linear with the HCl concentration (Table II). It is a function of some power of the HCl concentration and this dependence is more pronounced at higher

temperatures. The log k-pH plots in their acid branch vary from -1.4 at 60° to -1.8 at 80°. The ionic strength effect is of the type expected for a reaction between two like-charged species (Table VI), and the main product of the acid hydrolysis is benzyl hydrazine. These results suggest that acid-catalyzed hydrolysis of 3-benzylsydnone contains a contribution proportional to $[HCl]^2$. At constant acid concentrations the rate dependence of benzylsydnone solvolysis can be formulated (18) as:

$$-d[\text{benzylsydnone}]/dt = k_1[HCl][\text{benzylsydnone}] + k_2[HCl]^2[\text{benzylsydnone}] \quad \text{(Eq. 6)}$$

where the apparent first-order rate constant is:

$$k = k_1[HCl] + k_2[HCl]^2 \quad \text{(Eq. 7)}$$

and

$$k/[HCl] = k_1 + k_2[HCl] \quad \text{(Eq. 8)}$$

A plot of $k/[HCl]$ against [HCl] should give a straight line with a slope k_2, the apparent termolecular rate constant, where the intercept, k_1, should estimate the bimolecular rate constant for the HCl-catalyzed hydrolysis. The values of k_1 and k_2 obtained from such a plot (Fig. 6) show that, for 1.0 M HCl, the relative contribution of k_2 to the over-all rate constant, k, with respect to k_1, *i.e.*, the ratio k_2/k_1, increases from 60° to 80° (Table V). Plots of log k_1 and log k_2 against $1/T$ give activation energies of 27.5 and 32.0 Kcal./mole, respectively, and show that k_2 is favored over k_1 at the higher temperatures. Similar plots of the data in Table II for other dialkyl sydnones also show that there may be present, although to a much lesser extent, a concurrent first and second-order dependence on hydrogen ion of the type expressed by Eq. 8. The $k/[HCl]$ values showed a tendency to increase with [HCl], but the slopes of such plots were small and the major contribution to solvolysis was the first power of the HCl concentration. The compound other than benzylsydnone that showed the greatest such dependency on the $[HCl]^2$ was 3-ethyl-4-methylsydnone. However, Eq. 3 was reasonably adequate for quantitative characterization since the k_2 value was relatively small.

These kinetic observations can be interpreted in terms of two concurrent pathways, one discussed previously in Scheme I and the other involving a second protonation.

Second protonation is likely in cases similar to 3-benzylsydnones because of the possibility of mesomeric structures and delocalization of the positive charge through the many possible mesomeric structures mediated by the benzyl substituent and its labile α-hydrogens. A few typical examples are given in Scheme II before and after single protonation, *i.e.*, structures like IX, X, and XI can be represented where one positive charge can be delocalized.

It can be argued that the labile hydrogens of the benzylsydnone permit contributions of structures which may be similar to the V, VI, VII, and VIII of Scheme I. These may dilute out the effective concentration of an activated state as represented by II. Thus, an effective concentration of single protonated hybrids such as XI may occur which react further to products after a second protonation.

Confirmation of the existence of these additional

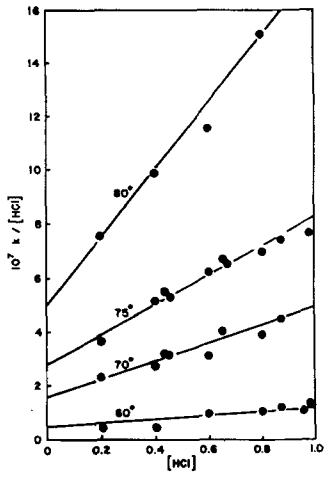
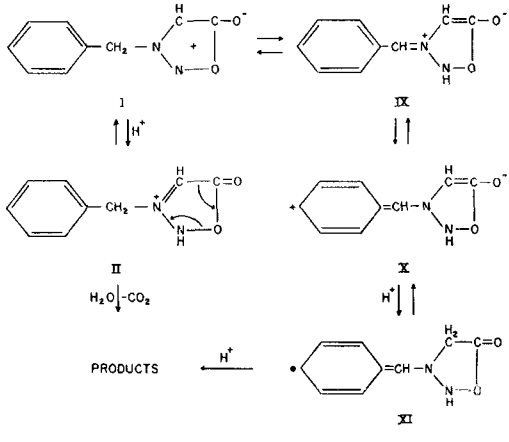


Fig. 6—Dependence of k/[*HCl*] *on* [*HCl*] *at different temperatures, according to* $k/[HCl] = k_1 + k_2[HCl]$, *for the apparent first-order rate constant values,* k, *of benzylsydnone.*

mesomeric structures with 3-benzylsydnone is obtained from its ultraviolet spectrum (Fig. 1). The 3-benzylsydnone, with one methylene group intervening between the benzene and sydnone rings, shows a single absorbance maximum at 292 mμ (ε = 7,000). However, 3-phenylethylsydnone, with two intervening methylene groups between the rings, exhibits two distinct absorbance maxima at 254 and 286 mμ (ϵ_{286} = 3,000) (12). This indicates that the benzene ring and the sydnone ring maintain their separate chromophores in 3-phenethylsydnone and that there is little or no conjugation between the π-electron systems of these rings. In the case of the 3-benzylsydnone, however, the solitary methylene group does not appear to prevent such conjugation. The ultraviolet absorption spectrum indicates that conjugation of the π-electron systems of the two rings exists, together with a bathochromic shift in the wavelength of maximum absorbance, as has been predicted for such cases (19). The partial positive charge, instead of remaining on the sydnone



Scheme II

ring only, may now be smeared over two rings. This results in the sydnone ring in 3-benzylsydnone having less partial positive charge than in the case of the other 3-alkyl sydnones. The results obtained in the study of the ionic strength effects (Table VI) also support this explanation of a smaller positive charge on the neutral sydnone ring in the 3-benzylsydnone, since the greater positive ionic strength effect on acid-catalyzed solvolysis implies a greater significant hydrogen ion attack on a positively charged species.

On the basis of energetic factors, hydrogen ion attack on the nonprotonated sydnones is the favored pathway at lower temperatures because of the smaller activation energy (Table VII). At higher temperatures, however, hydrogen ion attack on the protonated sydnone will be the major route (Scheme II).

It was previously anticipated (12) that the benzyl radical would behave similarly to the furfuryl radical, in that the intervening single methylene group does not insulate the rings and both these radicals may act as electron sinks with greater electron attracting properties on hydroxyl ion attack. In the general sense this anticipation was confirmed and the rates of alkaline solvolysis were equivalent. The hydroxyl-ion-catalyzed solvolyses of benzyl (Table III) and furfuryl sydnones (12) are more than twice the rates of other 3-alkyl sydnones at comparable temperatures (12).

However, the acid-catalyzed solvolysis of 3-benzylsydnone (Table II) is similar in magnitude to those values for the simpler 3-alkyl sydnones, whereas the 3-furfurylsydnone acid- and water-catalyzed solvolyses are anomalously high (12). Thus, the previously given prediction (12) that benzylsydnone would not be as readily solvolyzed in acid and neutral solutions as furfurylsydnone appears valid. This prediction was based on the feasible stabilization of the activated complex of furfurylsydnone by the oxygen of the furfuryl ring which is sterically positioned adjacent to the 2-nitrogen.

Extrathermodynamic Free Energy Relationships—Simple linear free energy relationships can also usually provide information as to whether any substituent effects are present. The best known of these is the Hammett $\sigma\rho$ equation. Use of the σ values obtained from the literature (20) did not provide any meaningful correlation in the Hammett plot (20). Since the sydnones were considered to be of an aromatic nature, these substituent values for phenyl systems were preferred over the Taft parameters.

It is interesting to note that when, according to Leffler and Grunwald (21), the derived ΔH^* values for the sydnones were plotted against the derived ΔS^* values (22) a reasonably linear relationship was obtained with a slope of 380°. This should represent the isokinetic temperature when all reactions of this series of compounds have the same free energy of activation. At this temperature, effects due to alkyl substituent should completely disappear (21). However, in our case the criticism of Petersen (23, 24) of this approach is valid since plots of log k/T *versus* $1/T$ had no common point of intersection, and the observation of an apparent isokinetic temperature was an artifact of the data in the Leffler and Grunwald method (21) of determining extrathermodynamic parameters.

REFERENCES

(1) Baker, W., and Ollis, W. D., *Chem. Ind. (London)*, **1955**, 910.
(2) Stewart, F. H. C., *Chem. Rev.*, **64**, 140(1964).
(3) Kier, L. B., and Roche, E. B., *J. Pharm. Sci.*, **56**, 149(1967).
(4) Davis, D., Becker, H. J., and Rogers, E. F., *Phytopathology*, **49**, 821(1959).
(5) Putter, R. W., and Wolfrom, G., Brit. pat. 823,001 (November 4, 1959); and subsequent patents.
(6) Kier, L. B., Fox, L. E., Dhawan, D., and Waters, I. W., *Nature*, **195**, 817(1962).
(7) Daeniker, H. U., and Druey, J., *Helv. Chim. Acta*, **40**, 918(1957).
(8) Greco, C. V., Nyberg, W. H., and Cheng, C. C., *J. Med. Pharm. Chem.*, **5**, 851(1962).
(9) Nyberg, W. H., and Cheng, C. C., *J. Med. Chem.*, **8**, 531(1965).
(10) Earl, J. C., and Mackney, A. W., *J. Chem. Soc.*, **1935**, 899.
(11) Fugger, J., Tien, J. M., and Hunsberger, I. M., *J. Am. Chem. Soc.*, **77**, 1843(1955).
(12) Garrett, E. R., *J. Pharm. Sci.*, **53**, 42(1964).
(13) Kier, L. B., and Dhawan, D., *ibid.*, **53**, 83(1964).
(14) Baker, W., Ollis, W. D., and Poole, V. D., *J. Chem. Soc.*, **1949**, 307.
(15) Harned, H. S., and Owen, B. B., "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold Publishing Co., New York, N. Y., 1958.
(16) Garrett, E. R., *J. Pharm. Sci.*, **51**, 811(1962).
(17) Baker, W., and Ollis, W. D., *Quart. Rev.*, **11**, 15(1957).
(18) Bruice, T. C., and Benkovic, S. J., *J. Am. Chem. Soc.*, **86**, 418(1964).
(19) Gillam, A. E., and Stern, E. S., "An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry," 2nd ed., Edward Arnold Publishers Ltd., London, England, 1960, pp. 75–78.
(20) McDaniel, D. H., and Brown, H. C., *J. Org. Chem.*, **23**, 420(1958).
(21) Leffler, J. E., and Grunwald, E., "Rates and Equilibria of Organic Reactions," John Wiley & Sons, Inc., New York, N. Y., 1963.
(22) Laidler, K. J., "Chemical Kinetics," 2nd ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1965, p. 90.
(23) Petersen, R. C., Markgraf, J. H., and Ross, S. D., *J. Am. Chem. Soc.*, **83**, 3819(1961).
(24) Petersen, R. C., *J. Org. Chem.*, **29**, 3133(1964).

Subcutaneous Absorption Kinetics of Benzyl Alcohol

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Benzyl alcohol (BA) was used as a model compound to study subcutaneous (s.c.) drug absorption kinetics in the rat. It has been used extensively as an adjuvant in pharmaceuticals intended for parenteral administration. Benzyl alcohol has a high lipid–water partition coefficient, and is a neutral, relatively nontoxic local anesthetic. The area of s.c. tissue exposed to the BA solution was controlled by a glass absorption cell affixed to the s.c. tissue by a silicone adhesive. The about 1 percent (w/v) BA in normal saline solution was stirred in the cell at an equilibrium temperature. Multiple doses of the drug were used to monitor any volume changes in the cell. Benzyl alcohol was extracted from the fluids at the site into *n*-hexane and assayed spectrophotometrically at 208 mμ. The BA mean absorption half-life in the s.c. cell was 1.6 hr.

THE PURPOSE of this study was to develop general experimental methods for studying the kinetics of drug absorption from the subcutaneous (s.c.) site following the administration of multiple doses of a drug. Schou (1) pointed out that the experimental method of choice where feasible for studying the rate of drug absorption from s.c. tissue is to record the clearance of injected drug from the local area. To date no investigators have used a direct method of studying drug absorption rate per unit area (p.u.a.) of s.c. tissue, very likely because of the difficulty of controlling the spreading of drug solution at the site.

In this work, benzyl alcohol (BA) was selected as a model drug for studying the kinetics of s.c. drug absorption because (*a*) it is a valuable pharmaceutical adjuvant in parenteral products intended for s.c. or intramuscular (i.m.) administration due to its local anesthetic and bacteriostatic properties; (*b*) it is relatively nontoxic (2–4) compared to other local anesthetics; (*c*) it is a lipid-soluble, neutral molecule not subject to the usual pH-partition effects expected of organic anionic and cationic drugs; and (*d*) it is quantitatively assayable in the biological fluids found at the s.c. region.

THEORETICAL

If one can assume the BA molecules penetrate the s.c. tissue by passive diffusion, then the penetration rate of the drug from the site of administration can be described by Fick's law in one direction:

$$dN/dt = \bar{D}\,A\,(dC/dx) \qquad \text{(Eq. 1)}$$

where dN/dt is the penetration rate, and N is the amount of drug penetrating the membrane. In a well-stirred system the rate is proportional to the mean diffusion coefficient (5) of drug in the membrane, $\bar{D}$, the area, A, of the solution exposed to the absorbing membrane, and the concentration gradient (dC/dx) of drug across the membrane. Under conditions of this experiment where the penetration rate is fairly rapid, Eq. 1 can be approximated (6) by:

$$dN/dt = \frac{\bar{D}AK}{\delta}(C_c - C_b) \qquad \text{(Eq. 2)}$$

where the dx term of Eq. 1 has been replaced by δ, the thickness of the thin s.c. membrane which is assumed to be constant for each animal, and the dC term of Eq. 1 has been replaced by the terms K and $(C_c - C_b)$. The term K is the equilibrium distribution ratio or partition coefficient of the drug between the membrane lipids and the aqueous phases of the absorption cell or body fluids. The term $(C_c - C_b)$ is the difference between the drug concentration in the absorption cell, C_c, and the drug concentration C_b, in the fluids, *e.g.*, blood and lymph, flowing past the absorption site at any time.

The term C_b can be ignored in these experiments, since it can be reasonably assumed that the drug's concentration in the fluids of distribution is negligible compared to its concentration at the absorption site at any time. Thus Eq. 2 can be written as:

$$dN/dt = \frac{\bar{D}AK}{\delta}(C_c) \qquad \text{(Eq. 3)}$$

When the mean volume of the drug solution at the absorption site, $\bar{V}_c$, remains nearly constant throughout the experiment, the rate of penetration will be equal to:

$$dN/dt = \left(\frac{\bar{D}AK}{\delta \bar{V}_c}\right)Ac \qquad \text{(Eq. 4)}$$

where Ac is the amount of drug in the cell at any time. The penetration rate per unit area (p.u.a.) equals:

$$\frac{dN/dt}{A} = \bar{P}\,\frac{Ac}{A} \qquad \text{(Eq. 5)}$$

where $\bar{P}$ is the mean penetration coefficient which includes the terms $\bar{D}$, A, K, δ, and $\bar{V}_c$, and has the

Received June 5, 1967, from the School of Pharmacy, University of California, San Francisco, CA 94122, and the *Hebrew University-Faculty of Medicine, School of Pharmacy, Jerusalem, Israel.

Accepted for publication August 9, 1967.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This study was supported in part by research funds of the University of California, a general research support grant FR 05453 from the Division of Research Facilities and Resources, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and additional research funds allocated by the Eastern Mediterranean Office of WHO, and approved by the Washington WHO Regional Office (January 13, 1966).

The authors are indebted to Mr. Silas Braley, Dow Corning Center for Aid to Medical Research, Midland, Mich., for supplying samples of the silicone medical adhesive used in this study, Dr. Louis A. Strait and Mr. Michael K. Hrenoff for their assistance in developing some of the spectrographic assay procedures used, and to Mr. Russell Vossbrink and Mr. John R. Cafiso for technical assistance.

units of time^{-1}. The mean penetration coefficient or absorption rate constant has the same magnitude as the clearance constant, but the opposite sign. Equation 5 may therefore be simplified to:

$$dB/dt = \bar{P}Bc \qquad \text{(Eq. 6)}$$

where dB/dt is the penetration rate p.u.a., and Bc is the amount of drug in the cell p.u.a. at any time. The term Bc equals the initial amount of drug p.u.a., Bi, less the amount of drug p.u.a., B, that has penetrated the membrane to any time, thus:

$$Bc = Bi - B \qquad \text{(Eq. 7)}$$

Substitution of Eq. 7 into Eq. 6 and integrating between the limits of zero and infinite time yields:

$$Bc = Bie^{-\bar{P}t} \qquad \text{(Eq. 8)}$$

When the same amount of BA p.u.a., B', is administered repeatedly at the times of zero, τ, 2τ, $3\tau \ldots$, the amount of drug in the cell p.u.a. after n doses will be:

$$Bc = B' + B'e^{-\bar{P}\tau} + B'e^{-\bar{P}2\tau} + B'e^{-\bar{P}3\tau} + \ldots + B'e^{-\bar{P}(n-1)\tau} \qquad \text{(Eq. 9)}$$

where the time τ is the dosing interval and is a constant. The amount of drug in the cell p.u.a. just after the administration of the nth dose p.u.a. can be calculated from:

$$B_c = \frac{B'(1 - e^{-\bar{P}n\tau})}{(1 - e^{-\bar{P}\tau})} \qquad \text{(Eq. 10)}$$

Equation 10 applies when Bi and the mean maintenance dose(s) p.u.a., $\overline{Bm}$, are both equal to B' in magnitude. When the initial and mean maintenance dose(s) p.u.a. are not equal in magnitude, then the term Bc at any time can be computed from Eq. 11:

$$Bc = Bie^{-\bar{P}t} + \left\{\overline{Bm}\left[\frac{1 - e^{-\bar{P}(n-1)\tau}}{1 - e^{-\bar{P}\tau}}\right]\right\}e^{-\bar{P}T} \qquad \text{(Eq. 11)}$$

where n, in this case, is an integer number of initial and maintenance doses p.u.a. administered, and T is another clock time starting at zero immediately after the administration of the last maintenance dose p.u.a.

When the number of doses p.u.a. approaches infinity (and the contribution of $Bie^{-\bar{P}t}$ becomes nil), the value of Bc as defined by Eq. 11 approaches a maximum limiting value, $Bc_{max.}$, just after the administration of the last mean maintenance dose p.u.a.:

$$Bc_{max.} = \overline{Bm}\left(\frac{1}{1 - e^{-\bar{P}\tau}}\right) \qquad \text{(Eq. 12)}$$

and a minimum limiting value, $Bc_{min.}$, just before the administration of the next mean maintenance dose p.u.a.:

$$Bc_{min.} = \overline{Bm}\left(\frac{e^{-\bar{P}\tau}}{1 - e^{-\bar{P}\tau}}\right) \qquad \text{(Eq. 13)}$$

Most of the equations described above have been discussed in connection with blood or serum drug concentration changes following intravenous, i.m., and oral drug administration (7–10), but have not been discussed in connection with changes in drug concentration at the s.c. absorption site as a function of time.

An equation for calculating the limiting value for $\overline{Bc}$, the asymptotic mean amount of drug in the cell p.u.a., can be derived using the values of $Bc_{max.}$ and $Bc_{min.}$ (11). The area under the curve from $Bc_{max.}$ to $Bc_{min.}$ over the time interval of one τ can be calculated from:

$$\text{area} = \frac{(Bc_{max.} - Bc_{min.})\tau}{\ln (Bc_{max.}/Bc_{min.})} \qquad \text{(Eq. 14)}$$

Substitution of Eqs. 12 and 13 into Eq. 14, and subsequent division by the dosing time interval, τ, yields:

$$\overline{Bc} = \frac{\overline{Bm}}{\bar{P}\tau} \qquad \text{(Eq. 15)}$$

Since $\bar{P}$ equals $(\ln 2/\overline{t_{0.5}})$, where $\overline{t_{0.5}}$ is the drug's mean absorption half-life in the cell, Eq. 15 can be written:

$$\overline{Bc} = \frac{\overline{Bm}}{(\tau/\overline{t_{0.5}})\ln 2} \qquad \text{(Eq. 16)}$$

A more general expression than Eq. 16 was derived by Wagner and co-workers (12) for drug blood levels at the equilibrium state after multiple dosing.[1] The term, $\overline{Bc}$, can also be calculated from:

$$\overline{Bc} = Bc_{max.}e^{-\bar{P}Z\tau} \qquad \text{(Eq. 17)}$$

where Z is the fractional part of τ at which time $\overline{Bc}$ is reached following the last maintenance dose p.u.a. given at a time infinite in terms of the experiment. Substitution of Eq. 12 into Eq. 17 yields:

$$\overline{Bc} = \overline{Bm}\left(\frac{1}{1 - e^{-\bar{P}\tau}}\right)e^{-\bar{P}Z\tau} \qquad \text{(Eq. 18)}$$

Substitution of Eq. 15 into Eq. 18, and solving for the term Z yields:

$$Z = \frac{1}{\bar{P}\tau}\ln\left(\frac{\bar{P}\tau}{1 - e^{-\bar{P}\tau}}\right) \qquad \text{(Eq. 19)}$$

Equation 19 can also be written in terms of the drug's absorption half-life:

$$Z = \frac{1}{(\tau/\overline{t_{0.5}})\ln 2}\ln\left[\frac{(\tau/\overline{t_{0.5}})\ln 2}{1 - e^{-(\tau/\overline{t_{0.5}})\ln 2}}\right] \qquad \text{(Eq. 20)}$$

The magnitude of the unitless Z term is a complex function of the ratio $\tau/\overline{t_{0.5}}$ and is dose independent. The significance of the term Z will be indicated under *Results and Discussion*.

[1] Equation 16 of this paper and Eq. 6 found in Wagner and co-workers' report (12) are mathematically similar, but are different in some important respects. Their Eq. 6 is $V\bar{C} = 1.44\,F\,D\,t_{1/2}/\tau$, where V is the apparent volume of distribution of the drug, $\bar{C}$ is the average asymptotic blood concentration, F is the fraction of each dose which is absorbed, $t_{1/2}$ is the biological half-life of the drug in a particular animal or individual, and τ is the length of the dosing interval (12). The two equations differ in terms of their definitions of half-life. In Eq. 16 the half-life is for absorption, while in Eq. 6 above it is the biological half-life. In Eq. 16 the term $\overline{Bm}$ equals the magnitude of the product of the terms F and D found in Eq. 6 above only when the fraction of each dose absorbed is equal to one. The term (VC) is the average amount of the drug in the "body" during a dosage interval at the steady state, whereas $\overline{Bc}$ is the asymptotic mean amount of drug in the s.c. cell p.u.a. during a dosing interval.

MATERIALS AND METHODS

Animals—Large Sprague-Dawley female rats weighing between 400 and 500 Gm. were deliberately chosen for this study because the drug's distribution volume in them would be large. After the animals were anesthetized with ethyl ether, a circular portion of the ventral skin approximately 3 cm. in diameter was carefully excised, exposing the s.c. connective tissue. The exposed region was anatomically at about the same location as that described in previous work with s.c. implants (13). As the experiment progressed, the amount of anesthetic had to be decreased to prevent death.

Reagents—The BA used was reagent grade (Merck) with a boiling point of 205°/752 mm. which agrees closely with that reported in the literature (14, 15). The complete solution of BA in 0.9% (w/v) aqueous sodium chloride or normal saline solution (NS) was effected by warming and stirring the mixture on a steam bath for about 1 hr. and then allowing it to cool to room temperature. The *n*-hexane used as the extraction solvent in the assay was of spectrographic grade[2] with an ultraviolet cut-off of 199 mμ or less.

Subcutaneous Absorption Cell—A cylindrical glass absorption cell was constructed to hold the drug solutions in contact with the s.c. tissue. It was about 21 mm. high and had an inside diameter of 17.2 mm. At one end of this cell was a rim having an outside diameter of about 26 mm. The part of the rim adjacent to the s.c. tissue was ground flat. The inner cross-sectional area of the cell was 2.322 cm.^{2}.

Adhesive—The rim of the absorption cell was attached to the moist s.c. tissue by a medical silicone adhesive[3] commercially available as a developmental aerosol formulation in a pressurized container. The use of silicones in biological research has been recently reviewed (16). According to the manufacturer, this adhesive appears to be completely inert to living tissue and will not cause irritation or sensitization. The adhesive qualities are retained in the presence of moisture. The only solvent present is a volatile fluorocarbon propellent,[4] which evaporates after a few minutes. The adhesive could not be sprayed accurately onto the cell's narrow glass rim. A special nozzle supplied by the company was inserted into the spray orifice. The nozzle was directed into an inverted 9.52 mm. diameter ophthalmic ointment tube with cap in place. The tube was nearly filled with the adhesive, allowed to stand for about 5 min., and placed in a dry oven at 104° until it reached a suitable semisolid consistency which usually took about 15 min. The ointment tube was cooled to room temperature and crimped in the usual manner. The adhesive could now be applied directly to the rim from the small orifice at the tube tip. Under experimental conditions this adhesive made the glass rim adhere to the moist tissue and formed a water-tight seal which prevented the solution from spreading onto the surrounding regions.

Assay—A method for determining the amount of BA in NS has been reported previously (15). However, the procedure is too tedious to perform routinely and not sufficiently sensitive for the present purposes. Instead, an assay procedure was developed which involved the extraction of BA from a small sample of the fluids present in the absorption cell into a suitable solvent and analyzing the resulting mixture by ultraviolet spectrophotometry. The extraction step was required because proteins qualitatively similar to those which could be present in the absorption cell shortly after the experiment had begun have chromophores that absorb in the same 205–210 mμ region of the U.V. as does BA in aqueous solution (17). According to the literature BA has a $\lambda_{max.}$ of 210 mμ (log ϵ = 3.88) in methanol (18) and 208 mμ (log ϵ = 3.83) in ethanol (19). Preliminary determinations using a Cary spectrophotometer, model 11, having a continuous recording attachment showed that BA dissolved in distilled water and spectrographic grade *n*-hexane had a $\lambda_{max.}$ of 208 mμ in both solvents. The values for log ϵ in water and *n*-hexane, respectively, were 3.82 and 3.88. Stock solutions were prepared containing 1 to 4% (w/v) BA dissolved in NS. Standard curves for the absorbance of BA extracted into *n*-hexane *versus* the BA concentration in NS were made by the method of least squares. The two instruments used were the Cary spectrophotometer, model 11, and the Beckman spectrophotometer, model DU, with a hydrogen lamp accessory. A 20-μl. sample of a saline blank and each stock solution was added to 50-ml. glass-stoppered volumetric flasks using a 25-μl. Hamilton syringe.[5] Three milliliters of NS was then added in order to rinse the drug sample down from the sides of the flask. Duplicate samples were run in each case. About 25 ml. of spectrographic grade *n*-hexane was added to each flask. It was stoppered and shaken for 0.5 hr. on a mechanical shaker. Afterward, sufficient *n*-hexane was added to bring the total volume up to the 50-ml. mark, and the shaking was repeated for another 0.5 hr. Samples of the *n*-

TABLE I—ULTRAVIOLET ABSORBANCE OF BENZYL ALCOHOL

Concn. of BA in NS, mg./ml.	Amt. of BA in Flask, μm.[a]	Absorbance in Hexane Extract at 208 mμ Cary Model 11[b]	Absorbance in Hexane Extract at 208 mμ Beckman Model DU[b]	Calcd. Concn. of BA in NS, mg./ml.[c]
9.9	1.83	...	0.223	9.6
10.0	1.85	0.205	...	10.0
15.1	2.79	...	0.342	15.1
19.5	3.61	...	0.454	20.1
20.0	3.70	0.425	...	19.9
24.6	4.55	...	0.554	24.7
30.0	5.55	0.655	...	30.1
35.0	6.47	...	0.778	34.9
40.0	7.40	0.875	...	40.0

[a] The amount of BA contained in 20 μl. of the solution listed in column 1 partitioned between 3 ml. of NS and sufficient *n*-hexane to make 50 ml. of total solution. [b] The equation for the standard curve by the least squares method in the Cary instrument is: concn. (mg./ml.) BA = (absorbance + 0.020)/0.0224 and that in the Beckman instrument is: concn. (mg./ml.) BA = (absorbance − 0.011)/0.0220. [c] The concentrations were computed using the appropriate standard curve equations for the respective instruments.

[2] Spectroquality reagent, Matheson, Coleman and Bell, East Rutherford, N. J.
[3] Dow Corning Medical Adhesive, type B, Midland, Mich.
[4] Freon. E. I. duPont de Nemours and Co., Wilmington, Del.
[5] Hamilton Co., Whittier, Calif.

hexane layer were removed from each flask and were read in the respective instruments at 208 mμ against the *n*-hexane blank similarly treated with NS solution. The absorbances in *n*-hexane were proportional to BA concentration in NS from 9.9 to 40 mg./ml. (See Table I.) Potential problems of stray radiation at this wavelength were investigated (20). Using the method described by Ofler (21), calculations were made with the intent to improve the linearity of the absorbance *versus* concentration standard curves mentioned above. No improvement in linearity could be achieved. Column 5 of Table I shows with both spectrophotometers that the calculated values of drug concentration using the respective standard curves agreed closely with the actual concentrations listed in column 1 of Table I.

The question of whether the silicone adhesive might interfere with the BA assay was investigated. The insides of two 1.9 cm. diameter test tubes were sprayed with a thin film of the silicone adhesive and allowed to stand until the volatile solvent had evaporated. Two milliliters of a 2.04% (w/v) BA stock solution in NS was added to each tube. One tube was shaken by hand intermittently for 4 hr. and the other for 24 hr. Then 20-μl. samples of these solutions, the original stock solution, and the NS blank solution were transferred to 50-ml. volumetric flasks and assayed according to the procedures described above. All determinations were made in triplicate. There was no significant difference between the absorbance values for the BA stock solutions exposed to the silicone adhesive and the BA stock solution not so exposed. It was concluded that the adhesive did not interfere with the assay at the BA concentration used.

Penetration Coefficient Determinations—Shortly after the absorption cell was securely affixed to the s.c. tissue, 0.5 ml. of NS preheated to 28° was introduced into the cell to ensure that the exposed tissue would remain moist before the introduction of the BA solution. A hollow glass rod 5.1 mm. in diameter and 34 cm. long was fastened by a rubber band to the metal spatula of a vibrating stirrer.[6] To avoid splashing of the test solution and injury to the s.c. tissue, one end of a piece of glass wool was twisted to a fine tip and the other end was attached to the glass rod by another rubber band. The strand of vibrating glass wool was introduced into the test solution. After about 10 min., 1.0 ml. of 2.04% (w/v) BA dissolved in NS also preheated to 28° was pipeted into the absorption cell. This was zero time in terms of this experiment. Four to five 20-μl. samples of solution were withdrawn each 0.5 hr. indicated in Table II. Each 20-μl. sample was transferred to a 50-ml. glass-stoppered volumetric flask and assayed for BA content according to the method described previously. At the end of each 0.5-hr. period, 0.1 ml. of 3.42% (w/v) BA in NS solution was added to the cell. From time to time the accuracy of the sampling technique with the Hamilton syringe was checked. The syringe containing the aliquot was weighed on a Sartorius analytical balance, and reweighed after the sample was transferred for the U.V. assay. In all cases the difference between the two weights corresponding to the volume of sample delivered (1 μl. = 1 mg.) agreed with that estimated from the syringe barrel markings within ±1%.

The magnitude of both the mean diffusion and partition coefficients and the resulting penetration coefficient as defined by Eq. 5 may be temperature dependent. It was therefore necessary to determine what the mean solution temperature was in the cell and the magnitude of possible fluctuations during the experiment. The temperature of the BA solution in one anesthetized animal was continuously recorded,[7] using a small thermocouple probe introduced into the cell. The thermocouple and accompanying equipment was specially designed[8] for routine pyrogen testing of parenteral solutions. After about 15 min., thermal equilibrium between the animal, the absorption cell contents, and the room environment was established. The temperature of the solution oscillated about a mean value of 28.3 ± 0.2°. In a parallel *in vitro* experiment the same absorption cell was affixed to a metal block heated to constant temperature, and no measurable amount of evaporation from the stirred solution used above was found when maintained at 28° for 3 hr. For each 0.5-hr. period, an estimate of the penetration coefficient, P, was made by plotting the logarithm of the amount of BA p.u.a. in the cell at any time, against the sampling times shown in Table II and by multiplying by −2.303 the resultant slope obtained by the least squares method. In this study all computations were programmed for the Wyle calculator.[9] In some cases in Tables II through IV extra digits obtained by computational means and indicated by subscripts, are listed to minimize rounding off errors in subsequent calculations, and do not imply that four significant figure experimental accuracy was achieved.

In a few cases each reading for the amount of BA p.u.a. was determined according to the weighting method described by Paradine and Rivett (22) to determine the value for P again. This latter method was discontinued, since the values for P calculated by this method seldom differed from the previous one by more than one digit in the third decimal place. The half-life values reported in Table III were calculated from the relationship: $t_{0.5} = \ln 2/P$.

Volume Determinations—The calculated intercepts of drug concentration in the cell at the beginning and end of each 0.5-hr. period were used to estimate any possible volume changes in the cell as a function of time. The values in Table II are expressed in terms of amount of BA p.u.a. and are reconvertible to units of concentration (mg./ml.) by the equation:

$$\text{concn. BA (mg./ml.)} = Bc\ (\text{mg./cm.}^2) \times \frac{A\ (\text{cm.}^2)}{\bar{V}_c\ (\text{ml.})} \quad \text{(Eq. 21)}$$

where A is the tissue area of 2.322 cm.^{2} exposed to the drug solution, and $\bar{V}_c$ is the mean volume of solution tabulated in column 9 of Table III for the respective animals. One could calculate the final concentration, C_f, of BA at the *end* of a given 0.5-hr. time from the 0.5-hr. intercepts of the regression line computed from the logarithm of BA concentra-

[6] Vibro Spatula, catalog No. 57900, Van Waters and Rogers, Brisbane, Calif.

[7] Speedomax, type G, Leeds and Northrup.

[8] Research and Development Laboratory, University of California, San Francisco Medical Center.

[9] Wyle Laboratories, El Segundo, Calif.

TABLE II—ABSORPTION DATA ON BENZYL ALCOHOL IN SOLUTION FROM THE SUBCUTANEOUS SITE

Clock Time, hr.	mg. BA at the Absorption Site/Unit Area of Exposed Tissue			
	Animal A	Animal B	Animal C	Animal D
		Second 0.5-hr. Run		
0.500[a]	7.52[b] (7.39$_4$; 6.86$_9$)[c]	6.67 (6.73$_6$; 6.55$_4$)	5.72 (5.68$_0$; 5.49$_5$)	5.51 (5.47$_4$; 5.31$_8$)
0.583	...	6.50 (6.43$_9$; 6.31$_1$)	...	...
0.600	...	...	5.24 (5.32$_5$; 5.19$_5$)	...
0.617	...	...	...	5.09 (5.15$_8$; 5.05$_3$)
0.633	6.95 (7.11$_1$; 6.75$_6$)	...	...	...
0.717	...	5.97 (5.98$_5$; 5.87$_3$)	5.01 (4.93$_7$; 4.84$_1$)	...
0.767	...	...	...	4.84 (4.77$_9$; 4.70$_1$)
0.800	6.74 (6.77$_0$; 6.44$_5$)	...	...	...
0.833	...	5.61 (5.61$_9$; 5.45$_8$)	4.55 (4.58$_0$; 4.47$_3$)	...
0.867	...	...	...	4.51 (4.54$_3$; 4.45$_2$)
0.967	6.50 (6.44$_6$; 5.96$_3$)	...	4.21 (4.19$_9$; 4.05$_4$)	...
0.984	...	...	...	4.29 (4.28$_2$; 4.16$_3$)
		Third 0.5-hr. Run		
1.000	7.44 (7.39$_9$; 6.93$_9$)	6.28 (6.33$_7$; 6.11$_0$)	5.32 (5.33$_1$; 5.31$_3$)	5.26 (5.24$_2$; 4.99$_9$)
1.100	...	...	...	4.86 (4.96$_6$; 4.79$_4$)
1.117	...	6.05 (6.04$_6$; 5.89$_1$)	4.92 (4.91$_7$; 4.90$_5$)	...
1.150	7.17 (7.14$_1$; 6.83$_7$)	...	...	...
1.233	...	...	...	4.71 (4.62$_2$; 4.49$_4$)
1.250	...	5.78 (5.73$_2$; 5.61$_3$)	...	...
1.267	...	...	4.43 (4.43$_2$; 4.42$_4$)	...
1.317	6.72 (6.86$_3$; 6.57$_7$)	...	...	...
1.350	...	...	...	4.39 (4.33$_8$; 4.19$_2$)

(*Continued on next page.*)

tions *versus* sampling times. This equals

$$C_f = B_f/V_f \qquad \text{(Eq. 22)}$$

where B_f is the final amount of BA dissolved in the final volume, V_f, at that time. The initial concentration, C_i, of drug at the *beginning* of the next 0.5-hr. period following the addition of more BA in the amount ΔB dissolved in a NS solution with a volume, ΔV, may be calculated from the zero intercept of the regression line for the next 0.5-hr. period. This equals:

$$C_i = (B_f + \Delta B)/(V_f + \Delta V) = B_i/V_i \qquad \text{(Eq. 23)}$$

where B_i is the initial amount of BA dissolved in the initial volume, V_i, at the initial time for the next 0.5-hr. period. If the values for ΔB and ΔV are

TABLE II—(*Continued.*)

Clock Time, hr.	mg. BA at the Absorption Site/Unit Area of Exposed Tissue			
	Animal A	Animal B	Animal C	Animal D
1.367	...	5.56 (5.46_9; 5.33_0)	4.14 (4.13_5; 4.12_6)	... ...
1.467	...	...	...	4.01 (4.07_1; 3.88_1)
1.484	6.68 (6.59_7; 6.17_6)	5.13 (5.21_8; 5.03_4)	3.81 (3.81_5; 3.80_2)	...
Fourth 0.5-hr. Run				
1.500	7.68 (7.68_3; 7.58_4)	6.05 (6.08_9; 5.94_5)	4.81 (4.79_6; 4.72_5)	5.09 (5.12_9; 4.90_4)
1.600	...	5.86 (5.87_2; 5.76_8)	4.41 (4.44_2; 4.39_2)	...
1.633	7.38 (7.38_9; 7.32_3)	...	...	4.81 (4.79_6; 4.65_2)
1.717	...	...	4.09 (4.06_0; 4.02_4)	...
1.734	...	5.64 (5.59_4; 5.51_4)	...	4.66 (4.56_2; 4.44_7)
1.800	7.07 (7.03_7; 6.97_6)	...	...	...
1.850	...	5.42 (5.36_2; 5.27_1)	3.66 (3.66_6; 3.62_5)	4.22 (4.30_5; 4.17_6)
1.967	6.68 (6.70_2; 6.61_3)	...	3.35 (3.35_2; 3.29_9)	...
1.984	...	5.05 (5.10_7; 4.98_2)	...	4.04 (4.02_6; 3.84_9)
Fifth 0.5-hr. Run				
2.000	7.58 (7.58_8; 7.46_6)	6.17 (6.14_4; 6.02_0)	4.43 (4.42_8; 4.33_5)	5.19 (5.18_2; 5.09_5)
2.100	...	...	4.06 (4.09_1; 4.02_6)	...
2.117	...	5.83 (5.88_8; 5.80_3)	...	...
2.133	7.32 (7.33_5; 7.25_2)	...	...	4.79 (4.82_6; 4.77_2)
2.233	...	5.64 (5.64_3; 5.57_7)	3.69 (3.68_1; 3.63_6)	4.61 (4.57_6; 4.53_2)
2.300	7.07 (7.03_1; 6.95_5)	...	...	...
2.334	...	5.50 (5.44_3; 5.36_8)	...	...
2.350	...	...	...	4.31 (4.30_1; 4.25_8)
2.367	...	...	3.35 (3.31_1; 3.25_9)	...
2.467	6.72 (6.73_8; 6.63_9)	...	3.03 (3.05_9; 2.99_5)	...
2.484	...	5.13 (5.15_3; 5.04_6)	...	3.99 (4.00_5; 3.93_9)

[a] The experimental points for the first and last 0.5-hr. periods are not included in this table. Consult text for discussion. [b] This value may be converted back to the original concentration of drug in the cell by Eq. 21. [c] The first number within the parentheses is the value for the amount of BA per cm.^{2} of absorption site as calculated from the equation for the regression line derived from the experimental values. The second number is the value for the lower limit of the 95% confidence limit. The upper and lower 95% confidence limits are equidistant from the first number in the parenthesis on a logarithmic scale (23).

known in Eq. 23, one can calculate the values for V_f and B_f from:

$$V_f = \left[\frac{\Delta B - (\Delta V)(C_i)}{C_i - C_f}\right] \quad \text{(Eq. 24)}$$

and

$$B_f = C_f\left[\frac{\Delta B - (\Delta V)(C_i)}{C_i - C_f}\right] \quad \text{(Eq. 25)}$$

The amounts of ΔB and ΔV added to the cell were kept constant and were 3.42 mg. and 0.10 ml., respectively. The mean volume, $\bar{V}'$, during any 0.5-hr. interval is:

$$\bar{V}' = (V_i' + V_f')/2 \quad \text{(Eq. 26)}$$

where the primes over each quantity indicate that

TABLE III—HALF-LIVES OF BA, MEAN VOLUMES OF SOLUTION IN THE ABSORPTION CELL, AND MEAN VALUES FOR Bm

Animal		0.5-hr. Intervals 1	2	3	4	5	6	Mean	$\overline{Bm}$[i] (mg./cm.²)
A	$t_{0.5}$[a]	(2.15)[b]	2.36	2.92	2.37	2.73	(2.88)[b]	2.58_4[c]	1.00_5
	$\overline{V}'$[d]	...	1.60	1.47	1.49	1.59	...	$1.53_8(0.10_4)$[e]	
	$B_{max.}$[f]	...	(7.39_4)	7.39_9	7.68_3	7.58_8	...	7.55_6[g]	
	$B_{min.}$[h]	...	6.46_6	6.47_1	6.71_7	...	...	6.55_1	
B	$t_{0.5}$	(1.37)	1.27	1.73	1.91	1.91	(1.96)	1.79_5	0.92_3
	$\overline{V}'$	...	1.28	1.45	1.56	1.41	...	$1.42_6(0.18_4)$	
	$B_{max.}$	...	(6.73_6)	6.33_7	6.08_9	6.14_4	...	6.19_0	
	$B_{min.}$	...	5.55_4	5.22_5	5.02_1	...	...	5.26_7	
C	$t_{0.5}$	(1.06)	1.07	1.00	0.90	0.79	(0.82)	0.95_7	1.18_3
	$\overline{V}'$	...	1.46	1.48	1.51	1.40	...	$1.46_2(0.07_9)$	
	$B_{max.}$	...	(5.68_0)	5.33_1	4.79_6	4.42_8	...	4.85_1	
	$B_{min.}$	...	3.95_5	3.71_1	3.33_9	...	...	3.66_8	
D	$t_{0.5}$	(1.10)	1.37	1.28	1.39	1.30	(1.61)	1.33_4	1.11_1
	$\overline{V}'$	...	1.31	1.30	1.24	1.23	...	$1.27_4(0.07_3)$	
	$B_{max.}$	...	(5.47_4)	5.24_2	5.12_9	5.18_2	...	5.18_4	
	$B_{min.}$	...	4.22_2	4.04_2	3.95_6	...	...	4.07_3	

[a] Absorption half-life (hr.) of drug in the cell. [b] The half-lives for the first and last 0.5-hr. runs appear in parentheses and were calculated on the assumption that the mean volume in the cell at these time periods was identical to the mean volume listed in column 9 for the 2nd through 5th half-hour intervals. [c] Mean half-life, $\overline{t_{0.5}}$, of intervals two through five. The mean penetration coefficient, $\bar{P}$, for animal D, for example, equals $\ln 2/1.33$ hr. or 0.52 hr.$^{-1}$. [d] Mean volume (ml.), $\overline{V}'$, calculated according to Eq. 26 for each 0.5-hr. interval. [e] Mean volume, $\overline{Vc}$, for intervals two through five followed by the plus and minus values for the 95% confidence limits about the mean in parentheses. [f] The values of $B_{max.}$ (mg./cm.²) are the calculated intercepts of the equation for the regression line and are identical to values shown in Table II at the beginning of the respective 0.5-hr. intervals. [g] Mean of the three values of $B_{max.}$. The value in parentheses is not included in this mean, but was used to calculate $B_{min.}$ at the end of the second interval. [h] The values of $B_{min.}$ (mg./cm.²) were calculated from Eq. 27. [i] The value of $\overline{Bm}$ was calculated by subtracting the mean of $B_{min.}$ from the mean of $B_{max.}$ shown in column 9.

TABLE IV—COMPUTED AMOUNTS OF BENZYL ALCOHOL IN THE ABSORPTION CELL AT VARIOUS TIMES AND OTHER RELEVANT INFORMATION

Constant		Animal A	B	C	D
$\tau/\overline{t_{0.5}}$[a]		0.193	0.278	0.522	0.375
Bi (mg./cm.²)[b]		7.39	6.74	5.68	5.47
$\overline{Bm}$ (mg./cm.²)[c]		1.00_5	0.92_3	1.18_3	1.11_1
$Bi/\overline{Bm}_{expt.}$[d]		7.35	7.30	4.80	4.92
$Bi/\overline{Bm}_{calcd.}$[e]		7.97	5.70	3.29	4.37
$\overline{Bc}_{max.}$ (mg./cm.²)[f]		8.00_3	5.25_8	3.89_4	4.85_7
$\overline{Bc}$ (mg./cm.²)[g]		7.49	4.78	3.27	4.28
Z[h]		0.494	0.493	0.485	0.490
$B_{min.}$ (mg./cm.²)[i] At end of	1 τ	6.46	5.55	3.96	4.22
	2 τ	6.53	5.34	3.58	4.11
	4 τ	6.64	5.02	3.13	3.96
$\overline{Bc}_{min.}$ (mg./cm.²)[j]		7.00_3	4.33_5	2.71_1	3.74_6
No. of total doses p.u.a. needed to approach within 1% of $\overline{Bc}_{min.}$ shown above[k]		>16	>18	>11	>10

[a] The value of τ is the experimental one of 0.5 hr. as shown in Table II, and the value of $\overline{t_{0.5}}$ is the mean value found in column 9 of Table III. [b] The initial amount of drug/cm.² at the absorption site as calculated from the regression line equation. This is the first number within the parentheses at 0.5 hr. in column 2 of Table II. [c] See column 10 of Table III. [d] The experimental value of the ratio calculated from items 2 and 3 of this column. [e] The calculated value of the ratio at infinite time from Eq. 31 using the ratio of $\tau/\overline{t_{0.5}}$ tabulated as item 1 in this column. [f] The calculated maximum value at infinite time from Eq. 12. [g] The calculated value for the asymptotic mean value at infinite time from Eq. 16, using the ratio of $\tau/\overline{t_{0.5}}$ found as item 1 of this column. [h] Calculated from Eq. 20. [i] Calculated minimum value using Eq. 11 up to four τ. [j] Calculated according to Eq. 13. [k] Calculated from the appropriate form of Eq. 11.

the initial and final calculated volumes are from the same 0.5-hr. time interval.

RESULTS AND DISCUSSION

The results of the *in vivo* absorption experiments are presented in Tables II through IV and Fig. 1. The experiment for each animal lasted 3 hr. Table II shows the experimental values for the amount of BA in the absorption cell p.u.a. of s.c. tissue exposed to the drug solution as a function of time. It also lists the calculated values of the amount of BA p.u.a. from the regression line for each 0.5 hr., and the lower value for the 95% confidence limit (23) for each calculated point all at the same sampling times. In Table II no data points are presented for the zero to 0.5 and the 2.5 to 3-hr. time intervals, although four to five samples were withdrawn from the absorption cell and analyzed for BA content in the same manner as samples taken during the intervening 0.5-hr. intervals. The main reason for excluding details of the data for the first and sixth 0.5-hr. intervals is that mean volume calculations for these two runs could not be made according to Eq. 26, because values for V_i and V_f were not determined. At zero time no estimate of V_i could be made according to the experi-

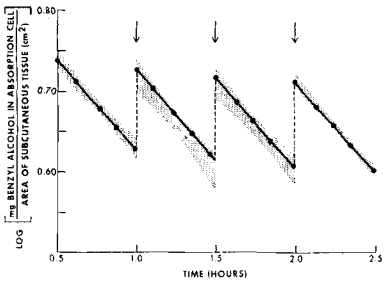

Fig. 1—The calculated logarithmic amount of BA present at the subcutaneous site per unit area as a function of dosing regimen and time for animal D. The solid lines were calculated using Eq. 11 where the value for $\bar{P}$ *is 0.52 hr.*$^{-1}$, B_i *is 5.47 mg./cm.*^{2}, $\bar{B}_m$ *is 1.11 mg./cm.*^{2}, τ *is 0.5 hr., and* n *is four. The arrows indicate the times of administration of maintenance doses. The upper and lower boundaries of the shaded areas are the logarithmic values for the 95% confidence limits* vs. *time about the regression line for the experimental points for each 0.5 hr.* (*See Table II.*)

mental procedures used, and at the end of 3 hr. no estimate of V_f could be made according to Eq. 24, since another maintenance dose was not given at that time.

In Table III the half-life of BA for each animal was calculated during each 0.5-hr. period from 0.5 to 2.5 hr. and is shown in columns 4 through 7. For each animal, the mean half-life, $\overline{t_{0.5}}$, shown in column 9 was calculated using *all* data points for the intervals from 0.5 to 2.5 hr. To do this, the data given in Table II were transposed in the following manner. The ordinate values at 0.5, 1.0, 1.5, and 2.0 hr. as obtained from the least square fit of the points for the 0.5-hr. intervals were divided into all the experimental points for that 0.5 hr. Thus, there was a common reference point for each of the four runs. Then, for purposes of calculation, the times of 0.5, 1.0, 1.5, and 2.0 hr. were all made equal to a zero clock time. For each animal $\overline{t_{0.5}}$ was computed by the least squares method from the slope of the plot of the logarithm of *Bc versus* time using all transposed experimental points listed in Table II. This mean half-life differed only slightly from the arithmetic mean half-life of the four runs because of weighting effects (22) and possible errors due to numerical rounding off. The half-lives for the first and last 0.5-hr. time periods are also included in Table II in parentheses. They were calculated on the assumption that the mean volume in the cell during these periods was identical to the mean volume shown in column 9 for the 0.5 to 2.5-hr. periods. The values of $\overline{t_{0.5}}$ for BA at the s.c. absorption site for all animals range from 0.96 to 2.58 with an average of about 1.6 hr. This mean is little affected even when one includes the values in parentheses.

In general, the variations in half-lives for the four runs in any one animal are less than those found among all animals. Probably the larger inter-animal variations in $\overline{t_{0.5}}$ are due to differences in membrane thickness. With the surgical procedures used in this study there was no way to ensure that all animals had the same membrane thickness at the absorption site. In Eq. 4, the penetration coefficient is inversely proportional to the membrane thickness, and therefore the half-life is directly proportional to membrane thickness. There is no easy way to explain the variations in half-lives with successive doses in any one animal, since membrane thickness in each case would be constant. Apart from analytical errors, the one possible reason for the half-life differences in successive 30-min. periods might be traceable to flow rate changes in the microcirculation supplying the s.c. site (24). It is not known at present how and to what extent the regional blood and lymph flows near the absorption site are affected at various times by changes in blood concentrations of ethyl ether and BA in the animal.

Table III shows the mean volume, $\bar{V}'$, of the solution in the cell during each 0.5-hr. period from 0.5 to 2.5 hr. in columns 4 through 7 as calculated from Eq. 26. The arithmetic mean values, $\bar{V}_c$, of these volumes for each animal are given in column 9 along with the plus and minus 95% confidence limits about the mean. The range of the 95% confidence limits for these volumes for the four animals is 0.07 to 0.18 ml. These small variations indicate that the volume of the BA solution in the cell remains essentially constant throughout the experiment. Equation 4 is therefore valid in so far as constancy of volume is concerned.

Table III also shows the calculated values of $B_{max.}$ and $B_{min.}$ The terms $B_{max.}$ are the initial intercepts of the equation for the regression line calculated from the logarithm of the BA amounts p.u.a. *versus* time at the beginnings of the third through fifth time intervals (see Table II). The terms $B_{min.}$ are the final intercepts during the second through fourth 0.5-hr. periods using the mean penetration coefficient, $\bar{P}$, in the equation:

$$B_{min.} = B_{max.}e^{-\bar{P}\tau} \qquad \text{(Eq. 27)}$$

where τ is equal to 0.5 hr. The mean value of the maintenance dose p.u.a., $\overline{Bm}$, was calculated by subtracting the mean values of $B_{min.}$ and $B_{max.}$ shown in column 9 of Table III. These values for $\overline{Bm}$ for each animal were used in Table IV to calculate the asymptotic values for $Bc_{max.}$ and $Bc_{min.}$ at times infinite in terms of these experiments.[10]

Multiple Dosing—Multiple dosing of the drug was used in this study for the following reasons: (*a*) the concentration of BA in the absorption cell could be maintained at a level much higher than that found at all times in the animal's fluids of distribution so that the assumption made in connection with Eq. 3 would be valid; (*b*) the volume of drug solution in the cell could be maintained nearly constant by replacing solution lost through the frequent sampling; (*c*) the drug could be used as its own "marker" for detecting possible volume changes in the cell as a function of time, and (*d*) several independent determinations of absorption half-life of BA in the cell could be made in the same animal under comparable experimental conditions.

There may be instances under multiple dosing

[10] Note that $Bc_{max.}$ and $Bc_{min.}$ are constant and can be calculated from Eqs. 12 and 13, respectively, whereas both $B_{max.}$ and $B_{min.}$ are not constant except at infinite time when $B_{max.} = Bc_{max.}$ and $B_{min.} = Bc_{min.}$ provided that the original maintenance dosing regimen is continued.

conditions where it is desirable to establish constant values for the maximum and minimum amounts of drug p.u.a. at the s.c. site at all times starting with the initial dose. At the beginning of the experiment, let:

$$Bc_{max.} = Bi \qquad \text{(Eq. 28)}$$

and at the end of the initial dosing interval of one τ, let:

$$Bc_{min.} = Bie^{-\bar{P}\tau} \qquad \text{(Eq. 29)}$$

Immediately after an infinite number of mean maintenance doses p.u.a. have been administered, the value for $Bc_{max.}$ can be calculated using Eq. 12. Substitution of Eq. 28 into Eq. 12 yields:

$$Bi = \overline{Bm}\left(\frac{1}{1 - e^{-\bar{P}\tau}}\right) \qquad \text{(Eq. 30)}$$

The same equation results when Eq. 29 is substituted into Eq. 13. The ratio of the respective doses p.u.a. is:

$$R = \frac{Bi}{\overline{Bm}} = \left(\frac{1}{1 - e^{-\bar{P}\tau}}\right) = \left[\frac{1}{1 - e^{-(\tau/\overline{t_{0.5}})\ln 2}}\right] \qquad \text{(Eq. 31)}$$

and is constant as long as the terms $\bar{P}$ and τ are constant.

Constant values for the maximum and minimum amounts of drug p.u.a. of tissue at the s.c. site can be maintained at all times by adjusting the ratio $Bi/\overline{Bm}$ in accordance to any fixed value of the ratio $\tau/\overline{t_{0.5}}$. For example, if the ratio $\tau/\overline{t_{0.5}}$ is fixed at unity, the ratio $Bi/\overline{Bm}$ must equal the value of two. In this case if the ratio $Bi/\overline{Bm}$ is greater than two, the amount of drug p.u.a. in the cell will decrease with time until the appropriate asymptotic values for $Bc_{max.}$ or $Bc_{min.}$ are reached. On the other hand, if the $Bi/\overline{Bm}$ ratio is less than two, the amount of drug p.u.a. in the cell will increase with time until the appropriate asymptotic values for $Bc_{max.}$ or $Bc_{min.}$ are reached.

One can see examples of this type of descending or ascending behavior in the data compiled for Table IV. With animal A the $\tau/\overline{t_{0.5}}$ ratio was 0.193 and the experimental $Bi/\overline{Bm}$ ratio was 7.35. This ratio was not large enough to maintain constant values for $B_{min.}$ (and $B_{max.}$) from the first dose on. Values for $B_{min.}$ increase gradually from a value of 6.46 at the end of the first dosing interval to the asymptotic value of 7.00 at the end of an infinite number of dosing intervals. If the $Bi/\overline{Bm}$ ratio of 7.97 calculated according to Eq. 31 had been used, then values for $Bc_{max.}$ and $Bc_{min.}$ would have remained at constant levels throughout the experiment on to an infinite number of doses. With animal D, the $\tau/\overline{t_{0.5}}$ ratio was 0.375 and the experimental $Bi/\overline{Bm}$ ratio was 4.92. This ratio was too large to maintain constant values for $B_{min.}$ (and $B_{max.}$) from the first dose on. Values for $B_{min.}$ decrease from a value of 4.22 at the end of the first dosing interval to the asymptotic value of 3.75 at the end of an infinite number of dosing intervals. According to calculations that can be made from Eq. 31, the correct $Bi/\overline{Bm}$ ratio should have been 4.37 instead of 4.92 to effect constant values for $Bc_{max.}$ or $Bc_{min.}$ from the first dose on. Results for animals B and C are similar in this regard to those for animal D.

The asymptotic mean amount of drug p.u.a., $\overline{Bc}$, can be calculated according to Eq. 15 when the number of doses p.u.a. approaches infinity. For animal A this value is 7.49. The value of $\overline{Bc}$ can be closely approximated by Eq. 32 only when the $\tau/t_{0.5}$ ratio is small:

$$\overline{Bc} \simeq \frac{Bc_{max.} + Bc_{min.}}{2} \qquad \text{(Eq. 32)}$$

From Eq. 17 one should be able to determine when the value of $\overline{Bc}$ is reached after the administration of the last mean maintenance dose p.u.a. at a time infinite in terms of the experiment. The value of $\overline{Bc}$ should be reached at some time, $Z\tau$, after $Bc_{max.}$ is reached. Solution of Eqs. 17 and 18 for Z yielded Eqs. 19 and 20. It should be evident from Eq. 20 that when the ratio $\tau/\overline{t_{0.5}}$ tends toward zero, or the drug is being administered by continuous infusion at a constant rate, the term Z tends toward a value of 0.5. On the other hand, when the ratio $\tau/\overline{t_{0.5}}$ tends toward infinity, or the drug is administered only once, the term Z tends toward zero. In Table IV the calculated values of Z for all animals is nearly equal to 0.5, since the $\tau/\overline{t_{0.5}}$ ratios are all small. Table IV also shows the total number of doses p.u.a. needed to approach within 1% of the values of $Bc_{min.}$ after the administration of an infinite number of doses p.u.a. By the use of Eq. 11, one can see that the value approaching 1% of $Bc_{min.}$ is reached with animals A through D in the range of more than 10 to 18 total doses.

Figure 1 represents some of the data given in Table II for animal D. The boundaries of the shaded regions represent plots of the logarithms of the calculated upper and lower 95% confidence limits of *Bc versus* time. The actual experimental points and the regression lines calculated from these points for each 0.5 hr. are omitted from this figure. The solid dots represent times at which samples were withdrawn from the absorption cell for analysis. The solid lines are plots of the logarithm of *Bc versus* time and were calculated from Eq. 11, where the initial dose p.u.a., Bi, is 5.47 mg./cm.^{2}, the mean value for the penetration coefficient, $\bar{P}$, is 0.52 hr.$^{-1}$, t is clock time from 0.5 to 2.5 hr., the mean value for the maintenance doses p.u.a., $\overline{Bm}$, is 1.11 mg./cm.^{2}, n is the total number of doses and ranges in whole numbers from one to four, τ is the dosing time interval of 0.5 hr., and the time, T, ranges from zero to 0.5 hr. after the last maintenance dose p.u.a. The arrows represent times at which the three mean maintenance doses p.u.a. were administered. The vertical dashed lines in Fig. 1 represent the magnitude of logarithm of $\overline{Bm}$. Figure 1 shows with animal D, when values for $\overline{Bm}$ and $\bar{P}$ are substituted into Eq. 11, that the straight lines generated by plotting the logarithm of *Bc versus t* all fall within the shaded areas. Qualitatively similar results are obtained with data treated in the same manner for animals A through C.

In conclusion, the procedures outlined in this report illustrate some quantitative methods of studying drug absorption from the s.c. site. Although BA in NS was used as a model drug system

for this work, one can reasonably expect that other drugs in the same or different vehicles could be similarly studied in various animal species in order to discover fundamental factors affecting drug absorption rate from the s.c. site.

REFERENCES

(1) Schou, J., *Pharmacol. Rev.*, **13**, 441(1961).
(2) "Industrial Hygiene and Toxicology," vol. II, Patty, F. A., ed., Interscience Publishers, Inc., New York, N. Y., 1949, pp. 874–876.
(3) Smyth, H. F., Jr., Carpenter, C. P., and Weil, C. S., *Arch. Ind. Hyg. Occupational Med.*, **4**, 119(1951).
(4) Jenner, P. M., Hagan, E. C., Taylor, J. M., Cook, E. L., and Fitzhugh, O. G., *Food Cosmet. Toxicol.*, **2**, 327 (1964).
(5) Robinson, R. A., and Stokes, R. H., "Electrolyte Solutions," 2nd ed., Academic Press Inc., New York, N. Y., 1959, pp. 256–261.
(6) Davson, H., "A Text Book of General Physiology," 3rd ed., Little, Brown and Co., Boston, Mass., 1964, pp. 275–280.
(7) Boxer, G. E., Jelinek, V. C., Thompsett, R., DuBois, R., and Edison, A. O., *J. Pharmacol. Exptl. Therap.*, **92**, 226(1948).
(8) Dost, F. H., "Der Blutspiegel. Kinetik der Konzentrationsabläufe in der Kreislaufflüssigkeit," George Thieme, Leipzig, Germany, 1953, pp. 224–251.
(9) Swintosky, J. V., Bondi, A., Jr., and Robinson, M. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 753(1958).
(10) Krüger-Thiemer, E., *J. Theoret. Biol.*, **13**, 212(1966).
(11) Riggs, D. S., "The Mathematical Approach to Physiological Problems," Williams and Wilkins Co., Baltimore, Md., 1963, pp. 161–163.
(12) Wagner, J. G., Northam, J. I., Alway, C. D., and Carpenter, O. S., *Nature*, **207**, 1301(1965).
(13) Ballard, B. E., and Nelson, E., *J. Pharmacol. Exptl. Therap.*, **135**, 120(1962).
(14) Costello, J. M., and Bowden, S. T., *Rec. Trav. Chim.*, **77**, 36(1958).
(15) Callaway, J., Jr., and Reznek, S., *J. Assoc. Offic. Agr. Chemists*, **16**, 285(1933).
(16) Braley, S., *Med. Electron. Biol. Eng.*, **3**, 127(1965).
(17) Goldfarb, A. R., Saidel, L. J., and Mosovich, E., *J. Biol. Chem.*, **193**, 397(1951).
(18) "Organic Electronic Spectral Data," vol. I, 1946–1952, Kamlet, M. J., ed., Interscience Publishers, Inc., New York, N. Y., 1960, p. 150.
(19) "Absorption Spectra in the Ultraviolet and Visible Region," vol. IV, Lang, L., ed., Academic Press Inc., New York, N. Y., 1963, pp. 541–542.
(20) Saidel, L. J., Goldfarb, R., and Kalt, W. B., *Science*, **113**, 683(1951).
(21) Ofler, A., *J. Opt. Soc. Am.*, **40**, 401(1950).
(22) Paradine, C. G., and Rivett, B. H. P., "Statistical Methods for Technologists," English Universities Press, London, England, 1962, pp. 183–186.
(23) Goldstein, A., "Biostatistics, An Introductory Text," Macmillan Co., New York, N. Y., 1964, pp. 139–143.
(24) Kety, S. S., *Am. Heart J.*, **38**, 321(1949).

Technical Articles

Automatic Determination of Ethinyl Estradiol in Pharmaceutical Preparations

By A. J. KHOURY* and L. J. CALI†

An automated method for determining ethinyl estradiol or ethinyl estradiol methyl ether is described. The method is based on the fluorescence exhibited by ethinyl estradiol when it is treated with 90 per cent sulfuric acid. The nonvarying time, temperature, and volume characteristics of an automatic analyzer (AutoAnalyzer) system have been used to study a number of variables which might affect the data. Specificity of the fluorescence method with respect to degradation products, interference from other estrogens and progestational agents, as well as repeatability and sensitivity of the method are discussed. Also presented are data on trade packages of a number of antifertility formulations. Samples can be analyzed using the automated method at a rate of 20 samples per hour as opposed to one or two samples per hour by manual techniques.

SINCE THE USE of progesterone and ethisterone (17α-ethinyltestosterone), was first described by Inhoffen and Hohlweg (1) a number of antifertility products have become available. All of these products contain ethinyl estradiol (EE) or its methyl ether (EEME) in combination with a progestational steroid hormone. EE is usually formulated into tablets at concentrations as low as 0.05% of the total tablet weight along with another steroid whose concentration is 50 to 500 times greater than that of EE. The analysis of EE in this environment has presented a difficult analytical problem. The U.S.P. method of analysis (2) is very time consuming, while the colorimetric method described by Urbanyi and Rehm (3) does not distinguish between EE and other estrogens, and also is not applicable to the methyl ether of EE. Paper chromatographic

Received January 20, 1967, from Wyeth Laboratories, Philadelphia, PA 19101
Accepted for publication May 22, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors gratefully acknowledge the technical assistance of Dr. Jay G. Rutgers during preparation of this paper.
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for this work, one can reasonably expect that other drugs in the same or different vehicles could be similarly studied in various animal species in order to discover fundamental factors affecting drug absorption rate from the s.c. site.

REFERENCES

(1) Schou, J., *Pharmacol. Rev.*, **13**, 441(1961).
(2) "Industrial Hygiene and Toxicology," vol. II, Patty, F. A., ed., Interscience Publishers, Inc., New York, N. Y., 1949, pp. 874–876.
(3) Smyth, H. F., Jr., Carpenter, C. P., and Weil, C. S., *Arch. Ind. Hyg. Occupational Med.*, **4**, 119(1951).
(4) Jenner, P. M., Hagan, E. C., Taylor, J. M., Cook, E. L., and Fitzhugh, O. G., *Food Cosmet. Toxicol.*, **2**, 327 (1964).
(5) Robinson, R. A., and Stokes, R. H., "Electrolyte Solutions," 2nd ed., Academic Press Inc., New York, N. Y., 1959, pp. 256–261.
(6) Davson, H., "A Text Book of General Physiology," 3rd ed., Little, Brown and Co., Boston, Mass., 1964, pp. 275–280.
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(8) Dost, F. H., "Der Blutspiegel. Kinetik der Konzentrationsabläufe in der Kreislaufflüssigkeit," George Thieme, Leipzig, Germany, 1953, pp. 224–251.
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(10) Krüger-Thiemer, E., *J. Theoret. Biol.*, **13**, 212(1966).
(11) Riggs, D. S., "The Mathematical Approach to Physiological Problems," Williams and Wilkins Co., Baltimore, Md., 1963, pp. 161–163.
(12) Wagner, J. G., Northam, J. I., Alway, C. D., and Carpenter, O. S., *Nature*, **207**, 1301(1965).
(13) Ballard, B. E., and Nelson, E., *J. Pharmacol. Exptl. Therap.*, **135**, 120(1962).
(14) Costello, J. M., and Bowden, S. T., *Rec. Trav. Chim.*, **77**, 36(1958).
(15) Callaway, J., Jr., and Reznek, S., *J. Assoc. Offic. Agr. Chemists*, **16**, 285(1933).
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(19) "Absorption Spectra in the Ultraviolet and Visible Region," vol. IV, Lang, L., ed., Academic Press Inc., New York, N. Y., 1963, pp. 541–542.
(20) Saidel, L. J., Goldfarb, R., and Kalt, W. B., *Science*, **113**, 683(1951).
(21) Ofler, A., *J. Opt. Soc. Am.*, **40**, 401(1950).
(22) Paradine, C. G., and Rivett, B. H. P., "Statistical Methods for Technologists," English Universities Press, London, England, 1962, pp. 183–186.
(23) Goldstein, A., "Biostatistics, An Introductory Text," Macmillan Co., New York, N. Y., 1964, pp. 139–143.
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methods (4) have been applied with some success but are limited due to the lack of accuracy and precision generally afforded by such procedures. Spectrofluorometric methods (5) while generally yielding specific data at low levels of concentration, can yield inaccurate data due to fluorescence quenching. Methods based on the Kober reaction (6) are inapplicable unless a separation technique is first applied. Talmage (7) described a gas chromatographic method for EE, but this procedure does not lend itself to analysis of large numbers of samples. A method which has not been reported previously but which is applicable to the analysis of EE in tablets of low dosage has been developed. The method is based on the chemically induced fluorescence which is manifested by EE in 90% H_2SO_4. The specificity afforded by this reaction makes it possible to directly apply this method to the analysis of EE in oral contraceptive preparations or in preparations containing similar estrogenic substances. Although the procedure may be carried out by conventional techniques, application of the method to the automatic analyzer[1] provides a method of analysis which can be used to obtain unit-to-unit variation of formulations containing as little as 5 mcg. of EE.

EXPERIMENTAL

Materials—Automatic analyzer equipment was used for the study. (*a*) Solid-prep sampler; volume set at 75 ml. and programmed at 20 samples per hour. (*b*) Proportioning pump. (*c*) Continuous filter; speed 3. (*d*) Fluorometer; primary filter (513 mμ) is a combination of Corning No. 1–60 (nearest lamp) and Wratten No. 58. Secondary filter (545 mμ) is a Wratten No. 23A. Sensitivity settings; activation source 30X; slit-3. (*e*) Linear recorder. (*f*) Acid flex tubing; conditioned by allowing the system to flush for 1 hr. Assemble the apparatus as shown in the flow diagram of Fig. 1.

Reagents and Solutions—Ninety per cent sulfuric acid in water (v/v); denatured alcohol (ethyl–methyl, 10:1) with water 40%.

Standard Ethinyl Estradiol Solutions—Prepare solutions of ethinyl estradiol in the denatured alcohol to contain 35, 40, 45, 50, and 55 mcg. of EE per ml. Place 1 ml. of the standards in the cups of the Solid-prep unit and obtain a standard curve.

Preparation of Samples—Place a weighed, finely powdered sample containing the equivalent of about 50 mcg. of EE in each sample cup. Obtain the reading from the recorder and calculate the number of micrograms of EE per weight of sample. For study of unit-to-unit variation, place 1 tablet in each of the sample cups.

RESULTS AND DISCUSSION

Linearity—An actual working curve which was obtained by analyzing standards of known concentration may be found in Fig. 2. A linear relationship exists for fluorescence and EE concentration in the range which was studied.

[1] AutoAnalyzer. Technicon Corp., Ardsley, N. Y.

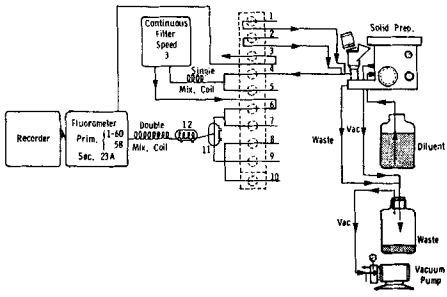

Fig. 1—Flow diagram for EE or EEME. Key: 1, 0.8 ml./min. air; 2, 4.06 ml./min. SDA No. 30; 3, 2.76 ml./min. debubbler; 4, 2.76 ml./min. sample; 5, 2.03 ml./min. SDA No. 30; 6, 2.76 ml./min.; 7, 2.0 ml./min. air; 8, 9, and 10, 2.76 ml./min. 90% H_2SO_4; 11, A-7 water-cooled TEE; 12, water-cooled mixing coil. All tubing is Acidflex.

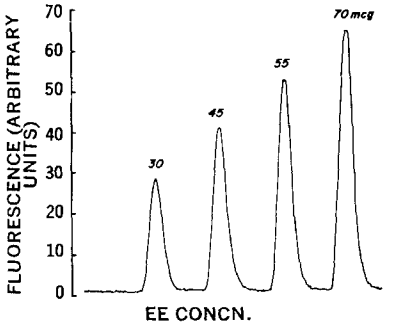

Fig. 2—Actual working curve for EE standards.

Precision—Repeatability of the procedure was demonstrated by assaying equal aliquots of an EE standard solution. These data, which may be found in Table I, indicate an average repeatability standard deviation of 1.3%

TABLE I—REPEATABILITY: TWENTY INDIVIDUAL ASSAYS ON EQUAL ALIQUOTS FROM AN EE STANDARD SOLUTION[a]

	Recovery, %		
1,	100.1	*11*,	102.1
2,	96.9	*12*,	104.0
3,	101.0	*13*,	100.7
4,	100.7	*14*,	101.0
5,	100.4	*15*,	98.9
6,	99.6	*16*,	100.6
7,	99.6	*17*,	99.4
8,	100.1	*18*,	100.0
9,	100.0	*19*,	101.0
10,	101.0	*20*,	100.0

[a] 1.0 mcg./ml., 95% alcohol.

Sensitivity—The automated method which has been developed was done so with the application of the method to individual EE dose variation in mind. The flow diagram of Fig. 1 is therefore applicable

to the analysis of samples which contain 25–100 mcg. of EE. The final concentration of the EE in the flow diagram indicated is about 0.06 mcg./ml. However, the practical limit of detectability has been shown to be 0.004 mcg./ml. of EE.[2]

Specificity—It has been known for some time that phenolsteroids (estrogens) when treated with sulfuric acid give rise to derivatives having a characteristic fluorescence. This effect was first observed in 1929 by Weiland, Straub, and Dorfmuller (8). The absorption and/or excitation spectra of these estrogen–H_2SO_4 chromogens exhibit maxima with broad peaks ranging from 410–450 mμ. The fluorescence characteristics of the EE–H_2SO_4 chromogen are vastly different from those of the other estrogens. EE has its activation maximum displaced at least 60 mμ toward the long wave region of the spectrum (λ_A = 513 mμ), and its fluorescence maximum at 545 mμ is displaced about 45 mμ. Figure 3 indicates the difference between

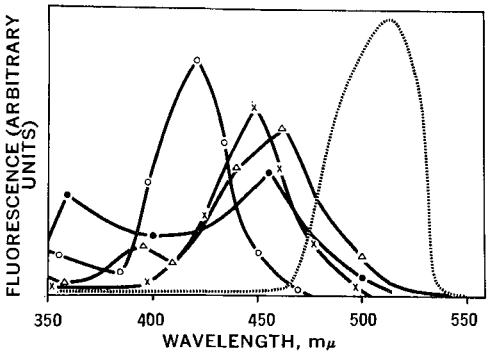

Fig. 3—Activation spectra of estrogen-sulfuric acid chromogens. Key: O, β-estradiol; ●, equilin; △ equilenin; X, estrone; ...., ethinyl estradiol.

the activation spectrum of the EE–H_2SO_4 chromogen and the activation spectra of other typical estrogen–H_2SO_4 chromogens.[3] This is quite significant if one considers that the only structural difference between EE and a typical estrogen, *e.g.*, β-estradiol, is the ethinyl moiety in position 17. When synthetic samples of EE plus a tenfold excess of equilin, equilenin, estrone, and β-estradiol were analyzed, less than 3% interference was contributed by these estrogenic substances.

The specificity of the reaction with respect to ethinyl estradiol degradation products was demonstrated by thin-layer chromatography.

Thin-layer plates were prepared from Silica Gel G HR after Stahl. The solvent system consisted of 1:1 hexane–ethylacetate. Two spray reagents, phosphomolybdic acid (9) and 90% sulfuric acid, were used.

Samples of thermally and photochemically degraded raw material exhibit a number of spots when phosphomolybdic acid detection is employed. This is illustrated in the chromatoplate on the left side of Fig. 4. Phosphomolybdic acid is a nonspecific reagent and is used only to indicate the absence or presence of components other than intact EE. When 90% H_2SO_4 was used for visualization of the same degraded raw material, one fluorescent component which corresponds in R_f to pure EE was detected. This is illustrated in the chromatoplate on the right side of Fig. 4. This indicates that the

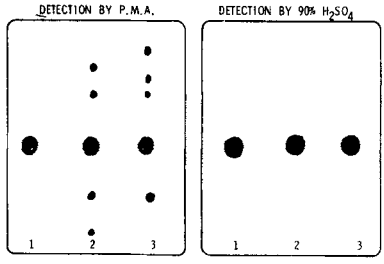

Fig. 4—Thin-layer chromatoplates of EE raw material. Key: 1, 20 mcg. of EE raw material; 2, 20 mcg. of the same raw material after exposure to direct U.V. light for 1 month; 3, 20 mcg. of the same raw material after storage at 100° for 1 month.

specificity of the sulfuric acid reagent eliminates the need for lengthy separation techniques. Further proof of H_2SO_4 specificity with respect to raw material EE decomposition products was obtained by quantitatively evaluating the degraded raw material by the automatic analyzer method and also by native fluorescence and the U.S.P. colorimetric methods. The data which are contained in Table II indicate that comparable results are obtained by all three assay procedures.

TABLE II—QUANTITATIVE ANALYSIS OF ETHINYL ESTRADIOL RAW MATERIAL BY THREE ASSAY PROCEDURES

Sample No.	Storage Condition	Native Fluorescence, % of Theory	Modified Kober, % of Theory	Automatic Analyzer, % of Theory
1	1 mo. 75° C.	67.6	69.0	69.8
2	1 wk. direct U.V. light exposure	94.3	94.6	93.9
3	1 mo. direct U.V. light exposure	88.8	89.1	88.9

Compressed tablets containing only EE (50 mcg./tablet) were subjected to accelerated conditions of heat and light. When these tablets were evaluated by the automated procedure and by the same two referee methods which were used previously, the results in Table III were obtained. These data indicate that the automatic analyzer method gives results which agree with native fluorescence and the colorimetric procedure.

The progestational type steroid hormone which is commonly found in oral contraceptive products

[2] Established on an Aminco-Bowman spectrophotofluorometer.

[3] These spectra are uncorrected and were obtained with an Aminco-Bowman spectrophotofluorometer.

TABLE III—COMPARISON OF DATA OBTAINED ON EE STABILITY SAMPLES BY THREE ASSAY PROCEDURES

Sample No.	Storage Condition	% of Claim: Native Fluorescence	Colorimetry	Automatic Analyzer
1	3 mo., D.S.	60.0	65.3	66.0
2	1 mo., D.S.	73.0	76.0	76.0
3	1 mo., 75°C.	89.9	90.0	90.5
4	1 mo., 75°C.	83.1	79.8	81.0
5	12 mo., R.T.	97.1	96.9	99.0
6	12 mo., R.T.	93.1	97.2	97.0

along with EE is generally of the Δ^4-3-keto structural variety. The concentration of this steroid is such that it may usually be determined by direct ultraviolet spectrophotometric methods, with no interference from EE. Usually the Δ^4-3-ketosteroid will be 50–500 times more concentrated than EE in an individual dosage unit. In spite of this large difference in concentration, significant interference was not encountered when a number of antifertility products were examined by the automated procedure. Table IV indicates a tabulation of data which were obtained when a number of commercial products were assayed by the automated procedure. These four products contained 3 different progestational steroids ranging in concentration from 2 to 10 mg. per tablet.

TABLE VI—COMPARISON OF RESULTS BY COLORIMETRIC, NATIVE FLUORESCENCE, AND SULFURIC ACID FLUORESCENCE OF EE-Δ^4-3-KETOSTEROID FORMULATION

Aged Sample No.	Ethinyl Estradiol, mcg.: Natural Fluorescence Before Extn.	Natural Fluorescence After Extn.	Colorimetric	Sulfuric Acid Fluorescence
1	36.2	46.7	46.8	47.2
2	33.0	44.8	45.0	44.7

In order to study Δ^4-3-ketosteroid interference with the method, EE was separated from the progestational steroid by solvent extraction, and then assayed by the automated method. The data in Table V show that results with and without extraction are equivalent, indicating little or no interference from the progestational steroid.

Two trade materials were not amenable to the automated sulfuric acid fluorescence assay. One contains a Δ^5-3-ketosteroid which interferes with the sulfuric acid fluorescence causing high results. Another product contains a material in the formulation which quenches the fluorescence and causes low results. In the latter case, a chloroform extraction removed the active ingredients away from the interfering substances. The chloroform was removed by evaporation and the residue was analyzed by the method described earlier. This separation technique was not successful for the Δ^5-3-ketosteroid containing products.

TABLE IV—DETERMINATION OF ETHINYL ESTRADIOL IN CURRENTLY MANUFACTURED CONTRACEPTIVES

% of Claim by Company No. 1[a]		2[a]		3		4	
100.0	103.0	99.0	101.0	104.0	106.1	97.0	102.0
104.0	104.0	93.9	100.0	100.5	100.5	97.0	101.2
99.0	105.0	99.0	91.7	101.0	94.5	98.0	100.0
102.0	105.0	94.6	98.0	96.5	101.0	102.0	88.6
99.0	102.0	96.5	100.0	98.0	100.5	101.0	104.0
104.0	99.0	105.0	94.5	100.0	96.0	112.0	97.8
105.0	98.0	97.0	97.0	100.0	103.4	101.0	99.8
102.0	103.5	99.2	97.0	96.5	99.1	99.2	100.6
103.0	103.0	95.8	93.5	95.0	95.9	97.8	99.8
99.0	104.0	99.0	97.0	99.5	96.7	98.2	99.2
Av. value (ϕ) = 102.2%		ϕ = 97.4%		ϕ = 99.3%		ϕ = 99.8%	
S. D. (σ) = 2.1%		σ = 3.1%		σ = 3.6%		σ = 4.2%	

[a] Ethinyl estradiol 3-methyl ether.

TABLE V—COMPARISON OF RESULTS WITH AND WITHOUT EXTRACTION ON SAMPLES CONTAINING 10 mg. OF A Δ^4-3-KETOSTEROID

No Extraction, mcg. EE	Extraction, mcg. EE
42.5	41.0
40.5	46.0
40.5	37.1
39.0	39.4
38.5	41.0
42.5	41.5
40.0	42.0
40.5	45.0
46.5	42.5

This sulfuric acid fluorescence method was used successfully in controlling developmental samples of a new Wyeth (10) antifertility product. This product contains 0.5 mg. of a new progestational steroid,[4] in combination with 50 mcg. of ethinyl estradiol. Since this quantity of progestational steroid is much lower than that found in other commercial products (2–25 mg.), attempts were made to analyze these products for EE by the native fluorescence method mentioned earlier. This provided a striking example of the problems that can occur when assaying by the native fluorescence procedure.

[4] WY-3707, 13-β-ethyl-17α-ethinyl-17-hydroxygon-4en-3-one.

Two aged samples of the combination tablets were assayed by the native fluorescence method, the colorimetric method, and the sulfuric acid fluorescence method. The data in Table VI show that the results by the native fluorescence method are much lower indicating that there may be a quenching effect operative. When the ethinyl estradiol was extracted from these sample mixtures and then assayed by the native fluorescence method, the results agreed with those obtained by the other two methods. This quenching effect was not noted with tablets containing only ethinyl estradiol.

SUMMARY

Application of the automatic analyzer to the analysis of ethinyl estradiol has been accomplished. The nonvarying volume, time, and temperature characteristics of this system have enabled the development of a repeatable assay procedure even for very low concentrations of EE. The procedure has been shown to be specific for intact EE in the presence of thermal and photochemical decomposition products. The procedure was also demonstrated to be specific for EE in combination with large amounts of the usual progestational steroid hormones and in the presence of large amounts of other similar estrogenic substances. A number of trade packages of antifertility products were successfully analyzed by the automated method. Twenty samples per hour can be handled easily as opposed to one to two samples per hour by conventional methods. This laboratory has found the automated method very convenient for determining unit-to-unit variation in formulations.

REFERENCES

(1) Inhoffen, H. H., and Hohlweg., W., *Naturwiss.*, **26**, 96(1938).
(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 245–247.
(3) Urbanyi, T., and Rehm, C. R., *J. Pharm. Sci.*, **55**, 501(1966).
(4) Kadin, H., Ugolini, M. S., and Roberts, H. R., *ibid.*, **53**, 1313(1964).
(5) Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S., *Arch. Biochem. Biophys.*, **68**, 1(1957).
(6) Kober, S. J., *Biochem. J.*, **32**, 357(1938).
(7) Talmage, J. M., Penner, M. H., and Geller, M., *J. Pharm. Sci.*, **54**, 1194(1965).
(8) Weiland, T., Straub, W., and Dorfmuller, M., *Ztschr. Physiol. Chem.*, **97**, 146(1929).
(9) Kritchevsky, D., and Kirk, M. R., *Arch. Biochem. Biophys.*, **35**, 346(1952).
(10) O'Roark, H. C., Lock, F. R., and Smith Foushee, J. H., "Clinical Study of WY-3707 and Ethinyl Estradiol as an Oral Contraceptive," Fifth World Congress on Fertility and Sterility, Stockholm, Sweden, June 1966.

New System of Disk Electrophoresis Using Large Acrylamide Gels

By ROBERT C. PETERSON

The disk electrophoretic method has been modified to use gels of 22-mm. diameter. A gel tube base was designed to produce a gel with a convex base, and a special buffer cell was provided. With the future goal of preparative electrophoresis, a quenched fluorescence technique was developed which locates the major protein zones without dyeing. A transverse destainer was designed for rapid removal of unbound dye from stained protein patterns.

THE THEORY, methods, and applications of gel electrophoresis have been well reviewed (1, 2). It has been our purpose to develop a simple, convenient method of columnar electrophoresis which could be adapted to preparative electrophoresis. Preliminary work has indicated that this approach is very promising.

The method of Ornstein (3) and Davis (4) has been modified to give gels 22 mm. in diameter by about 11.4 cm. in length.

METHODS AND MATERIALS

The pilot compound for this work was N.F. trypsin crystallized reference standard, lot 6040, 3226 N.F. units/mg. (5). The pH 5 buffer, pH 4.3 gel, and solutions for a gel concentration of 15% have been described.[1] Acrylamide, *N,N'*-methylenebisacrylamide, and β-alanine were of good purity.[2] *N,N,N',N'*-Tetramethylethylenediamine was practical grade, redistilled.[3] The ammonium persulfate and riboflavin were reagent grade.[3] The acetic acid was reagent grade.[4]

The gel tube was placed in the new base (see Fig. 1), and 40 ml. of lower gel ingredients[1] were introduced with the usual water layering. Teflon was selected for the base material in order to give less wear and a minimum of contamination. The 12.7-cm. long glass tube fits snugly within the O-ring and on the shelf in plug C.

When plug C is threaded into block B, the concavity in plug C forms the gel base for glass tube A. This rounded gel base (convex on the gel) is neces-

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[1] Canal Industrial Corp., Bethesda, Md.
[2] Eastman Organic Chemicals, white label.
[3] Matheson Coleman and Bell.
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Two aged samples of the combination tablets were assayed by the native fluorescence method, the colorimetric method, and the sulfuric acid fluorescence method. The data in Table VI show that the results by the native fluorescence method are much lower indicating that there may be a quenching effect operative. When the ethinyl estradiol was extracted from these sample mixtures and then assayed by the native fluorescence method, the results agreed with those obtained by the other two methods. This quenching effect was not noted with tablets containing only ethinyl estradiol.

SUMMARY

Application of the automatic analyzer to the analysis of ethinyl estradiol has been accomplished. The nonvarying volume, time, and temperature characteristics of this system have enabled the development of a repeatable assay procedure even for very low concentrations of EE. The procedure has been shown to be specific for intact EE in the presence of thermal and photochemical decomposition products. The procedure was also demonstrated to be specific for EE in combination with large amounts of the usual progestational steroid hormones and in the presence of large amounts of other similar estrogenic substances. A number of trade packages of antifertility products were successfully analyzed by the automated method. Twenty samples per hour can be handled easily as opposed to one to two samples per hour by conventional methods. This laboratory has found the automated method very convenient for determining unit-to-unit variation in formulations.

REFERENCES

(1) Inhoffen, H. H., and Hohlweg., W., *Naturwiss.*, **26**, 96(1938).
(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 245–247.
(3) Urbanyi, T., and Rehm, C. R., *J. Pharm. Sci.*, **55**, 501(1966).
(4) Kadin, H., Ugolini, M. S., and Roberts, H. R., *ibid.*, **53**, 1313(1964).
(5) Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S., *Arch. Biochem. Biophys.*, **68**, 1(1957).
(6) Kober, S. J., *Biochem. J.*, **32**, 357(1938).
(7) Talmage, J. M., Penner, M. H., and Geller, M., *J. Pharm. Sci.*, **54**, 1194(1965).
(8) Weiland, T., Straub, W., and Dorfmuller, M., *Ztschr. Physiol. Chem.*, **97**, 146(1929).
(9) Kritchevsky, D., and Kirk, M. R., *Arch. Biochem. Biophys.*, **35**, 346(1952).
(10) O'Roark, H. C., Lock, F. R., and Smith Foushee, J. H., "Clinical Study of WY-3707 and Ethinyl Estradiol as an Oral Contraceptive," Fifth World Congress on Fertility and Sterility, Stockholm, Sweden, June 1966.

New System of Disk Electrophoresis Using Large Acrylamide Gels

By ROBERT C. PETERSON

The disk electrophoretic method has been modified to use gels of 22-mm. diameter. A gel tube base was designed to produce a gel with a convex base, and a special buffer cell was provided. With the future goal of preparative electrophoresis, a quenched fluorescence technique was developed which locates the major protein zones without dyeing. A transverse destainer was designed for rapid removal of unbound dye from stained protein patterns.

THE THEORY, methods, and applications of gel electrophoresis have been well reviewed (1, 2). It has been our purpose to develop a simple, convenient method of columnar electrophoresis which could be adapted to preparative electrophoresis. Preliminary work has indicated that this approach is very promising.

The method of Ornstein (3) and Davis (4) has been modified to give gels 22 mm. in diameter by about 11.4 cm. in length.

METHODS AND MATERIALS

The pilot compound for this work was N.F. trypsin crystallized reference standard, lot 6040, 3226 N.F. units/mg. (5). The pH 5 buffer, pH 4.3 gel, and solutions for a gel concentration of 15% have been described.[1] Acrylamide, *N,N'*-methylenebisacrylamide, and β-alanine were of good purity.[2] *N,N,N',N'*-Tetramethylethylenediamine was practical grade, redistilled.[3] The ammonium persulfate and riboflavin were reagent grade.[3] The acetic acid was reagent grade.[4]

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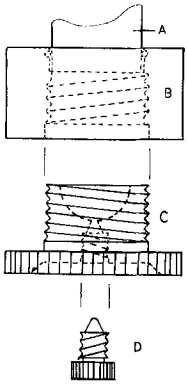

Fig. 1—Gel tube base. Key: A, glass gelation tube; B, Teflon block fitted with O-ring to hold glass tube and threaded to take plug C; C, Teflon plug milled to form base for gelation tube, threaded to fit block B and also to admit air plug D; D, plug to admit air.

sary. During polymerization, larger gels such as this tend to contract at the bottom and become concave. This cavity will collect gases during electrophoresis which stop the flow of current. A convex gel bottom as we have prepared will not hold these gases.

Plug C is made removable from block B in order to leave a minimum of surface holding the gel tube; this reduces the chances of detaching the gel from the tube when the tube is removed. Plug D is necessary to provide access of air to plug C before it is removed; otherwise, suction is created when plug C is removed which detaches the gel. This base has worked very satisfactorily.

The critical dimensions of this gel tube base assembly can be furnished by the author upon request.

The polymerization of these larger gels produces considerable heat; this heat can form cavities in the gel from dissolved gases which separate the gel from the tube. However, if polymerization is carried out in a refrigerator (about 6°), it is successful very nearly 100% of the time. Gelation is accomplished in about 1 hr.; this can be detected by the disappearance of heat from the gel tube.

For use with trypsin, the gels were pre-run overnight without spacer gel to remove possible interfering contaminants. Fresh buffer was used for the actual run. It was found empirically that this treatment improved resolution and definition. The electrophoresis assembly is illustrated in Fig. 2. The vertical glass tube buffer compartment is 40 mm. in diameter (o.d.) by 153 mm. The lower rectangular buffer compartment is made from 1/4-in. Lucite and measures 152 mm. long, 114 mm.

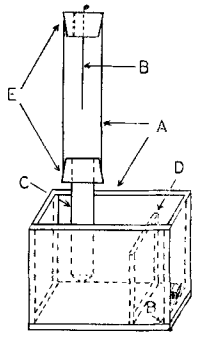

Fig. 2—Electrophoresis assembly. Key: A, buffer compartments; B, platinum electrodes; C, glass gel tube; D, baffle plate; E, rubber stoppers.

wide, and 108 mm. deep. The baffle in this compartment is just covered when the volume is 1100 ml. This baffle aids in keeping electrophoresis gases away from the gel.

Before the actual run, 8 ml. of spacer gel solution was added in the usual manner[1] above the small pore gel and photopolymerized for about 20 min. Trypsin, 5 mg., was added in 0.5 ml. of pH 5 buffer[1] containing 30% sucrose w/v.

Movement of trypsin components is downward when the upper electrode is made the anode. The d.c. power source was variable voltage regulated.[5] The fractionation was carried out for 9 hr. at 200 v. and about 24 ma. Both the pre-run and fractionation were done in the refrigerator (about 6°). Removal of the gel was accomplished by rimming with a 22-gauge hypodermic needle through which distilled water was continually forced from a syringe. Immediately upon removal, the gel was placed on a fluorescent plate (see Fig. 3) under a low wavelength (253.7 mμ) mercury lamp.[6] The gel must be lying over the slot with its long axis horizontal to the slot. Zones of highly concentrated protein appear as medium to dark blue zones where the fluorescence of the green fluorophor[7] is quenched. Figure 4 shows a sketch of the quenched pattern above the stained pattern of the same gel. The dye solution is 1% naphthol blue black[8] w/v in methanol–acetic

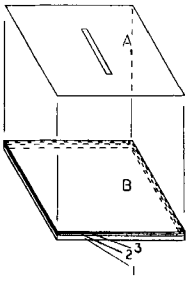

Fig. 3—Fluorescent plate. Key: A, black paper mask with slot (4 1/4 in. × 3/16 in.); B, glass plate with indicated layers (1, 6-in. square glass; 2, sprayed coating of fluorophor; 3, dipped coating of paraffin).

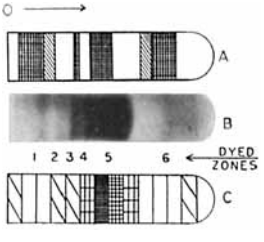

Fig. 4—Key: A, pattern from quenched fluorescence; B, same gel as A after staining protein zones; C, pattern of proteolytic activity of similar, unstained gel (see text).

acid–water (5:1:5 by vol.). The gel is kept in the dye solution for 48 hr. before destaining. The authors have found that an aqueous acetic acid solution of the dye is insufficient in its complexing of the protein to prevent zone removal during destaining. Also in this figure is shown a sketch of the pattern of proteolysis of gelatin[9] as given by a gel column run in identical fashion. The gel was not stained, and transverse sections about 0.25 in.

[5] Heath, model PS-3, Benton Harbor, Mich.
[6] Chromato-Vue Cabinet, model C-3, Ultra-Violet Products, San Gabriel, Calif.
[7] Luminescent Chemical No. 161 (p-1), lot CEX-3B; this was kindly donated by Sylvania Electric Products, Inc.
[8] C. I. No. 20470, Allied Chemical Corp., New York, N. Y.
[9] Peter Cooper Corp., Oak Creek, Wis.

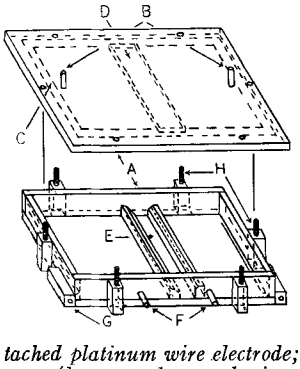

Fig. 5—Transverse destainer. Key: A, *Lucite cover and electrolyte compartment;* B, *gas vents and electrolyte inlets;* C, *rubber gasket (1/8 in. × 5/16 in.);* D, *Lucite strip (1/8 in. × 1 in. × 4-9/16 in.);* E, *gel compartment (see text);* F, *drains for removing electrolyte;* G, *banana plug socket with attached platinum wire electrode;* H, *brass bolts to hold cover (brass washers and wing nuts are not shown).*

thick were incubated on the plate of gelatin at room temperature for 25 hr. The gelatin, approximately 15% w/v, had been dissolved with heat in pH 7.6, 0.067 *M* phosphate buffer and hardened in a refrigerator before use. Correlation of most zones is seen with the dyed pattern and the quenched fluorescence and proteolytic pattern. These will be discussed later.

When using the transverse destainer, it was noticed that after most of the blue dye was removed some red dye remained. Multiplicity of the dye was suspected. Paper chromatography did indeed show 2 distinct zones, blue and red. Further investigation by cellulose acetate[10] electrophoresis showed 4 zones. The buffer was formic acid (90%)–pyridine–water (15:2.5:982.5 by vol.), pH 2.65. The main blue pigment stayed at the origin; at its tip was a small spot of brown pigment. Both of these pigments were preceded by 2 red pigments. Obviously this dye consists of at least 4 pigments. It appears that, in particular, those investigators who are quantitating proteins with this dye for clinical study should consider the possible variability in dye absorption by various proteins and the variability in wavelength of absorption among these pigments.

A transverse destainer has been designed and used in these laboratories for nearly 2 years. It is shown in Fig. 5. Other destainers using the same principle have been made (6–10), but to the author's knowledge these have been for 5–6-mm. gels or slabs. Although not shown in the figure, the 2 strips on either side of the gel compartment are each drilled through with about 100 holes, $^1/_{32}$ in. in diameter. The holes are staggered to give uniform current transfer. Destaining of the gel can be accomplished within 4 to 8 hr., or if more convenient, overnight at 50 to 100 v. (50 to 100 ma.). Four per cent acetic acid was the electrolyte.

RESULTS AND DISCUSSION

This system gives 6 to 7 zones in the dyed pattern of trypsin N.F. The electrophoretic pattern can be duplicated. This resolution can be compared with 2 zones by paper electrophoresis (11) and 4 zones by cellulose acetate electrophoresis (12). The variation in number of zones occurs with the light, lead zones in the region of zone 6. They vary from 1 to 2. These, to the best of the author's knowledge, have not been noted before. No attempt was made to identify these proteins, but from electrophoresis on cellulose acetate, followed by reaction with specific substrate (12), one would predict that the dark and largest zone would contain the tryptic activity.

Acrylamide gel electrophoresis of this same trypsin in the commonly used 5-mm. gels and with the same electrolyte system has shown only 4 or 5 zones. It seems that the larger gel gives much greater resolution partly due, no doubt, to the larger amount of material (5–15 mg.) which can be run and which results in being able to see what are probably mcg. quantities in a separated zone. It is felt that this approach is ideal for preparative electrophoresis. Since, in most cases, the desired compound already will be 80–90% pure, the major zone can be located by quenched fluorescence and sectioned out, and the purified compound can be removed by various means from the gel for further purification, if needed. Also, it is felt that this method will give better separation of zones both mechanically and electrically than the familiar continuous electrophoresis methods.

From Fig. 4, good correlation is seen for the major zone in quenching of fluorescence, staining, and proteolytic activity. A small amount of proteolytic activity is found in other areas of the gel. This indicates the probable presence of other proteolytic enzymes, such as chymotrypsin. The considerable quenching of fluorescence which is seen in the region of zone 6 routinely is found in the electrophoresis of trypsin. Its cause is not understood.

The broad, quenched area in the region of dyed zones 1 and 2 is considered to be the effect of scattered light from these zones.

Any possibility of the zones, other than the main one, being artifacts seems very unlikely. The pattern can be duplicated. In addition, the proteolytic pattern indicates the difference in activities shown by the various zones. Possibly more conclusive is an experiment in which the major zone (zone 5) was cut out using the fluorescent plate (a butter and cheese cutter is excellent) and placed on top of a new gel column. Conditions were the same except that the run time was slightly longer. The stained pattern had 2 zones, the major one and a very light following zone which probably was a portion of zone 4 that was cut out with it. The gel section which had been placed on top also was dyed and destained. It showed no protein zones.

It has not been a purpose here to quantitate by densitometry or other means the stained protein zones of these larger gels. The aim is to use large gels for preparative electrophoresis of practical amounts of various compounds of high purity, and the work noted in this paper is considered to be a step in this direction. With the fluorescent plate method of locating the major zone, the greatest hurdle has been eliminated. It should be mentioned that gels 2 in. in diameter have been used with good results.

[10] Oxoid. Colab Laboratories, Inc., Chicago Heights, Ill.

REFERENCES

(1) Morris, C. J. O. R., and Morris, P., "Separation Methods in Biochemistry," 1st ed., Interscience Publishers, Inc., New York, N. Y., 1963, vol. 1, pp. 620–770.

(2) Whipple, H. E., ed., *Ann. N. Y. Acad. Sci.*, **121**, 305 (1964).
(3) Ornstein, L., *ibid.*, **121**, 321(1964).
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(6) Matson, C. F., *Clin. Chem.*, **10**, 644(1964).
(7) Schrauwen, J. A. M., *J. Chromatog.*, **15**, 256(1964).
(8) Stanton, M. G., *Anal. Biochem.*, **12**, 310(1965).
(9) Burger, W. C., and Wardrip, W. O., *ibid.*, **13**, 580 (1965).
(10) Schwabe, C., *ibid.*, **17**, 201(1966).
(11) Nikkila, E., Ekholm, K., and Silvola, H., *Acta Chem. Scand.*, **6**, 617(1952).
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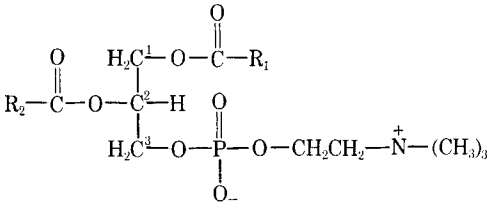
Notes

Solubilization and Rate of Dissolution of Drugs in the Presence of Physiologic Concentrations of Lysolecithin

By THEODORE R. BATES*, SONG-LING LIN, and MILO GIBALDI†

The micellar solubilizing properties of physiologic concentrations of lysolecithin for hexestrol, dienestrol, and griseofulvin are demonstrated. The extent of solubilization was found to decrease in the following order: hexestrol > dienestrol ≫ griseofulvin. Dissolution rate studies showed that lysolecithin significantly enhances the rate of solution of hexestrol, dienestrol, and griseofulvin. A role for this physiologic surfactant in the absorption process of hydrophobic drug molecules is explored.

HUMAN BILE is chiefly composed of cholesterol, calcium, bile salts, and phospholipids. Lecithin is the major phospholipid component of bile. It belongs to the class of compounds referred to as phosphatidylcholines (I).



R_1, R_2 = alkyl chains of fatty acids

I

The enzyme, phospholipase A, is capable of removing only one of the fatty acids from the lecithin molecule to form either 1- or 2-acyl lysolecithins. Phospholipase A is found in abundance in snake venom (*Crotalus adamanteus*) and also occurs in humans and animals. Its presence has been established in the mucous membrane of the small intestine (1), in duodenal fluid (2, 3), in the pancreas (4–6), and in blood serum (7). Commercially available lysolecithin is usually prepared by the enzymatic action of snake venom on purified egg lecithin. This material has palmitic or stearic acid in the 1-position of the glycerol moiety, since snake venom phospholipase A specifically catalyzes the hydrolysis of the fatty acid linkage at the 2-position (8–11).

Aqueous solutions of lysolecithin exhibit a more or less abrupt change in their physical properties over a narrow concentration range suggesting the formation of aggregates or micelles. The marked surface activity of this compound as well as other physical properties of its solutions have been reported by a number of investigators (9, 11–18). Surface tension *versus* concentration curves indicate that the critical micelle concentration (CMC) for this compound is in the concentration range of 1–2 $\times 10^{-3}$% (11, 14).

One of the most interesting properties of surface-active agents is their ability to bring into aqueous solution, at concentrations above the CMC, otherwise water-insoluble compounds. This phenomenon is known as micellar solubilization (19). There have been only a limited number of reports demonstrating the solubilizing ability of lysolecithin. Robinson and Saunders (20) found that aqueous solutions of lysolecithin possess a marked solubilizing power for cholesterol, triolein, and monostearin.

Lysolecithin appears to occur in small concentrations in human bile (21) and in high concentrations in the contents of the duodenal region of the small intestinal lumen (2). On examination of human small intestinal contents, following a test meal free of phospholipids, Borgström found that most of the phospholipids contained therein were in the form of monopalmitoyl-lysolecithin. This material was identical to that prepared by the action of snake venom on purified egg lecithin. The presence of phos-

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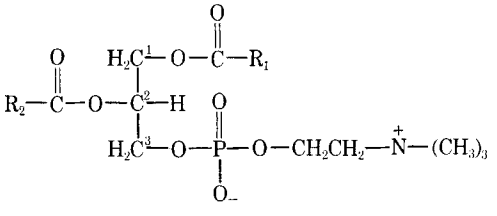

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pholipase A (3) and lysolecithin (2) in duodenal fluids suggests that lysolecithin in the lumen results from the action of phospholipase on bile lecithin.

Borgström *et al.* (22) have reported that the human gall bladder is emptied during the first 30 min. after a balanced test meal, giving rise to high concentrations of bile salts and phospholipid in the content of the duodenum and proximal jejunum. The main part of the test meal is mixed with liver bile and digested and absorbed from a mixture with concentrations of bile acid of about 1 to 3 mg./ml. and of phospholipids of about 0.5 to 1 mg./ml. chiefly as lysolecithin.

The role of physiologic surface-active agents in the absorption mechanism of water-insoluble drug molecules has received scant attention. Recent reports have demonstrated the effect of physiologic bile salt solutions on the solubilization (23, 24) and dissolution rate (25) of water-insoluble drugs. On the basis of these *in vitro* findings, it was proposed that bile salts play a role in the dissolution step of the absorption process from the small intestine.

The present communication is concerned with the interaction between relatively water-insoluble drug molecules and lysolecithin, another component of the intestinal fluids, and the effect of lysolecithin on the rate of solution of these compounds.

EXPERIMENTAL

Materials—Dienestrol,[1] hexestrol,[1] and griseofulvin[2] were used as received. Chromatographically pure lysolecithin[3] was dried *in vacuo* for 24 hr. prior to use.

Equilibration—An excess quantity of drug was added to ampuls containing varying concentrations of lysolecithin solutions. The ampuls were sealed and rotated in a constant-temperature water bath at 37° until equilibrium was established. Equilibrium was determined by repetitive sampling. The solutions were then rapidly filtered through a Millipore filter assembly (0.45 μ pore size) to remove undissolved drug, and the filtrate was appropriately diluted with anhydrous reagent methanol and assayed spectrophotometrically. (See under *Assay Procedure.*)

Dissolution Rate Determination—The method employed was essentially that of Higuchi and Shefter (26). One hundred milligrams of drug in excess of its predetermined solubility in 0.05% lysolecithin solution was added to exactly 100 ml. of dissolution medium maintained at 37 ± 1° in a water-jacketed vessel. Water or a 0.05% lysolecithin solution was used as the dissolution medium.

The solution was agitated by means of an overhead stirrer (2.5-cm. blade). The agitation intensity[4] was high (800 r.p.m.) to insure complete wetting of the hydrophobic drug when water served as the dissolution medium. At appropriate time intervals a 5-ml. sample was withdrawn from the vessel and replaced by the same volume of dissolution medium. The sample was treated as outlined under *Equilibration.* A cumulative correction was made to account for the previously removed samples.

Assay Procedure—Beer's law curves were constructed for hexestrol (278 mμ), dienestrol (228 mμ), and griseofulvin (292 mμ). A methanol–water (4:1) solvent system was employed for each drug. A Beckman DB recording spectrophotometer was used to obtain absorbance readings. Lysolecithin was found not to interfere with the spectrophotometric analysis in the concentrations present in the diluted samples.

RESULTS AND DISCUSSION

The Influence of Lysolecithin Concentration on Solubilization—The solubilization curves for hexestrol and dienestrol in varying concentrations of lysolecithin at 37° are depicted in Fig. 1. It can be seen from these curves that the solubility of both drugs increases, in a linear fashion, after a certain minimum concentration (about 2.5 × 10^{-3}%) of lysolecithin has been exceeded (*i.e.*, the CMC).

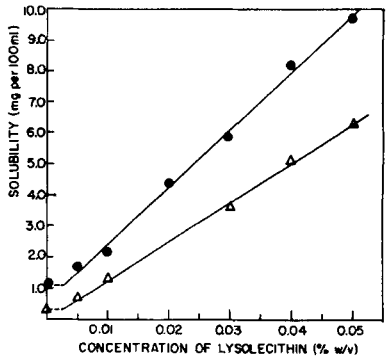


Fig. 1—Solubilization of hexestrol and dienestrol in dilute solutions of lysolecithin at 37°. Key: ○, hexestrol; △, dienestrol.

The apparent saturation capacities or ratios of the lysolecithin micelles for the drug-solubilizate molecules were obtained from the least squares slopes of the linear portion of the solubilization curves. These values, expressed as moles of micellar solubilized drug per mole of lysolecithin,[5] were found to be 0.34 and 0.23 for hexestrol and dienestrol, respectively.

Figure 2 shows the solubility of griseofulvin in solutions of lysolecithin at 37°. The saturation capacity determined for this solubilizate was 0.042.

Based on the saturation ratios found for the three solubilizates under investigation it can be seen that the lysolecithin micelle displays a somewhat similar affinity for hexestrol and dienestrol and a significantly lower affinity for griseofulvin. These differences are examples of the well established fact that the extent of micellar solubilization, in any surfactant solution, is influenced by the nature of the solubilizate molecule (19).

Lysolecithin forms essentially spherical micelles in solution, with its polar group oriented away from the

[1] Obtained from Gallard-Schlesinger Chemical Mfg. Co., New York, N. Y.

[2] Generously supplied by Schering Co., Bloomfield, N. J.

[3] Obtained from Nutritional Biochemicals Co., Cleveland, Ohio. Reported to be >99% pure by paper chromatography. Elemental analysis of the lysolecithin sample—Calcd. for monopalmitoyl-lysolecithin: C, 58.1; H, 10.2; N, 2.83; P, 6.25. Found: C, 58.4; H, 10.8; N, 2.86; P, 6.08. Elemental analysis of the fatty acid removed by heating lysolecithin in 2 *N* HCl for 48 hr. at 110° in sealed tubes—Calcd. for $C_{16}H_{32}O_2$: C, 74.9; H, 12.6. Found: C, 74.5; H, 12.7.

[4] Constant agitation intensity was achieved by utilizing a Servodyne constant torque system.

[5] Based on a theoretical molecular weight of 495.6 for monopalmitoyl-lysolecithin.

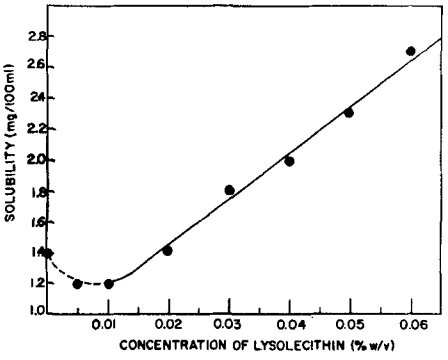


Fig. 2—Solubilization of griseofulvin in dilute solutions of lysolecithin at 37°.

"hydrocarbon" center of the micelle (14, 17, 27). Its isoelectric point is about 6.7 and in aqueous solutions it functions primarily like a nonionic surfactant (11). The lower degree of interaction of griseofulvin with the lysolecithin micelle can probably be attributed to steric effects. The volume available within the micelle for the solubilizate molecule is limited. As a result, griseofulvin being the largest of the three solubilizate molecules would encounter the greatest difficulty in packing within this restricted space.

The presence of free hydroxyl groups in the hexestrol and dienestrol molecules permits strong dipole interactions with the polar head group of the lysolecithin molecules in the micelle. This interaction may act as a driving force for the solubilization process.

Dienestrol and hexestrol are quite similar in chemical structure. The sole difference between the two molecules is the presence of double bonds in the 2 and 4 position of dienestrol, whereas the alkyl chain of hexestrol is saturated. The reduced affinity of lysolecithin for dienestrol relative to the affinity for hexestrol may be related to the fact that dienestrol is a more rigid molecule (because of conjugation) and may be restricted in its ability to pack within the micelle.

It is also of interest to compare the inverse saturation ratios (*i.e.*, moles of solubilizer/mole of drug) found with lysolecithin in the present study and with various bile salts in previous studies (23). Approximately 3 moles and 24 moles of lysolecithin were required to solubilize 1 mole of hexestrol and 1 mole of griseofulvin, respectively. The affinity of the various bile salts for hexestrol was similar to the affinity of lysolecithin for the drug. A range of about 4–6 moles of bile salt was needed to solubilize 1 mole of hexestrol. A marked difference exists, however, in the relative affinity for griseofulvin. The bile salts were much less effective solubilizers; a range of about 160–200 moles of bile salt was needed to solubilize 1 mole of griseofulvin. Nevertheless, it is apparent that both surface-active components of intestinal fluid have marked detergent properties.

The Influence of Lysolecithin on Dissolution Rate—For a drug to be absorbed across the gastrointestinal mucosa it must be in solution. In many instances the rate-limiting step in the absorption process of drugs administered in a solid form is the rate of dissolution in the gastrointestinal fluids.

Only one report has appeared in the literature concerning the influence of bile components on the dissolution rate of water-insoluble drugs (25). These investigators demonstrated that bile salts markedly enhance the dissolution characteristics of the drugs investigated.

Figures 3–5 show the dissolution behavior, in water and 0.05% lysolecithin solution, of hexestrol, dienestrol, and griseofulvin, respectively. Each curve is drawn through points which represent an average of two dissolution runs. It can be seen from these curves that the presence of lysolecithin in the dissolution medium significantly enhances the rate of dissolution of these drugs compared to the rate found in water. Inspection of these curves shows that after 2 min. the ratios of the amount of drug dissolved in the 0.05% lysolecithin solution

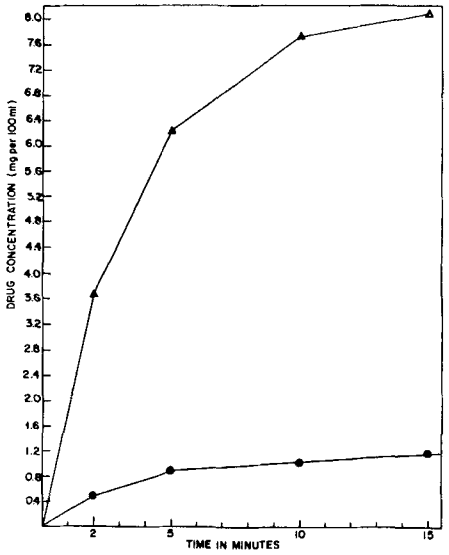


Fig. 3—Dissolution rates of hexestrol in water (●) and 0.05% lysolecithin (△) at 37°.

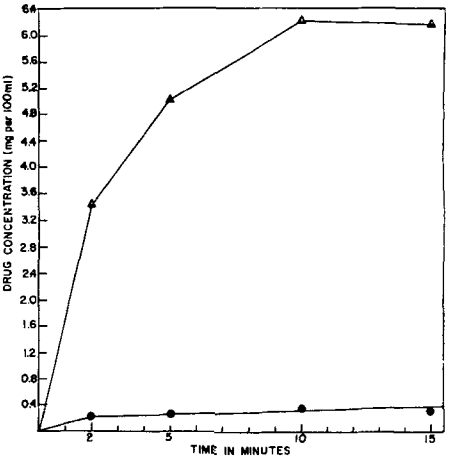


Fig. 4—Dissolution rates of dienestrol in water (●) and 0.05% lysolecithin (△) at 37°.

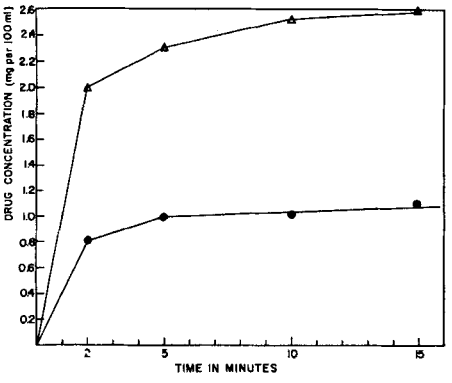


Fig. 5—Dissolution rates of griseofulvin in water (●) and 0.05% lysolecithin (△) at 37°.

to that amount dissolved in water are, 7.0, 15.6, and 2.4 for hexestrol, dienestrol, and griseofulvin, respectively. Preliminary studies indicate a threefold increase in the amount of reserpine dissolved in 0.05% lysolecithin solution at the 2-min. interval as compared to that present *at equilibrium* in water. The increased dissolution rate of these water-insoluble drugs in the presence of lysolecithin can be attributed essentially to micellar solubilization.

The presence of physiologic surfactants (*i.e.*, conjugated bile salts and lysolecithin) in the fluids of the upper region of the small intestine coupled with the strong interactions observed between these surfactants and certain poorly soluble drugs suggests that these surfactants play a role in the dissolution step of the absorption process of these pharmaceuticals.

It is anticipated that such physicochemical studies of the nature of the interactions of drugs with normal constituents of the gastrointestinal tract will significantly contribute to a greater understanding of the complex process of drug absorption.

REFERENCES

(1) Epstein, B., and Shapiro, B., *Biochem. J.*, **71**, 615 (1959).
(2) Borgström, B., *Acta Chem. Scand.*, **11**, 749(1957).
(3) Vogel, W. C., and Zieve, L., *J. Clin. Invest.*, **39**, 1295 (1960).
(4) Magee, W. L., and Thompson, R. H. S., *Biochem. J.*, **77**, 526(1960).
(5) Rimon, A., and Shapiro, B., *ibid.*, **71**, 621(1959).
(6) Shapiro, B., *Nature*, **169**, 29(1952).
(7) Zieve, L., and Vogel, W. C., *J. Lab. Clin. Med.*, **57**, 586(1961).
(8) DeHass, G. H., Daemen, S. J. M., and van Deenen, L. L. M., *Biochim. Biophys. Acta*, **65**, 260(1962).
(9) Saunders, L., *Ann. Pharm. Franc.*, **24**, 89(1966).
(10) van Deenen, L. L. M., *J. Am. Oil Chemists' Soc.*, **43**, 296(1966).
(11) Robinson, N., *J. Pharm. Pharmacol.*, **13**, 321(1961).
(12) Niemiro, R., *Progr. Biochemistry*, **7**, 317(1962).
(13) Robinson, N., and Saunders, L., *J. Pharm. Pharmacol.*, **10**, 384(1958).
(14) *Ibid.*, **11**, 115T(1959).
(15) *Ibid.*, **10**, 227T(1958).
(16) *Ibid.*, **10**, 755(1958).
(17) Perrin, J. H., and Saunders, L., *Biochim. Biophys. Acta*, **84**, 216(1964).
(18) Elworthy, P. H., *J. Chem. Soc. (Part 4)*, **1961**, 5385.
(19) McBain, J. W., and Hutchinson, E., "Solubilization," Academic Press Inc., New York, N. Y., 1955.
(20) Robinson, N., and Saunders, L., *J. Pharm. Pharmacol.*, **11**, 346(1959).
(21) Spitzer, H. L., Kyriakides, E. C., and Balint, J. A., *Nature*, **204**, 288(1964).
(22) Borgström, B., Dahlquist, A., Lundh, G., and Sjovall, J., *J. Clin. Invest.*, **36**, 1521(1957).
(23) Bates, T. R., Gibaldi, M., and Kanig, J. L., *J. Pharm. Sci.*, **55**, 191(1966).
(24) *Ibid.*, **55**, 901(1966).
(25) Bates, T. R., Gibaldi, M., and Kanig, J. L., *Nature*, **210**, 1331(1966).
(26) Shefter, E., and Higuchi, T., *J. Pharm. Sci.*, **52**, 781 (1963).
(27) Saunders, L., Thomas, J. L., and Robinson, N., *Nature*, **181**, 782(1958).

Ultraviolet and Attenuated Total Reflectance Infrared Spectra of Chlorpromazine Metabolites

By R. J. WARREN, W. E. THOMPSON, J. E. ZAREMBO, and I. B. EISDORFER

Ultraviolet absorption and attenuated total reflectance infrared spectra of chlorpromazine and 14 possible metabolites have been recorded and analyzed. Structural correlations of the absorption bands are presented and discussed. This collection provides a positive means of identifying chlorpromazine metabolites extracted from body fluids and tissues and separated by chromatographic techniques. Spectral identification can be made on microgram samples.

IDENTIFICATION of the metabolites of chlorpromazine has been the subject of considerable research effort over the past decade. As a result of this research, many, if not most, of its important metabolites have been identified. The usual procedure for identifying chlorpromazine metabolites has been comparison of either paper or thin-layer chromatograms of the metabolites, and of standards run at the same time. Very few investigators have used infrared spectroscopy for metabolite identification, principally no doubt because of the small amounts of metabolites available. With the advent of attenuated total reflectance spectroscopy (ATR), this obstacle has been effectively removed. The technique of ATR was developed by Fahrenfort (1) and has been described in detail by him and by Harrick (2). Its application to the identification of metabolites has been reported by several investigators. The ATR method provides infrared spectra from samples of the order of 10 mcg. In most cases, the unchanged sample can be recovered easily for further study. The combination of ATR and ultraviolet absorption spectroscopy provides an effective and positive means of identifying chlorpromazine metabolites and other materials from biological systems.

The purpose of this paper is to report the results of a spectral study of 14 possible metabolites of chlorpromazine. The ATR and ultraviolet spectral data presented here make possible a positive identification of these compounds.

EXPERIMENTAL

The infrared spectra were recorded on a Perkin-Elmer model 521 spectrometer equipped with a Wilks Scientific model 12 attenuated total reflectance attachment. The samples were prepared and run as natural films dispersed over KRS-5 plates by evaporation from chloroform solution. The average sample size was of the order of 75 to 100 mcg.

The ultraviolet spectra were recorded between 200 and 400 mμ with a Cary model 14 spectrophotometer using matched fused silica cells with a 1 cm. path length.

All spectral data were obtained from the same samples of analytical standards of a purity of 98% or greater.

RESULTS AND DISCUSSION

Infrared Spectra (ATR)—Although it is possible that transmission spectra and ATR spectra of the same materials may show slight differences (*e.g.*, band shape, relative intensities), no significant differences between transmission and ATR spectra have been found in this study. Figure 1 shows a comparison of the I.R. transmission spectrum of chlorpromazine (top) and the ATR spectrum of the same material (bottom). It is readily apparent that, for all practical purposes, the two spectra are identical. The remaining spectra of the reference materials are ATR spectra rather than transmission spectra because it is believed that, in most cases of metabolite study, the use of ATR rather than transmission spectra will be dictated by the size of the sample.

For infrared study the compounds can be separated into six groups: four groups are distinguished by the type of side chain on the nitrogen atom; the fifth group is made up of hydroxylated derivatives; and the sixth of *N*-oxides.

Figure 2 shows the spectrum of 2-chlorophenothiazine-5-oxide. This is the only member of the series without a side chain. The spectrum is typical of a 2-substituted phenothiazine (3). The strong S=O absorption band in the vicinity of 1000 cm.$^{-1}$ identifies the material as a sulfoxide.

Figure 3 shows the spectra of chlorpromazine derivatives with a primary amine side chain on the nitrogen atom. The side chain is the same in each case but the compounds shown from top to bottom are the sulfide, sulfoxide, and sulfone analogs. The spectra agree with the expected pattern of a 2,10-disubstituted phenothiazine (3); and a strong S=O absorption band at 1000 cm.$^{-1}$ ($\pm$20) and SO_2 bands at 1250–1300 cm.$^{-1}$ and 1140–1160 cm.$^{-1}$ identify the sulfoxide and sulfone derivatives.

Figure 4 shows the spectra of chlorpromazine analogs with a secondary amine side chain. Again, the order of spectra from top to bottom is sulfide, sulfoxide, sulfone.

Figure 5 shows chlorpromazine sulfoxide, chlorpromazine sulfone, and promazine. Chlorpromazine itself is shown in Fig. 1. The comments regarding 2,10-disubstituted phenothiazines in Figs. 3 and 4 apply to the sulfoxide and sulfone. Promazine gives the expected pattern of a 10-substituted phenothiazine, *i.e.*, the strong absorption band in the 720–770 cm.$^{-1}$ region of the 4 adjacent ring hydrogens (4).

Figure 6 shows the spectra of the 7- and 8-hydroxy derivatives of chlorpromazine, which differ in several respects from those of other metabolites. Since these two materials have ring substituents in addition to the 2-position, the aromatic substitution pattern would be expected to be different, and in fact it is. The peak in the 770–735 cm.$^{-1}$ region which is

Received March 31, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication June 5, 1967.

The authors are grateful to Mrs. Eleanor E. Cherry for her assistance in obtaining the spectra presented here and to the National Institutes of Health for generously supplying samples of 7- and 8-hydroxychlorpromazine.

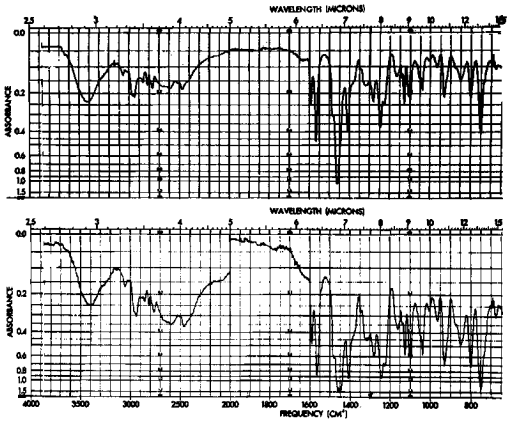


Fig. 1—I.R. transmission spectrum (top) and ATR spectrum (bottom) of chlorpromazine.

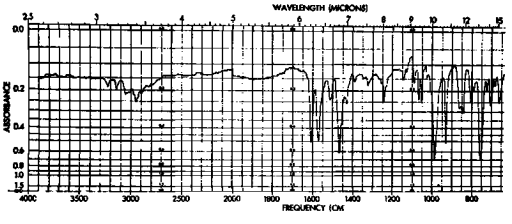


Fig. 2—ATR spectrum of 2-chlorophenothiazine-5-oxide.

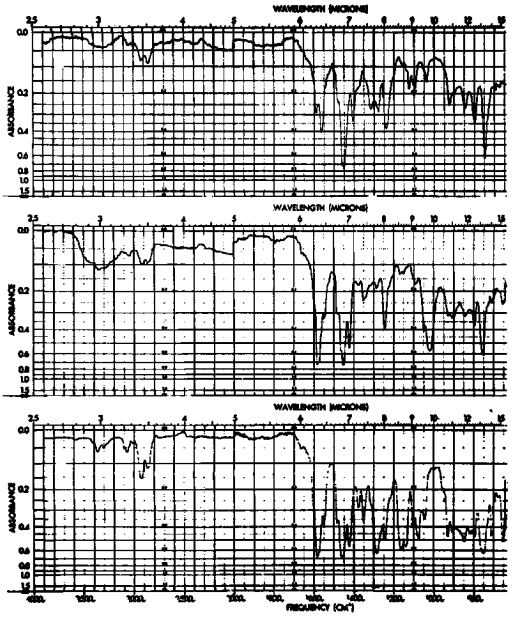


Fig. 3—ATR spectra of 2-chloro-10-(3'-aminopropyl)-phenothiazine (top), 2-chloro-10-(3'-aminopropyl)-(middle), and 2-chloro-10-(3'-aminopropyl) phenothiazine-5,5-dioxide (bottom).

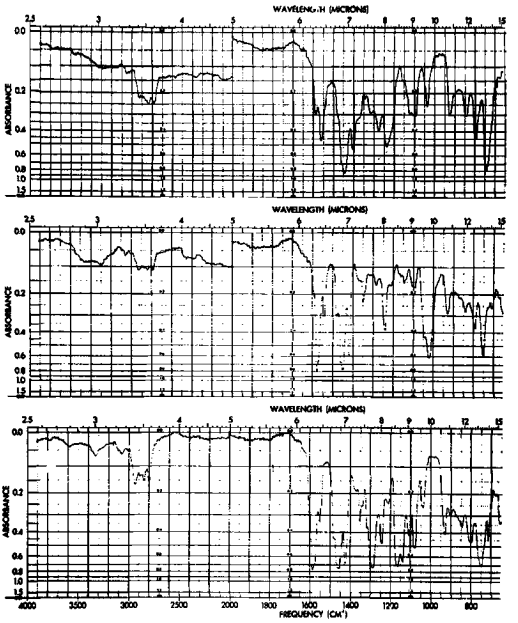


Fig. 4—ATR spectra of 2-chloro-10-(3'-methylaminopropyl)phenothiazine (top), 2-chloro-10-(3'-methylaminopropyl)phenothiazine-5-oxide (middle), and 2-chloro - 10 - (3' - methylaminopropyl)phenothiazine-5,5-dioxide (bottom).

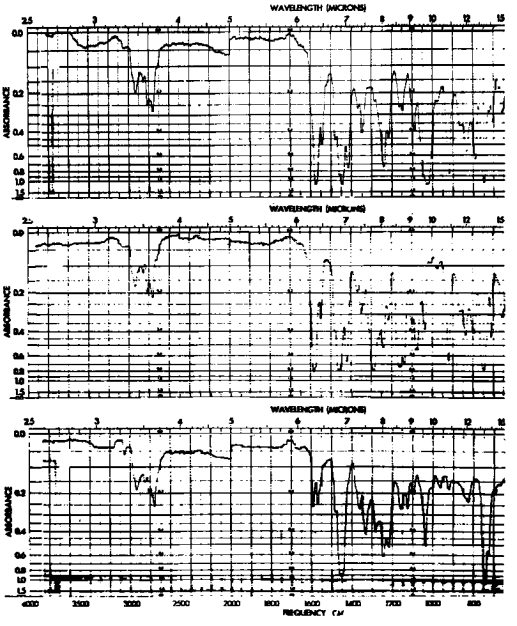


Fig. 5—ATR spectra of 2-chloro-10-(3'-dimethylaminopropyl)phenothiazine-5-oxide (top), 2-chloro-10-(3' - dimethylaminopropyl)phenothiazine - 5,5 - dioxide (middle), and 10-(3'-dimethylaminopropyl)phenothiazine (bottom).

attributed to four adjacent hydrogens (4) in 2-substituted phenothiazines disappears, and absorption bands are found at higher frequencies. This is what one should expect from the number of adjacent hydrogens in the hydroxy derivatives. The ATR spectra of the 7- and 8-hydroxy derivatives are

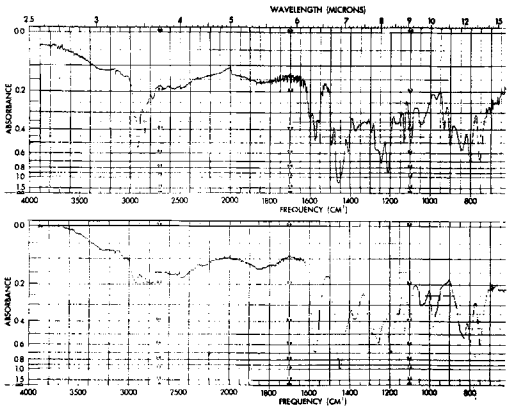


Fig. 6—ATR spectra of 2-chloro-10-(3′-dimethylaminopropyl)-7-hydroxyphenothiazine (top) and 2-chloro-10-(3′-dimethylaminopropyl)-8-hydroxyphenothiazine (bottom).

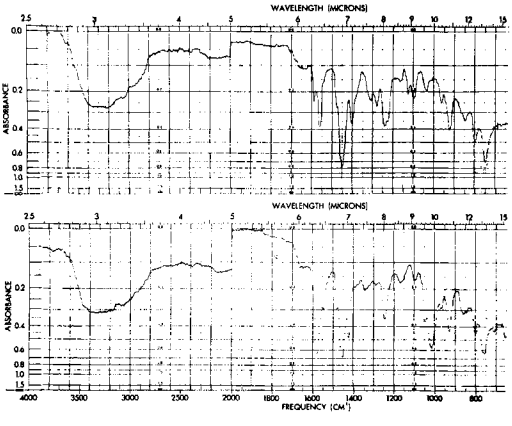


Fig. 7—ATR spectra of 2-chloro-10-(3′-dimethylaminopropyl)phenothiazine-N-oxide (top) and 2-chloro-10-(3′-dimethylaminopropyl)phenothiazine-N-oxide-5-oxide (bottom).

characteristic enough to allow differentiation from each other as well as from the other metabolites.

Figure 7 shows the ATR spectra of *N*-oxide derivatives of chlorpromazine. The *N*-oxide absorption band is located at 1200–1250 cm.$^{-1}$ and, in the case of the *N*-oxide-sulfoxide derivative, this absorption band and the strong band in the 1000 cm.$^{-1}$ region confirm the structure.

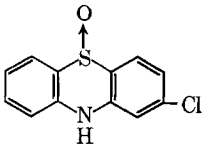
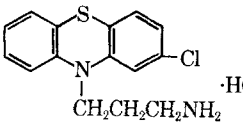
Ultraviolet Spectra—The ultraviolet spectra of chlorpromazine, chlorpromazine sulfoxide, and sulfone have been previously reported (3) by these laboratories. In addition to the ultraviolet data on these compounds, Table I gives data on the primary and secondary amine side chain analogs of these materials as well as data on hydroxy and *N*-oxide derivatives. As expected, the derivatives within a single group have the same over-all ultraviolet spectrum; thus, with the exception of hydroxy derivatives, identification of a specific sulfide, sulfoxide, or sulfone could not be made on the basis of the ultraviolet spectrum alone. Nevertheless, the ultraviolet spectra of these analogs assume greater importance when coupled with the ATR spectra, which provide positive identification of the material in question. Once identity has been established, the ultraviolet spectra provide quantitative data on the amount of material present.

The over-all differences between sulfide, sulfoxide, and sulfone are well known, as has been mentioned above. Not yet reported are the ultraviolet spectra and their differentiation of the 7- and 8-hydroxy and of the *N*-oxide derivatives. The ultraviolet data for the hydroxy compounds in acidic and basic media are given in Table I, which shows that the spectrum of either of these materials can be easily distinguished from the other metabolites listed. The shift in peak locations on change in pH is a ready identification, even in mixtures of two or more of the metabolites.

The ultraviolet spectra of the *N*-oxide derivatives are not much different from those of chlorpromazine and the sulfoxide. With the exception of a slight shift of the long wavelength peak, the spectrum of the *N*-oxide is very similar to that of chlorpromazine itself. A similar relationship is found also between the spectra of the *N*-oxide sulfoxide and chlorpromazine sulfoxide (see Table I).

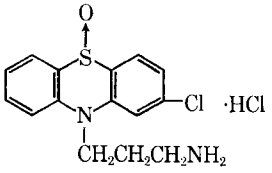
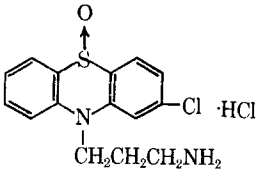
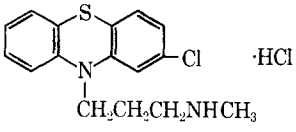
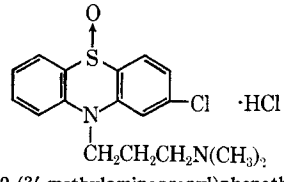
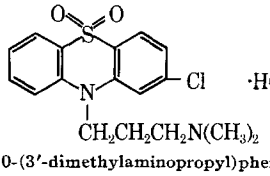
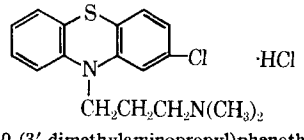
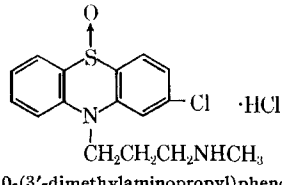
The value of these ultraviolet data is increased immeasurably after identity has been confirmed by ATR spectra, and relative amounts of the various metabolites can be calculated from standard reference data.

TABLE I—ULTRAVIOLET ABSORPTION DATA FOR CHLORPROMAZINE AND ITS METABOLITES

Compd.	$\lambda_{max.}$, mμ	Log ε	Solvent	ATR Spectrum (Free Base)
2-Chlorophenothiazine-5-oxide 	233 257 sh 273.8 304 339	4.55 4.38 4.19 3.95 3.75	95% EtOH	Fig. 2
2-Chloro-10-(3′-aminopropyl)phenothiazine hydrochloride 	256 306	4.57 3.65	95% EtOH	Fig. 3 (top)

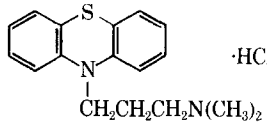
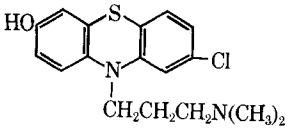
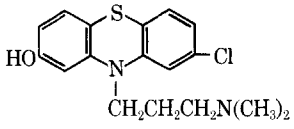
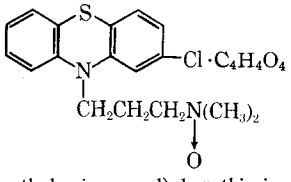
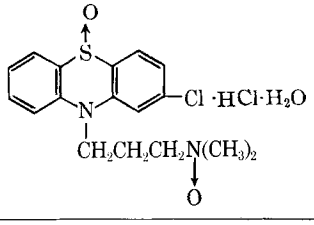
(*Continued on next page.*)

TABLE I—(*Continued.*)

Compd.	$\lambda_{max.}$, mμ	Log ε	Solvent	ATR Spectrum (Free Base)
2-Chloro-10-(3′-aminopropyl)phenothiazine-5-oxide hydrochloride 	239 275 298 340	4.56 4.10 3.94 3.78	95% EtOH	Fig. 3 (middle)
2-Chloro-10-(3′-aminopropyl)phenothiazine-5,5-dioxide hydrochloride 	233 271.5 294.5 332	4.52 4.16 3.90 3.37	95% EtOH	Fig. 3 (bottom)
2-Chloro-10-(3′-methylaminopropyl)phenothiazine hydrochloride 	255.5 306	4.57 3.65	95% EtOH	Fig. 4 (top)
2-Chloro-10-(3′-methylaminopropyl)phenothiazine-5-oxide hydrochloride 	239 275 298 341	4.56 4.08 3.92 3.76	95% EtOH	Fig. 4 (middle)
2-Chloro-10-(3′-methylaminopropyl)phenothiazine-5,5-dioxide hydrochloride 	233 271 294 332	4.55 4.18 3.91 3.75	95% EtOH	Fig. 4 (bottom)
2-Chloro-10-(3′-dimethylaminopropyl)phenothiazine hydrochloride 	256 310	4.54 3.60	95% EtOH	Fig. 1
2-Chloro-10-(3′-dimethylaminopropyl)phenothiazine-5-oxide hydrochloride 	240 275 298 342.5	4.52 4.03 3.88 3.72	95% EtOH	Fig. 5 (top)
2-Chloro-10-(3′-dimethylaminopropyl)phenothiazine-5,5-dioxide hydrochloride	233 271 294 332	4.54 4.16 3.89 3.76	95% EtOH	Fig. 5 (middle)

(*Continued on next page.*)

TABLE I—(*Continued.*)

Compd.	$\lambda_{max.}$, mμ	Log ε	Solvent	ATR Spectrum (Free Base)
10-(3′-Dimethylaminopropyl)phenothiazine hydrochloride  ·HCl	254 306	4.53 3.64	95% EtOH	Fig. 5 (bottom)
2-Chloro-10-(3′-dimethylaminopropyl)-7-hydroxyphenothiazine 	261 322 255 310	4.41 3.86 4.47 3.79	pH 13 pH 1	Fig. 6 (top)
2-Chloro-10-(3′-dimethylaminopropyl)-8-hydroxyphenothiazine 	262 312 256 308	4.40 3.68 4.40 3.68	pH 13 pH 1	Fig. 6 (bottom)
2-Chloro-10-(3′-dimethylaminopropyl)phenothiazine-*N*-oxide maleate 	255.5 307.5	4.51 3.63	95% EtOH	Fig. 7 (top)
2-Chloro-10-(3′-dimethylaminopropyl)phenothiazine-*N*-oxide-5-oxide hydrochloride monohydrate 	239 274 296 332 sh 340	4.50 4.04 3.88 3.68 3.72	95% EtOH	Fig. 7 (bottom)

SUMMARY

Attenuated total reflectance infrared spectra and ultraviolet absorption spectra of chlorpromazine and 14 metabolites have been presented. On the basis of these data, it is possible to make an identification of any of the materials listed.

REFERENCES

(1) Fahrenfort, J., *Spectrochem. Acta*, **17**, 698(1961).
(2) Harrick, N. J., *Phys. Rev. Letters*, **4**, 224(1960).
(3) Warren, R. J., Eisdorfer, I. B., Thompson, W. E., and Zarembo, J. E., *J. Pharm. Sci.*, **55**, 144(1966).
(4) Bellamy, L. J., "Infrared Spectra of Complex Molecules," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958.

Uropepsin Excretion and the Effect of Antacid Materials

By D. D. ABBOTT, J. W. E. HARRISSON, and R. C. BROGLE*

The effect of a series of antacid materials upon uropepsin output was evaluated in a group of 15 normal adults. The antacids, namely, sodium bicarbonate, calcium carbonate, magnesium hydroxide, hydrated magnesium aluminate-sulfate activated (HMAS), and bismuth subsalicylate, were administered on the comparable basis of acid consuming power (ACP). Urine specimens, both control and test, were evaluated for total proteolytic activity. Statistical analysis of the results showed all test materials, except calcium carbonate, to have elicited significant decreases ($P < 0.05$) in uropepsin excretion.

THE THERAPEUTIC usefulness of gastric antacids in the treatment of gastritis and peptic ulcer is a result of their capacity to both reduce the acidity and inactivate pepsin of gastric secretions (1). This latter action has been mainly attributed to a denaturation of pepsin at pH values of 4 or higher. This has been demonstrated for aluminum hydroxide, in animals, by Komarov and Komarov (2), Komarov and Kruegar (3), and Schiffrin and Komarov (4).

A previous investigation (5), in these laboratories, studied dihydroxyaluminum sodium carbonate (DASC), aluminum hydroxide, calcium carbonate, and sodium bicarbonate for antisecretory and antipeptic activity in histamine stimulated rabbits. Therein the aluminum containing antacids elicited greater antisecretory effects than did calcium carbonate and sodium bicarbonate. *In vivo* decreases in proteolytic activity for the various antacids were in relation to the degree of pH elevation. However, under *in vitro* conditions DASC was observed to decrease proteolytic activity to a greater degree than could be anticipated from pH changes alone.

The present study in humans was conducted in an effort to study further the antisecretory effect of antacids using uropepsin excretion as an indirect means of determination. Janowitz and Hollander (6) have estimated that the proteolytic activity of urine, attributed to uropepsin, approximates 1% of gastric peptic output. This relationship is dependent upon many variables but mainly the rate at which pepsinogen enters the general circulation from the chief cells of the gastric mucosa and its subsequent excretion, into the urine, through the glomerular apparatus. Although uropepsin as an index of gastric pepsin secretion is subject to many inconsistencies, the authors have attempted to employ this parameter in the evaluation of a series of antacid materials.

EXPERIMENTAL

Materials—Sodium bicarbonate; calcium carbonate; magnesium hydroxide (as milk of magnesia); hydrated magnesium aluminate-sulfate activated (HMAS); HMAS, 4.22% and bismuth subsalicylate 4.54% (liquid); bismuth subsalicylate.

Dosages—The dosages of the antacid test materials, given t.i.d., after meals, were based upon the acid consuming power (ACP) of the individual items. The ACP values and the individual doses for each of the antacid materials appear in Table I.

Procedure—A total of 15 subjects (13 males and 2 females) ranging in age from 20 to 55 years were utilized. None of the subjects claimed any past history of gastroenterological condition that would preclude their participation.

During the course of study a total of six 24-hr. control urine specimens were obtained from each subject at weekly intervals. Comparable urine specimens were obtained on the day following the collection of the control specimens, during which each subject took the assigned test material, three times a day after each meal. The subjects were assigned to each of the individual test materials in a random fashion.

TABLE I—ACP AND DOSAGE (t.i.d.) OF ANTACID MATERIALS

Material	ACP[a]	Dose[b]
Sodium bicarbonate	124/Gm.	1.303 Gm.
Calcium carbonate	222/Gm.	0.728 Gm.
Magnesium hydroxide (milk of magnesia)	26.6/ml.	6.08 ml.
Hydrated magnesium aluminate-sulfate activated (HMAS)	248.6/Gm.	0.65 Gm.
HMAS and bismuth subsalicylate, 2.3% (liquid)	17.6/ml.	9.2 ml.
Bismuth subsalicylate	0	0.210 Gm.[c]

[a] Milliters 0.1 *N* HCl neutralized. [b] Amount required to neutralize 161.6 ml. 0.1 *N* HCl. [c] Amount contained in HMAS–bismuth subsalicylate combination.

The total volume of each 24-hr. urine specimen was measured prior to the determination of uropepsin activity in accordance with the method of Grey *et al.* (7) as described by Green and Power (8). A unit of uropepsin activity is defined as that amount which during 30 min. of incubation at 37°, under the conditions of assay, releases 0.04 mg. of tyrosine-like substances from a hemoglobin substrate.

RESULTS

The total 24-hr. uropepsin excretion levels during drug administration and during the paired control periods are presented in Table II. Both the mean values and the ranges are given. The results for the individual subjects with each of the test materials are presented in Fig. 1.

The significance of the observed differences in uropepsin excretion between the individual pairs of control and test specimens for each subject on each test material was assessed using a one-sided *t* test.

Received April 14, 1967, from LaWall and Harrisson Research Laboratories, Inc., Philadelphia, PA 19103
Accepted for publication July 27, 1967.
Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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TABLE II—UROPEPSIN EXCRETION
(TOTAL UNITS/24 hr.)
$N = 15$

Material	Test Values Range	Test Values Mean	"Paired" Control Values Range	"Paired" Control Values Mean
Sodium bicarbonate	470–3770	1696	91–4590	2385
Calcium carbonate	122–4460	2034	175–3305	1775
Magnesium hydroxide	128–3086	1637	550–3950	2209
HMAS	240–4340	1467	494–4240	2225
HMAS and bismuth subsalicylate	225–3558	1609	0–6265	2449
Bismuth subsalicylate	0–3474	1309	238–4430	2057

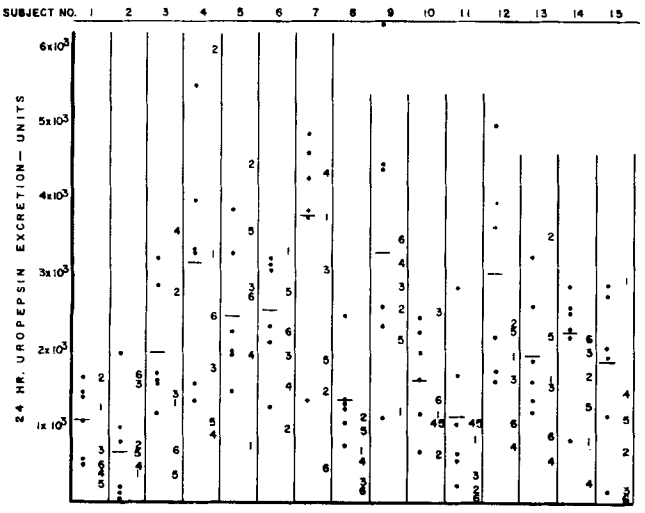

Fig. 1—Uropepsin excretion (units/24 hr.) test and control data. Key: 1, sodium bicarbonate; 2, calcium carbonate; 3, magnesium hydroxide; 4, HMAS; 5, HMAS and bismuth subsalicylate; 6, bismuth subsalicylate; ●, individual control values; —, mean values.

When the results were so evaluated, all test materials, except calcium carbonate, elicited significant decreases ($P < 0.05$) in uropepsin excretion.

An evaluation of the data showed a significant correlation ($P < 0.001$) within treatment groups between the magnitude of the control uropepsin level and the decrease in level upon treatment with the test materials. Higher control levels tended to be associated with greater decreases. Therefore, using an analysis of covariance, the decreases were adjusted for the differences in control levels. Based on Duncan's new multiple range test and owing to the large residual variances, none of the differences among the adjusted mean decreases for the various test treatments are statistically significant.

DISCUSSION

Of major consequence to the over-all evaluation of the uropepsin results obtained in this study is the extremely large variation in the 24-hr. excretion values by the individual subjects under the control conditions. The majority of subjects showed a variation in control uropepsin values of approximately two to fivefold. The over-all range of the control uropepsin values was 0–6265. These findings are in keeping with normal range by Corazzo and Myerson (9).

Notwithstanding the wide variations observed in the normal control values, both within and among the test subjects, there were statistically significant decreases in uropepsin excretion associated with the administration of all the test materials, except calcium carbonate. The basic reason for this occurrence remains to be elucidated. The decrease could result from inhibition of over-all gastric secretion, selective inhibition of pepsinogen formation, change in the partition of pepsinogen between gastric secretion and plasma, or a decrease in the excretion of pepsinogen by the glomerular apparatus. The results of earlier studies in animals (5) tend to support the initial possibility.

It is apparent from these findings that the usefulness of uropepsin excretion as a parameter in the evaluation of antacids is limited. This limitation is accentuated by the large variations noted in uropepsin excretion both within and among the test subjects.

SUMMARY

The effect of six antacid materials upon uropepsin excretion was evaluated in 15 test subjects. The effect was assessed by comparing total uropepsin excretion during a 24-hr. control period with that excreted in the next 24-hr. period during which a test material was taken three times a day after meals. The doses of the antacid were comparable, based upon their acid consuming power. All test materials evaluated, except calcium carbonate, resulted in decreases in uropepsin excretion that were significant at $P < 0.05$. The rationale of this effect upon uropepsin excretion requires further study of the many factors influencing uropepsin excretion.

REFERENCES

(1) Modell, W., "Drugs of Choice," C. V. Mosby Co. St. Louis, Mo., 1960, p. 357.
(2) Komarov, S. A., and Komarov, O., *Am. J. Digest. Diseases*, **7**, 177(1940).
(3) Komarov, S. A., and Kruegar, L., *ibid.*, **7**, 170(1940).
(4) Schiffrin, M. J., and Komarov, S. A., *ibid.*, **8**, 215 (1941).
(5) Packman, E. W., Abbott, D. D., Trabin, B., and Harrisson, J. W. E., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 46 (1959).
(6) Janowitz, H. D., and Hollander, F., *Gastroenterology*, **17**, 591(1951).
(7) Grey, S. J., Ramsey, C. G., and Reifenstein, R. W., *New Engl. J. Med.*, **25**, 835(1954).
(8) Green, P. A., and Power, M. H., *Staff Meetings Mayo Clinic*, **32**, 6(1957).
(9) Corazzo, L. J., and Myerson, R. M., *J. Am. Med. Assoc.*, **165**, 146(1957).

Demonstation of Enhanced Lethality of Drugs in Hypoexcretory Animals

By JAMES E. GIBSON and BERNARD A. BECKER

Hypoexcretory states were produced in mice: anuria by penile ligation, extrahepatic cholestasis by bile duct ligation, intrahepatic cholestasis by phenylisothiocyanate treatment, and reduced bile flow by α-naphthylisothiocyanate treatment. Intraperitoneal administration of ouabain to mice with no or diminished bile flow resulted in enhanced mortality rates. Digoxin and digitoxin effected enhanced lethality in anuric mice. Lanatoside-C was not more toxic to hypoexcretory mice. The use of hypoexcretory mice in toxicologic evaluations of pharmacologic agents is suggested.

THE PURPOSE of this paper is to compare the acute lethalities of certain drugs in hypoexcretory animals with acute lethalities obtained in normal animals. While drugs may be excreted by several routes, the major routes of excretion are the urinary and biliary routes. Urinary excretion can be prevented in male mice by the production of anuria (1). Biliary excretion may be totally blocked by ligation of the common bile duct which produces extrahepatic cholestasis. A condition resembling intrahepatic cholestasis can be induced by treatment with aryl isothiocyanates in mice (2) or rats (3). Only two aryl isothiocyanates, phenylisothiocyanate (PIT) and α-naphthylisothiocyanate (ANIT), possess cholestatic activity (4); both ANIT and PIT have been used in these investigations. The drugs selected as test agents in these studies are four cardiac glycosides: ouabain, lanatoside-C, digoxin, and digitoxin. The excretion of these agents has been studied by Cox and Wright (5), who show that ouabain is excreted primarily *via* bile, lanatoside-C mainly *via* bile with a small degree of urinary excretion, digoxin excreted approximately equally in the bile and urine, and digitoxin excreted primarily in the urine.

METHODS

Adult, male, Swiss-Webster-type mice weighing 25–40 Gm., were housed in groups of 10 in stainless steel "shoebox" cages and allowed food and water *ad libitum* before and after treatment. Groups of 20 mice were anesthetized with ether and rendered anuric by the penile ligation (PL) method of Becker and Gibson (1). Other groups of 20 mice, anesthetized and sham-ligated (PL-sham), served as controls. Other mice were anesthetized with ether and the common bile duct isolated. The common bile duct was ligated near its junction with the duodenum and the incision closed with wound clips. Cholestasis or reduced bile flow was also induced by treating groups of 20 mice with 85 mg./Kg. of PIT[1] or ANIT.[1]

Total cholestasis was demonstrated 24 hr. after treatment by the method of Plaa and Becker (6) in which treated mice are injected intravenously with a saturated solution of fluorescein in isotonic sodium bicarbonate. Fifteen minutes after fluorescein was injected, the bile ducts were exposed and examined under ultraviolet light. A cholestatic animal showed no fluorescence in the bile duct. Actual flow of bile in ANIT treated mice was determined by cannulation of the common bile duct according to the method of Plaa and Becker (6) 24 hr. after oral administration of ANIT.

Groups of PL, PL-sham, and normal animals were injected intraperitoneally 2 hr. after operation with varying concentrations of the selected glycoside. Twelve hours after glycoside treatment, the number dead in each group was counted. Ouabain was dissolved in water, the other glycosides were dissolved in ethanol, and the concentration adjusted so that the desired drug dosage was administered intraperitoneally in 0.1 ml. of 47.5% ethanol and the final average dosage (mg./Kg.) was calculated from the average body weight per group. Preliminary tests showed that 0.1 ml. of 47.5% ethanol was not lethal to mice. In other experiments, groups of 20 BDL and 20 BDL-sham mice were treated 24 hr. after surgery, along with groups of 20 PIT, ANIT, corn oil vehicle treated, and normal mice, by intraperitoneal injection of varying dosages of the selected glycosides. The number dead in each group 24 hr. after glycoside treatment was recorded.

For both the anuria and cholestasis studies, the number dead in each group was expressed as percentage of the number treated and the standard error calculated by the binomial expansion method. Statistical significance ($P < 0.05$) between test groups and sham-operated and normal groups was also found by the binomial expansion method (7). Statistical analysis of bile flow data was made by Student's t test with the level of significance chosen as $P < 0.05$.

RESULTS

Cholestasis and Impaired Bile Flow—Twenty four hours after PIT, 85 mg./Kg., ten treated mice were tested for bile flow by the fluorescein method; nine (90%) showed no fluorescein, indicating total cholestasis. On the other hand, ANIT, 85 mg./Kg., effected total cholestasis in only 12 of 33 animals (35%). However, actual bile flow as measured in 4 mice 24 hr. after ANIT treatment was found to be definitely reduced in all but one animal (Table I). The percentage of animals shown in Table I which produced no bile agrees well with the 35% value obtained by the indirect (fluorescein) method.

Lethality in Anuric Mice—Digitoxin in three dosages, 2.5, 3.5, and 6.0 mg./Kg., effected significantly higher mortality rates in anuric mice than in sham-operated or normal mice. Similarly, digoxin in two dosages, 5.0 and 9.2 mg./Kg., also

Received June 5, 1967, from the Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52240

Accepted for publication July 20, 1967.

This investigation was supported by a grant from the Iowa Heart Association and grant PHS-5-TO1-GM1308-2 from the U. S. Public Health Service, Bethesda, Md.

[1] K&K Laboratories, Inc., Jamaica, N. Y.

TABLE I—EFFECT OF ANIT, 85 mg./Kg. ORALLY, ON BILE FLOW IN THE MOUSE 24 hr. AFTER ADMINISTRATION

Mouse No.	Bile Flow, μl./hr. hr. After Bile Duct Cannulation 1	2	3	4
		Control[a]		
1	115	105	85	90
2	115	110	100	95
3	60	90	115	85
4	65	80	130	55
$\bar{X}$ ± S.E.	89 ± 15	96 ± 7	108 ± 10	81 ± 9
		Anit		
1	75	60	30	55
2	75	0	0	0
3	150	90	85	70
4	0	0	0	0
$\bar{X}$ ± S.E.	75 ± 31	38 ± 22[b]	29 ± 20[b]	31 ± 18[b]

[a] Control animals received corn oil p.o., 0.01 ml./Gm. body weight, 24 hr. before bile duct cannulation. [b] Significantly less than controls at $P < 0.05$.

TABLE II—TWELVE-HOUR ACUTE LETHALITY OF OUABAIN, LANATOSIDE-C, DIGOXIN, AND DIGITOXIN IN ANURIC AND NORMAL MALE MICE

Dosage,[a] mg./Kg.	Lethality[b] Anuric	Normal[c]	Significance (P)
	Ouabain		
2.2	15.0 ± 8.0	5.0 ± 4.8	N.S.
4.2	25.0 ± 9.9	25.0 ± 9.9	N.S.
8.5	45.0 ± 11.3	50.0 ± 11.2	N.S.
	Lanatoside-C		
3.4	33.3 ± 15.7	8.4 ± 8.0	N.S.
6.8	70.0 ± 14.5	70.0 ± 14.5	N.S.
10.2	78.0 ± 14.0	90.0 ± 9.5	N.S.
	Digoxin		
4.0	35.0 ± 10.6	10.0 ± 6.7	N.S.
5.0	50.0 ± 11.2	10.0 ± 6.7	<0.01
9.2	75.0 ± 6.9	37.5 ± 7.7	<0.001
	Digitoxin		
2.5	70.0 ± 14.5	0.0	<0.001
3.5	80.0 ± 12.7	10.0 ± 9.5	<0.002
6.0	86.0 ± 6.6	43.0 ± 9.0	<0.001

[a] Intraperitoneal administration. Minimum group size: 20 mice. [b] Per cent dead ± standard error. [c] Sham operated groups were not significantly different from the normal groups. [d] Not significantly different ($P > 0.05$).

ouabain or lanatoside-C (Table II). Lethality in sham-operated animals did not differ significantly from that in normal mice.

Lethality in Cholestatic or Impaired Bile Flow Mice—Ouabain was the only cardiac glycoside tested which effected increased mortality in mice with experimentally induced extrahepatic (BDL), intrahepatic cholestasis (PIT), or reduced bile flow (ANIT). Results are given in Table III.

DISCUSSION

The enhanced lethality of ouabain in animals with poor or no bile flow is a reasonable result as ouabain is believed to be excreted only in the bile. Similarly, the enhanced lethality of digitoxin in anuric animals is reasonable since digitoxin is excreted primarily *via* the urinary route. The increase in mortality rates seen in anuric animals treated with digoxin suggests that the urinary route of excretion is more important in mice than the biliary route, although the drug is believed to be excreted by both routes in the normal animal. Lanatoside-C, which according to Cox and Wright (5) is about 70% excreted in the bile and to a small degree in the urine, failed to effect enhanced lethality in either biliary or urinary hypoexcretory mice. This unexpected and challenging finding indicates that lanatoside-C can be eliminated, apparently, by either route of excretion with equal facility. The mechanism of the change in excretory routes remains to be investigated. The polarity of lanatoside-C is approximately that of ouabain as both compounds have five free hydroxyl groups. Another question which arises, then, is why the biliary hypoexcretory mouse cannot shift the elimination of ouabain to the urinary route.

Enhancement of toxicity of drugs in hypoexcretory animals other than the mouse has also been demonstrated. Enhanced lethality of ouabain in bile duct ligated cats has been reported by Kusakari and Uchida (8). Marcus *et al.* (9) studied the distribution of digitoxin in anuric dogs and humans in

TABLE III—TWENTY-FOUR HOUR ACUTE LETHALITY OF OUABAIN, LANATOSIDE-C, DIGOXIN, AND DIGITOXIN IN CHOLESTATIC AND NORMAL MALE MICE

Drug	Dosage,[a] mg./Kg.	Lethality[b] ANIT[c]	PIT[c]	BDL[d]	Normal[e]	Significance[f] (P)
Ouabain	2.2	33.3 ± 12.2	26.6 ± 4.4	26.6 ± 11.4	0.0	<0.05
	4.2	47.0 ± 12.1	60.0 ± 12.9	67.0 ± 10.3	16.0 ± 4.6	<0.05
	8.5	96.0 ± 4.2	92.0 ± 5.4	74.0 ± 9.4	52.0 ± 10.0	<0.05
Lanatoside-C	3.4	15.0 ± 8.0	5.0 ± 4.8	20.0 ± 9.0	10.5 ± 7.0	N.S.[g]
	6.8	52.6 ± 12.0	45.0 ± 11.0	52.6 ± 12.0	55.5 ± 12.0	N.S.
	10.2	85.0 ± 8.0	75.0 ± 10.0	85.0 ± 8.0	85.0 ± 8.0	N.S.
Digoxin	4.0	26.3 ± 10.1	0.0	29.4 ± 11.1	5.6 ± 5.4	N.S.
	5.0	42.0 ± 11.3	45.0 ± 11.1	26.4 ± 10.1	35.0 ± 10.6	N.S.
	9.2	63.2 ± 11.1	65.0 ± 10.6	63.2 ± 11.1	35.0 ± 10.6	N.S.
Digitoxin	2.5	53.0 ± 12.1	16.7 ± 8.8	25.0 ± 9.7	22.2 ± 10.4	N.S.
	3.5	67.0 ± 9.6	60.0 ± 12.7	58.0 ± 11.3	50.0 ± 7.1	N.S.
	6.0	87.0 ± 5.5	85.0 ± 8.0	85.0 ± 8.0	80.0 ± 9.0	N.S.

[a] Intraperitoneal administration. Minimum group size: 20 mice. [b] Per cent dead ± standard error. [c] Eighty-five mg./Kg. in corn oil by mouth, 24 hr. before test drug administration. [d] Twenty-four hours before test drug administration. [e] Sham-treated animals of the various cholestatic groups were not significantly different from normals. [f] Probability of no difference between the smallest lethality in any cholestatic group and the normal group. [g] N.S., no significant differences between the various groups ($P > 0.05$).

effected enhanced lethality in anuric mice. The lowest digoxin dosage, 4.0 mg./Kg., effected higher but nonsignificant increases in lethality. No significant changes in mortality rates were effected by renal failure and found increased storage of digitoxin in these species when urinary flow was diminished.

The possibility of enhanced lethality of therapeutic agents in hypoexcretory humans, therefore,

exists. Physicians have intuitively recognized the need to reduce dosages of some agents, notably certain antibiotics, in patients with renal insufficiency. Toxicologists might properly concern themselves with the need to evaluate the toxicity of new pharmacologic agents in hypoexcretory animals as the mouse preparations described here. Such evaluation might be made as a part of drug safety evaluation during the preclinical testing period.

REFERENCES

(1) Becker, B. A., and Gibson, J. E., *Proc. Soc. Exptl. Biol. Med.*, **124**, 296(1967).

(2) Becker, B. A., and Plaa, G. L., *Toxicol. Appl. Pharmacol.*, **7**, 680(1965).

(3) Goldfarb, S., Singer, E. J., and Popper, H., *Am. J. Pathol.*, **40**, 685(1962).

(4) Becker, B. A., and Plaa, G. L., *Toxicol. Appl. Pharmacol.*, **7**, 804(1965).

(5) Cox, E., and Wright, S. E., *J. Pharmacol. Exptl. Therap.*, **126**, 117(1959).

(6) Plaa, G. L., and Becker, B. A., *J. Appl. Physiol.*, **20**, 534(1965).

(7) Goldstein, A., "Biostatistics: An Introductory Text," Macmillan Co., New York, N. Y., 1964, p. 93.

(8) Kusakari, I., and Uchida, S., *Sapporo Med. J.*, **26**, 1 (1964).

(9) Marcus, F. I., Peterson, A., Salel, A., Scully, J., and Kapadia, G. G., *J. Pharmacol. Exptl. Therap.*, **152**, 372 (1966).

Determination of Benzoyl Peroxide Stability in Pharmaceuticals

By MURRAY P. GRUBER and ROBERT W. KLEIN

Spectrophotometric, titrimetric, and polarographic techniques were compared for stability testing of commercially available benzoyl peroxide pharmaceuticals. Initial testing revealed comparable results but significant discrepancies were observed on prolonged testing. The titrimetric procedure employed showed little degradation of the benzoyl peroxide at elevated temperatures, while both the spectrophotometric and polarographic methods demonstrated radical potency losses under the conditions of testing. Agreement was excellent between the polarographic and spectrophotometric procedures above 70 per cent of the original assay with a significant divergence being noted only below this level. A rapid, reproducible procedure of sufficient accuracy for pharmaceutical product stability and control purposes is presented.

WITH THE INTRODUCTION of benzoyl peroxide as a topical medicinal, this laboratory began an investigation into the published methods of analysis with a view to pharmaceutical stability evaluation. Previous methods have been employed to establish the absence of residual peroxides and associated by-products in the manufacture of plastics, polymers, fats, oils, breads, and flours. Since the primary purpose of any pharmaceutical test is specificity, it was believed that a more intensive investigation of the available methods was necessary prior to the adoption of any particular technique.

Titrimetric, spectrophotometric, and polarographic methods were compared for the determination of benzoyl peroxide stability in the dry form and in commercially available pharmaceutical preparations. The titrimetric method employed in this comparison was a modification of Wheeler's (1) procedure, a variation of the technique originally proposed by Lea (2).

Many spectrophotometric procedures have been described for the determination of organic peroxides, and the relative merits and disadvantages of these techniques have been thoroughly evaluated by Kolthoff and Medalia (3) and others (4, 5). The spectrophotometric procedure employed in this comparison was a modified form of the method of Banerjee and Budke (6). They adapted the iodimetric procedure of Heaton and Uri (7), using a 2 to 1 mixture of acetic acid and chloroform, instead of the proportions suggested by Lea (2). This technique proved to be the most promising due to the reduction of any tendency toward continued autooxidation, catalyzed oxidation, and/or induced oxidation. These properties were associated with the higher polarity of the solvent system. This procedure was designed for trace quantities of peroxide, but it was found applicable, by judicious choice of sample size and dilutions, to pharmaceutical preparations containing up to 10% benzoyl peroxide, with excellent accuracy and reproducibility.

A polarographic method was utilized for comparison as a result of the work of Ricciuti, Coleman, and Willets (8), who performed a statistical evaluation of a modified Wheeler procedure compared to a polarographic method for the analysis of tetralin hydro-peroxide and found the polarographic technique to be more reliable where impure products were encountered, due to its greater specificity. The various methods for polarographic measurement of organic peroxides are closely related in procedure and similar in results; therefore, a modification of the procedure of Bezuglyi and Dmitrieva (9) was employed.

Many pharmaceutical laboratories do not have polarographic instrumentation available because of the limited applications of polarography in this field. For this reason, a rapid, accurate, and reproducible spectrophotometric method is proposed for laboratory control and stability testing of benzoyl peroxide in pharmaceutical preparations.

EXPERIMENTAL

Spectrophotometric Assay

Apparatus—Beckman model DU spectrophotometer: 1-cm. matched cells were used.

Reagents—Nitrogen, research grade; iodine, A.R.; glacial acetic acid, A.R.; chloroform, A.R.;

Received March 15, 1967, from the Analytical Research Department, Dermik Laboratories, Inc., Syosset, NY 11791
Accepted for publication July 17, 1967.
The authors are indebted to Professor Mary J. Kingkade, Department of Chemistry, Hunter College, City University of New York, for suggestions and a review of this manuscript.

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REFERENCES

(1) Becker, B. A., and Gibson, J. E., *Proc. Soc. Exptl. Biol. Med.*, **124**, 296(1967).

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(3) Goldfarb, S., Singer, E. J., and Popper, H., *Am. J. Pathol.*, **40**, 685(1962).

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potassium iodide solution: dissolve 50.0 Gm. of potassium iodide, A.R., in 50 ml. of freshly deionized water in a 100-ml. graduated cylinder. Dilute to 100 ml. with freshly deionized water. This solution must be made up fresh, and should be colorless.

Procedure—*Standard Curve Preparation*—Weigh accurately about 635 mg. of iodine and dissolve in approximately 300 ml. of 2:1 glacial acetic acid–chloroform. Care should be taken to assure complete solution of the iodine. This is best accomplished by warming the iodine in the solvent on a water bath with frequent stirring. Keep the solution covered to avoid any loss of iodine. Dilute the solution to 500.0 ml. with the solvent mixture. Pipet 0, 1-, 2-, 3-, 4-, and 5-ml. aliquots into glass-stoppered test tubes; dilute to 25.0 ml. with the 2:1 acetic acid–chloroform solution. Insert a glass capillary tube into the bottom of the test tube and purge solution for 1.5 min. with nitrogen. Add 1 ml. of the potassium iodide solution, purge for 1 min. with nitrogen, stopper, and mix. Measure the absorbance of the solution immediately at 470 mμ in a 1-cm. covered cell *versus* a 2:1 acetic acid–chloroform blank. Plot the mg. of benzoyl peroxide (121.1/126.9 × mg. iodine) *versus* the absorbance at 470 mμ.

Sampling Procedure for Stability Assays—This section would ordinarily not be included. However, it is the authors' opinion that other investigators may lose valuable time in setting up stability tests, only to find that the samples under consideration had irreversibly separated during storage at elevated temperatures, thereby invalidating any further exploration.

Accurately weigh a sample equivalent to approximately 50 mg. of benzoyl peroxide into a screw-top glass vial. Record the exact weight and place an identifying mark on the vial. Repeat this procedure for as many analyses as required for the entire study. Place the vials in a controlled temperature oven at the desired test temperature. Two vials should be removed from the oven at predetermined intervals for (duplicate) stability assays.

For initial assays, weigh the sample into a tared 150-ml. beaker and proceed as directed under *Sample Preparation and Analysis*, beginning with "Bring the volume of liquid . . . ."

Sample Preparation and Analysis—To the sample vial, add 2:1 glacial acetic acid–chloroform and carefully wash entire contents into a 150-ml. beaker. Repeat four additional times, taking care that no residue remains in vial. Bring the volume of liquid in beaker to approximately 50 ml. with the 2:1 acetic acid–chloroform. Stir for 5 min. Quantitatively transfer the solution to a 100-ml. volumetric flask, filtering through a Whatman No. 4 filter paper (previously washed with the 2:1 acetic acid–chloroform). Wash the beaker with two additional portions of the 2:1 acetic acid–chloroform, filtering these into the volumetric flask; bring to volume with same. Mix well. Pipet a 5-ml. aliquot of the above into a 50-ml. glass-stoppered test tube. Pipet in 20.0 ml. of the 2:1 acetic acid–chloroform, mix, and purge the solution with nitrogen for 1.5 min. Immediately add 1.0 ml. of the 50% w/v KI solution and purge with nitrogen for 1 min. Stopper immediately. Place in the dark for exactly 1 hr. Then rapidly read the absorbance at 470 mμ in a covered 1-cm. cell *versus* a 2:1 acetic acid–chloroform blank. Determine the weight of benzoyl peroxide from the standard curve.

$$\text{benzoyl peroxide } \%(\text{w/w}) = \frac{\text{mg. benzoyl peroxide (from curve)} \times 2}{\text{sample wt. (Gm.)}}$$

Titration Assay

Reagents—Sodium iodide solution: dissolve a sufficient quantity of sodium iodide, A.R., in 20 ml. of freshly deionized water to make a saturated solution. This solution must be made up fresh and should be colorless. Glacial acetic acid, A.R.; methylene chloride, A.R.; carbon dioxide, research grade; and standardized sodium thiosulfate solution, 0.1 *N*.

Procedure—*Sampling Procedure for Stability Assays*—Accurately weigh a sample equivalent to 250–275 mg. of benzoyl peroxide into a screw-top glass vial. Record the exact weight and place an identifying mark on the vial. Repeat this procedure for as many analyses as required for the entire study. Place the vials in a controlled temperature oven at the desired test temperature. Two vials should be removed from the oven at predetermined intervals for (duplicate) stability assays.

For initial assays, weigh the sample into a tared 500-ml. iodine flask. Pipet in 20.0 ml. of glacial acetic acid and proceed as directed under *Sample Preparation and Analysis*, beginning with "Add 10 ml. of methylene chloride . . . ."

Sample Preparation and Analysis—To the sample vial, add 5.0 ml. (pipeted) glacial acetic acid and carefully wash the entire contents into a 500-ml. iodine flask. Repeat three additional times (total, 20.0 ml. acetic acid), taking care that no residue remains in vial. Using a graduated cylinder, add 10 ml. methylene chloride and swirl to dissolve sample (lotions and creams may leave a turbid solution). Purge the solution for exactly 2 min. with carbon dioxide. Immediately pipet in 5.0 ml. of saturated sodium iodide solution and stopper. Swirl and stand solution in the dark for 30 min. After 28 min. have elapsed, purge 200 ml. of deionized water for 2 min. with carbon dioxide. Add 50 ml. of this deaerated water to the solution. Swirl and titrate to a colorless end point with 0.1 *N* sodium thiosulfate solution. Near the end point, deaerated starch T.S. may be used as an indicator.

$$\text{benzoyl peroxide } \%(\text{w/w}) = \frac{(\text{ml. thiosulfate}) \times (N \text{ thiosulfate}) \times 0.1211 \times 100}{\text{Gm. of sample}}$$

where 0.1211 = meq. wt.

Polarographic Assay

Apparatus—Polarograph (recording polarograph preferred) equipped with an H cell, using a dropping mercury electrode (2–7 sec. drop time) as recording electrode and a saturated calomel electrode as reference electrode.

Reagents—Benzene, A.R.; methanol, A.R.; ammonia, A.R. concentrated; methyl red, 0.2% w/v in methanol.

Procedure—Accurately weigh a sample equivalent to 10 mg. of benzoyl peroxide into a 250-ml. volumetric flask. Add 50 ml. of benzene, shake for 15 min. on a shaker, add 100 ml. of methanol, and swirl. Add, in order, 5 ml. of ammonia and 2.5 ml.

of 0.2% methyl red solution. Swirl, dilute to volume with methanol, and mix thoroughly. Transfer a portion to the sample side of the H cell. Bubble nitrogen or hydrogen through the solution for 10 min. Polarograph the solution from +0.45 v. to −0.2 v. . . $E_{1/2}$ = +0.27 v. *versus* S.C.E.

Calculate the diffusion current i_d in the conventional manner [limiting current (i_l) − residual current (i_r)].

Prepare a standard by dissolving an accurately weighed sample of approximately 100 mg. of benzoyl peroxide in benzene and diluting to 100 ml. Transfer a 10-ml. aliquot to a 250-ml. volumetric flask, add 40 ml. of benzene, and continue as directed above, beginning with "Add 100 ml. of methanol. . ."

$$\text{benzoyl peroxide } \%(w/w) = \frac{\frac{i_d \text{ sample}}{i_d \text{ std.}} \times \text{concn. BP std. (mg./ml.)} \times 25}{\text{Gm. of sample}}$$

where BP = benzoyl peroxide.

RESULTS AND DISCUSSION

A comparison of the titrimetric and spectrophotometric methods was performed to determine the accuracy and precision of the two procedures. Analyses were performed on a reagent grade benzoyl peroxide and a nonflammable pharmaceutical grade (35% active). Known concentrations were incorporated in an oil-in-water lotion and analyses repeated. Table I indicates the results of duplicate assays and demonstrates that all three methods are interchangeable for pure or freshly compounded mixtures. The polarographic procedure was eliminated as an analysis of reagent grade benzoyl peroxide. The diffusion current constant obtained, however, was used for the calculation of peroxide content of the other preparations.

TABLE I—COMPARISON OF RESULTS BY TITRIMETRIC, SPECTROPHOTOMETRIC, AND POLAROGRAPHIC METHODS ON PURE BENZOYL PEROXIDE POWDER AND FRESHLY PREPARED MIXTURES

	Method		
	Titrimetric, %	Spectrophotometric, %	Polarographic, %
Benzoyl peroxide, 99%	98.91	98.94	. . .
Benzoyl peroxide, 35%	35.00	35.01	35.00
Oil-in-water lotion (= 5.29% benzoyl peroxide)	5.25	5.33	5.28

Based on these findings, stability studies involving the three methods were instituted on commercial preparations containing 5 and 10% benzoyl peroxide. Two of these are lotions manufactured in the United States; the third is a cream produced in Canada.

One of these commercial formulations differs from the others in two respects. First, the benzoyl peroxide is packaged separately as a nonflammable powder containing 35% peroxide which is added by the pharmacist at the time the prescription is dispensed. Second, the pharmacist is directed to place an expiration date on the product at the time of compounding. The other formulations bear no expiration date, implying indefinite stability.

Additional tests were performed on the separately packaged powder to determine the stability of the peroxide in the mixture. The powder was tested at 49° C. and 60° C., while the finished formulations were tested at 46° C. The temperature of 46° C. (115° F.) was chosen since it is not uncommon for this temperature to be encountered in transit and in storage areas during the summer months, and in warmer climates for even longer periods. The powder was tested at higher temperatures because of the increased stability of benzoyl peroxide in the dry form. The results for the dry material, as determined by spectrophotometric analysis and polarographic assay, are indicated in Table II.

TABLE II—STABILITY ANALYSIS OF BENZOYL PEROXIDE POWDER

Temp.	Benzoyl Peroxide Content— Initial, %	8 Wk., %	27 Wk., %
49°C. (120° F.)	34.2	. . .	33.6
60°C. (140° F.)	34.4	33.1	. . .

This benzoyl peroxide appears to be quite stable at 60° C. after 8 weeks and at 49° C. even after 27 weeks The stability may be attributable to the filler employed (dicalcium phosphate), but no attempt has been made to ascertain the reasons for this since they are outside the scope of this study.

In order to test all three medications under the same condition, the 35% benzoyl peroxide mixture was added to its lotion base (lotion 1) as directed. Figures 1–3 summarize the results of 6.5 weeks of accelerated testing at 46 °C.

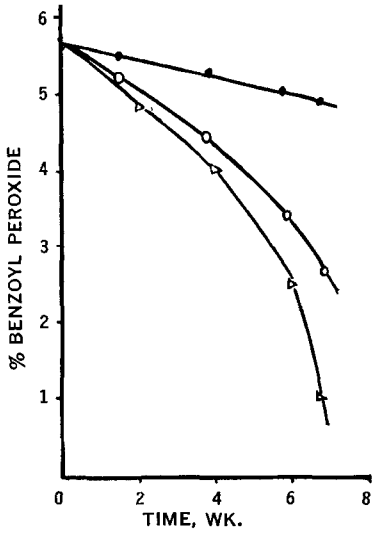


Fig. 1—Lotion 1. Key: ●, titrimetric; ○, spectrophotometric; △, polarographic.

The results of the three methods indicate some rather interesting discrepancies. According to the titrimetric procedure, both lotion formulations contained more than 90% of their original peroxide concentrations at the end of the 6.5-week testing period.

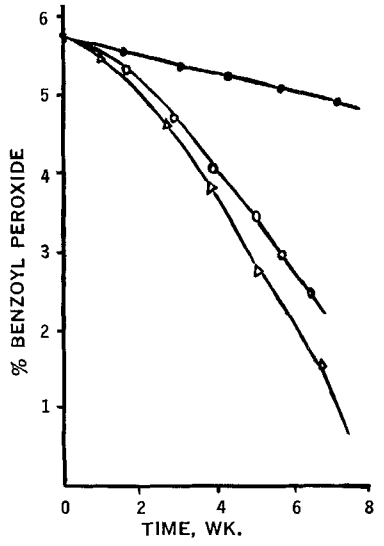


Fig. 2—Lotion 2. Key: ●, titrimetric; O, spectrophotometric; △, polarographic.

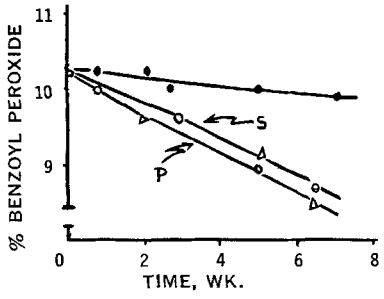


Fig. 3—Cream. Key: ●, titrimetric; O, spectrophotometric (S); △, *polarographic* (P).

Similarly, the cream formulation yielded nearly 97% of the benzoyl peroxide initially found. The spectrophotometric method, however, indicates a loss of more than 50% potency for both lotions, while the cream showed a loss of 17%. The polarographic method indicated losses, similar to those found spectrophotometrically, to approximately 70% of original potency. Below this point the divergence becomes significant and increases to yield a nearly 70% loss in one lotion and 78% in the other after 6.5 weeks' storage at 46°C. It is also noteworthy that a secondary wave appeared with $E_{1/2}$ values in the region of 0 v. *versus* S.C.E. after an apparent peroxide loss of 30%. This wave(s) exhibited sharp fluctuations in recorded microamperages. The intensity of the fluctuations and size of this wave increased with longer storage and decreasing benzoyl peroxide concentration. Continued investigation into the nature of the waves(s) and isolation of the component(s) causing this phenomenon is under way at the time of this writing.

From the available data, it seems that the titration procedure apparently does not differentiate some of the degradation products associated with benzoyl peroxide from the parent compound. The polarographic procedure appears to be the most accurate due to its specificity, particularly where significant breakdown has occurred. These results are in line with the finding of Ricciuti *et al.* (8) as previously noted. The spectrophotometric procedure indicates a significant amount of decomposition under prolonged storage conditions, but does not exhibit the full extent of this deterioration as exposed by the polarographic technique, the disparity becoming significant only below the 70% potency level. Values below this level are of little significance for pharmaceutical purposes since the usually accepted minimum limit of potency is 90% of the specified concentration. The method is simple, straightforward, and of sufficient accuracy above 70% potency to be extremely useful for pharmaceutical control and stability testing.

SUMMARY

Several commercially available benzoyl peroxide pharmaceuticals were subjected to quantitative analysis by a conventional titration method, a spectrophotometric procedure, and a polarographic assay. Although the titration and spectrophotometric methods compared favorably with each other for analysis of the pure benzoyl peroxide substance, the titration procedure proved inadequate for accurately determining the stability of benzoyl peroxide formulations, as confirmed by polarographic investigation. It is ascertained that a dry combination of benzoyl peroxide (35%) and dicalcium phosphate (65%) forms a stable mixture with long storage life. Both the polarographic and spectrophotometric procedures indicate that benzoyl peroxide dispersed in a lotion or cream vehicle has a limited shelf-life and should bear an expiration date. Because of its failure to discriminate between benzoyl peroxide and some of its associated decomposition products, the titrimetric technique cannot be applied with reliability to stability studies of these products.

The preferred method of analysis for adequate control and stability evaluation of pharmaceutical lotions and creams containing benzoyl peroxide (simulating elevated temperature conditions as commonly encountered in pharmaceutical transit and storage), where potency levels must be maintained at 90% or more of the label claim, is a simple spectrophotometric procedure. This method has been demonstrated to be accurate at levels of 70% or more of the original concentration.

REFERENCES

(1) Wheeler, D. H., *J. Am. Oil Chemists' Soc.*, **25**, 144 (1948).
(2) Lea, C. H., "Rancidity in Edible Fats," D.S.I.R. Sp. Rep. No. 46, Her Majesty's Stationery Office, London, England, 1938, p. 106.
(3) Kolthoff, I. M., and Medalia, A. I., *Anal. Chem.*, **23**, 595(1951).
(4) Barnard, D., and Hargrave, K. R., *Anal. Chem. Acta*, **5**, 476(1951).
(5) Martin, A. J., "Organic Analysis," Interscience Publishers, Inc., New York, N.Y., 1960, vol. 4, p. 3.
(6) Banerjee, D. K., and Budke, C. C., *Anal. Chem.*, **36**, 792(1964).
(7) Heaton, F. W., and Uri, N., *J. Soc. Food Agr.*, **9**, 781 (1958).
(8) Ricciuti, C., Coleman, J. E., and Willits, C. O., *Anal. Chem.*, **27**, 405(1955).
(9) Bezuglyi, V. D., and Dmitrieva, V. N., *Zh. Priklad. Khim.*, **31**, 298(1958).

Separation of Some Estrogen Sulfates from Their Oxidation Products

By A. R. HURWITZ, H. J. BURKE, and R. A. MARRA

Quantitative separations of sodium equilin sulfate from sodium equilenin sulfate and sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate were obtained on dextran gel columns. It appears that these separations were the result of differences in adsorption character.

ALTHOUGH many quantitative methods are available for the determination of estrogens, it is generally necessary that the free form, the phenolic species, be used. Thus, naturally occurring sulfate or glucuronide esters have been submitted to hydrolysis of one form or another prior to analysis. In many instances direct separation of the conjugates would be preferred.

Steroid conjugates have been isolated from biological fluids by extraction with organic solvents (1), extraction as pyridinium salts into chloroform (3), gel filtration (4–7), and adsorption on charcoal or alumina–diatomaceous earth[1] (2, 8). Group separations of steroid sulfates and glucuronides have been accomplished by adsorption chromatography on alumina (9, 10) or diatomaceous earth (11), paper chromatography (12), thin-layer chromatography (13), and ion exchange (14). Steroid sulfates have been separated by partition chromatography on diatomaceous earth (15) or on methylated dextran gel (16), by ion exchange (14), and steroid glucuronides have been partially separated by gel filtration on dextran gel (7).

Procedures for the separation of estrogen conjugates have been generally concerned with estrone, estradiol, and estriol. Where other estrogens such as equilin, dihydroequilin, equilenin, and dihydroequilenin are also of interest, hydrolytic procedures have been employed prior to analysis.

The objective of this study was the separation of sodium equilin sulfate from sodium equilenin sulfate and sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate. These separations were achieved on dextran gel[2] and were found to be quantitative and readily adaptable to ultraviolet determination. It was also possible to separate colored oxidation products of equilenin by this method. Observations made previously and independently elsewhere (17) have shown dextran gels to be useful in the qualitative separation of complex mixtures of estrogen conjugates. Observations on individual estrogens appear to indicate that quantitative separations of complex mixtures may now be possible. Apparently the separations are the result of differences in adsorption on the gel.

EXPERIMENTAL

Reagents—The sodium sulfate derivatives of the various estrogens used were prepared by D. Irvine, Ayerst Laboratories, Division of Ayerst, McKenna and Harrison Limited, Montreal, Canada. Dextran gel filtration media of various degrees of cross linking, G10, G15, and G25, were obtained from Pharmacia Fine Chemicals. Retention of sodium equilin sulfate on G10 and G15 was about the same and greater than that on G25. After these initial trials, G15 was used exclusively. The water used throughout the study was finally distilled from acid permanganate. All other reagents used were of analytical reagent grade and were used without further purification.

Preparation and Operation of Columns—The dextran gel was soaked in an excess of water for at least 4 hr. to allow for complete swelling. The slurried material was added to a 50-ml. buret, 70 cm. × 1.1 cm. i.d., containing a pledget of glass wool. While the water was allowed to drain out and as settling progressed, more slurry was added. Where salt or buffer solutions were used in elution, the column was prepared using these solutions. The columns were left to stand for several hours for completion of gel settling. The excess eluant above the gel surface was removed and the sample solution was carefully pipetted on to the top of the bed. After the sample had passed into the column bed, the eluant was added and maintained at an approximately steady level using a reservoir to decrease the chances of a change in flow rate, about 18 ml. per hour, during the course of the separation.

Eluate fractions of about 2 ml. were collected using an automatic siphon fraction collector[3] or a Volumeter collector.[4] For initial trials an ultraviolet absorbance spectrum, as shown in Fig. 1, was obtained for each fraction using a Cary 14 recording spectrophotometer, whereby the identity and concentration of the eluted materials could be determined. Chromatograms were obtained by plotting observed absorbance, corrected for dilution, against volume eluted.

Preparation of Samples—Fresh solutions of the sodium estrogen sulfate salts were prepared in a minimum amount of water prior to addition to the column. Samples of sodium equilin sulfate in phosphate buffer solution prepared for oxidation studies were applied to the column directly. Partially hydrolyzed samples were extracted with chloroform to remove free estrogens and the aqueous layer containing the conjugates was applied to the column.

RESULTS AND DISCUSSION

The column chromatographic procedure described permits the separation, isolation, and direct quantitative determination of two estrogen conjugates and their oxidation products. Initially, on

Received April 14, 1967, from the Pharmaceutical Development Laboratories, Ayerst Laboratories, Inc., Rouses Point, NY 12979

Accepted for publication July 19, 1967.

Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors thank Dr. Desmond Beall and Dr. George Milosovich for their interest and helpful discussions and Mr. Palmer Neil for colorimetric analyses.

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

[2] Marketed as Sephadex G15 by Pharmacia Fine Chemicals, Piscataway, N. J.

[3] Marketed by G. M. Instrument Co., Greenville, Ill.

[4] Marketed as model V Volumeter with model 270 collector by Instrumentation Specialties Co., Lincoln, Nebr.

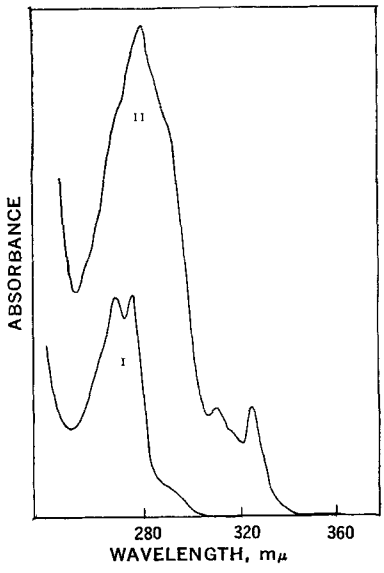


Fig. 1—Comparison of ultraviolet spectra of the sodium salts of equilin sulfate, dihydroequilin sulfate, equilenin sulfate, and dihydroequilenin sulfate. Key: I, equilin and dihydroequilin sulfates; II, equilenin and dihydroequilenin sulfates.

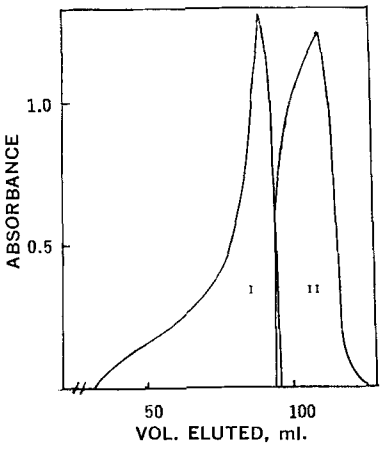


Fig. 3—Separation of sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate on dextran gel (14 Gm.) with water as eluant. Key: I, sodium 17β-dihydroequilin, absorbance 275 mμ; II, sodium 17β-dihydroequilenin sulfate, absorbance 325 mμ. Recovery data shown in Table I, 2.

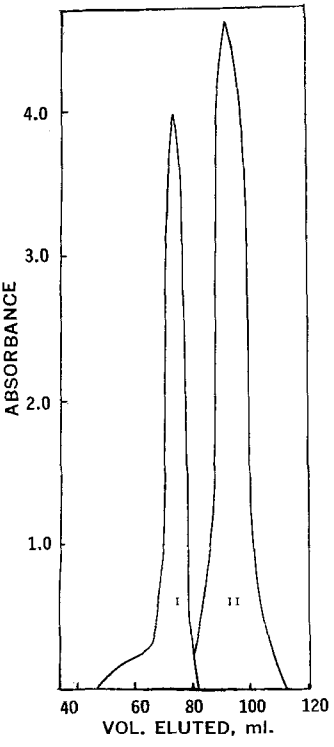


Fig. 2—Separation of sodium equilin sulfate from sodium equilenin sulfate on dextran gel (14 Gm.) with water as eluant. Key: I, sodium equilin sulfate, absorbance at 275 mμ; II, sodium equilenin sulfate, absorbance at 325 mμ. Recovery data shown in Table I, 1.

TABLE I—PER CENT RECOVERED FOR SEVERAL STANDARD SODIUM ESTROGEN SULFATE SAMPLES (ELUANT: WATER)

Estrogen Moiety	Added, mg.	Found, mg.	Recovery, %
1, Equilin	19.2	19.1	99.5
Equilenin	18.9	18.1	95.8
2, 17β-Dihydroequilin	10.8	10.4	96.3
17β-Dihydroequilenin	5.7	6.0	105
3, Equilenin	3.9	3.9	100
4, Estrone	27.3	28.2	103
5, 17β-Dihydroequilin	17.3	17.1	98.9
6, Equilin	940	873	92.9
Equilenin (trace impurity)		11.9	1.3
7, Equilin	235	219	93.2
Equilenin (trace impurity)		3.0	1.3

a 7-Gm. column with water as the eluant, after the elution of the traces of inorganic salts present in the samples, sodium equilin sulfate and sodium equilenin sulfate were eluted with only partial separation. By increasing the column length with twice the amount of dextran gel, these two compounds were separated, as shown in Fig. 2. Samples subjected to accelerated oxidation gave elution patterns in which the appearance of equilin and equilenin was preceded by the elution of colored oxidation products of equilenin. Similar chromatograms were obtained for sodium 17β-dihydroequilin sulfate and sodium 17β-dihydroequilenin sulfate, as shown in Fig. 3.

A compilation of some theoretical and recovered weight data for various trials is given in Tables I and II. From the data presented in Tables I and II it can be seen that the procedure should also offer a means of purifying large amounts of individual estrogen conjugates. Purification methods would necessitate modification of column dimensions, bed volume, and eluent composition in order to improve capacity. Data of this type will be reported at a later date.

TABLE II—PER CENT RECOVERED FOR STANDARD SODIUM ESTROGEN SULFATE SAMPLES (ELUANT: SALT OR BUFFER SOLUTION)

Estrogen Moiety	Eluant	Added, mg.	Found, mg.	Recovery, %
1, Equilin	5×10^{-4} *M*	10.4	10.7	103
Equilenin	NaCl	2.0	2.2	109
2, Equilin	0.001 *M* pH 4.3 Phosphate	14.4	14.0	97.2
3, Equilin	0.001 *M* pH 10.0 Phosphate	22.8	21.7	95.2
4, Equilin	0.01 *M* NaCl	94.3	59.8	63.4[a]
Equilenin		19.3	...	...

[a] Elution incomplete after 150 ml. of eluate collected.

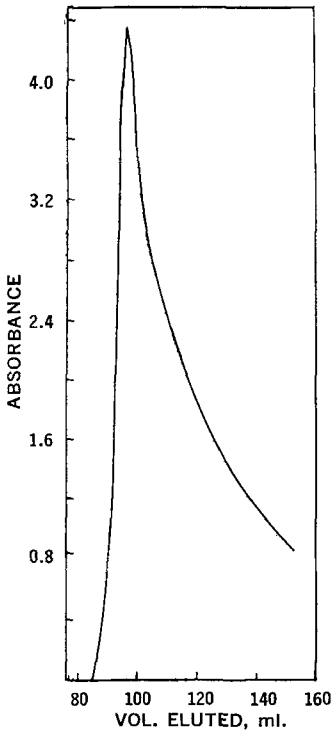


Fig. 4—Elution of sodium equilin sulfate from dextran gel (14 Gm.) with 0.01 M *sodium chloride as eluant. Recovery data shown in Table II,* 4.

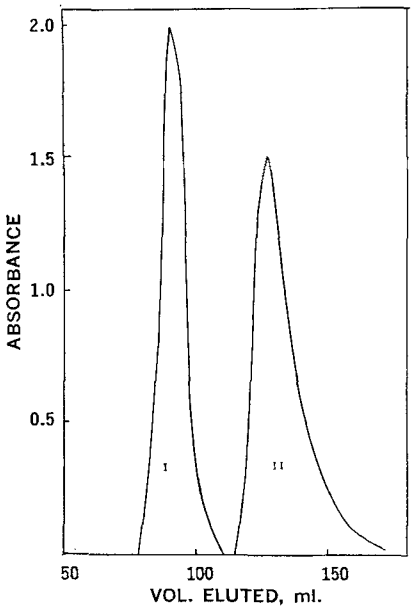


Fig. 5—Separation of sodium equilin sulfate from sodium equilenin sulfate on dextran gel (14 Gm.) with 5×10^{-4} M *sodium chloride as the eluant. Key:* I, *sodium equilin sulfate, absorbance 275 mμ;* II, *sodium equilenin sulfate, absorbance 280 mμ. Recovery data shown in Table II,* 1.

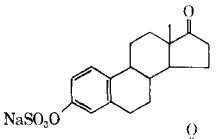
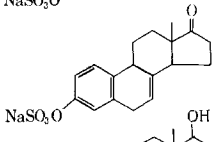
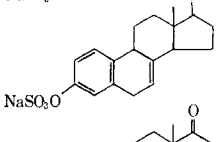
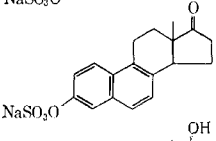
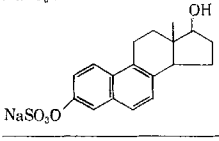
There are three possible factors to be considered when interpreting elution patterns on dextran gels: gel filtration, adsorption, and ion exchange. Simple gel filtration depends on the molecular size with the larger molecules being eluted first. Aromatic, heterocyclic, or basic compounds have been observed to adsorb reversibly with the degree of adsorption increasing with the amount of cross linking in the gel. Dextran gels also exhibit a weak ion-exchange property where the active functional groups are available carboxylic acid groups.

In separations that were performed with small amounts of sodium chloride or buffer present using water as the eluant, the electrolyte was eluted prior to the elution of the estrogen conjugate. Varying the pH (4.3 and 10.0, 0.001 *M* phosphate) of the eluant had little or no effect on the elution pattern. When 0.01 *M* sodium chloride was used as the eluant, the retention of sodium equilin sulfate on the gel was increased, as shown in Fig. 4, while higher salt concentrations resulted in nearly complete retention. From these observations, it would appear that the major factor operative in the observed separations was adsorption.

Proper selection of eluant electrolyte concentration slightly improves the separation of sodium equilin sulfate from sodium equilenin sulfate, as shown in Fig. 5. A sample of sodium equilin sulfate was prepared and subjected to accelerated oxidation conditions until only sodium equilenin sulfate and further oxidation products remained as determined by equilin and equilenin color tests. To this sample, 33.8 mg. of sodium equilin sulfate was added and the mixture placed on a dextran gel column for separation using 5×10^{-4} *M* sodium chloride as the eluant. Colored oxidation products were either irreversibly adsorbed on the gel or were eluted initially. Sodium equilin sulfate was recovered at 33.5 mg. or 99%.

The difference in the adsorption and elution be-

TABLE III—APPROXIMATE ELUATE VOLUME AT PEAK APPEARANCE IN THE CHROMATOGRAMS OF SEVERAL SODIUM ESTROGEN 3-SULFATE SALTS (14-GM. COLUMN, BED VOLUME, 45. ML.)

Estrogen	Vol. at Peak (ml.) Eluant: H_2O	Eluant: 5×10^{-4} M NaCl
 Estrone	70	73
 Equilin	75	90
 17β-Dihydroequilin	90	100
 Equilenin	95	125
 17β-Dihydroequilenin	110	160

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With due consideration of experimental conditions, it should be possible to modify this chromatographic procedure for the quantitative separation of complex mixtures of sodium estrogen sulfate salts. Preliminary work toward this end is already under way (17).

REFERENCES

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By GEORGIA J. PERSINOS* and MAYNARD W. QUIMBY

Fifty species of Nigerian plants were evaluated for the presence of alkaloids, saponins, and tannins. Of these, 24 contained alkaloids, 43 contained tannins, and 16 contained saponins.

THIS WORK is a continuation of the investigation carried out on plants used as medicinals by a pagan tribe, the Anagutas, living on the Jos Plateau in northern Nigeria, and the Hausas, living throughout northern Nigeria. Since plants occupy an important and sometimes central role in the religious beliefs and social practices of these peoples, an investigation of certain of these plants[1] has been undertaken

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* Present address: Research Pathology Associates, Division of Phytochemistry, Rockville, MD 20851
[1] The plant specimens were authenticated by Dr. Quimby and were made a part of the botanical collections of the Massachusetts College of Pharmacy.

EXPERIMENTAL

Preparation of Extracts—The screening procedures for alkaloids, saponins, and tannins were adapted from those described by Wall *et al.*(1, 2). An extract of each plant was prepared by refluxing 10 Gm. of the air-dried milled plant sample with 100 ml. of 80% ethanol for 1 hr. Each extract was then cooled to room temperature, suction-filtered, and washed with sufficient 80% ethanol to bring the volume of filtrate to 100 ml.

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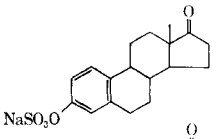
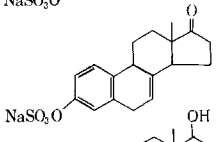
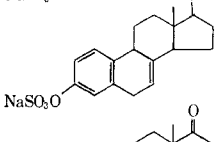
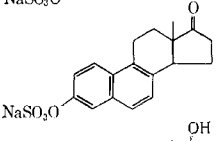
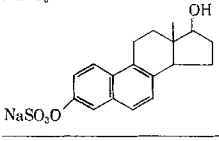
 Estrone	70	73
 Equilin	75	90
 17β-Dihydroequilin	90	100
 Equilenin	95	125
 17β-Dihydroequilenin	110	160

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TABLE I—PHYTOCHEMICAL SCREENING RESULTS FOR 50 SPECIES OF NIGERIAN PLANTS

Scientific Name	Part[a] Used	Alkaloids[b] M[c]	Alkaloids[b] STA[d]	Alkaloids[e] Fraction I	Alkaloids[e] Fraction II	Tannins Gelatin Salt	Tannins $FeCl_3$[f]	Saponins
Ampelidaceae								
Cissus hochstetteri Planch.	r	−	−	−	−	+	bk	+
Anacardiaceae								
Mangifera indica Linn.	l	−	−	−	−	+	bk/g	−
Annonaceae								
Annona senegalensis Pers.	sb	+	+	+	+	+	b/g	+
Uvaria chamae P. Beauv.	r	−	−	+	−	+	bk/g	−
Uvaria chamae P. Beauv.	sb	−	−	+	−	+	bk/g	−
Araliaceae								
Cussonia nigerica Hutch.	s	−	−	−	−	−	bk/g	+
Bignoniaceae								
Stereospermum kunthianum Cham.	sb	−	−	−	−	−	−	−
Bombacaceae								
Adansonia digitata Linn.	s	+	+	+	−	−	−	−
Celastraceae								
Maytenus senegalensis Exell.	r	+	+	+	−	+	b/g	−
Cochlospermaceae								
Cochlospermum tinctorium A. Rich.	rz	−	−	−	−	+	bk/g	−
Combretaceae								
Terminalia avicennoides Guill. and Perr.	sb	−	−	−	−	+	bk/g	+
Terminalia avicennoides Guill. and Perr.	rb	−	−	−	−	−	b/b	−
Terminalia avicennoides Guill. and Perr.	r	−	−	−	−	+	b/b	−
Terminalia avicennoides Guill. and Perr.	l	−	−	−	−	−	b/b	−
Compositae								
Echinops longifolius A. Rich.	l	−	−	−	−	+	g	−
Convulvulaceae								
Ipomea sp.	l	+	+	+	−	+	g	−
Dioscoreaceae								
Dioscorea abyssinica Hochst. ex Kunth.	Yam	+	+	+	−	−	−	+
Dioscorea sp.	Corms	−	−	−	−	+	bk/g	+
Euphorbiaceae								
Bridelia ferruginea Benth.	l	−	−	−	−	+	bk	−
Elaeophorbia drupifera Stapf	r	+	+	+	−	+	bk	−
Elaeophorbia drupifera Stapf	sb	−	−	−	−	−	br	−
Hymenocardia acida Tul.	r	−	−	+	−	−	dg	−
Hymenocardia acida Tul.	sb	−	−	−	−	+	br	−
Hymenocardia acida Tul.	l	−	−	−	−	+	g	−
Gramineae								
Vetiveria nigritana Stapf	wp	−	−	−	−	+	bk	+
Hypericaceae								
Harungana madagascariensis Lam. ex Poir.	sb	−	−	−	−	+	br	−
Harungana madagascariensis Lam. ex Poir.	l	−	−	−	−	+	bk/g	−
Psorospermum corymbiferum Hochst. var. *kerstingii* (Engl.) Keay ex Milne-Redh.	l	+	+	−	−	+	bk	+
Psorospermum febrifugum var. *ferrugineum* (Hook. f.) Keay ex Milne-Redh.	sb	+	+	−	−	+	bk/g	+
Leguminosae								
Abrus precatorius Linn.	wp	+	+	+	−	−	bk	−
Adenodolichos paniculatus (Hua) Hutch. and Dalz.	sb	+	+	+	−	+	bk/g	−

(*Continued on next page.*)

TABLE I—(*Continued.*)

Scientific Name	Part[a] Used	Alkaloids[b] M[c]	Alkaloids[b] STA[d]	Alkaloids[e] Fraction I	Alkaloids[e] Fraction II	Tannins Gelatin Salt	Tannins $FeCl_3$[f]	Saponins
Bauhinia reticulata DC.	sb	—	—	—	—	+	bk/g	—
Bauhinia reticulata DC.	l	—	—	+[g]	—	+	bk/g	—
Cassia goratensis Fres.	r	—	—	—	—	+	b/b	—
Cassia occidentalis Linn.	s	—	—	—	—	+	bk	—
Cassia siberiana DC.	r	—	—	—	—	+	br	—
Desmodium gangeticum DC.	r	—	—	—	—	+	g	—
Desmodium salicifolium DC.	r	—	—	—	—	+	g	—
Erythrina senegalensis DC.	sb	—	—	—	—	—	br	—
Erythrophleum guineense G. Don.	sb	+	+	—	—	+	br	—
Mucuna puriens (Linn.) DC.	sb	—	—	+	—	+	bk/g	—
Parkia filicoidea Welw.	sb	+	+	+	—	+	bk	+
Parkia filicoidea Welw.	l	—	—	—	—	+	bk/g	—
Liliaceae								
Asparagus schroederi Engl.	s	+	+	+	—	+	dg	+
Loranthaceae								
Loranthus sp.	l	—	—	—	—	+	bk/g	—
Loranthus sp.	s	—	—	—	—	+	bk/g	—
Melastomataceae								
Dissotis sp.	s	—	—	—	—	+	b/g	—
Dissotis sp.	r	—	—	—	—	+	bk	—
Meliaceae								
Pseudocedrela kotschyi (Schweinf.) Harms	sb	—	—	—	—	+	b/b	—
Moraceae								
Ficus indica Hochst. ex Walp.	r	+	+	+	—	+	b/b	—
Ficus gnaphalocarpa (Miq.) Steud. ex A. Rich.	sb	+	+	+	—	+	b/g	—
Myrtaceae								
Eugenia owariensis Beauv.	sb	—	—	—	—	+	b/b	+
Eugenia owariensis Beauv.	l	—	—	—	—	+	b/b	—
Psidium guajava Linn.	l	—	—	—	—	+	bk	—
Pedaliaceae								
Rogeria adenophylla J. Gay	r	+	+	+	+	—	—	—
Polygalaceae								
Polygala arenaria Willd.	r	—	—	+	—	+	bk/g	—
Rubiaceae								
Fadogia agrestis Schweinf.	r	—	—	+	—	+	b/g	—
Fadogia agrestis Schweinf.	s	—	—	—	—	+	br	+
Fadogia agrestis Schweinf.	l	—	—	—	—	+	bk/g	—
Gardenia erubescens Stapf ex Hutch.	l	—	—	—	—	+	bk/g	—
Gardenia erubescens Stapf ex Hutch.	sb	—	—	—	—	—	g	+
Pavetta sp.	r	+	—	—	—	—	br	—
Pavetta sp.	s	—	—	—	—	+	b/g	—
Tiliaceae								
Grewia mollis Juss.	rb	—	—	—	—	+	g	—
Grewia mollis Juss.	sb	—	—	—	—	+	g	—
Grewia mollis Juss.	l	—	—	+[h]	—	+	g	—
Ulmaceae								
Trema guineensis (Schum. and Thonn.) Ficalko	r	—	—	—	—	+	g	—
Umbelliferae								
Steganotaenia araliacea Hochst.	l, s	—	—	—	—	+	bk	+

[a] sb, stem bark; s, stem; r, root; rb, root bark; rz, rhizome; l, leaves; wp, whole plant. [b] Wall *et al.* procedure (1), based on confirmatory test. [c] Mayer's reagent. [d] Silicotungstic acid. [e] Farnsworth and Euler procedure (3). [f] bk/g, back-green; g, green; b/b, blue-black; br, brown; dg, dark green; bk, black; bl/g, blue-green. [g] Positive with 60 μl. [h] Positive with 70 μl.

in the following manner with all extracts giving a preliminary positive test for alkaloids.

Two milliliters of the acidic aqueous extract, prepared as described above, was treated with 28% ammonium hydroxide solution until the solution was distinctly alkaline to litmus paper, and then extracted several times with chloroform. The chloroform extracts were combined and concentrated *in vacuo* to about 2 ml., and then extracted with an equal volume of 1% aqueous hydrochloric acid.

One milliliter of the separated acid extract was treated with a few drops of Mayer's reagent, and a second 1-ml. portion was treated with silicotungstic acid reagent as previously described. Turbidity or precipitation after the addition of either of these reagents was taken as a confirmed positive test for the presence of alkaloids in the extract. The results of these tests are presented in Table I.

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Tannins—Twenty milliliters of the original 80% ethanol extract from each plant sample was evaporated to dryness on a steam bath and the residue was stirred with 5 ml. of distilled water and filtered. Two milliliters of the filtrate was treated with a few drops of gelatin–salt reagent (1), and precipitation was taken as evidence for the presence of tannins. The addition of ferric chloride reagent to extracts of plants giving positive gelatin–salt tests served to further categorize the tannin present as to hydrolyzable (blue, blue-black), or condensed types (green, blue-green). These results are presented in Table I.

SUMMARY

Of the 50 species screened for alkaloids, 24 showed the presence of tertiary alkaloids, representing 14 families of plants. Only two species exhibited the presence of quaternary alkaloids, one in the *Annonaceae*, and the other in the *Pedaliaceae*. Sixteen species, distributed in 13 families, contained saponins. The 43 species giving positive tests for tannins were in 29 different families.

REFERENCES

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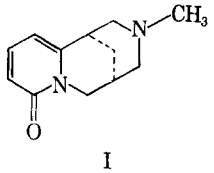
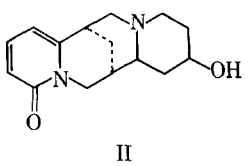


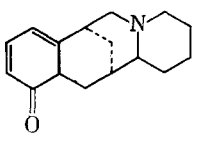
I

(−) Methylcytisine



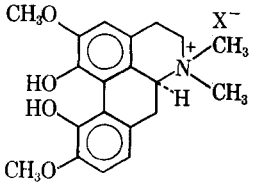
II

(−) Baptifoline



III

(−) Anagyrine



IV

(+) Magnoflorine

One milliliter of the separated acid extract was treated with a few drops of Mayer's reagent, and a second 1-ml. portion was treated with silicotungstic acid reagent as previously described. Turbidity or precipitation after the addition of either of these reagents was taken as a confirmed positive test for the presence of alkaloids in the extract. The results of these tests are presented in Table I.

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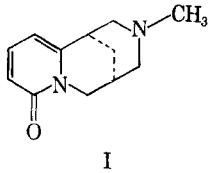
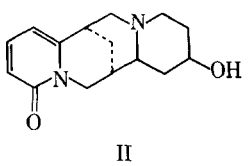


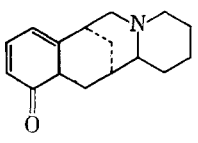
I

(−) Methylcytisine



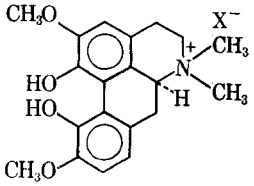
II

(−) Baptifoline



III

(−) Anagyrine



IV

(+) Magnoflorine

EXPERIMENTAL[1]

Plant Material—The roots and rhizomes of *Caulophyllum thalictroides* (L.) Michx. used in this investigation were obtained from S. B. Penick and Co. (lot No. 668 BA W-80576).

Extraction and Initial Separation—The plant material (13.7 Kg.) was extracted by percolation at room temperature with ethanol U.S.P. requiring about 74 L. of solvent to completely exhaust the material of alkaloids as evidenced by a negative Valser's test. Evaporation of the solvent at reduced pressure at 40° left 2.24 Kg. of residue. This was partitioned between chloroform and 2% aqueous citric acid in 100-Gm. quantities requiring 300 ml. of each phase. The citric acid phase was extracted three times with equal volumes of chloroform and then brought to pH 9 with concentrated ammonium hydroxide and then extracted with chloroform. This extract was dried over anhydrous sodium sulfate and on evaporation of the solvent left 30.6 Gm. of crude tertiary alkaloids.

Thin-layer chromatography of the tertiary alkaloids on Silica Gel G[2] with chloroform–methanol–ammonium hydroxide (200:20:1) indicated the presence of six tertiary alkaloids when detected with Dragendorff's reagent (2).

The ammonium hydroxide solution after removal of the tertiary alkaloids was acidified with 10% hydrochloric acid producing a gelatinous precipitate which was collected by filtration. From this precipitate it was possible to isolate caulosaponin, m.p. 257–259° [lit. values m.p. 250–255° (1), 254–255° (3)] by the procedure of Power and Salway (1). Hydrolysis of caulosaponin with aqueous alcoholic hydrochloric acid yielded hederagenin, m.p. 333–335°, $[\alpha]_D^{29}$ + 80° (c, 0.10% in ethanol), ultraviolet end absorption, λ = 210 mμ (ϵ = 4380); the literature values (3) are m.p. 332–333°, $[\alpha]_D^{20}$ + 78° (c, 0.10% in ethanol), ultraviolet absorption $\lambda_{max.}$ = 210 mμ (ϵ = 2860). Refluxing hederagenin in acetic anhydride for 1.5 hr. gave the acetate, m.p. 172–174° [lit. value (3) m.p. 173–174°]. The acidic filtrate was treated with a saturated aqueous solution of ammonium reineckate (2 Gm. reagent/100 ml. solution) while stirring. The precipitate of alkaloid reineckate was collected by suction filtration, sucked dry, and then washed with ether giving a yield of 285 Gm. TLC examination of the crude quaternary chloride (see below) on Silica Gel G with *n*-propanol–ammonium hydroxide–water (4:1:1) as solvent system showed one spot (R_f 0.55) when sprayed with Dragendorff's reagent.

Isolation of Magnoflorine Chloride—The alkaloid reineckate (190 Gm.) was dissolved in 3 L. of acetone–water (1:1) and the solution passed through a column containing 1700 ml. of Amberlite IRA-410 (Cl) resin. The crude quaternary alkaloid chloride (81 Gm.) from the effluent was chromatographed on Woelm neutral alumina,[3] activity V. The mixture was placed on the column as a methanol solution and eluted with methanol. The effluent was examined by TLC and the alkaloid fraction collected. Crystallization of the alkaloid fraction (72 Gm.) from ethanol gave magnoflorine chloride, m.p. 240–241° dec., $[\alpha]_D^{27}$ + 208° (c, 0.144% in methanol). The reported values (4) are m.p. 242–243° dec. and $[\alpha]_D^{31}$ + 213°. The infrared spectrum of the isolated substance and that of an authentic sample of magnoflorine chloride were superimposable. Mobility of the two samples was the same on TLC plates.

Separation of the Tertiary Alkaloids—The mixture of tertiary alkaloids, which on TLC indicated six components, was separated on a partition chromatographic column according to the procedure of Brown and Kupchan (5). The solvent system was Skellysolve B–ethylene dichloride–methanol–water (10:6:2.5:0.5). A column, 3.7 cm. (i.d.) × 47 cm., was prepared by mixing 182 ml. of the stationary phase (upper phase) with 300 Gm. of diatomaceous earth[4] and packing the material in 1-cm. segments with the aid of a ramrod. The mobile phase was passed through the packed column till all of the air had been removed (about 2 days). The holdup volume of the column was 395 ml.

The tertiary alkaloid mixture of 1.32 Gm. in 20 ml. of the mobile phase was passed into the column. The flow rate was 30 ml./hr. Fractions of effluent (20 ml.) after passage of the holdup volume were collected and analyzed by TLC. A summary of the separation obtained is given in Table I along with the tubes pooled according to the materials present.

Of the fractions examined only fractions 2, 3, and 8 yielded crystalline product or a crystalline salt. All the other fractions remained as heavy oils. These latter fractions account for only about 9% of the crude tertiary alkaloids. (Table I.)

Methylcytisine (I)—From pooled fraction No. 3, the 343 mg. of crude material yielded 194 mg. of crystalline product from benzene–Skellysolve B, m.p. 137° [lit. value (1) m.p. 137°]. The perchlorate salt was prepared by the usual procedure, m.p. 280–281° [lit. value (6) m.p. 282°] as well as the crystalline picrate, m.p. 221–222° [lit. value (1) m.p. 228°]. The specific rotation was $[\alpha]_D^{30}$ − 224° (c, 1.05% in water) [lit. value (1) $[\alpha]_D$ − 221.6°]. The ultraviolet absorption spectrum showed $\lambda_{max.}$ = 309 mμ (ϵ = 7,560); 234(6,860). In addition, a known sample of cytisine was methylated by the Eschweiler-Clarke procedure of the Leuckart reaction (7) to give a product identical with the isolated material (I.R., U.V., and mixed melting point determination).

Baptifoline (II)—The pooled column fraction No. 8 (241 mg.) was crystallized from benzene to yield 118 mg. of product, m.p. 210°. [Baptifoline lit. value (8) m.p. 210°.] The crystalline perchlorate salt, m.p. 286–287°, and the picrate which sinters at 145° then melts at 252° were prepared by the usual methods. The values in the literature are m.p. 286–287° (8) and m.p. 145° (sinters), then 256° (6). The optical rotation was $[\alpha]_D^{27}$ − 133° (c,

[1] Melting points were determined with a Thomas-Hoover Uni-Melt capillary melting point apparatus in sealed evacuated capillaries and were corrected. The infrared spectra were taken in chloroform and as KBr pellets on a Perkin-Elmer model 237 infrared spectrophotometer. The ultraviolet spectra were determined on a Cary model 15 recording spectrophotometer, in ethanol.

[2] Made by E. Merck (Darmstadt, West Germany), obtained from Brinkmann Instruments, Inc., Westbury, N. Y.

[3] Obtained from Alupharm Chemicals, New Orleans, La.

[4] Celite 545, a product of Johns-Manville Corp., New York, N. Y., which was purified by dispersing in 6 *N* HCl, three times, then filtering by suction and washing successively with distilled water, methanol, benzene, Skellysolve B, and methanol. Skellysolve B is a petroleum ether fraction, b.p. 60–80°. Ethylene dichloride, C.P., was from Union Carbide Co., and methanol A.R. was from Mallinckrodt Chemicals.

TABLE I—SEPARATION OF TERTIARY ALKALOID MIXTURE

Fraction No.	Tube No., 20 ml.	TLC R_f	Pooled Wt., mg.	Crystalline Product, mg.	% Yield[a]	Compd.
1	12–14	0.74	35	...	...	...
2	16–19	0.67	144	109[b]	0.012	Anagyrine
3	21–28	0.60	343	194	0.033	Methylcytisine
4	43–50	0.38	25	...	...	...
5	54–58	0.28	14	...	...	...
6	71–85	0.27	22	...	...	...
7	91–104	0.24	22	...	...	...
8	105–165	0.28	241	118	0.020	Baptifoline

[a] Based on dry plant material. [b] As perchlorate salt.

0.325% in ethanol). [Lit. value $[\alpha]_D^{18}$ − 147.7° (6).] The ultraviolet absorption spectrum showed $\lambda_{max.}$ = 309 mμ (ε = 7,540); 234(7,150). The infrared spectrum of authentic baptifoline[5] taken in a Nujol mull was identical to that of our compound determined in the same way.

Anagyrine (III)—The oil (144 mg.) in the pooled fraction No. 2 provided a crystalline perchlorate from methanol, m.p. 315°, and a picrate from methanol, m.p. 247–249°. Anagyrine perchlorate melts at 315° (8) and the picrate at 249–251° (6). The ultraviolet absorption spectrum of the perchlorate salt showed $\lambda_{max.}$ = 308 mμ (ε = 7,460); 234(6,900) and an infrared absorption spectrum with peaks at $\nu_{max.}$ 1650, 1570, and 1560 cm.$^{-1}$ when taken in a Nujol mull, and are consistent with the reported values for anagyrine (9).

DISCUSSION

C. thalictroides has been known for over 50 years to contain methylcytisine. With the application of modern isolation and analytical methods this source has been found to contain many more alkaloids of which three not previously recognized have been isolated in pure form and identified.

The three lupin tertiary alkaloids, methylcytisine, baptifoline, and anagyrine, were readily separated by partition column chromatography and are the first example of the isolation of the latter two alkaloids from the family *Berberidaceae*. The other alkaloids (at least three) in this fraction were present in very small amounts, remained as oils, and could not be induced to crystallize or to form crystalline salts, although separated by the column method so as to show only one spot reacting with Dragendorff's reagent. Further identification of the minor tertiary alkaloids must await the processing of large amounts of plant material. Undoubtedly they will also be of the lupin type.

The presence of magnoflorine in *Caulophyllum robustum* Maxim., has been reported by Tomita and Takahashi (10). The presence of this ubiquitous alkaloid has now been confirmed in *C. thalictroides*.

[5] Kindly provided by Professor Shigenobu Okuda, University of Tokyo, Tokyo, Japan.

REFERENCES

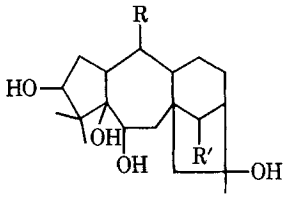
(1) Power, F. B., and Salway, A. H., *J. Chem. Soc.*, **103**, 191(1913).
(2) Block, R. J., Durrum, E. L., and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press Inc., New York, N. Y., 1958, p. 361.
(3) McShefferty, J., and Stenlake, J. B., *J. Chem. Soc.*, **1956**, 2314.
(4) Winek, C. L., Beal, J. L., and Cava, M. P., *Lloydia*, **27**, 111(1964).
(5) Brown, K. S., Jr., and Kupchan, S. M., *J. Chromatog.*, **9**, 71(1962).
(6) Marion, L., and Cockburn, W. F., *J. Am. Chem. Soc.*, **70**, 3472(1948).
(7) Moore, M. L., in "Organic Reactions," vol. V, Adams, R., ed., John Wiley & Sons, Inc., New York, N. Y., 1949, p. 301.
(8) Marion, L., and Turcotte, F., *J. Am. Chem. Soc.*, **70**, 3253(1948).
(9) Okuda, S., Murakoshi, I., Kamata, H., Kashida, Y., Haginawa, J., and Tsuda, K., *Chem. Pharm. Bull. (Japan)*, **13**, 482(1965).
(10) Tomita, M., and Takahashi, J., *Yakugaku Zasshi*, **78** 680(1958).

Grayanotoxin I. Occurrence in Additional *Ericaceae* Species

By G. H. CONSTANTINE, JR., KIRTI SHETH*, and PHILIP CATALFOMO

The title compound was identified in five of seven ericaceous species not previously investigated but listed as being poisonous. Thin-layer chromatography and spectral analyses established the presence of grayanotoxin (acetylandromedol) in *Rhododendron macrophyllum*. Comparative chromatography and U.V. spectral analysis indicated that the compound was present in four additional species.

THE GRAYANOTOXINS (I) are known to occur only in the family *Ericaceae* (1). Several members of this family, which occur in the Pacific Northwest and are reported to be poisonous (2–4), have never been investigated for the presence of these compounds. This study was undertaken to determine if the grayanotoxins could be identified in certain native species thereby verifying, at least in part, the agent responsible for the reported toxicity.



	R	R′
Grayanotoxin I	CH_3, OH	OAc
Grayanotoxin II	CH_2	H, OH
Grayanotoxin III	CH_3, OH	H, OH

EXPERIMENTAL

Chromatographic Screening—The plants were collected in Oregon during the summer of 1966.[1] The leaves were dried in a forced-air dryer, ground to a coarse powder, and stored in plastic bags until time for extraction.

One hundred-gram samples of each plant were defatted with petroleum ether in a Soxhlet apparatus and then exhaustively extracted with ethanol U.S.P. The ethanol extracts were concentrated *in vacuo* to a lesser volume (about 50 ml.) and diluted with an equal volume of distilled water. These mixtures were subjected to liquid–liquid extraction with chloroform U.S.P. The chloroform extracts were concentrated to dryness *in vacuo*, redissolved in a minimum amount of chloroform, and examined chromatographically.

Thin-layer chromatography on Silica Gel G plates, prepared according to Stahl, was employed as the preliminary screening procedure. Three solvent systems were found to separate authentic samples of the grayanotoxins.[2] The results of the co-spotting experiments are recorded in Table I. Grayanotoxin I (G-I) was verified as being present in the following: *Rhododendron albiflorum* Hook, *R. macrophyllum* D. Don., *R. occidentale* (T. and G.) Gray, *Kalmia polifolia* Wang. var. *microphylla* Hook, and *K. polifolia* Wang. var. *polifolia*. The toxic principle could not be detected in either *Ledum glandulosum* Nutt or *Menziesea ferruginea* Smith.

A vacuum-zone extractor was used to remove samples from unsprayed chromatoplates for U.V. analyses. Areas corresponding in R_f to G-I for each of the five plants, as well as G-I, were then removed from the extractor with boiling methanol (spectral grade). Aliquots were scanned in a Beckman DB recording spectrophotometer and all gave a characteristic maximum at 255 mμ.

TABLE I—CO-SPOTTING OF PLANT EXTRACTS

Compd.	Solvent Systems[a] CAW	EIW	TEF
1, Grayanotoxin I	0.86[b]	0.73	0.18
2, Grayanotoxin II	0.90	0.79	0.22
3, Grayanotoxin III	0.80	0.69	0.12
4, *R. albiflorum*	0.86	0.73	0.18
5, Co-spot (*1* and *4*)	0.86	0.73	0.18
6, *R. macrophyllum*	0.85	0.73	0.18
7, Co-spot (*1* and *6*)	0.85	0.73	0.18
8, *R. occidentale*	0.87	0.71	0.17
9, Co-spot (*1* and *8*)	0.87	0.70	0.17
10, *K. poli.* var. micro.	0.86	0.71	0.19
11, Co-spot (*1* and *10*)	0.86	0.71	0.20
12, *K. poli.* var. poli.	0.87	0.72	0.17
13, Co-spot (*1* and *13*)	0.87	0.70	0.18
14, *L. glandulosum*	...	...	...
15, Co-spot (*1* and *14*)	0.86	0.71	0.18
16, *M. ferrugenea*	...	...	...
17, Co-spot (*1* and *16*)	0.88	0.74	0.18

[a] Solvent systems: CAW, chloroform–acetic acid–water (35:50:17.5); EIW, ethyl acetate–isopropanol–water (65:24:11); TEF, toluene–ethyl acetate–water (5:4:1). [b] Detection: sprayed with $SbCl_3$ (10% w/w in $CHCl_3$), visualized under U.V. light, and sprayed again with Godin's reagent (1% vanillin in EtOH, 3% H_2SO_4, equal parts.) Heated to 110° for 10 min. after each spray. $SbCl_3$ gives a red-pink coloration, U.V. a red fluorescence, and Godin's reagent a characteristic blue coloration.

Isolation of Grayanotoxin I—A 1-Kg. sample of *R. macrophyllum* was extracted in a similar manner as previously described. The resulting hydroalcoholic solution was then extracted with ethyl acetate in a liquid–liquid extractor for 48 hr. The ethyl acetate fraction on drying *in vacuo* yielded 50 Gm. of a dark green residue which was initially

Received May 8, 1967, from the Department of Pharmacognosy, Oregon State University, Corvallis, OR 97331

Accepted for publication July 26, 1967.

Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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This investigation was partially supported by grant GM-10963-03 from the U. S. Public Health Service, Bethesda, Md.

Appreciation is extended to Mr. Eugene Lee and Mr. William Frenzel for technical assistance.

[1] Plant voucher specimens have been placed in the Oregon State University Herbarium.

[2] Supplied through the courtesy of Professor H. Kakisawa, Tokyo Kyoiku University, Otsuka, Japan.

TABLE II—COLUMN CHROMATOGRAPHY OF *Rhododendron* EXTRACT

Solvent	Fractions	Vol., ml.	R_f Value[a]
1, EtOAc	4	250	...
2, *n*-Propanol (10%) in EtOAc	4	250	0.33[b]
3, *n*-Propanol (50%) in EtOAc	4	250	0.33
4, *n*-Propanol	4	250	...
Grayanotoxin I	...	...	0.33

[a] Solvent system: TEFF, toluene–ethyl formate–formic acid (5:4:1). [b] Detection: see Table I.

TABLE III—COLUMN CHROMATOGRAPHY OF *Rhododendron* FRACTION

Solvent	Fractions	Vol., ml.	R_f Value[a]
1, Benzene	1	500	...
2, EtOAc	6	250	0.34[b]
3, *n*-Propanol (5%) in EtOAc	4	250	0.34
4, *n*-Propanol (10%) in EtOAc	4	250	...
Grayanotoxin I	...	...	0.34

[a] Solvent system: TEFF, toluene–ethyl formate–formic acid (5:4:1). [b] Detection: see Table I.

purified on three identical 450-Gm. alumina columns (5 × 50 cm.).[3] The column was eluted first with twelve 250-ml. fractions of ethyl acetate followed by six 250-ml. fractions of 10% methanol in ethyl acetate.

The progress of the column was monitored by using the solvent system TEF (see Table I). The third and fourth fractions of the second solvent clearly demonstrated the presence of G-I. The combined fractions yielded 4.5 Gm. of greenish residue which was placed upon another 450-Gm. alumina column and eluted as shown in Table II.

The G-I containing fractions thus collected were combined, concentrated to dryness, washed with boiling *n*-hexane, and dissolved in ethyl acetate–*n*-hexane (1:1) in an attempt to precipitate the compound. This procedure yielded a dark green oil which was placed upon a 100-Gm. alumina column (2.5 × 25 cm.) and eluted as shown in Table III.

The positive fractions were combined, dried, and crystallized from *n*-hexane–acetone to yield a green-white precipitate. Two recrystallizations from *n*-hexane–ethyl acetate yielded 5 mg. of pure white needles, m.p. 256–258°.[4] [Reported m.p. 240–243° (5), 259–260° (6), and 264–266° (7).]

Co-chromatography using known G-I and the isolated compound was employed as an additional verification procedure. The co-spot did not separate in the same three solvent systems as in Table I and had an R_f value equivalent to both controls.

The I.R. (KBr pellet) and U.V. spectra (spectral grade methanol) of the isolated as well as the reference G-I were identical.

SUMMARY

Seven species of reportedly poisonous ericaceous plants were screened by thin-layer chromatography in order to detect the presence of the grayanotoxins. Grayanotoxin I was isolated and identified from one of the species. Five of the plants screened were found to contain grayanotoxin I whereas grayanotoxins II and III were not detected. Poisoning reportedly caused by *Ledum glandulosum* and *Menziesea ferruginea* cannot be attributed to the grayanotoxins because of their apparent absence in these species.

REFERENCES

(1) Hegnauer, R., "Chemotaxonomie der Pflanzen, IV," Birkhauser, Basel, Switzerland, 1966, p. 551.
(2) Fleming, C. E., *Ann. Rept., Nev. Agr. Expt. St.*, **39**, 1919(1920).
(3) Marsh, C. D., *USDA Dept. Bull.*, **1924**, 1245.
(4) Sampson, A. W., and Malmstead, H. E., *Calif. Agr. Expt. St. Bull.*, **1942**, 593.
(5) White, J. W., and Riethof, M. L., *Arch. Biochem. Biophys.*, **79**, 165(1959).
(6) Takemoto, T., Nishimoto, Y., Meguri, H., and Katayama, K., *J. Pharm. Soc. Japan*, **75**, 1441 (1955).
(7) Kakisawa, H., Kozima, T., Yanai, M., and Nakanishi, K., *Tetrahedron*, **21**, 3091(1965).

[3] Alumina, activated, chromatographic grade; Matheson, Coleman and Bell, East Rutherford, N. J.

[4] Kofler micro-melting point apparatus, C. Reichert A. G., Vienna, Austria; melting point was uncorrected.

3-Aminopiperidones IV. 2-(*N*,*N*-Diethylamino)-2-*p*-chlorophenylglutarimide and 2-(*N*,*N*-Diethylamino)-2-*p*-tolylglutarimide

By S. K. ARYA, K'UN CHANG, and C. F. MARTIN

The syntheses of two new modifications of the model compound, 2-(*N*,*N*-diethylamino)-2-phenylglutarimide (V) bearing chloro- and methyl-substituents on the *para* position of the phenyl group are described. The preparations of these compounds are extensions of work designed to provide a basis for studies of structure *versus* antiepileptic activity relationships among 3-aminopiperidones.

PREVIOUS WORK, which led to the synthesis and pharmacological screening of the model compound, 2-(*N*, *N*-diethylamino)-2-phenylglutarimide (V) (1, 2), suggested that of several possibilities, *p*-hydroxylation of the phenyl group, with subsequent glucuronide conjugation of the molecule in the biological system, could account in part for its short duration of action. The present communication deals, therefore, with routes to the synthesis of modifications V*a* and V*b* of the model compound (V) designed not only to block this position from metabolic attack, but also to provide a basis for studies of the effect on activity of substituents that would reasonably be expected to have opposite electronic influences on the phenyl ring and, in turn, on the molecular stability, drug–receptor interactions, and biotransportation of the compounds.

DISCUSSION

The syntheses of 2-(*N*, *N*-diethylamino)-2-*p*-chlorophenylglutarimide (V*a*) and of 2-(*N*, *N*-diethylamino)-2-*p*-tolylglutarimide (V*b*) were accomplished by following the reaction sequence shown in Scheme I. In both instances, ethyl α-bromo-*p*-chlorophenylacetate (I*a*) and ethyl α-bromo-*p*-tolylacetate (I*b*) were prepared by modifications of the procedure of Anschütz (3). In the subsequent displacement reaction (I→II) using diethylamine as the nucleophile, aminolysis of the ester was avoided by using a short-term, high-temperature procedure as reported in earlier work (1, 4).

Based upon experience in this laboratory with the unsubstituted phenyl compound of this series (1), the next step in the reaction sequence, that of adding the 3-carbon chain to the appropriate amino ester, was accomplished by employing the general procedure developed by Zaugg *et al.* (5) using β-bromopropionitrile. Treatment of III*a* and III*b* with a 1:1 mixture of concentrated sulfuric and glacial acetic acids at steam-bath temperature for 30 min. afforded a fair yield of the amido ester (IV*a* and IV*b*).

The cyclizations of IV*a* and IV*b* were accomplished by adaptations of the procedure used in the alkylation step (II→III). It was observed in both instances that upon heating IV*a* and IV*b* in the sodium hydride–dimethylformamide system, hydrogen abstraction (as evidenced by cessation of gas evolution) was essentially complete after 2.5 hr. of heating at 55°. However, if the reaction was stopped at this point, the starting material was recovered. Continued heating of the reaction mixture beyond this point at a higher bath temperature (100–105°) did effect the desired cyclizations to V*a* and V*b*.

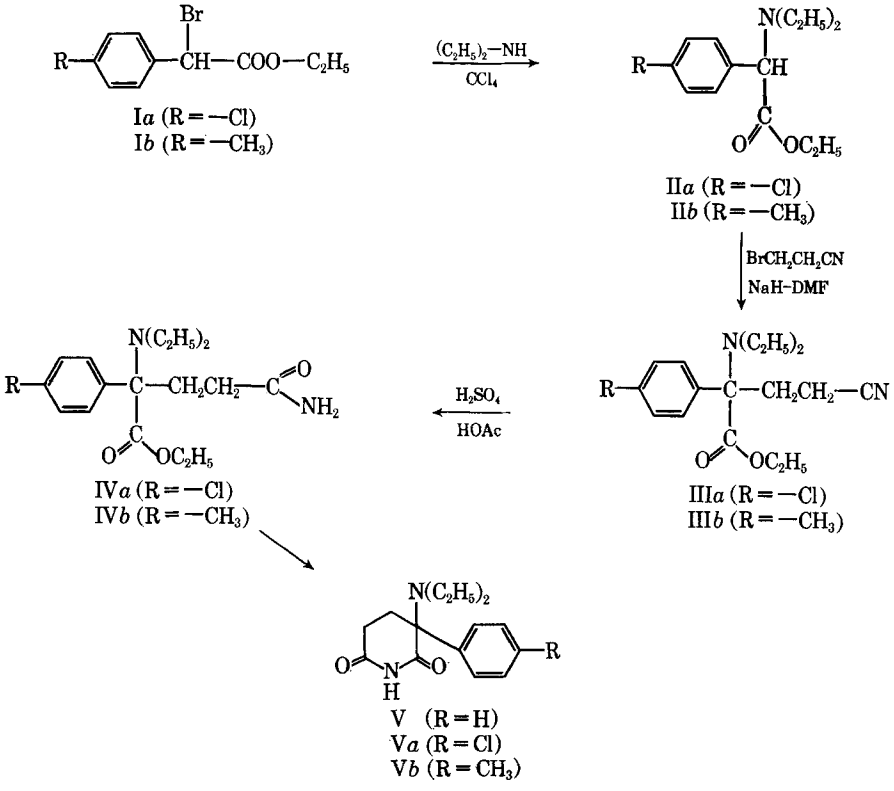
As is the case with the unsubstituted phenyl derivative, it is expected that the imide portions of both V*a* and V*b* are relatively flat (6), and as both the substituted phenyl and diethylamino groups are large, they will tend to assume quasi-equatorial and quasi-axial positions with respect to the adjacent carbonyl (7).

EXPERIMENTAL

Melting points were determined on a Fisher-Johns melting point block and are uncorrected. Infrared spectra were obtained with a Beckman IR-5 spectrophotometer; all were run on 10% solutions in a cell with a 0.1-mm. path length unless otherwise stated. Nuclear magnetic resonance spectra were determined on the 60 Mc. NMR manufactured by Varian Associates. The Beckman DB spectrophotometer was used for the ultraviolet absorption spectra reported. Microanalyses were performed by the Weiler and Strauss Microanalytical Laboratory, Oxford, England, or by the Galbraith Laboratories, Inc., Knoxville, Tenn.

Ethyl α-Bromo-p-chlorophenylacetate (Ia)—A modification of the procedure described by Anschütz (3) was used. To a stirred mixture of 34.1 Gm. (0.2 mole) of *p*-chlorophenylacetic acid and 1.2 Gm. of red phosphorus, 64.0 Gm. (0.4 mole) of bromine was added at 75–85° over a period of 5 hr. The mixture was stirred for an additional 5 hr. at 80°. After cooling, 12.5 Gm. (0.25 mole) of absolute ethanol was added slowly with stirring and the reaction mixture was heated at 75–80° for 10 hr. At the end of the period, the mixture was poured into ice water, the ester layer separated with ether, washed successively with 10% sodium bisulfite solution and water, and dried (Na_2SO_4). After filtering and flash evaporating the ether, the oil was distilled to yield 44.5 Gm. (80.5%) of liquid, b. p. 96° (0.06 mm.). Redistillation gave a center cut of product I*a*, b. p. 94–95° (0.06 mm.); infrared ($CHCl_3$): 1732 (C=O ester); NMR (CCl_4): triplet 1.25 p.p.m. (methyl protons of ester ethyl); quartet 4.20 p.p.m.

Received April 14, 1967, from the College of Pharmacy, Washington State University, Pullman, WA 99163
Accepted for publication August 1, 1967.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported in part by funds provided for medical and biological research by the State of Washington Initiative Measure No. 171, and by research grant NB 05032-01 and -02, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
Abstracted from portions of theses submitted by S. K. Arya (1964) and K'un Chang (1966) to the Graduate School, Washington State University, in partial fulfillment of Master of Science degree requirements.
Previous paper: Narayanan, V. L., and Martin, C. F., *J. Med. Chem.*, **9**, 615(1966).



Scheme I

(methylene protons of ester ethyl); singlet 5.15 p.p.m. (proton on α-carbon) with phenyl protons centered at 7.45 p.p.m.

Anal.—Calcd. for $C_{10}H_{10}BrClO_2$: C, 43.28; H, 3.63; Br, 28.54; Cl, 12.77. Found: C, 43.50; H, 3.82; Br, 28.54; Cl, 12.59.

Ethyl α-Bromo-p-tolylacetate (Ib)—To a stirred mixture of 150.2 Gm. (1.0 mole) of *p*-tolylacetic acid and 6.0 Gm. of red phosphorus, 320.0 Gm. (2.0 moles) of bromine was added at 60–70° over a period of 12 hr. The mixture was stirred for an additional 5 hr. at 70°. After cooling, 62.5 Gm. (1.25 moles) of absolute ethanol was added slowly with stirring and the reaction mixture was heated at 75–80° for 10 hr. At the end of the period, the mixture was poured into ice water, the ester layer separated with ether, washed successively with 10% sodium bicarbonate solution, water, 10% sodium bisulfite solution and water, and dried (Na_2SO_4). After filtering and flash evaporating the ether, the oil was distilled to yield 194.0 Gm. (75.4%) of liquid, b. p. 125–129° (2.1 mm.). Redistillation twice yielded product *Ib*, b.p. 124.5° (2.1 mm.); infrared ($CHCl_3$): 1745 (C=O ester).

Anal.—Calcd. for $C_{11}H_{13}BrO_2$: C, 51.38; H, 5.09; Br, 31.08. Found: C, 51.59; H, 5.13; Br, 31.20.

Ethyl α-(N,N-Diethylamino)-p-chlorophenylacetate (IIa)—A well-stirred solution of 55.5 Gm. (0.2 mole) of freshly distilled *Ia* and 58.5 Gm. (0.8 mole) of diethylamine in 50 ml. of dry CCl_4 was heated gently under reflux for 10 hr. The mixture of precipitated hydrobromides was filtered, washed with CCl_4, dissolved in water, the solution adjusted to pH 9.5 (cooling), and extracted with ether. The CCl_4 solution was extracted with 5% HCl, and after adjustment of the pH of the acidic extract to 9.5 (cooling), it was also extracted with ether. The ethereal extracts were combined and dried (Na_2SO_4). The solvent was flash evaporated and the product distilled to yield 48.5 Gm. (95%) of pale yellow mobile liquid, b. p. 102–105° (0.1 mm.). Redistillation twice yielded product *IIa*, b. p. 102° (0.1 mm.); infrared ($CHCl_3$): 2975 (—CH arom.); 2800–2950 (—CH aliph.); 1725 $cm.^{-1}$ (C=O ester).

Anal.—Calcd. for $C_{14}H_{20}ClNO_2$: C, 62.34; H, 7.41; Cl, 13.14; N, 5.19. Found: C, 62.48; H, 7.39; Cl, 13.16; N, 5.18.

Ethyl α-(N,N-Diethylamino)-p-tolylacetate (IIb)—A well-stirred solution of 124.7 Gm. (0.5 mole) of freshly distilled *Ib* and 146.0 Gm. (2.0 moles) of diethylamine in 130 ml. of dry CCl_4 was heated gently under reflux for 10 hr. The mixture of precipitated hydrobromides was filtered, washed with CCl_4, dissolved in water, the solution adjusted to pH 9.5 (cooling), and extracted with ether. The CCl_4 solution was extracted with 10% HCl, and after adjustment of the pH of the acidic extract to 9.5 (cooling), it was also extracted with ether. The ethereal extracts were combined and dried (Na_2SO_4). The solvent was flash evaporated and the product distilled to yield 80.9 Gm. (64.8%) of pale yellow mobile liquid, b. p. 104–107° (0.4 mm.). Redistillation twice yielded product *IIb*, b. p. 102° (0.35 mm.); infrared ($CHCl_3$): 2850–2970 (—CH aliph.); 1730 (C=O ester).

Anal.—Calcd. for $C_{15}H_{23}NO_2$: C, 72.25; H, 9.29;

N, 5.62. Found: C, 72.36; H, 9.40; N, 5.71.

4 - Carbethoxy - 4 - (N,N - diethylamino) - 4 - p-chlorophenylbutyronitrile (IIIa)—All of the glassware used in this reaction was flame-dried. Spectroquality dimethylformamide (DMF) was used, and the entire reaction was run under a blanket of dry nitrogen.

To a well-stirred suspension of 2.5 Gm. (0.11 mole) of sodium hydride in 50 ml. of dry DMF, 27.7 Gm. (0.1 mole) of freshly distilled II*a* was added at such a rate as to maintain an internal temperature of 45–50° (addition time, 3 hr.). The resulting thick red liquid was cooled in ice (0–5°) and 15.5 Gm. (0.11 mole) of freshly distilled β-bromopropionitrile was added dropwise, with continuous stirring, over a period of 4 hr. The temperature was not allowed to exceed 5° during the addition. The mixture, containing the precipitated NaBr, was stirred overnight at room temperature under dry N_2. The residue left after removal of the solvent was taken up in 100 ml. of ice water and extracted with ether. The ether extract was washed with water and dried ($MgSO_4$). After removal of the ether, the residual product was fractionally distilled to yield 19.7 Gm. (60%) of III*a* as a pale yellow viscous liquid, b. p. 162° (0.01 mm.). An analytical sample was obtained by an additional distillation, b. p. 159–160° (0.01 mm.); infrared ($CHCl_3$): 2975 (—CH arom.); 2800–2950 (—CH aliph.); 2250 (—CN); 1720 $cm.^{-1}$ (C=O ester).

Anal.—Calcd. for $C_{17}H_{23}ClN_2O_2$: C, 63.25; H, 7.20; Cl, 10.99; N, 8.64. Found: C, 63.37; H, 7.25; Cl, 11.31; N, 8.43.

4 - Carbethoxy - 4 - (N,N - diethylamino) - 4 - p-tolylbutyronitrile (IIIb)—The use of flame-dried glassware, the maintenance of a dry, inert atmosphere, and the use of spectroquality dimethylformamide (DMF) were criteria for success in this reaction as they were for the synthesis of III*a*.

To a well-stirred suspension of 5.5 Gm. (0.23 mole) of sodium hydride in 100 ml. of dry DMF, 49.8 Gm. (0.20 mole) of freshly distilled II*b* was added at such a rate as to maintain an internal temperature of 40–45° (addition time, 3 hr.). The resulting thick red liquid was cooled in ice (0–5°) and 29.5 Gm. (0.22 mole) of freshly distilled β-bromopropionitrile was added dropwise, with continuous stirring, over a period of 3 hr. The temperature was not allowed to exceed 5° during the addition. The mixture, containing the precipitated NaBr, was stirred overnight at room temperature under dry N_2. The mixture, in this case, instead of first being stripped of solvent was poured into 150 ml. of ice water and extracted with ether. The ether extract was washed with water and dried ($MgSO_4$). After removal of the ether, the residue was fractionally distilled to yield 20.8 Gm. (34.4%) of III*b* as a pale yellow viscous liquid, b. p. 136–139° (0.06 mm.). An analytical sample was obtained by an additional distillation, b.p. 135° (0.06 mm.); infrared (neat film): 2975 (—CH arom.); 2850–2950 (—CH aliph.); 2250 (—CN); 1720 $cm.^{-1}$ (C=O ester).

Anal.—Calcd. for $C_{18}H_{26}N_2O_2$: C, 71.49; H, 8.67; N, 9.27. Found: C, 71.44; H, 8.63; N, 9.44.

4 - Carbethoxy - 4 - (N,N - diethylamino) - 4 - p-chlorophenylbutyramide (IVa)—A mixture of 32.3 Gm. (0.1 mole) of III*a* and 30 ml. of glacial acetic acid was stirred at room temperature for 20 min. Concentrated H_2SO_4 (30 ml.) was added slowly and the mixture heated on a steam bath with stirring for 30 min. The brownish-red liquid obtained was poured into stirred ice water, and 30% aqueous NaOH was added until the mixture was alkaline (pH 8.5). The product was extracted with chloroform, washed with water, dried (Na_2SO_4), and concentrated. Because attempts to solidify the resulting thick oil by rubbing with *n*-hexane and with ligroin were unsuccessful, the product was distilled to yield 23.8 Gm. (70.5%) of light yellow viscous liquid, b. p. 190° (0.05 mm.); infrared ($CHCl_3$): 3490, 3390, 3150 (NH amide, 2 free, 1 bonded); 1720 (C=O ester); 1680 (amide I); 1595 (amide II); no nitrile band at 2250 $cm.^{-1}$.

Anal.—Calcd. for $C_{17}H_{25}ClN_2O_3$: C, 59.94; H, 7.34; Cl, 10.42; N, 8.22. Found: C, 59.83; H, 7.19; Cl, 10.15; N, 8.41.

4 - Carbethoxy - 4 - (N,N - diethylamino) - 4 - p-tolylbutyramide (IVb)—A mixture of 30.2 Gm. (0.1 mole) of III*b* and 30 ml. of glacial acetic acid was stirred at room temperature for 20 min. Concentrated H_2SO_4 (30 ml.) was added slowly and the mixture heated on a steam bath for 30 min. The brownish-red liquid obtained was poured into stirred ice water, and 30% NaOH was added until the mixture was alkaline (pH 8.5). The product was extracted with chloroform, washed with water, dried (Na_2SO_4), and concentrated. The thick oil which could not be crystallized or distilled without decomposition, was obtained as a crude product in a yield of 31.8 Gm. (99.1%); infrared ($CHCl_3$): 3500, 3400, 3160 (NH amide, 2 free, 1 bonded); 1720 (C=O ester); 1680 (amide I); 1595 (amide II); no nitrile band at 2250 $cm.^{-1}$. It was used in the final step without further purification.

2 - (N,N - Diethylamino) - 2 - p - chlorophenylglutarimide (Va)—Spectroquality DMF was used. All glassware was flame-dried. The reaction was carried out under an atmosphere of dry nitrogen.

A solution of 10.0 Gm. (0.028 mole) of freshly distilled 4 - carbethoxy - 4 - (*N, N* - diethylamino) - 4 - *p* - chlorophenylbutyramide (IV*a*) in 25 ml. of dry DMF (warming aids the dissolution process) was added dropwise to a stirred suspension of 0.8 Gm. (0.03 mole) of NaH in 50 ml. of dry DMF. The mixture was stirred at 55° for 2.5 hr., by which time the formation of anion was complete as evidenced by the cessation of H_2 evolution. The bath temperature was raised to 105° and maintained at this point for 15 hr. The residue, left after distilling off the solvent under reduced pressure, was well stirred with ice water, the solution adjusted to pH 8.5 (cooling), and the product extracted with chloroform, washed with water, and dried (Na_2SO_4). Filtration and removal of the chloroform left a thick oil which was washed with *n*-hexane and ligroin. The partially purified product was redissolved in chloroform, extracted with 10% HCl, and the acid extract, upon evaporation on a steam bath, yielded a residue which, after rubbing with a chloroform–ether mixture, gave 4.2 Gm. (46.0%) of V*a* as the solid hydrochloride. Recrystallization of the product from hot chloroform yielded a white powder, m.p. 132–135°; infrared (Nujol): 3150, 3050 (NH); 1710 with shoulder at 1730 (C=O); 2535, 2450 $cm.^{-1}$ (amine salt); ultraviolet (95% ethanol): 220 mμ (flat shoulder) and evidence of peak below 200 mμ out of

range of instrument; ($10^{-2}M$ ethanolic KOH): bathochromic shift to 225 mμ (flat shoulder) and 215 mμ (peak).

Anal.—Calcd. for $C_{15}H_{20}Cl_2N_2O_2$: C, 53.39; H, 6.08; Cl, 21.20; N, 8.45. Found: C, 53.03; H, 5.96; Cl, 20.21; N, 8.81.

2-(N,N-Diethylamino)-2-p-tolylglutarimide (Vb)—Spectroquality DMF was used. All glassware was flame-dried, and the reaction was carried out under an atmosphere of dry nitrogen.

A solution of 16.0 Gm. (0.05 mole) of crude, but dried, 4 - carbethoxy - 4 - (*N, N* - diethylamino) - 4 - *p* - tolylbutyramide (IV*b*) in 50 ml. of dry DMF was added dropwise to a stirred suspension of 1.4 Gm. (0.057 mole) of NaH in 100 ml. of dry DMF. The mixture was stirred at 55° for 2.5 hr., by which time the formation of anion was complete as evidenced by the cessation of H_2 evolution. The bath temperature was raised to 105° and maintained at this point for 15 hr. The residue, left after distilling off the solvent under reduced pressure, was well stirred with ice water, the solution adjusted to pH 8.5 (cooling), and the product extracted with chloroform, washed with water, and dried (Na_2SO_4). Filtration and removal of the chloroform left a thick oil which solidified on rubbing with dry *n*-hexane. Recrystallizations from dry chloroform–hexane[1] yielded 1.62 Gm. (11.8%) of V*b* as white crystals, m. p. 112°; infrared ($CHCl_3$): 3375, 3200 (NH); 1720 $cm.^{-1}$ (C=O).

Anal.—Calcd. for $C_{16}H_{22}N_2O_2$: C, 70.04; H, 8.08; N, 10.21. Found: C 69.95; H, 8.04; N, 10.21.

[1] Using decolorizing carbon (Norit).

REFERENCES

(1) Narayanan, V. L., and Martin, C. F., *J. Med. Chem.*, **9**, 615(1966).
(2) Fink, G. B., and Juchau, M. R., *J. Pharm. Sci.*, **53**, 325 (1964).
(3) Anschütz, R., *Ann.*, **354**, 127(1907).
(4) Weizman, C. H., Sulzbacher, M., and Bergmann, E., *J. Am. Chem. Soc.* **70**, 1153(1948).
(5) Zaugg, H. E., Horrom, B. W., and Borgwardt, S., *ibid.*, **82**, 2895(1960).
(6) Lee, C. M., and Kumler, W. D., *ibid.*, **83**, 4586(1961).
(7) Kumler, W. D., personal communication, 1962.

Segregation Kinetics of Particulate Solids Systems IV. Effect of Particle Shape on Energy Requirements

By E. G. RIPPIE, M. D. FAIMAN*, and M. K. PRAMODA

The relative energy requirements of the processes of segregation and mixing, which occur in systems of particulate solids, are shown to be a function of the shape and size of the particles. These energy requirements, as reflected by agitation coefficients, have been determined for several idealized systems. Granular solids, as represented by crushed quartz and pyrite, were found to behave in conformity with predictions based on the idealized systems.

PARTICLE SHAPE is known to exert a marked influence on the behavior of particulate solids. This is true, moreover, when powdered or granular material is either in a dynamic state of flow or is at rest. Little is presently known regarding the specific cause and effect relationships between bulk behavior and particle shape, a situation which arises because of difficulties in expressing the latter as a single scalar quantity.

The problem is frequently approached by the use of shape factors as a quantitative expression of the average shape of particles in a system. The function of shape factors, however, is not to express shape, but rather to relate the results of particle size measurements which have been obtained by several different methods (1). Consequently, it is possible to have different particulate solids with quite different characteristic shapes, but which all possess a similar value of a given shape factor. Further complications result when average values are used to describe particulate variables without regard for the deviation of individual values from the mean.

The present work is intended to give quantitative information on the effects of particle shape on segregation and mixing in idealized systems where an intuitive appraisal of shape is possible. Such idealized systems, which are made up of regularly shaped particles (spheres, paired spheres, cubes, rods, and hexagonal rods), have the advantage that they may be described exactly as to shape which is uniform from particle to particle. For purposes of comparison, crushed quartz and pyrite were also studied.

Results indicate that substantial differences in bulk behavior can result from relatively minor changes in particle shape. Particle size is also shown to influence the change in rate of segregation and mixing with a change in agitation intensity.

EXPERIMENTAL

Materials—Chrome steel balls of the type and grade commonly employed in the manufacture of commercial ball bearings were used. Paired balls were prepared by spot welding two similar sized balls together. The effective distance of separation of the centers of mass was reduced by approximately 1% by the welding operation. Steel cubes and cylinders of circular and hexagonal cross section were made of cold rolled steel and case hardened in

Received May 26, 1967, from the Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455
Accepted for publication July 21, 1967.
This investigation was supported by grants GP-1820 and GK-471 from the National Science Foundation.
Previous paper: Faiman, M. D., and Rippie, E. G., *J. Pharm. Sci.*, **54**, 719(1965).
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range of instrument; ($10^{-2}M$ ethanolic KOH): bathochromic shift to 225 mμ (flat shoulder) and 215 mμ (peak).

Anal.—Calcd. for $C_{15}H_{20}Cl_2N_2O_2$: C, 53.39; H, 6.08; Cl, 21.20; N, 8.45. Found: C, 53.03; H, 5.96; Cl, 20.21; N, 8.81.

2-(N,N-Diethylamino)-2-p-tolylglutarimide (Vb)—Spectroquality DMF was used. All glassware was flame-dried, and the reaction was carried out under an atmosphere of dry nitrogen.

A solution of 16.0 Gm. (0.05 mole) of crude, but dried, 4 - carbethoxy - 4 - (*N, N* - diethylamino) - 4 - *p* - tolylbutyramide (IV*b*) in 50 ml. of dry DMF was added dropwise to a stirred suspension of 1.4 Gm. (0.057 mole) of NaH in 100 ml. of dry DMF. The mixture was stirred at 55° for 2.5 hr., by which time the formation of anion was complete as evidenced by the cessation of H_2 evolution. The bath temperature was raised to 105° and maintained at this point for 15 hr. The residue, left after distilling off the solvent under reduced pressure, was well stirred with ice water, the solution adjusted to pH 8.5 (cooling), and the product extracted with chloroform, washed with water, and dried (Na_2SO_4). Filtration and removal of the chloroform left a thick oil which solidified on rubbing with dry *n*-hexane. Recrystallizations from dry chloroform–hexane[1] yielded 1.62 Gm. (11.8%) of V*b* as white crystals, m. p. 112°; infrared ($CHCl_3$): 3375, 3200 (NH); 1720 $cm.^{-1}$ (C=O).

Anal.—Calcd. for $C_{16}H_{22}N_2O_2$: C, 70.04; H, 8.08; N, 10.21. Found: C 69.95; H, 8.04; N, 10.21.

[1] Using decolorizing carbon (Norit).

REFERENCES

(1) Narayanan, V. L., and Martin, C. F., *J. Med. Chem.*, **9**, 615(1966).
(2) Fink, G. B., and Juchau, M. R., *J. Pharm. Sci.*, **53**, 325 (1964).
(3) Anschütz, R., *Ann.*, **354**, 127(1907).
(4) Weizman, C. H., Sulzbacher, M., and Bergmann, E., *J. Am. Chem. Soc.* **70**, 1153(1948).
(5) Zaugg, H. E., Horrom, B. W., and Borgwardt, S., *ibid.*, **82**, 2895(1960).
(6) Lee, C. M., and Kumler, W. D., *ibid.*, **83**, 4586(1961).
(7) Kumler, W. D., personal communication, 1962.

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molten potassium cyanide. All dimensions of these three forms were 0.125 in.

Natural quartz and pyrite were crushed and sieved through U. S. Standard sieves. Following sieving, the quartz was purified by washing with 5% HCl after soaking overnight in the acid. The material was oven dried following a distilled water wash. The crushed pyrite was washed with distilled water and oven dried. After drying, both materials were again sieved before use.

Procedure—Equipment and procedure were essentially as reported previously (2–4) with regard to particle size analysis, calculation of system standard deviation, and computation of the rate constants for segregation and mixing. Agitation coefficients are defined as the slope of a plot of the natural logarithm of the rate constant for a given process *versus* the square of the reciprocal of the velocity imparted to the particles by the shaker.

The particulate systems were subjected to a vertical sine wave agitation in brass cylinders of 1-in. internal diameter which were hard chrome plated, when used with quartz and pyrite, to resist abrasion. Amplitudes and frequencies of agitation motion were held to within ±0.001 in. and ±5 c.p.m. of the stated values, respectively.

Systems comprised of steel particles were 304 Gm. in total weight and those of quartz and pyrite were 92 Gm. and 150 Gm., respectively.

RESULTS AND DISCUSSION

Agitation coefficients, as defined earlier, represent proportionality constants between the natural logarithm of the rate constant under consideration and the reciprocal of the energy imparted to the particles by the shaker. Large values of agitation coefficients indicate a high degree of dependency of the rate process on agitation and, therefore, a relatively high energy requirement for the process.

No well defined trend is apparent between the particle size ratio, V_r, (the ratio of particulate volumes of the two components) and the agitation coefficients of segregation and mixing, as shown on lines 4–7 in Table I. It is apparent, however, that the equilibrium agitation coefficients of these same systems tend to decrease with a decrease in the average particle size.

Paired Spheres—Unexpected results were obtained for the mixtures of single and paired balls, and are shown on lines 8 and 9 of Table I. The energy requirements for both segregation and mixing are less than in the same systems containing no paired particles, as shown on lines 6 and 7.

In addition to the results reported in Table I, binary mixtures of $^3/_{32}$-in. paired spheres and $^7/_{32}$-in. paired spheres, and $^3/_{32}$-in. paired spheres and $^7/_{32}$-in. single spheres were agitated at an amplitude of 0.141 in. and a frequency of 1030 c.p.m. The data indicated that segregation either had not occurred, or that the rate was immeasurably small. In contrast, binary systems of the same size single balls (Table I, line 7) segregate fairly rapidly. Binary mixtures of single and paired balls of similar size ($^3/_{32}$-, $^5/_{32}$-, and $^7/_{32}$- in.) exhibited no tendency to segregate.

It appears, from these observations, that in a binary mixture where the smaller component was paired, segregation of the system either decreased or did not occur. When the larger component only was paired, segregation was found to occur. This is consistent with the theory that the large and small components compete for void space created by particulate motion.

TABLE I—AGITATION COEFFICIENTS FOR IDEALIZED BINARY SYSTEMS

System	V_r	Segregation Coeff.	Mixing Coeff.	Equilibrium Coeff.
$^1/_{16}$-in. cube	15.3	21	...	...
$^1/_{16}$-in. hex.	14.3	24	...	...
$^1/_{16}$-in. cyl.	12.0	36	...	...
$^1/_{16}$-in.–$^1/_8$-in.	8.0	41	84	−42
$^1/_{16}$-in.–$^5/_{32}$-in.	15.6	46	74	−28
$^3/_{32}$-in.–$^5/_{32}$-in.	4.6	79	106	−27
$^3/_{32}$-in.–$^7/_{32}$-in.	12.7	51	46	5
$^3/_{32}$-in.–$^5/_{32}$-in.[a]	9.3	42	45	−3
$^3/_{32}$-in.–$^7/_{32}$-in.[a]	25.4	34	31	4

[a] Paired balls.

Nonspherical Particles—Over-all segregation of systems containing particles other than spheres or paired spheres showed an atypical pattern. A typical graph of such a system undergoing segregation is shown in Fig. 1, which represents a mixture of $^1/_{16}$-in. steel balls and $^1/_8$-in. steel cubes agitated at an amplitude of 0.141 in. and a frequency of 1030 c.p.m. The standard deviation is seen to increase, decrease, and then increase again. Similar results occurred with binary mixtures of $^1/_{16}$-in. spheres with both of the other nonspherical shapes studied.

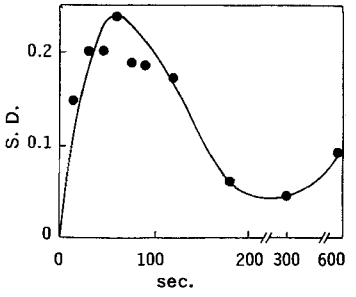


Fig. 1—Standard deviation as a function of agitation time for a 1:1 w/w mixture of $^1/_{16}$-in. steel balls and $^1/_8$-in. steel cubes agitated at a frequency of 1030 c.p.m. and an amplitude of 0.141 in.

The reason for this anomalous behavior became apparent when a Lucite cylinder was used, permitting the process to be viewed. It was found that the angular particles, when originally mixed with the balls, had a random orientation. After a short period of segregation in which the angular particles migrated to the top, they were found to orient themselves with their flat surfaces to the sides of the cylinder and to move in a reverse direction down the sides. In effect, convection currents were set up which accounted for the cycling behavior of the standard deviation. This behavior was not observed for systems other than those containing angular particles.

Segregation rate constants were obtained for these systems by fitting a quadratic equation to the data

TABLE II—AGITATION COEFFICIENTS FOR CRUSHED QUARTZ AND PYRITE

System 12/14–70/80 Mesh	Segregation Coefficients	Mixing Coefficients	Equilibrium Coefficients
Quartz	19	2	17
Pyrite	50	49	1

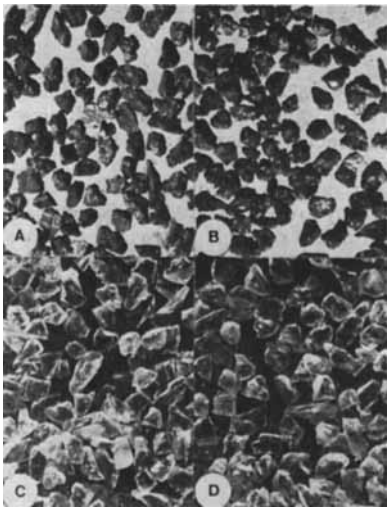


Fig. 2—Key: A, 12/14 mesh pyrite; B, 70/80 mesh pyrite; C, 12/14 mesh quartz; D, 70/80 mesh quartz. Materials are magnified to the same apparent size to show their characteristic particle shapes.

over the initial period of segregation. The coefficient of the linear term in the equation is thus proportional to the initial segregation rate and can be used to compute the segregation agitation coefficients. These are listed on lines 1–3 in Table I.

This coefficient can be seen to increase as the particles become more nearly spherical. The volumes of the angular particles lie between those of the $^1/_8$ and $^5/_{32}$-in. balls so that comparisons can be made among the first 5 lines of Table I. The lower energy apparently required for segregation of the systems containing the more angular particles cannot be explained on the basis of the data at hand.

The agitation coefficients for the equilibrium values in the last column of Table I can be seen to be the numerical difference between the agitation coefficients for segregation and mixing. Apparent discrepancies are due to rounding to a reasonable number of significant figures.

Results of studies on two granular systems, crushed quartz and pyrite, are presented in Table II. These data agree qualitatively with those relating to the angular shapes. While no attempt was made to measure shape, it was readily apparent (Fig. 2) that crushed quartz of a given mesh size is more angular than pyrite of the same size. The higher values of the agitation coefficients of pyrite for both segregation and mixing can be expected on the basis of data in Table II.

TABLE III—AGITATION AMPLITUDES AND FREQUENCIES EMPLOYED AND THEIR CORRESPONDING PARTICULATE VELOCITIES

Amplitude, in.	Frequency, c.p.m.	Particle Velocities, in./sec.
0.200	1030	10.18
0.195	1030	9.89
0.174	1030	8.68
0.174	925	7.43
0.141	1238	8.64
0.141	1119	7.58
0.141	1030	6.71
0.141	935	5.66
0.141	856	4.63
0.100	1400	6.84
0.100	1030	4.04

Conditions under which the foregoing data were obtained are summarized in Table III. With the exception of the data on lines 6 and 7 of Table I which were determined from studies at all 11 agitation conditions, agitation coefficients were calculated from data obtained under the three conditions represented by lines 1, 7, and 11 in Table III.

SUMMARY

A useful indication of the relative energy requirements for the processes of segregation and mixing, as they occur in idealized particulate systems of the type studied here, may be obtained from the determination of agitation coefficients. Such coefficients are somewhat analogous to activation energies for molecular processes but possess certain important fundamental differences from the latter as indicated previously (4).

The energy requirements of both segregation and mixing have been shown to depend on the shape and relative size of the particles involved. Two granular systems possessing different particulate characteristics were found to behave in conformity with predictions based on the idealized systems. The general method appears to be applicable to the study of other particulate variables as they influence diffusive segregation and mixing.

REFERENCES

(1) Herdan, G., "Small Particle Statistics," 2nd ed., Academic Press Inc., New York, N. Y., 1960, pp. 26, 173–179.
(2) Olsen, J. L., and Rippie, E. G., *J. Pharm. Sci.*, **53**, 147 (1964).
(3) Rippie, E. G., Olsen, J. L., and Faiman, M. D., *ibid.*, **53**, 1360(1964).
(4) Faiman, M. D., and Rippie, E. G., *ibid.*, **54**, 719(1965).

Field Screening Test for Lysergic Acid Diethylamide

By JAMES LOOK

A rapid and sensitive field screening test for lysergic acid diethylamide (LSD), which requires little manipulation and equipment, is described. The method is only a presumptive test, and the sample must be taken back to the laboratory for confirmatory tests. (The method is also applicable as a screening test for other psychotomimetic drugs such as dimethyltryptamine, diethyltryptamine, bufotenin, serotonin, and psilocybin.)

THE CLASSICAL Van Urk test is specific for α- or β-unsubstituted indoles (1). In the presence of strong acid these indoles condense with *p*-dimethylaminobenzaldehyde to form colorless, violet-red, and blue compounds, the relative amounts depending on the concentration of reagents and the presence or absence of oxidizing agents (1–6).

This procedure has been modified to provide a sensitive rapid field screening test for LSD which requires no oxidizing agent other than air. In addition, several observations have been made which support the reaction scheme and structures previously proposed by other workers (2–4).

PROCEDURE

Reagents—Filter paper (*e.g.*, Whatman No. 1) saturated with a solution of 2% *p*-dimethylaminobenzaldehyde in 95% alcohol, then air-dried, cut into strips, and stored in a tightly capped amber glass bottle.

Screening Test—The amount of LSD found in a dosage form usually varies from 75 to 250 mcg. A quantity equivalent to one-tenth of the dosage gives a strong, rapid test.

Macerate a portion of the sample with sufficient alcohol to provide some liquid extract. Keep the volume of the liquid extract to a minimum in order to keep the presumed concentration of LSD high. Transfer the extract to a strip of the prepared filter paper and allow the alcohol to evaporate. Add 1 drop of concentrated hydrochloric acid and let the paper stand, supporting it in such a way that the acidified spot does not touch any surface. A violet-red or violet-blue spot develops if LSD or other psychotomimetic agents of indole derivatives are present; the color slowly diffuses to the edge of the spot to form a colored ring.

The test can be applied directly by placing a portion of the crushed suspected material directly on the prepared paper and then adding a drop of concentrated hydrochloric acid. A violet-red or violet-blue color will develop if LSD or other psychotomimetic indole derivative is present, but the color may or may not edge out to form a ring.

The test can be applied to aqueous solutions if several mcg. of LSD is present in a few drops of the solution. Apply a few drops of the liquid to the filter paper, let it air-dry, and proceed as above. The reaction occurs slowly if the paper is not allowed to dry thoroughly before the hydrochloric acid is added. If the suspected material is in a dilute aqueous solution, make a portion alkaline with a drop or two of concentrated ammonium hydroxide. Add 1–3 ml. chloroform (or enough to provide some liquid extract without greatly lowering the LSD concentration) and shake well in a stoppered vial. Place a tight wad of cotton on the mouth of the vial, quickly invert it, and let the chloroform extract settle into the cotton. Place a few drops of the chloroform extract on a piece of the prepared filter paper, let the paper dry, and add a drop of concentrated hydrochloric acid to the spot.

DISCUSSION

Screening Test—This test as given in the procedure is convenient, requires little equipment, is completed in a few minutes, and is sensitive to as little as 1 mcg. LSD. The *p*-dimethylaminobenzaldehyde-saturated paper is stable for several months.

Concentrated hydrochloric acid is found to give a rapid test and a clearly defined ring. However, 1:1 hydrochloric acid solutions in water or alcohol are also satisfactory developing agents. More dilute acid solutions are unsatisfactory. When they are used, the reaction is very slow, and the color remains diffused throughout the spot rather than migrating and concentrating on the edge of the spot to form a ring.

The color changes observed in the usual air atmosphere depend on which reagent is present in excess (Scheme I). Usually, *p*-dimethylaminobenzaldehyde is in excess and in less than 1 min. a violet-red or violet-blue color develops; however, on standing, the initially formed ring loses its violet tinge and the color becomes less intense but bluer; then it gradually fades through a series of color changes: pale blue, pale blue-gray, and pale green. Another drop of hydrochloric acid regenerates the violet-blue color; upon standing, this fades again through the same sequence of colors. This observation is consistent with the expected dependence of acid concentration on the interconversion of IV and V. The color can be restored many times by the addition of more acid, but it is fainter each time. The color changing phenomena observed depend on the concentration of LSD, proportion of reagents, temperature, and other variables.

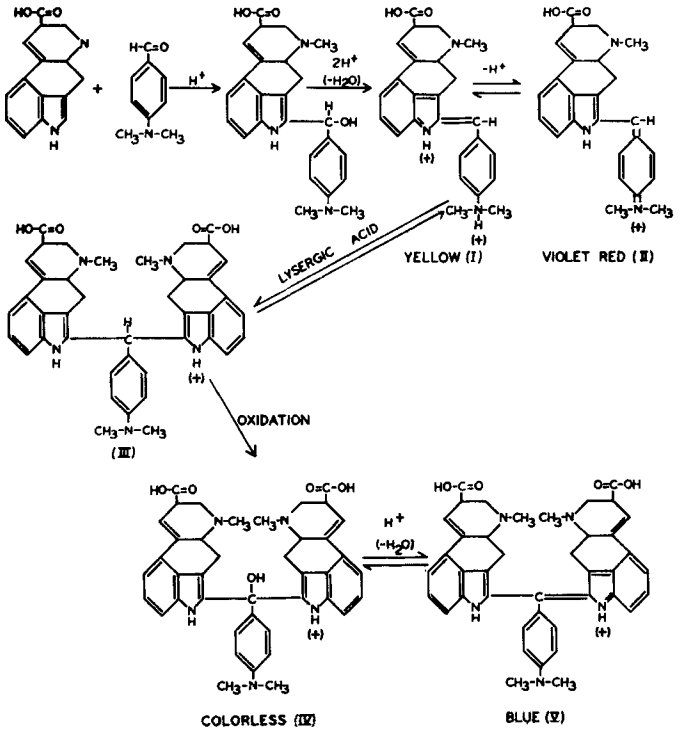
Since positive tests are obtained for any compound containing an indole ring with an open α or β position, any positive results should be tested for possible confirmation in the laboratory by thin-layer (7–9) or paper (10) chromatography or by ultraviolet and infrared spectrophotometry.

Chemistry of the Test—The reaction scheme and color assignments given in Scheme I have been generally agreed upon by previous workers (2–4), and we have made certain qualitative observations with lysergic acid which support the reaction sequence. In the first step of the scheme, the reagents condense in a 1:1 mole ratio to form a base

Received March 17, 1967, from the San Francisco District, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, San Francisco, CA 94102

Accepted for publication August 2, 1967.

The author thanks Dr. Frederick R. Jensen, San Francisco District, Food and Drug Administration, for his advice on this project.



Scheme I

which has a protonated yellow form (I) and a violet form (II). If excess lysergic acid is present, further condensation occurs to give III which is colorless. However, III is oxidized very readily to yield IV (colorless) or its ionic form (V) (intense blue) depending on acid concentration.

Under a helium atmosphere (nonoxidative conditions), the following observations were made.

(*a*) In the presence of excess *p*-dimethylaminobenzaldehyde, a strong violet color is instantly obtained, which is in accord with the expected formation of II under these conditions.

(*b*) With excess lysergic acid, the reaction mixture is initially colorless but slowly turns slightly violet, which supports the postulated predominant formation of III under these conditions.

(*c*) The observed colors are consistent with the proposal that regardless of which reagent is present in excess, compound II is formed and not V. The formation of II is rapid with an excess of aldehyde and slow with an excess of lysergic acid.

On addition of NH_4OH to compounds II and V, the intense colors disappear. These colors can be restored by adding excess concentrated HCl to the compounds.

Interferences—In the present procedure, any α- or β-unsubstituted indole gives a color reaction. The psychotomimetic drugs dimethyltryptamine, diethyltryptamine, bufotenin, serotonin, and psilocybin also give a color reaction, but the color which develops is more red than violet-red or violet-blue. Experience with this test procedure allows one to tentatively distinguish these compounds from LSD.

REFERENCES

(1) Van Urk, H. W., *Pharm. Weekblad*, **66**, 473(1929).
(2) Pohm, M., *Arch. Pharm.*, **286**, 509(1953).
(3) Von Dibbern, H. W., and Rochelmeyer, H., *Arzneimittel-Forsch.*, **13**, 7(1963).
(4) French, W. N., *J. Pharm. Sci.*, **54**, 1726(1965).
(5) Schulek, E., and Vastagh, G., *Dansk Tidsskr. Farm.*, **13**, 101(1939); Schulek, E., and Vastagh, G., *Quart. J. Pharm. Pharmacol.*, **12**, 611(1939).
(6) Spies, J. R., and Chamber, D. C., *Anal. Chem.*, **20**, 30(1948).
(7) Genest, K., and Farmilo, C. G., *J. Pharm. Pharmacol.*, **16**, 250(1964).
(8) French, W. N., and Wherli, A., *J. Pharm. Sci.*, **54**, 1515(1965).
(9) Dal Cortivo, L. A., and Broich, J. R., *Anal. Chem.*, **38**, 1959(1966).
(10) Reio, L., *J. Chromatog.*, **4**, 458(1960).

Chemistry of the Ephedrine Spot Test Based on Its Pyrolytic Oxidation to Acetaldehyde by Sodium Bismuthate

By LESTER CHAFETZ, MILTON ELEFANT, and PRAMOD R. AMIN

The presence of acetaldehyde in the pyrolysis products resulting from heating ephedrine with sodium bismuthate can be explained by a hydramine fission reaction or a periodate-type α-aminoalcohol mechanism. Model experiments using gas chromatographic analysis indicate that the reaction is analogous to a periodate oxidation.

FEIGL AND SILVA (1) described a sensitive and selective spot test for ephedrine based on the detection of acetaldehyde produced by heating the alkaloid or its salts with sodium bismuthate. They rationalized the reaction as a hydramine fission of the phenethanolamine to methylamine and propiophenone catalyzed by the alkalinity of the bismuthate reagent (Scheme I) followed by oxidation of the latter to acetaldehyde by excess bismuthate. The authors have found that propiophenone responds to the ephedrine spot test with a detection limit of less than 3 mcg., which lends credence to their explanation of the reaction route; however, an alternative explanation is possible.

$$\phi CHOHCH(NHCH_3)CH_3 \rightarrow \phi COCH_2CH_3 + CH_3NH_2$$

Scheme I

Rigby (2) described the use of sodium bismuthate in acid solution for the facile cleavage of 1,2-glycols to aldehydes and ketones and for related reactions analogous to those effected by lead tetraacetate and periodic acid. The periodate oxidation of ephedrine was first reported by Wickstrøm (3), who identified the products as benzaldehyde, acetaldehyde, and methylamine (Scheme II). He showed that the oxidation was highly dependent on pH,

$$\phi CHOHCH(NHCH_3)CH_3 \rightarrow \phi CHO + CH_3CHO + CH_3NH_2$$

Scheme II

proceeding quantitatively in minutes at room temperature at pH 7.5, but at an immeasurably slow rate at pH 6 or lower. Chafetz (4) indicated that periodate oxidation of phenethanolamines appears to require the availability of an unshared electron pair on the amine nitrogen. Assuming that the bismuthate and periodate reactions proceed by analogous mechanisms, one would not expect the acidic reagent described by Rigby to react with ephedrine; however, the spot test is performed with dry sodium bismuthate, an aqueous suspension of which gives a strongly alkaline reaction.

The problem of discriminating between the alternative reaction routes resolves itself to a matter of determining whether benzaldehyde or propiophenone is produced by the reaction of ephedrine with bismuthate. The experiments described here were designed to provide elucidation.

EXPERIMENTAL

Equipment and Supplies—Ephedrine HCl N.F., sodium bismuthate, propiophenone, benzaldehyde, isooctane, methylene chloride, and 2,4-dinitrophenylhydrazine were the best available laboratory grades used without further purification. A Beckman DK-2 recording spectrophotometer was used with 1-cm. silica cells for U.V. measurements. A Perkin-Elmer model 801 gas chromatograph was employed using the following experimental parameters.

Column: a 2 M. glass helix of 0.25 in. o.d. packed with 3% HI-EFF 8 BP on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa.); injector temperature: 200°; helium flow: 40 ml./min.; hydrogen flow: 30 ml./min.; column temperature: 80°, programmed immediately after injection at 10°/min. to a final temperature of 150°; sample collection: a Perkin-Elmer 008-0525 sample collector chilled in a Dewar flask of dry ice–acetone was used with a collection ratio of 4 parts effluent to the collector and 1 part to the flame ionization detector.

Reaction of Ephedrine with Aqueous Bismuthate—A solution of 2.0 mg. of ephedrine HCl in 5.0 ml. of water was mixed with a suspension of 330 mg. of sodium bismuthate in 1 ml. of water and 20.0 ml. of isooctane in a 50-ml. glass-stoppered centrifuge tube. The stoppered mixture was heated 30 min. in a boiling water bath and cooled at the tap. The isooctane layer was filtered through Whatman No. 1 paper, and its ultraviolet spectrum was scanned from 300 to 205 mμ *versus* solvent. The extent of conversion of ephedrine to benzaldehyde was calculated from the ratio of the molar absorptivity obtained in this experiment to the molar absorptivity of benzaldehyde in isooctane determined concomitantly.

A mixture of 2 Gm. of ephedrine HCl and 3.5 Gm. of sodium bismuthate was heated at reflux with 5 ml. of water in a round-bottom flask for 15 min. The cooled mixture was extracted with ether twice, and the ether was allowed to evaporate. The residue was taken up in 20 ml. of alcohol and treated with 10.0 ml. of 2,4-dinitrophenylhydrazine reagent prepared by dissolving 800 mg. of 2,4-dinitrophenylhydrazine in 4 ml. of concentrated sulfuric acid to which 6 ml. of water and 10 ml. of alcohol are added after cooling. The resulting 2,4-dinitrophenylhydrazone was recrystallized from alcohol. Its melting point was determined by the capillary method in a Thomas-Hoover apparatus and compared with that of authentic benzaldehyde 2,4-dinitrophenylhydrazone.

Pyrolysis and Gas Chromatography Experiments—Pyrolysis was effected in a test tube fitted with a sidearm. The vapors were collected through a pipet secured with rubber tubing in 1 ml. of methylene chloride in a second test tube. The pyrolysis tube was heated with a free flame until the vapors were observed to have been driven into

Received April 17, 1967, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950
Accepted for publication July 31, 1967.

the collection tube (no more than 5 min.). The following mixtures were pyrolyzed: 500 mg. of ephedrine HCl and 1.65 Gm. sodium bismuthate, 200 mg. propiophenone and 800 mg. sodium bismuthate, 500 mg. ephedrine HCl and 1.5 Gm. sodium hydroxide pellets, 0.5 ml. acetaldehyde and 1.5 Gm. sodium bismuthate, 0.5 ml. acetaldehyde and 1.5 Gm. sodium hydroxide pellets.

The methylene chloride solutions obtained were injected into the gas chromatograph, using 5-μl. volumes. Peaks with retention times corresponding to benzaldehyde and propiophenone were collected, and the trapped fractions were washed into 1-cm. cells with methanol and scanned in the ultraviolet range with the recording spectrophotometer.

RESULTS AND DISCUSSION

Ephedrine Reaction with Aqueous Bismuthate—A conversion of 2% of the ephedrine to benzaldehyde was obtained under the conditions used. The low yield is not unexpected, for the bismuthate is both insoluble and unstable in aqueous medium. The ultraviolet spectra of benzaldehyde and propiophenone are closely similar, and for this reason the identity of the product was checked by derivative formation. The 2,4-dinitrophenylhydrazone obtained in the second experiment melted at 236°. Authentic benzaldehyde 2,4-dinitrophenylhydrazone melted at 236°, and the mixed melting point was not depressed. The literature (5) melting point for benzaldehyde 2,4-dinitrophenylhydrazone is 237°, while the propiophenone derivative melts at 191°. The reaction in aqueous medium appears analogous to periodate oxidation, and it might be expected to proceed by a similar mechanism.

Pyrolysis Experiments—Dry-heating ephedrine with sodium bismuthate led to six major peaks. The peak with the retention time of benzaldehyde (4.0 min.) constituted 13.8% of the total detector response as determined by the internal normalization technique. The methanol solution of the isolated peak showed an absorption maximum at 244 mμ, and its spectrum corresponded with that of benzaldehyde in methanol. Other peaks were found with retention times of 2.4, 2.8, 6.0, 7.5, 8.4, 12.4, 14.4, and 16.0 min. The major peak, which amounted to nearly 75% of the total response, had a retention time of 12.4 min. It was not identified. The peak which eluted at 7.5 min. amounted to 2.5% of the total and eluted at about the same time as propiophenone. However, its ultraviolet spectrum after elution did not correspond with propiophenone; it had an absorption maximum at 270 mμ. Sodium bismuthate is a very alkaline material, and complex aldol and Cannizzaro products might well be expected. Heating ephedrine with sodium hydroxide led to a typical pyrolysis chromatogram with a large number of peaks and no major components. Pyrolysis of propiophenone with sodium bismuthate leads to a positive test for acetaldehyde under the Feigl and Silva conditions; however, the gas chromatogram showed no peak corresponding to benzaldehyde. This indicates that propiophenone is not an intermediate in the formation of benzaldehyde from ephedrine. Heating acetaldehyde with either sodium hydroxide or sodium bismuthate led to only minor amounts of products.

SUMMARY AND CONCLUSIONS

The Feigl and Silva rationalization of the spot test they described for ephedrine (1), detection of acetaldehyde after pyrolysis of the alkaloid with sodium bismuthate, was hydramine fission to propiophenone and methylamine followed by oxidation of propiophenone to acetaldehyde and other products. No propiophenone was detected in the reaction of ephedrine with bismuthate either during heating in aqueous medium or under pyrolytic conditions, which militates against acceptance of the hydramine fission explanation for the reaction. An alternative rationalization is presented: periodate-type cleavage of the ethanolamine side chain of ephedrine to benzaldehyde, acetaldehyde, and methylamine. Evidence supporting the alternative explanation includes the analogous behavior of bismuthate with periodate on glycols, identification of benzaldehyde as a product of oxidation of ephedrine by bismuthate under both aqueous and pyrolysis conditions, and the absence of benzaldehyde as a pyrolysis product of propiophenone with bismuthate. Gas chromatographic analysis of the ephedrine pyrolyzate showed it to be a complex mixture of products, of which benzaldehyde amounted to 14% and a major component accounted for nearly 75% of the total detector response.

REFERENCES

(1) Feigl, F., and Silva, E., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 460(1958).
(2) Rigby, W., *J. Chem. Soc.*, **1950**, 1907.
(3) Wickstrøm, A., *Ann. Pharm. Franç.*, **8**, 86(1950).
(4) Chafetz, L., *J. Pharm. Sci.*, **52**, 1193(1963).
(5) Vogel, A. I., "A Text-Book of Practical Organic Chemistry," Longmans, Green and Co., London, England, 1951, pp. 687, 706.

Rapid Analysis of Chlorinated Hydantoin by Gas Chromatography

By ARVIN P. SHROFF and R. E. HUETTEMANN

Gas chromatographic separation of a number of chlorinated hydantoins has been carried out using a split column. A quantitative method for the determination of 3-trichloromethylthio-5-(1-ethylamyl)hydantoin (chlordantoin) in suppositories has been developed. The accuracy of the method and results of the assay are reported.

A NUMBER OF trichloromethylthio derivatives of the phthalimide and hydantoin series have been investigated (1, 2) for their microbiological activity. Kupferberg and Doscher (2) found one of these hydantoin compounds, 3-trichloromethylthio-5-(1-ethylamyl)hydantoin (chlordantoin) to be the most active topical agent against *C. albicans* infections. A simple and rapid method of chemical analysis was required for this compound formulated in suppositories.

Hydantoins have been quantitated volumetrically in tablets by either compleximetric or precipitation techniques (3, 4). Borkowski and Dluzniewska (5) have used spectrophotometric procedures in biological fluids. These methods are sensitive but they lack the specificity required in the presence of other hydantoins.

Gas–liquid chromatography was considered as a desirable technique for estimating these halogenated hydantoins because it offered quantitation with concurrent fractionation. Chlorinated compounds used in pharmaceutical, pesticide, and fumigant areas have successfully been subjected to gas–liquid chromatography using either flame ionization or electron capture detectors (6–15).

The work reported here describes an analytical method utilizing a preliminary solvent extraction followed by a direct gas chromatographic determination using a flame ionization detector.

EXPERIMENTAL

Apparatus—A modified F and M model 1609 gas chromatograph equipped with flame ionization detector was used throughout this study. (The injection port was modified by F and M Scientific to eliminate the potential for inlet sample splitting.) The column used was a 12 in. long and 1/4 in. o.d. copper tube, half of which (injection port end) was packed with 4% Carbowax 20M on Gas Chrom Z, 100/120 mesh, and the other half with 2% phosphoric acid on Diatoport S, 60/80 mesh. The injection port and detector temperatures were approximately 250° and 235°, respectively. The column was conditioned at 210° and operated at 130° and 190°. Nitrogen was used as a carrier gas with a flow rate of 45 ml. per minute. Hydrogen and oxygen flow were maintained at approximately 45 and 400 ml. per minute, respectively. The attenuation and range throughout the determinations were at 32 and 10, respectively. The chart speed was maintained at 15 in. per hour.

Analytical Procedure—Two 100-ml. standard solutions containing 25 mg./ml. of chlordantoin and 3 mg./ml. of 9-anthraldehyde, respectively, were prepared in chloroform. A 2.0-ml. aliquot of the standard chlordantoin solution was evaporated in a 10 dr. vial and 2.95 Gm. of suppository base was added. To this and another vial containing two suppositories (equivalent to 50 mg. chlordantoin) were added 5.0 ml. of distilled pentane and 10.0 ml. of nitromethane. The mixture was shaken to effect complete solution followed by centrifugation for 2 min. A 5.0-ml. aliquot of the nitromethane solution from each vial was transferred to two other vials and evaporated. One milliliter of standard 9-anthraldehyde solution (internal standard) was added to each vial, mixed, and a 3-μl. injection was made into the gas chromatograph. The retention times for chlordantoin and 9-anthraldehyde were 1 and 2 min., respectively. The concentration of chlordantoin in each suppository was obtained as follows:

$$\text{mg. of chlordantoin/suppository} = \frac{A_s}{B_s} \times \frac{S_b}{S_a} \times 25$$

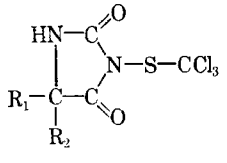
where

A_s, peak height of chlordantoin in sample,
B_s, peak height of 9-anthraldehyde in sample,
S_a, peak height of chlordantoin in standard,
S_b, peak height of 9-anthraldehyde in standard,
25, milligram of chlordantoin in standard.

RESULTS AND DISCUSSION

Table I gives the retention times of a number of hydantoins at 130°. It can be seen that the increase in bulkiness of the alkyl groups at C_5 does not correspondingly increase the retention time. Theoretically, any hydantoin in Table I can serve as an internal standard for chlordantoin (No. 4). However, because of the unavailability of these hydantoins and the time it would take to carry out each determination (130°), we chose to use an internal standard from another chemical series, namely 9-anthraldehyde, and carry out the determination at 190°. This speeds up the determination by a factor of four and is quite useful for control purposes.

TABLE I—RETENTION TIME OF HYDANTOINS AT 130°



No.	R_1	R_2	Time, min.
1	CH_3	C_2H_5	3.05
2	CH_3	i-C_3H_7	2.95
3	CH_3	i-C_4H_9	3.80
4	H	$CH(C_2H_5)(C_4H_9)$	14.00
5	H	$CH_2CH(CH_3)(CH_2)_3CH(CH_3)_2$	3.05

Received May 4, 1967, from the Analytical Research Group, Division of Organic Chemistry, Ortho Pharmaceutical Corp., Raritan, NJ 08869
Accepted for publication July 26, 1967.

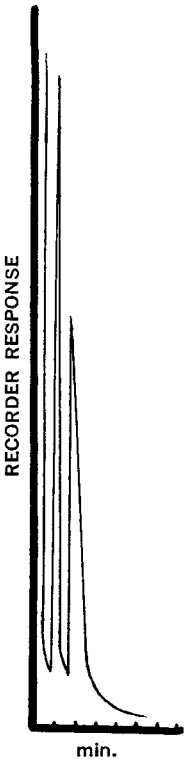


Fig. 1—A typical gas chromatogram for a mixture of chlordantoin and 9-anthraldehyde.

The analytical procedure utilizes extraction by two organic solvents, namely pentane and nitromethane. Thus, a modified extraction *p*-value determination (fractional amount of a solute partitioning into the nonpolar phase of an equal volume two-phase system) for chlordantoin was carried out. According to the procedure of Bowman and Beroza (16), *p*-values are determined by analysis of the nonpolar solution of the solute before and after extraction. However, we determined the *p*-value by analyzing both the polar and nonpolar phase and found it to be 1.0 in the nitromethane. Next the "binding" of chlordantoin to the suppository base was investigated in the same manner as the determination of *p*-value with different proportions of the suppository base in the pentane. It was found by analyzing the polar phase that there was a definite amount of retention in the pentane layer containing the base. This observation necessitated the addition of a fixed amount of base to the standard solution for the sake of relative comparison of available chlordantoin in samples.

Figure 1 illustrates a typical gas chromatogram of chlordantoin containing 9-anthraldehyde. The ratio of peak heights obtained for various concentrations of hydantoin in the standard solutions is given in Table II. A plot of these data is a straight line. The means for a series of injections of extracted samples containing 25 mg. of chlordantoin were ±1.5% (Table III) with a standard deviation of ±0.26 mg. (Table IV). This procedure affords a rapid means of comparative assay for chlordantoin.

TABLE II—CONCENTRATION *Vs.* RATIO OF PEAK HEIGHT

No.	Chlordantoin, mg.	Ratio of Peak Ht.
1	20	0.854
2	25	1.069
3	30	1.320

TABLE III—DETERMINATION OF PER CENT RECOVERY

Sample	mg. Added	mg. Found	% Recovery (X)
1	25.0	24.4	97.6
2	25.0	25.2	100.8
3	25.0	25.0	100.0
4	25.0	25.4	101.6
5	25.0	25.0	100.0

$\bar{X} = 100.0$

$\sigma = \pm 1.5\%$

TABLE IV—DETERMINATION OF CHLORDANTOIN IN SUPPOSITORIES[a]

Sample	Found (x)	$(\bar{X} - x)$	$(\bar{X} - x)^2$
1	25.1	0.1	0.01
2	24.9	0.1	0.01
3	25.2	0.2	0.04
4	24.7	0.3	0.09
5	25.2	0.2	0.04
6	24.6	0.4	0.16

$\bar{X} = 25.0$

$$\sigma = \sqrt{\frac{\Sigma (X - x)^2}{n - 1}}$$

$= \pm 0.26$ mg.

[a] Each suppository contains theoretical amount of 25 mg.

REFERENCES

(1) Daines, R. H., *Am. Fruit Grower*, **73**, 16(1953).
(2) Kupferberg, A. B., and Doscher, G. E., *Antibiot. Chemotherapy*, **11**, 73(1961).
(3) Przyborowski, L., *Lubin-Polania Sect. D.*, **17** 25 (1962); through *Chem. Abstr.*, **59**, 13769(1963).
(4) Kalinowska, Z. E., Podkowska, L., and Mieszczakowska, J., *Farm. Polska*, **19**, 329(1963); through *Chem. Abstr.*, **60**, 10478(1964).
(5) Borkowski, T., and Dluzniewska, A., *Acta Polon. Pharm.*, **18**, 235(1961); through *Anal. Abstr.*, 1963.
(6) Koshy, K. T., Conwell, R. C., and Duvall, R. N., *J. Pharm. Sci.*, **56**, 269(1967).
(7) Archer, T. E., Bevenue, A., and Zweig, G., *J. Chromatog.*, **6**, 457(1961).
(8) Beckman, H., and Bevenue, A., *ibid.*, **10**, 231(1963).
(9) *Ibid.*, **12**, 109(1963).
(10) Beckman, H. F., and Berkankolter, P., *Anal. Chem.*, **35**, 242(1963).
(11) Bosin, W. A., *ibid.*, **35**, 833(1963).
(12) Coulson, D. M., Cavagnagh, L. A., DeVries, J. E., and Walther, B., *J. Agr. Food. Chem.*, **8**, 399(1960).
(13) Hannon, C. L., Angelini, J., and Wolford, R. J., *J. Gas Chromatog.*, **1**, 27(1963).
(14) Hollis, O. L., and Hayes, W. V., *Anal. Chem.*, **34**, 1223 (1962).
(15) Reynolds, H. L., *J. Gas Chromatog.*, **2**, 219(1964).
(16) Bowman, M. C., and Beroza, M., *Anal. Chem.*, **38**, 1427(1966).

Peptides Synthesis: Coupling of Pentachlorophenyl Active Esters of *N*-Carbobenzoxy Amino Acids and Peptides to the Pentachlorophenyl Active Ester Hydrochlorides of Amino Acids

Sir:

Pentachlorophenyl active esters afford a satisfactory method for the synthesis of peptides and polypeptides with the ordered sequence of amino acids (1–3). One of the main advantages in the use of pentachlorophenyl esters is their relatively higher melting points which lead to easy crystallization and purification. The other very significant advantage lies in the fact that pentachlorophenyl esters are stable to controlled hydrogenation conditions, thereby allowing the selective removal of the *N*-carbobenzoxy protecting group (3). During the course of our work for the synthesis of biologically active peptide hormones, we extended the use of pentachlorophenyl esters by directly coupling them with amino acids which were C-protected by suitable salt formation. The aim in mind was to develop a procedure whereby aqueous alkaline treatment and the problems brought about by it could be avoided. The alkaline hydrolysis which is usually employed for the removal of methyl or ethyl C-protection in peptides is associated with a number of problems, *e.g.*, racemization (4), transpeptidation (3, 5), and formation of urea and hydantoin derivatives from the carbobenzoxy protecting group (6). In addition, hydrolysis becomes more difficult as the number of amino acids increases in the peptide chain (7).

We recently reported that *N*-carbobenzoxy amino acid or peptide pentachlorophenyl esters couple in good yields with amino acids which are C-protected by salt formation with dicyclohexylamine (8). The salt protection from the resulting peptides was removed by mild acid treatment, thereby avoiding the alkaline treatment. Stepwise lengthening of the peptide chain was achieved by using the combination of mixed anhydride and pentachlorophenyl active ester methods.

We now wish to report an interesting reaction sequence which we have found to be very rewarding as a complementary approach to the method previously described (8). Using the new approach, we have been able to couple pentachlorophenyl active esters of amino acids or peptides with pentachlorophenyl active ester hydrochlorides of amino acids in satisfactory yields. The advantage of this approach is significant since all the amino acids or peptides used in the coupling reaction are in the form of their pentachlorophenyl esters; therefore, as mentioned above, they are crystalline derivatives and easily purified (3). The outlines of the reaction conditions for the synthesis of *N*-carbobenzoxy-glycyl-serine pentachlorophenyl ester[1] are given below.

To a solution of *N*-carbobenzoxy-glycine pentachlorophenyl ester, (0.02 mole) and a catalytic amount (0.2–0.3 Gm.) of 2-hydroxypyridine in 75 ml. of methylene chloride, was added one equivalent of triethylamine. A suspension of serine pentachlorophenyl ester hydrochloride (0.02 mole) in 10 ml. of methylene chloride was added to the above solution over a period of 1 hr. After 24 hr. of stirring at room temperature, the reaction mixture was freed from solvents under reduced pressure. The residue was taken up in ethyl acetate and extracted with 1 *N* hydrochloric acid, 5% aqueous sodium bicarbonate, and water. From the ethyl acetate solution Z-Gly-Ser-OPCP was isolated in 75% yield after crystallization from methanol, m.p. 160–161°.

Anal.—Calcd. for $C_{19}H_{15}Cl_5N_2O_6$: C, 41.87; H, 2.75; N, 5.15. Found: C, 41.73; H, 2.69; N, 5.21.

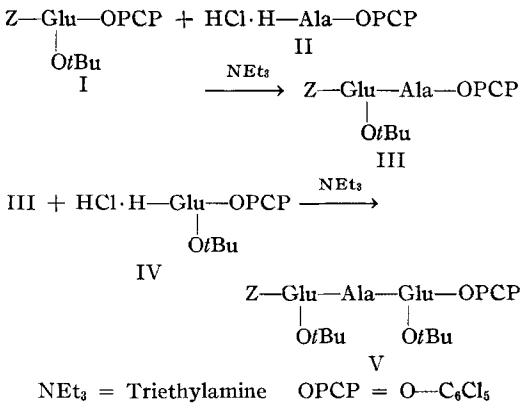
Z-Gly-Ser-OPCP was coupled following the above conditions with alanine pentachlorophenyl ester hydrochloride to afford Z-Gly-Ser-Ala-OPCP, in 69% yield, m.p. 169–170°.

Anal.—Calcd. for $C_{22}H_{20}Cl_5N_3O_7$: C, 42.91; H, 3.5; N, 6.83. Found: C, 43.12; H, 3.46; N, 6.78.

In order to further establish the usefulness of the above method, we selected a tripeptide active ester, *N*-carbobenzoxy-γ-*tert*-butyl-glutamyl-alanyl-γ-*tert*-butyl-glutamic acid pentachlorophenyl ester which was previously synthesized using the conventional methods (3). The yields for each step in Scheme I were above 62%.

The tripeptide (V) had melting point and ele-

[1] Abbreviations used in this paper for amino acids and peptides are those recommended in "Proceedings of the 5th European Peptide Symposium, Oxford, September, 1962," Young, G. T., ed., The Macmillan Co., New York, N. Y., 1963. Amino acids used in this work were all of L-configuration.



Scheme I

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(2) Kovacs, J., Kovacs, N. H., Chakrabarti, J. K., and Kapoor, A., *Experientia*, **21**, 20(1965).
(3) Kovacs, J., Giannoti, R., and Kapoor, A., *J. Am. Chem. Soc.*, **88**, 2282(1966).
(4) Schröder, E., and Lübke, K., "The Peptides," vol. 1, Academic Press Inc., New York, N. Y., 1965, p. 56.
(5) Katchalski, E., and Sela, M., *Advan. Protein Chem.*, **13**, 243(1958).
(6) MacLaren, J. A., *Australian J. Chem.*, **11**, 360(1958).
(7) Erlanger, B. F., and Brand, E., *J. Am. Chem. Soc.*, **73**, 3508(1951).
(8) Kapoor, A., and Davis, E. J., *Experientia*, **23**, 253 (1967).

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Received June 21, 1967.
Accepted for publication August 23, 1967.

Assay of Chlorpromazine Glucuronides in Human Urine

Sir:

Recently we reported a routine assay for conjugated and unconjugated chlorpromazine metabolites in human urine (1). The group of conjugated chlorpromazine metabolites was assayed by passing a small volume of urine through an ion-exchange resin to remove unconjugated drug metabolites and some contaminating endogenous urinary constituents. The eluate was then made up to 50% sulfuric acid content, and the conjugated drug metabolites were estimated spectroscopically. The procedure was satisfactory for conditions of chronic drug administration of 100 to 1400 mg. chlorpromazine per day. However, studying the excretion of chlorpromazine glucuronides in patients during the initial 4 days of low dosage chlorpromazine therapy (300 mg. or less per day), the method was found lacking in specificity and therefore inaccurate. In a typical determination, *i.e.*, with adequate amounts of chlorpromazine glucuronides, a certain amount of endogenous urinary chromogens can be compensated for by the background cancellation technique. At a starting level of 300 mg. chlorpromazine per day, an adequate urinary level of chlorpromazine glucuronides was reached only after about 1 week. A more selective assay was needed to measure the initial low levels of chlorpromazine glucuronides in the presence of excessive amounts of endogenous urinary chromogens. The following modification of the original procedure was found satisfactory.

Instead of adding 5 ml. of concentrated sulfuric acid to 5 ml. of effluent from the ion-exchange column, as previously specified, it was found practical to add 5 ml. of 50% sulfuric acid solution containing 75 mcg. $FeCl_3 \cdot 6H_2O$ per ml., in a 10-ml. volumetric flask immersed in an ice water bath. After thorough mixing under cooling, the solution was removed from the ice water bath, made up to volume with 25% H_2SO_4, and the absorption spectrum recorded between 400 and 700 mμ, when the mixture had reverted to room temperature. Maximum absorption was read as previously specified, at 550 mμ against a standard calibration curve prepared from 7-methoxychlorpromazine.

Sharper maxima, cleaner purple colors, and lower background readings were obtained by this modification of color development which lowered the over-all acidity from the previously specified 50% to 25%. Maximum color development was completed at 5 min., and colors remained stable for 60 min., the longest period tested.

The modification was therefore adopted for de-

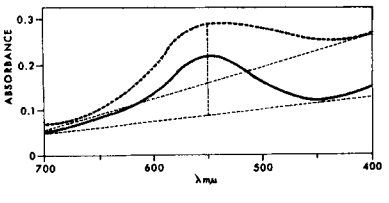
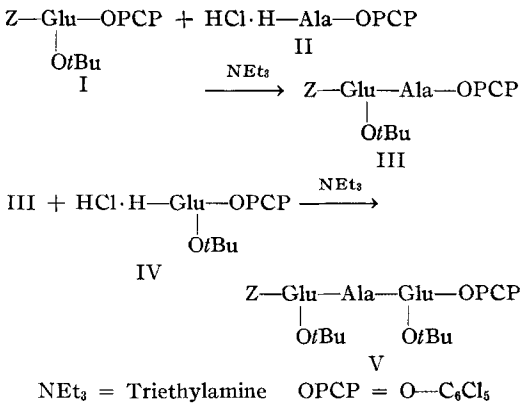


Fig. 1—Comparison of two reagents. Determination of chlorpromazine O-glucuronides in 0.5 ml. urine of a patient dosed with 4.8 mg. chlorpromazine/Kg. per day. Key: - - -, previously reported determination in 50% sulfuric acid medium; ——, proposed modification using 25% sulfuric acid medium in the presence of ferric chloride.



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(5) Katchalski, E., and Sela, M., *Advan. Protein Chem.*, **13**, 243(1958).
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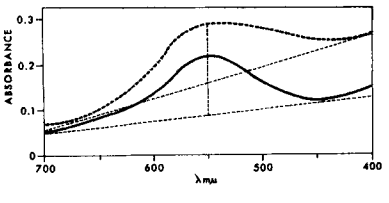

Fig. 1—Comparison of two reagents. Determination of chlorpromazine O-glucuronides in 0.5 ml. urine of a patient dosed with 4.8 mg. chlorpromazine/Kg. per day. Key: - - -, previously reported determination in 50% sulfuric acid medium; ——, proposed modification using 25% sulfuric acid medium in the presence of ferric chloride.

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Figure 1 illustrates the determination of chlorpromazine glucuronides in the urine of a patient chronically dosed with 4.8 mg. chlorpromazine per Kg. daily, according to the previously reported procedure (broken line) and the present modification (solid line). Using the background cancellation technique, the proposed modification yields an insignificantly higher reading for absorbance, but a lower background and a sharper maximum. As a rule, differences in absorbance due to the method of determination amounted to less than 5%, and did not exceed ±10% in any specimen examined.

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Received June 29, 1967.
Accepted for publication August 29, 1967.

This investigation was supported in part by grant HD-02693-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and by a research grant from Smith Kline & French Laboratories.

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In order to obtain an exact molecular weight for the isolate, it was subjected to mass spectral analysis and was found to be a mixture of nine *n*-alkanes.[1] All members of the C_{25}–C_{33} series were present with the odd carbon atom compounds being predominant. This finding is in agreement with recent extensive studies on plant *n*-alkanes reported by others (2–4).

Furthermore, the sample was subjected to a gas chromatographic analysis using a Packard FID instrument fitted with a 4 mm. × 2 M. glass column and packed with 3% SE-30 on Shimalite W (80–100 mesh). Argon (60 ml./min.) was the carrier gas, and a flame ionization detector with a sensitivity setting of 1×10^{-7} was used. The column temperature was maintained at 230° and the injection port was 235°. Estimation of the concentration of *n*-alkanes in the sample was by planimeter (5).

Under these conditions, it was found that the sample separated into nine distinct peaks, each corresponding to the mass spectral data. These peaks were identified as *n*-pentacosane, $C_{25}H_{52}$ (<1%); *n*-hexacosane, $C_{26}H_{54}$ (<1%); *n*-heptacosane, $C_{27}H_{56}$ (3%); *n*-octacosane, $C_{28}H_{58}$ (4%); *n*-nonacosane, $C_{29}H_{60}$ (17%); *n*-triacontane, $C_{30}H_{62}$ (5%); *n*-hentriacontane, $C_{31}H_{64}$ (60%); and *n*-dotriacontane, $C_{32}H_{66}$ (6%); as well as *n*-tritriacontane, $C_{33}H_{68}$ (5%). No peaks representative of *iso*alkanes were observed. An infrared absorption spectrum (KBr) of the original sample was typical for *n*-alkanes, with no evidence of OH or carbonyl absorption.

The qualitative and relative quantitative distribution of *n*-alkanes in the mixture is consistent with data reported for other ericaceous species (3).

Therefore, on the basis of the data reported at this time, the material reported earlier as "*n*-nonacosane" is a mixture containing *n*-hentriacontane (60%) as the major constituent (1).

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

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VOLUME 56

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JANUARY TO DECEMBER, 1967

JOURNAL OF Pharmaceutical Sciences

December 1967 volume 56, number 12

Review Article

Pharmacology and Toxicology of Toad Venom

By K. K. CHEN and ALENA KOVAŘÍKOVÁ

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HISTORY

FOR CENTURIES the toad has been known to produce a poisonous secretion. The Roman women used the toad, although unsuccessfully, as a murdering agent for their unfaithful husbands (1). William Shakespeare, born about 400 years ago, pictured the toad as wearing a precious jewel in its head. In Macbeth, Shakespeare made the witch throw a toad into the hell-broth. It has been long believed that handling of toads causes warts. In a small area of the state of Illinois, folklore has 300 references to warts of toad origin, particularly their cures (2). These superstitions can of course be disproved by laboratory experience. Houssay (3) operated on 15,000 toads for endocrine studies and we handled more than 10,000 toads for the collection of their poisons—with no ill effects. In form of a votive animal, the toad is associated with the uterus and various gynecological diseases (4) in Central Europe. Bronze toads of the Perm culture of Northeastern Russia were among the archaeological findings dating from the middle of the 1st century A.D.

Toad medicine has been advocated all over the world. For many years the Chinese have used a preparation of toad venom, ch'an su, for the treatment of canker sores, toothache, sinusitis, and local inflammations (5). During the 15th century a European physician wrote a book, "De Venenis," in which he mentioned that the toad's blood was of value in the treatment of difficult breathing (6). Pharmacopeias and dispensatories of early dates gave the dried toad a prominent place among healing agents (7, 8). The South American Indians have made use of the skin secretion of the toad as arrow poison. These arrows are shot into game or enemies from blowtubes.

The English word "toad" is applied to several tailless amphibians, but the various species under

Received from the School of Medicine, Indiana University, Indianapolis, IN 46202
This investigation was supported in part by grant HE-07714 from the U. S. Public Health Service, Bethesda, Md., and an Indiana Heart Association grant.

discussion belong to the genus *Bufo* of the *Bufonidae* family. The members of this genus all have a pair of well-defined skin glands behind the eyes, which secrete venom and are called "parotid" glands. The latter, however, have no relation to salivary glands. The longitudinal axis of the toad's parotid glands is parallel with the body axis, although that of exceptional species may be perpendicular to it—*B. peltocephalus* and *B. asper* (9, 10). Additional glands may appear on arms and legs, *e.g.*, *B. alvarius* (11). The venom can be expressed from the parotid gland between the thumb and index finger, and into a glass evaporating dish held over the toad's head. The toad has warts over the dorsal part of the body, and their secretion is difficult to obtain and may or may not be identical with the parotid secretion. Vulpian (12, 13) discovered that the toad venom had a digitalis-like action and that the toad's heart was relatively immune to its own poison. The histology of parotid glands and warts of European, South and North American toads has been documented by several workers (14–16).

Ugly and venomous, one species of toads, *B. marinus*, has saved sugar crops and other cultivated fields in the Hawaiian Islands and Puerto Rico by devouring the insects which do damage to the plantations. Although the toad is reputed to enjoy longevity, it lives in the laboratory from 8.5 to almost 16 years (17).

Attempts to isolate pharmacologically active substances from the European common toad were initiated over a half century ago (18). Abel and Macht (8) succeeded in isolating "bufagin" and epinephrine from the parotid venom of *B. marinus*. This was followed by the eventual identification of bufotalin and bufotoxin from *B. bufo bufo* by Wieland and Weil (19) and cinobufagin and cinobufotoxin from ch'an su (20). Marked advances were made at the University of Basel (21, 22), for numerous cardioactive substances were isolated from ch'an su, and later from the venoms of other species by elegant modern methods. It is now clear that the chemical composition of the venom of each species of *Bufo* differs from one another, although there are many similarities. Most species elaborate in addition, noncardiotonic sterols, indolealkylamines, and several, catecholamines.

CHEMICAL INGREDIENTS OF TOAD VENOM

Pharmacology of Bufadienolides

The cardioactive sterols are naturally the center of interest. In all instances the lactone ring is 6-membered of α-pyrone type, attached to C_{17}. They have a free secondary hydroxy group at C_3, and are known as bufagins corresponding to aglycones of plants. The structural formulas shown in Fig. 1 represent the better known substances which are chemically bufadienolides (23) and synonymous with bufagins. Because different species of toads do not synthesize identical bufadienolides, prefixes have been employed for distinction. The Japanese toad apparently can produce all of them (24). In our laboratory gamabufagin and gamabufotalin are used interchangeably.

None of the bufadienolides in the toad venom conjugates with a carbohydrate to form a glycoside, but a few couple with suberylarginine to become bufotoxins. The first bufotoxin was isolated from the European common toad (25). To distinguish it from other bufotoxins, Wieland's compound has been renamed vulgarobufotoxin (26), and a structure of 14-suberylarginine bufotalin has been proposed (22–27). Among the amphibians, *Bufo* is the only genus elaborating bufagins and bufotoxins (28). Recently it has been reported that a North African grasshopper, *Poekilocerus bufonius*, feeding on milkweeds, is capable of storing glycosides of cardenolide type (29).

Bufagins—The action of bufagins upon the heart follows almost the same pattern as that of digitalis glycosides. Perfusion by various methods with appropriate concentrations in the frog results in systolic standstill. The absorption is generally poor. The positive inotropic action is easily demonstrated in mammals, such as the cat, by slow infusion of a dilute solution of regularobufagin. Electrocardiographic changes are most instructive. Figure 2 serves as an example. The prolongation of P-R interval, bradycardia, the diphasic T-wave, the nodal rhythm, the A–V dissociation, and the idioventricular rhythm are obvious. The slowing of heart rate can be abolished by atropinization but not by vagotomy. The effect of bufagins on the heart is less cumulative than that of digitoxin, being more like that of ouabain. There is a low order of absorption through the gastrointestinal tract in mammals. Thus, no more than 15% of cinobufagin is absorbed by oral administration in anesthetized cats, and 50% of bufalin (36) by intrajejunal injection in nonanesthetized dogs.

Because the chemical structures of bufagins differ from one another, their cardiac activity varies. Table I shows the potencies of 27 compounds. Their mean (geometric) lethal doses (LD's) are determined in groups of 10 or more etherized cats. It should be noted that bufota-

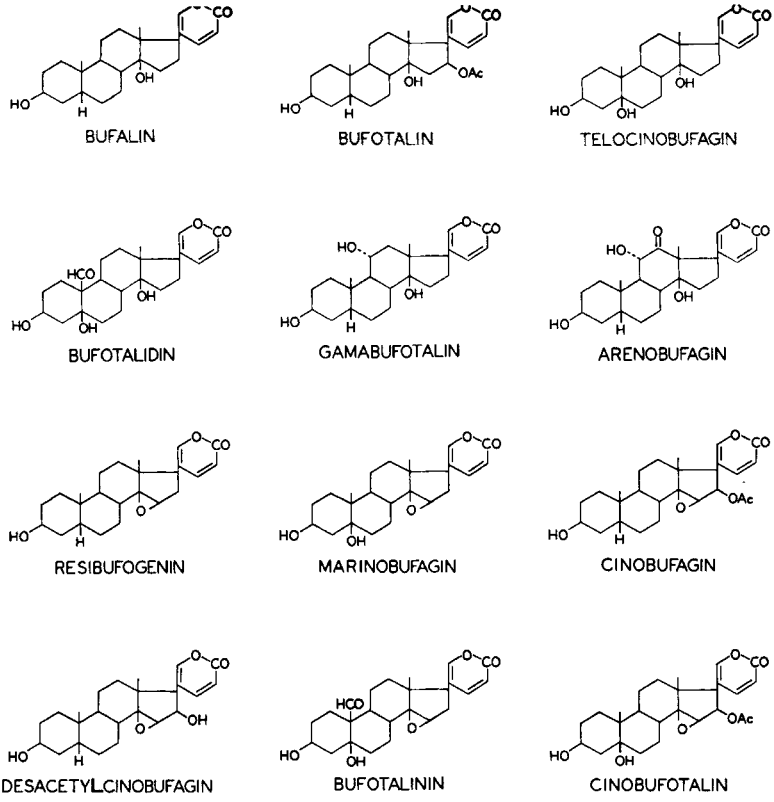

Fig. 1—Structural formulas for better known substances which are chemically bufadenolides (23) and synonymous with bufagins.

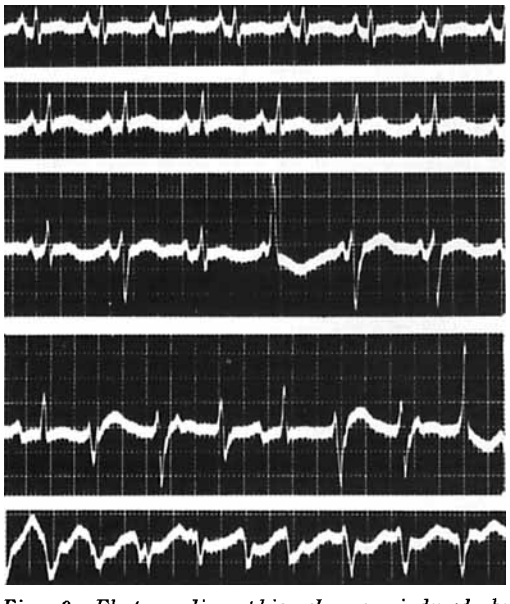

Fig. 2—Electrocardiographic changes induced by regularobufagin in a cat. Regularobufagin in percent of fatal dose (top to bottom): 0, 57, 60, 67, 99.

lidin is identical with hellebrigenin, an aglycone of the root of *Helleborus niger* (34). That toads can synthesize hellebrigenin and hellebrigenol of plant origin is exemplified by several species (37–39). When the means are converted to their reciprocals, they become the number of LD's per mg. as given in Table I, expressing the direct relationship of activity. The very high potency (toxicity) of some of these substances can be appreciated if one realizes that 1 Gm. of acetyl bufotalidin may have 15,600 cat LD's.

The contributions of the Basel scholars make it possible to arrive at certain trends of structure-activity relationship (30). If we consider bufalin the simplest bufadienolide, substitution of H with 5β-OH in telocinobufagin, or with 11α-OH in gamabufagin increases the activity (Table I). A 12-keto group in arenobufagin further enhances the activity. The presence of a free 16-OH in desacetyl bufotalin reduces the toxicity, but 16-acetyl in bufotalin brings back the potency of bufalin. In other cases, 16-desacetylation abolishes the cardiac effect of cinobufagin and cinobufotalin. 3-Acetylation of cinobufotalin, marinobufagin, and bufotalidin (hellebrigenin) has a favorable influence on the potency to various degrees, but the same reaction with cinobufagin produces the opposite effect. A surprising feature is that 14β:15β-epoxides preserve a substantial amount of cardiac activity

TABLE I—ACTIVITY OF BUFAGINS AS MEASURED BY LD'S IN CATS

Name	Mean (Geo.) LD, mg./Kg.	No. of LD's/mg.	Ref.
Arenobufagin	0.08 ± 0.01	13.0 ± 1.14	(30)
Bufotalin	0.13 ± 0.01	7.6 ± 0.40	(31)
Desacetylbufotalin	0.26 ± 0.02	3.8 ± 0.28	(32)
Cinobufagin	0.20 ± 0.02	5.0 ± 0.44	(33)
Acetylcinobufagin	0.59 ± 0.04	1.7 ± 0.11	(30)
Desacetylcinobufagin	inactive	...	(30)
Cinobufotalin	0.20 ± 0.02	5.0 ± 0.62	(33)
Acetylcinobufotalin	0.18 ± 0.01	5.6 ± 0.40	(30)
Desacetylcinobufotalin	inactive	...	(30)
Marinobufagin	1.49 ± 0.09	0.7 ± 0.03	(32)
Acetylmarinobufagin	0.95 ± 0.05	1.1 ± 0.05	(30)
12β-Hydroxymarinobufagin	3.00	0.3	unpublished
Bufotalidin (hellebrigenin)	0.08 ± 0.01	13.0 ± 0.93	(34)
Acetylbufotalidin	0.06 ± 0.00	15.6 ± 0.70	(30)
Resibufogenin	inactive	...	(30)
Acetylresibufogenin	inactive	...	(30)
12β-Hydroxyresibufogenin	4.16	0.2	unpublished
Bufalin	0.14 ± 0.01	7.3 ± 0.55	(33)
Telocinobufagin	0.10 ± 0.02	9.8 ± 0.64	(33)
Bufotalinin	0.62 ± 0.12	1.6 ± 0.31	(30)
Artebufogenin	inactive		(35)
Gamabufotalin	0.10 ± 0.01	9.9 ± 0.50	(31)
Vallicepobufagin	0.20 ± 0.02	5.0 ± 0.43	(31)
Quercicobufagin	0.10 ± 0.04	10.3 ± 0.39	(31)
Viridobufagin	0.11 ± 0.01	9.0 ± 0.66	(31)
Regularobufagin	0.15 ± 0.01	6.5 ± 0.25	(31)
Fowlerobufagin	0.22 ± 0.01	4.6 ± 0.24	(31)

as compared with their free 14β-OH analogs—marinobufagin *versus* telocinobufagin, cinobufagin *versus* bufotalin, and bufotalinin *versus* bufotalidin. It is difficult to explain the absence of activity of resibufogenin and artebufogenin (35). Telocinobufagin with a methyl group at C_{10} is less potent than the aldehyde derivative of the same structure, bufotalidin. The cardenolides of plants—digitoxigenin, periplogenin, oleandrigenin, sarmentogenin, and strophanthidin, corresponding to bufalin, telocinobufagin, bufotalin, gamabufotalin, and bufotalidin—have lower toxicities. The same order of difference has been demonstrated on the contractility of guinea pigs' auricles (40). The superiority of α-pyrone at C_{17} to the cyclobutenolide ring at the same position is unequivocal.

The nature of cardiotonic action is not yet clearly elucidated. An enormous amount of data with cardiac glycosides on tissue respiration, carbohydrate metabolism, high-energy phosphate bonds, ion equilibrium, and contractile proteins is probably also applicable to bufagins (41, 42).

Like ouabain, any of the bufagins causes vomiting in cats or pigeons following intravenous injection of one-half LD. The mechanism of action is presumably also due to the excitement of the chemoreceptor trigger zone where the impulse is mediated to the vomiting center (43).

Restlessness and convulsions occur in frogs with cinobufagin. The action appears to be on the spinal cord. Convulsant action has also been observed with acetyl cinobufagin, marinobufagin and its acetyl derivative, and bufotalinin, not only in frogs but also in cats (30).

As a class bufagins constrict arterial blood vessels and stimulate isolated rabbit's intestines and uterus (44–46). The action must be directly on the smooth muscles.

Some of the bufagins, if not all, have a local anesthetic action. Cinobufagin in 0.1–0.5% solutions produces numbness of the tongue when applied locally. With the stronger solution, sweet and bitter tastes may be lost. There is disappearance of tactile sensation. Local anesthesia can also be demonstrated on the rabbit's cornea after instillation and the guinea pig's skin following intracutaneous injections. Its action is not on nerve fibers, but is confined to sensory nerve endings. So far tested, bufalin, cinobufotalin, and gamabufagin also have a local anesthetic effect.

Bufotoxins—The principal action of a bufotoxin is also upon the heart. The electrocardiographic changes during the slow infusion of cinobufotoxin in a cat are illustrated in Fig. 3. They are similar to those with bufagins or cardiac glycosides. Their potencies are lower than those of corresponding bufagins. Thus vulgarobufotoxin is weaker than bufotalin. The data on

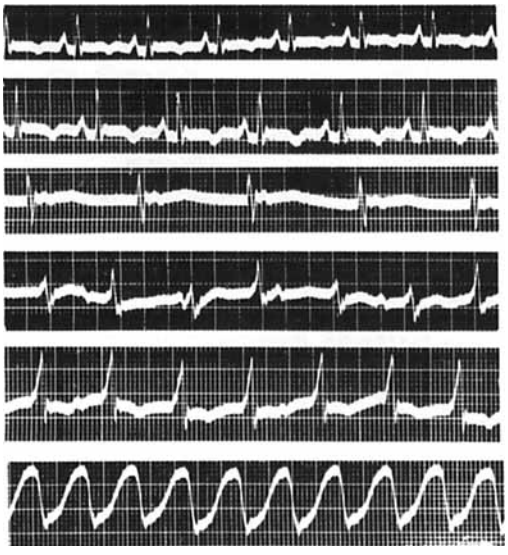

Fig. 3—Electrocardiographic changes caused by cinobufotoxin in a cat. Cinobufotoxin in percent of fatal dose (top to bottom): 0, 19, 37, 75, 77, 100.

nine bufotoxins are given in Table II. All of them are easily dissipated in the animal organism.

Extracardiac effects are repetitious of those of cardiotonic substances—emesis, stimulation of smooth muscle organs, and bitter taste when applied locally.

Clinical Trials—Because of the wide interest in the therapeutic value of toad venom in folklore, a clinical study of one of the bufadienolides was carried out. Cinobufagin can be used as a model, since it is easily available (47). Cases of auricular fibrillation are most suitable for this purpose. Indeed intravenous injection of 1 mg. slows the ventricular rate in some patients, and that of 2 mg. causes a marked fall of pulse rate. The effect lasts for about 5 hr. Nausea and vomiting occur in the majority of instances with the larger dose. It is obvious that cinobufagin offers no advantage over ouabain due to its brevity of action and frequency of side effects. In spite of such evidence, "bufomarine" has been recommended for the treatment of heart ailments of the aged (48). Aside from the curiosity, none of the toad cardiotonic principles can be justifiably employed in cardiology.

TABLE II—ACTIVITY OF BUFOTOXINS AS MEASURED BY LD'S IN CATS (31)

Name	Mean (Geo.) LD, mg./Kg.	No. of LD's/mg.
Viridobufotoxin	0.27 ± 0.01	3.70 ± 0.16
Vulgarobufotoxin	0.29 ± 0.02	3.43 ± 0.21
Cinobufotoxin	0.36 ± 0.02	2.79 ± 0.19
Gamabufotoxin	0.37 ± 0.03	2.67 ± 0.19
Arenobufotoxin	0.41 ± 0.01	2.46 ± 0.07
Marinobufotoxin	0.42 ± 0.02	2.40 ± 0.12
Regularobufotoxin	0.48 ± 0.03	2.10 ± 0.12
Alvarobufotoxin	0.76 ± 0.08	1.32 ± 0.13
Fowlerobufotoxin	0.79 ± 0.05	1.26 ± 0.09

Catecholamines

Epinephrine—The discovery of epinephrine in the venom of *B. marinus* marks the first extramedullary source of a very potent sympathomimetic amine (8). What is more remarkable is the large quantity present in a pair of parotid glands. By a pressor method in pithed cats the dried secretion contains more than 5% of this agent. Not all species of toads produce epinephrine in the venom. To date *B. marinus*, *B. bufo gargarizans*, *B. regularis*, *B. arenarum*, *B. formosus*, *B. blombergi*, and *B. peltocephalus* are known to elaborate epinephrine in the parotid glands (9, 47, 49), while *B. bufo bufo*, *B. viridis viridis*, *B. alvarius*, *B. americanus*, *B. valliceps*, *B. fowleri*, *B. quercicus*, *B. melanostictus*, and *B. asper* are devoid of it (10). The pharmacology of epinephrine is so frequently reviewed (50, 51) that it will be superfluous to give an account here.

Norepinephrine—The identification can be made on the cat's blood pressure and nictitating membrane as well as by paper chromatography. Norepinephrine occurs in the epinephrine of *B. marinus* (52, 53) in amounts of 2–5%. The *l*-isomer of this catecholamine has also been found as an impurity from the sample of epinephrine, isolated from ch'an su (54). The detection of such a small quantity cannot be achieved by using the toad venom without isolating the bulk of epinephrine (55, 56). A monograph on the pharmacology of norepinephrine is available for reference (57).

Indolealkylamines

The first indole base was isolated from the venom of *B. bufo bufo* (58). Its structure is 5-hydroxy-dimethylaminotryptamine (59). Indolethylamine derivatives occur in other species of toads (9, 10, 60). As shown in Fig. 4, cinobufotenine is the methylbetaine of bufotenine. 5-Hydroxytryptamine or serotonin and dehydrobufotenine are present in the parotid secretion of *B. marinus* (61, 62). Bufothionine occurs in the skin secretions of *B. viridis* (63).

The pressor activity in pithed cats can serve to distinguish indoleamines of different species (26). Cinobufotenine is the most potent. In fowls intravenous injection of these substances is followed by a fall of blood pressure (64). Application of the toad indoleamines stimulates

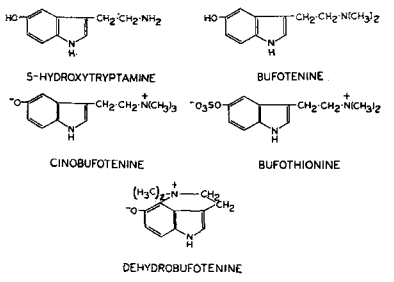

Fig. 4—Substances appearing in the parotid secretions of the toad.

the isolated uterus of the rabbit, the guinea pig, and the rat, and contracts the isolated ileum of the rabbit and the guinea pig. An extensive review on 5-hydroxytryptamine and its related indolealkylamines is available (65, 66).

Noncardiotonic Sterols

The petroleum ether fraction of the ethanol extract contains a mixture of noncardiotonic sterols.

Cholesterol—The bulk can be identified as cholesterol by melting point, spectrogram, and color reactions in addition to digitonin precipitation.

Provitamin D—An admixture of the cholesterol sample of different species of toads is a phytosterol which varies from zero to 14 parts per thousand. The spectrogram is characteristic of ergosterol and after irradiation the material causes bone healing in rachitic rats.

γ-Sitosterol—Sterols of the plant kingdom occur in the venom of toads. γ-Sitosterol has been isolated from *B. bufo bufo*, *B. formosus*, and *B. arenarum* (67).

Miscellaneous Substances—The fresh venom of the different species of toads contains an average of 51–61% of water (11). During squeezing a typical scent is emitted. After drying and extraction for active principles, the residue swells in water, suggestive of mucoproteins. Free suberic acid and arginine are identifiable. The significance of these inert products have not been explored.

USE OF THE VENOM TO THE TOAD

Protection from Enemies—The venoms of most animals are *a priori* intended for self-protection. If the toad finds it possible to introduce its poison into the enemy's bloodstream, it would be fatal to the latter. But the toad does not have teeth to bite, nor does it realize a danger by accurately squirting the venom. It blows itself up when approached by a human hand; when a dog or a cat bites a toad, prolonged salivation takes place but vomiting rarely follows (68). In our experience, one dog was induced to swallow a toad (*B. valliceps*) with complete recovery (47). Some snakes and rats feed on toads (69). The death of a little terrier from biting a toad (*B. alvarius*) was recorded (70). The bitter taste and local irritation of the nose and the eye when one comes in contact with the toad venom is well known (71, 72).

The process of regeneration of the venom is slow. If the pair of parotid glands (*B. marinus*) are manually expressed, it takes about 11 weeks for the toad to restore two-thirds of the original amount. It would be impossible for the animal to furnish large quantities of the poison repeatedly for defensive purposes.

It appears that the toad (*B. marinus*, *B. valliceps*) can dispense with the parotid glands. Surgical removal of these skin organs is compatible with the normal life of the animal. Observations of such an experiment were extended over a year (47).

Significance to Behavior—Recent work on psychopharmacology in higher mammals is centered around the amines of the brain. There is no information about the significance of 5-hydroxytryptamine and catecholamines to the toad. If a large toad, *B. marinus*, is dissected under local anesthesia for the blood supply and nerve innervation of a parotid gland, no epinephrine is demonstrable in the vein following electric stimulation of the glandular nerve which is a branch of the jugular ganglion. The result is not comparable to the splanchnic stimulation in mammals, liberating medullary epinephrine of adrenal glands. The toad's serum cholesterol averages 153 mg.%, lower than that of human adults. Is it possible that γ-sitosterol regulates it to this depressed level?

In short, toads of various species are capable of synthesizing in the parotid glands sterols of either *trans-* or *cis*-configuration, catecholamines, and indolealkylamines. All are pharmacologically active, but apparently serve little purpose to the toads.

NATURAL TOLERANCE TO CARDIAC GLYCOSIDES AND AGLYCONES

For more than a century it has been known that the toad's heart is resistant to its own venom and to digitalis. This is a distinct example of pharmacogenetics. By comparison, the frog, *Rana pipiens*, is many times more susceptible to scillaren, ouabain, cymarin, and coumingine

HCl than the toad, *B. valliceps* (73, 74). Similar differences can be demonstrated by perfusions with gamabufagin, viridobufagin, vulgarobufotoxin, or regularobufotoxin. The exact mechanism of the toad's cardiac resistance is not known, but it offers a great challenge for elucidation. A suggestion has been made that the toad resistance is not due to the fate of cardiotonic substances, but determined by the reactivity of "digitalis receptors" (75). Aside from the classical studies, certain methods employed in electrophysiology may throw light upon this subject (76, 77).

RESPONSE OF OTHER TISSUES TO DRUGS

Epinephrine on one hand and cinobufotenine and fowlerobufotenine on the other increase the tone and contraction of the frog's and the toad's heart to the same degree when perfused through the inferior vena cava (73, 78).

The central nervous system of the toad is almost equally susceptible as that of the frog to strychnine, picrotoxin, cocaine, caffeine, pentobarbital, and curare. The toad shows similar responses to gonadotropins as *Xenopus laevis* and *Rana esculenta* (79). The exhaustive investigations of Houssay (3) on the Argentine toad, *B. arenarum*, reveal more resemblance than exceptions to higher vertebrates. The natural tolerance of the toad's heart to cardiotonic steroids is unique and is only shared by rodents such as the rat. More studies to search for the reason will contribute to our understanding of this biological anomaly.

REFERENCES

(1) Faust, E. S., "Handbuch der Experimentellen Pharmakologie," vol. 2, Springer Verlag, Berlin, Germany, 1924, p. 1815.
(2) Hyatt, H. M., "Folk-Lore from Adams County, Illinois," Memoirs of the Alma Egan Hyatt Foundation, New York, N. Y., 1935.
(3) Houssay, B. A., *New Engl. J. Med.*, **214,** 913(1936).
(4) Born, W., *Ciba Symposia*, **1,** 129(1939).
(5) Li, S. C., "Pentsao Kang Mu," chap. 42, 1596.
(6) Podolsky, E., *Am. Scholar*, **3,** 201(1934).
(7) Abel, J. J., and Macht, D. I., *J. Am. Med. Assoc.*, **56,** 1531(1911).
(8) Abel, J. J., and Macht, D. I., *J. Pharmacol. Exptl. Therap.*, **3,** 319(1912).
(9) Henderson, F. G., Welles, J. S., and Chen, K. K., *Proc. Soc. Exptl. Biol. Med.*, **104,** 176(1960).
(10) Henderson, F. G., Welles, J. S., and Chen, K. K., *Science*, **136,** 775(1962).
(11) Chen, K. K., and Chen, A. L., *J. Pharmacol. Exptl. Therap.*, **47,** 281(1933).
(12) Vulpian, E. F. A., *Compt. Rend. Soc. Biol.*, **1,** 133 (1854).
(13) *Ibid.*, **3,** 125(1856).
(14) Calmels, G., *Arch. Phys. Norm. Pathol. 3 Sér.*, **1,** 321(1883).
(15) Muhse, E. F., *Am. J. Anat.*, **9,** 322(1909).
(16) Shipley, P. G., and Wislocki, G. B., "Contributions to Embryology," vol. 3, Carnegie Institution, 1915, p. 71.
(17) Pemberton, C. E., *Science*, **110,** 512(1949).
(18) Phisalix, C., and Bertrand, G., *Bull. Soc. Pharmacol.*, **5,** 211(1902).
(19) Wieland, H., and Weil, F. J., *Ber. Deut. Chem. Ges.*, **46,** 3315(1913).
(20) Chen, K. K., Jensen, H., and Chen, A. L., *J. Pharmacol. Exptl. Therap.*, **43,** 13(1931).
(21) Meyer, K., *Pharm. Acta Helv.*, **24,** 222(1949).
(22) Meyer, K., *Helv. Chim. Acta*, **32,** 1993(1949).
(23) Ciba Conference, *ibid.*, **34,** 1680(1951).
(24) Iseli, E., Kotake, M., Weiss, E., and Reichstein, T., *ibid.*, **48,** 1093(1965).
(25) Wieland, H., and Alles, R., *Ber. Deut. Chem. Ges.*, **55,** 1789(1922).
(26) Chen, K. K., Jensen, H., and Chen, A. L., *J. Pharmacol. Exptl. Therap.*, **47,** 307(1933).
(27) Urscheler, H. R., Tamm, C., and Reichstein, T., *Helv. Chim. Acta*, **38,** 883(1955).
(28) Daly, J. W., and Myers, C. W., *Science*, **156,** 970 (1967).
(29) Euw, J. v., Fishelson, L., Parsons, J. A., Reichstein, T., and Rothschild, M., *Nature*, **214,** 35(1967).
(30) Chen, K. K., and Henderson, F. G., *J. Med. Pharm. Chem.*, **3,** 111(1960).
(31) Chen, K. K., *Ann. Rev. Physiol.*, **7,** 677(1945).
(32) Chen, K. K., *Proc. First Intern. Pharmacol. Meeting*, **3,** 27(1963).
(33) Chen, K. K., Anderson, R. C., and Henderson, F. G., *Proc. Soc. Exptl. Biol. Med.*, **76,** 372(1951).
(34) Chen, K. K., Henderson, F. G., and Anderson, R. C., *J. Pharmacol. Exptl. Therap.*, **99,** 395(1950).
(35) Chen, K. K., and Henderson, F. G., *ibid.*, **111,** 365 (1954).
(36) Herrmann, R. G., Parker, R. J., Henderson, F. G., and Chen, K. K., *Proc. Soc. Exptl. Biol. Med.*, **109,** 646(1962).
(37) Rees, R., Schindler, O., Deulofeu, V., and Reichstein, T., *Helv. Chim. Acta*, **42,** 2400(1959).
(38) Barbier, M., Schröter, H., Meyer, K., Schindler, O., and Reichstein, T., *ibid.*, **42,** 2486(1959).
(39) Barbier, M., Bharucha, M., Chen, K. K., Deulofeu, V., Iseli, E., Jäger, H., Kotake, M., Rees, R., Reichstein, T., Schindler, O., and Weiss, E., *ibid.*, **44,** 362(1961).
(40) Förster, W., *Acta Biol. Med. Germ.*, **9,** 341(1962).
(41) Hajdu, S., and Leonard, E., *Pharmacol. Rev.*, **11,** 173(1959).
(42) Wilbrandt, W., *Proc. First Intern. Pharmacol. Meeting*, **3,** 3(1963).
(43) Borison, H. L., and Wang, S. C., *Proc. Soc. Exptl. Biol. Med.*, **76,** 335(1951).
(44) Kodama, K., *Acta Schol. Med. Univ. Imp. Kioto*, **3,** 299(1920).
(45) *Ibid.*, **4,** 355(1922).
(46) Raymond-Hamet, *Compt. Rend. Soc. Biol.*, **137,** 178(1943).
(47) Chen, K. K., and Chen, A. L., *Arch. Intern. Pharmacodyn.*, **47,** 297(1934).
(48) Süttinger, H., *Arzneimittel-Forsch.*, **9,** 256(1955).
(49) Novaro, V., *Compt. Rend. Soc. Biol.*, **88,** 371(1923).
(50) "Pharmacological Basis of Therapeutics," Goodman, L. S., and Gilman, A., eds., 3rd ed., Macmillan Co., New York, N. Y., 1965.
(51) Kopin, I. J., *Pharmacol. Rev.*, **16,** 179(1964).
(52) Fischer, P., and Lecomte, J., *Arch. Intern. Pharmacodyn.*, **81,** 387(1950).
(53) Lasagna, L., *Proc. Soc. Exptl. Biol. Med.*, **78,** 876 (1951).
(54) Lee, H. M., and Chen, K. K., *J. Pharmacol. Exptl. Therap.*, **102,** 286(1951).
(55) Bacq, Z. M., and Lecomte, J., *Compt. Rend. Soc. Biol.*, **141,** 861(1947).
(56) Pereira, J. R., *Rev. Brasil. Biol.*, **14,** 123(1954).
(57) Euler, U. S. v., "Noradrenaline," Charles C Thomas, Springfield, Ill., 1956.
(58) Handovsky, H., *Arch. Exptl. Pathol. Pharmakol.*, **86,** 138(1920).
(59) Wieland, H., Konz, W., and Mittasch, H., *Liebigs Ann. Chem.*, **513,** 1(1934).
(60) Jensen, H., and Chen, K. K., *Ber. Deut. Chem. Ges.*, **65,** 1310(1932).
(61) Udenfriend, S., Clark, T. C., and Titus, E., *Experientia*, **8,** 379(1952).
(62) Märki, F., Robertson, A. V., and Witkop, B., *J. Am. Chem. Soc.*, **83,** 3341(1961).
(63) Erspamer, V., *Biochem. Pharmacol.*, **2,** 270(1959).
(64) Powell, C. E., Swanson, E. E., and Chen, K. K., *J. Am. Pharm. Assoc., Sci. Ed.*, **44,** 399(1955).
(65) Erspamer, V., *Progr. Drug Res.*, **3,** 151(1961).
(66) Heinzelman, R. V., and Szmuszkovicz, J., *ibid.*, **6,** 75(1963).
(67) Hüttel, R., and Behringer, H., *Z. Physiol. Chem.*, **245,** 175(1937).
(68) Knowles, R., *Vet. Med.*, **59,** 38(1964).
(69) Smith, H. M., and White, F. N., *Herpetologica*, **11,** 137(1955).
(70) Musgrave, M. E., *Copeia*, (No. 173), 96(1930).
(71) Epstein, D., *S. African Med. J.*, **6,** 403(1932).
(72) Ross, A. E., and Neill, W. T., *Herpetologica*, **12** 150(1956).
(73) Chen, K. K., and Chen, A. L., *J. Pharmacol. Exptl. Therap.*, **47,** 295(1933).
(74) Chen, K. K., Hargreaves, C. C., and Winchester, W. W., *J. Am. Pharm. Assoc.*, **27,** 307(1938).
(75) Portius, H. J., Herrmann, I., and Repke, K., *Arch. Exptl. Pathol. Pharmakol.*, **241,** 534(1961).
(76) Hoffman, B. F., and Cranefield, P. F., "Electrophysiology of the Heart," McGraw-Hill Book Co., New York, N. Y., 1960.
(77) Azuma, T., Hayashi, H., and Matsuda, K., *Science*, **138,** 895(1962).
(78) Lutz, B. R., *Biol. Bull.*, **64,** 299(1933).
(79) Thorborg, J. V., *Acta Endocrinol.*, **4,** 16(1950).

Release Rates of Solid Drug Mixtures Dispersed in Inert Matrices I

Noninteracting Drug Mixtures

By PARVINDER SINGH*, SAURABH J. DESAI*, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

The theory for the simultaneous release of a mixture of two noninteracting drugs dispersed in an inert plastic matrix has been developed. Quantitative relationships based on a physical model were derived that express the diffusion-controlled rates of release of both drugs as functions of the amounts of each drug, the solubilities of the drugs, their diffusional coefficients, and the porosities and tortuosities of the tablet. The theoretical relationships were applied to the salicylic acid–benzoic acid–plastic mixture. The experimental data were found to be in close agreement with the theoretically calculated values.

RECENTLY the drug release rate of a single species dispersed in inert plastic matrices has been extensively investigated (1–4). It was felt that it would be of interest to explore the more complex situation in which more than one drug is present in the tablet matrix. This report is involved with matrices containing an ideal mixture of drugs where the presence of one drug species does not alter the physicochemical properties of the other. The salicylic acid–benzoic acid mixture was chosen to represent the nonreactive case. A subsequent paper will report the results of the benzocaine–caffeine mixture representing the reactive system.

A physical model for the simultaneous release of a mixture of two drugs embedded in an insoluble matrix is developed and theoretical equations derived from it. The equations permit the independent quantitative description of the release rates of both drugs. All the parameters operative in these equations were independently determined, and after substitution into the appropriate theory, the theoretical release rates were obtained. These were then compared with the experimentally determined release rates to test the validity of the proposed model.

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication July 17, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This research project was supported in part by grants from the Ciba Pharmaceutical Co., Summit, N. J., and Parke-Davis Co., Detroit, Mich.

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THEORETICAL MODEL

The diffusion-controlled model used to develop the mixture release equations derived under the *Appendix* is shown in Fig. 1. Figure 1(a) depicts initial conditions, *i.e.*, no solvent penetration, and both drugs exist solely as solids embedded in the matrix, region 3. Figure 1(b) depicts the conditions existing at a finite time, t. The boundary of the matrix solvent interface is at $x = 0$ and separates region 0 (solvent) and region 1 which corresponds to the completely leached portion of the matrix. The solid–liquid boundary of the slower moving drug is at $x = s_1$ and separates region 1 and region 2 which contains solid drug A in equilibrium with its saturated solution and drug B solely in solution. Finally the solid–liquid boundary of the faster

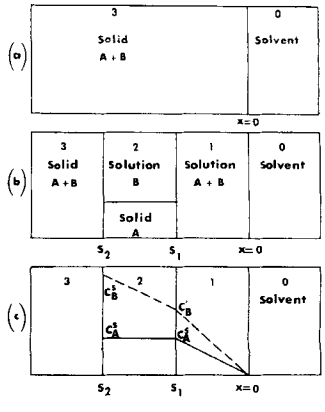

Fig. 1—The physical model that describes the release of a mixture of two noninteracting drugs from an inert matrix. Key: (a) *conditions existing at time* $t = 0$; (b) *conditions existing at finite time,* t; (c) *an illustration of the concentration gradients for all species.*

moving drug is at $x = s_2$ and separates region 2 and region 3 (solid drugs only). The concentration gradients of drugs A and B are shown in Fig. 1(c).

The relative boundary movement of drugs A and B will be a function of their solubilities, diffusion coefficients, and concentration of their solids in the matrix. If both drugs are present in equal concentrations and have equal diffusion coefficients, the more soluble drug will have the faster moving boundary. If both drugs have equal solubilities and are present in equal concentrations, the drug with the larger diffusion coefficient will move faster. Finally, if both drugs have equal solubilities and diffusion coefficients, the drug present in the smaller amount will have a faster moving boundary. It is evident from the above that the slower moving drug can be made to have a faster moving boundary by sufficiently decreasing its concentration in the matrix.

Inspection of Fig. 1 shows that the porosity and tortuosity of region 1 and region 2 will differ due to the presence of solid A in region 2 but not in region 1. The porosity of region 1 is given by the sum of the porosities due to the presence of drug A, drug B, and residual air in the matrix. The porosity of region 2, on the other hand, is given by the sum of porosity contributions of only drug B and residual air. In addition, the presence of solid drug A in region 2 will cause its tortuosity to be equal to or greater than that of region 1.

It is evident from the model that as neither solid A nor solid B is present in region 1, the release of the slower moving drug A can be described by the Higuchi equation (5):

$$\frac{dQ_A}{d(t^{1/2})} = \left[D_A \frac{\epsilon_1}{\tau_1} (2A_A - \epsilon_1 C_A^s) C_A^s \right]^{1/2} \quad \text{(Eq. 1)}$$

For the release of the faster moving drug, B, however, the following more complex equation must be utilized:

$$\frac{dQ_B}{d(t^{1/2})} = \frac{2D_B\epsilon_1}{\frac{k_A}{A_A}\tau_1} \left[\frac{\frac{\epsilon_2}{\tau_2} C_B^s}{\frac{\epsilon_2}{\tau_2} + \frac{\epsilon_1}{\tau_1} \left[\frac{\frac{k_B}{A_B} - \frac{k_A}{A_A}}{\frac{k_A}{A_A}} \right]} \right] \quad \text{(Eq. 2)}$$

Here, subscripts 1 and 2 symbolize parameters operative in regions 1 and 2, respectively, and subscripts A and B refer to the drugs A and B, respectively. The symbols used in the above equations are defined as follows: Q_i is the grams of drug i released per unit area of surface at time, t; D_i is the diffusion coefficient of the drug i in the release medium; A_i is the concentration of the drug i in the tablet; C_i^s is the solubility of the drug in the release medium; ϵ_j is the porosity of the region j and τ_j is the tortuosity of the region j. K_A is the slope of the Q_A *versus* $t^{1/2}$ plot (from Eq. 1) and K_B is the slope of the Q_B *versus* $t^{1/2}$ plot (from the *Appendix*).

The evaluation of these parameters and the application of these equations are discussed at length in later portions of the text.

EXPERIMENTAL

In previous papers the release behavior of drugs from polyethylene matrices as well as polyvinyl chloride (PVC) matrices were reported (3, 4). Both matrices provided nonideal release profiles using water as the release medium. It was found, however, that the inclusion of surfactant in the release medium provided ideal release profiles from polyethylene matrices but not for PVC matrices. The PVC matrices, on the other hand, provided ideal release profiles if previously subjected to vacuum treatment before introduction of solvent.

For these series of studies, both mutually interacting and noninteracting drug mixtures studies were anticipated. It would be highly undesirable, therefore, to include any surfactant in the release medium due to its possible direct or indirect interactions with the drug mixture systems. The use of vacuum techniques, on the other hand, always poses threats of technical difficulties.

Due to the possibility that a combination of these two plastics might lead to a matrix system that would eliminate the need for either of these treatments, a systematic series of matrices was studied in which the two plastics were mixed in different ratios from PVC: polyethylene ratios of 9:1 to 1:9 on a weight-to-weight basis. Both caffeine and potassium acid phthalate were separately used as drugs. In both instances the PVC–polyethylene ratio of 7:3 provided the most ideal matrix. The release rates in water, surfactant, and under vacuum conditions gave identical results for these drugs embedded in this matrix system. It was therefore decided to use this mixture as the inert matrix to investigate the release behavior for these series of drug mixtures.

The release rates from these matrices containing solid drug mixtures as well as solutions were studied using the techniques and the evaluation of parameters described previously (1, 2). The diffusion coefficients and solubilities of both drugs used in each mixture were determined individually as well as in the presence of each other.

For this study the simultaneous release of salicylic and benzoic acid from the above matrix was obtained by spectrophotometric assay of the release medium as a function of time at 302 mμ and 259 mμ and by solving the two simultaneous equations for their respective concentrations.

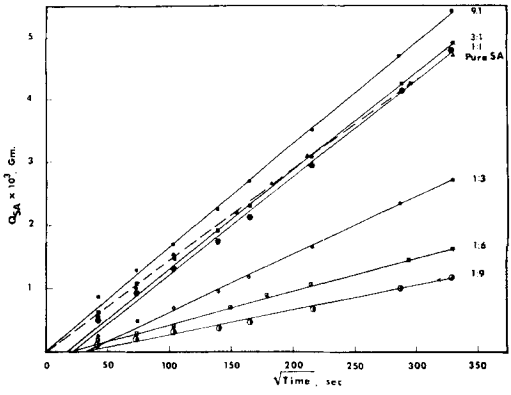

Fig. 2—Release of salicylic acid from matrices containing different ratios of salicylic acid-to-benzoic acid into 0.1 N HCl. The total amount of drug in all cases was 20% w/w, and the numbers indicate salicylic acid-to-benzoic acid ratios.

RESULTS AND DISCUSSION

Six different weight-to-weight ratios of salicylic acid and benzoic acid were homogeneously dispersed in the PVC–polyethylene 7:3 mixture and compressed. The total was maintained at 20% w/w. Release profiles were obtained in 0.1 *N* HCl to assure that both acids would exist primarily in their undissociated form. The results of these release studies are displayed in Figs. 2, 3, and 4. As predicted by the theoretical equations, a linear square root of time dependence is observed, both for the total as well as for the individual drugs when they are simultaneously released from the matrix.

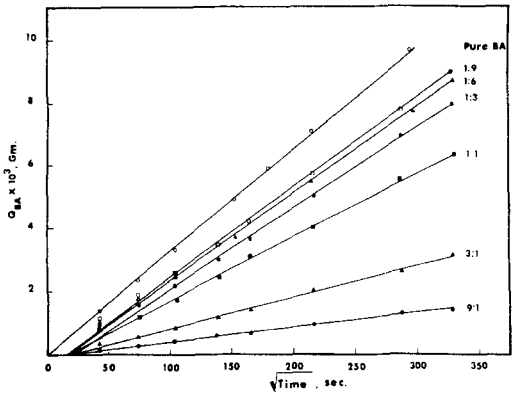

Fig. 3—Data showing benzoic acid release in the same experiments as in Fig. 2.

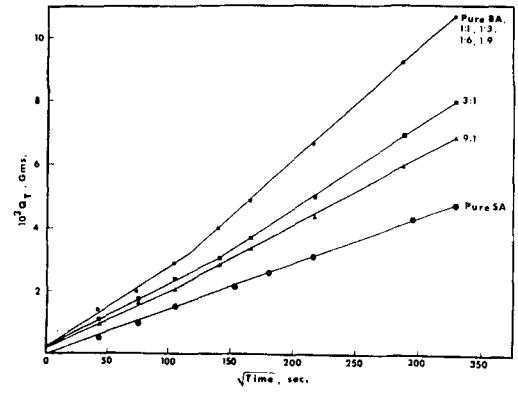

Fig. 4—The sum of the salicylic acid and benzoic acid release in the same experiments as in Figs. 2 and 3.

Examination of Fig. 1 indicates that the release profiles shown in Figs. 2 and 3 occur through region 1 for the slower moving drug and through region 2 for the faster moving drug. When $t = 0$, the relative rates of the two solid-liquid boundary movements can be given by the ratio of their respective release rates as indicated by Eq. 1; *i.e.*,

$$\frac{\text{salicylic acid boundary}}{\text{benzoic acid boundary}} = \frac{Q_{BA}/A_{BA}}{Q_{BA}/A_{BA}} = \left[\frac{D_{BA}C_{BA}A_{BA}}{D_{BA}C_{BA}A_{BA}}\right]^{1/2} \quad \text{(Eq. 3)}$$

All other factors in Eq. 1 are equal and cancel. The above indicates that both boundaries will move at an equal rate (no region 2) at a ratio of:

$$\frac{A_{SA}}{A_{BA}} = \frac{D_{BA}C_{BA}}{D_{BA}C_{BA}} = \frac{(1.16 \times 10^{-5})(2.0 \times 10^{-3})}{(1.22 \times 10^{-5})(3.6 \times 10^{-3})} = \frac{1}{1.9}$$

This analysis indicates that for ratios of salicylic to benzoic acid which are larger than 1:1.9, the benzoic acid drug–liquid boundary is moving more rapidly, but at ratios smaller than 1:1.9 it is moving more slowly.

ANALYSIS OF REGION 1 PARAMETERS

The previous discussion has indicated that Eq. 1 is valid for the release of the drug whose boundary is moving at a slower rate and therefore can be used to describe the salicylic acid release profile for drug ratios greater than 1:1.9 and for benzoic acid release at smaller ratios. The total porosity of the matrix (due to air and both drugs), the diffusion coefficient, solubility, and concentration in the matrix of the predicted slower moving drug were substituted in Eq. 1, and apparent tortuosity values, τ_1, for region 1 were calculated and tabulated in columns *7* and *8* of Table I. A similar treatment was used to calculate the tortuosity of region 1 using the parameters of the faster moving drug. Although Eq. 1 is not valid for the faster moving drug, these calculations were made only as a basis for comparison and are therefore listed in parentheses in Table I. An examination of columns *7* and *8* indicates that the bracketed values of τ are generally significantly higher when analyzing the extreme ratios and are in closer agreement as the ratio of drugs approaches the predicted ratio of equal boundary movement. This is reasonable because region 2 is relatively large at the extreme ratios and becomes smaller, completely disappearing when the boundaries move at an equal rate.

An independent test of the tortuosity values of region 1 can be made by obtaining the release profile of the completely leached matrix resaturated with a drug solution. Tortuosity values obtained in this manner are shown in column *9* of Table I. It is seen that these tortuosity values are independent of the drug mixture ratio. This would be expected as both drugs have been leached from the matrix. In addition, these values are in close agreement with those calculated using Eq. 1 for the slower moving drug and confirm the previous analysis.

ANALYSIS OF REGION 2 PARAMETERS

The drug with the faster moving boundary must diffuse both through regions 1 and 2, and it is obvious that Eq. 2 (not Eq. 1) must be used to describe its release. The porosity of region 2 is due to air and the faster moving drug. All other parameters in Eq. 2 have been independently determined except the tortuosity, τ_2, and allow its calculation. These values are listed in column *5* of Table II. To verify the above tortuosity values, an independent study of these matrices was made by obtaining release profiles of salicylic acid using saturated benzoic acid solution as the release

TABLE I—CALCULATION OF THE TORTUOSITY OF REGION 1 AS DETERMINED BY THE SOLID AND LIQUID RELEASE EXPERIMENTS

SA/BA Ratio	1 ϵ^a Total	2 A_{SA}	3 A_{BA}	4 $10^5 Q_{SA}/t^{1/2}$ Solid Release	5 $10 Q_{BA}/t^{1/2}$ Solid Release	6 $10^5 Q/t^{1/2}$ Liquid Release	7 τ_{1SA}	8 τ_{1BA}	9 τ_1 Liquid Release
Pure BA	0.319	...	0.210	...	3.30	3.32	...	4.4	3.5
1:9	0.303	0.021	0.193	0.42	3.12	3.82	(14.5)[b]	4.2	2.8
1:6	0.295	0.031	0.186	0.54	2.84	3.76	(12.4)[b]	4.7	2.5
1:3	0.299	0.054	0.162	0.90	2.57	3.28	(4.0)[b]	5.1	3.7
1:1	0.268	0.107	0.107	1.60	2.00	5.36	4.5	(5.0)[b]	2.7
3:1	0.294	0.163	0.054	1.64	1.00	4.44	7.1	(11.0)[b]	3.6
9:1	0.286	0.197	0.055	1.70	0.53	3.36	7.8	(38.5)[b]	3.7
Pure SA	0.299	0.216	...	1.46	...	3.54	12.0	...	4.5

[a] ϵ total = $\epsilon_{SA} + \epsilon_{BA} + \epsilon_{air}$. [b] These tortuosity values are incorrect, but have been included for discussion purposes only.

TABLE II—CALCULATION OF THE TORTUOSITY OF REGION 2

SA/BA Ratio	1 $\epsilon_{BA + air}$	2 $\epsilon_{SA + air}$	3 $10^5 Q_{SA}/t^{1/2}$	4 τ_2 Expt. (Using Eq. 1)	5 τ_2 Calcd. from Eq. 2
1:9	0.288	0.150	0.18	41.1	31.7
1:6	0.274	0.148	0.27	24.6	34.4
1:3	0.262	0.171	0.34	32.5	17.5
1:1	0.194	0.183	...	...	7.9
3:1	0.180	0.251	...	...	10.0
9:1	0.151	0.245	...	...	9.8

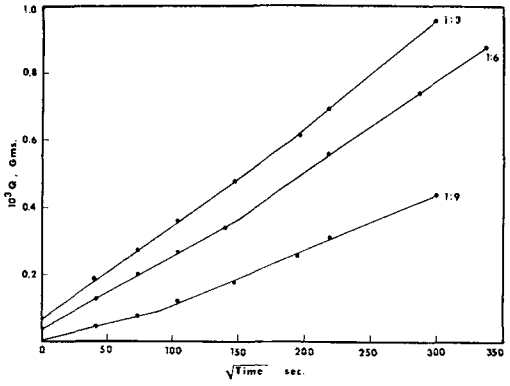

Fig. 5—Release of salicylic acid from matrices containing different ratios of salicylic acid to benzoic acid into saturated benzoic acid solutions containing 0.1 N HCl.

medium and are shown in Fig. 5. Since the benzoic acid embedded in the matrix is insoluble in this release medium, the conditions of region 2 are duplicated and permit its parameters to be determined using Eq. 1. The tortuosity values, τ_2, obtained in this manner are listed in column 4 in Table II and agree reasonably well with the values obtained using Eq. 2 and the release profiles in 0.1 *N* HCl.

The above procedure was not duplicated for benzoic acid release into saturated salicylic acid solution because of the difficulties in accurately determining small concentrations of benzoic acid in the presence of relatively high concentrations of salicylic acid. Furthermore, it was felt that the agreement between the τ_2 values obtained for the 1:9, 1:6, and 1:3 drug ratio systems sufficiently established the validity of this approach.

COMPLEMENTARY SURFACTANT AND VACUUM STUDIES

It was interesting to note, however, that the apparent tortuosity values for region 1 listed in Table I indicate an apparent tendency to increase with an increasing ratio of salicylic acid to benzoic acid. In contrast they were lower and relatively constant when obtained using the solution saturated matrices as compared to matrices containing the solid drug. The tortuosity values for region 2, on the other hand, indicate an apparent tendency to decrease rather than increase with an increasing ratio of salicylic acid to benzoic acid. Furthermore, they are relatively high for the lower ratios.

Although the potassium acid phthalate and caffeine studies indicated that there would not be any surfactant or vacuum effect when using this matrix system, the above observations prompted the study of their effect in the salicylic acid–benzoic acid system.

The surfactant effect was determined by obtaining release rates of matrices containing 20% salicylic acid in 0.1 *N* HCl solution containing 0.1% dioctyl sodium sulfosuccinate[1] (AOT) and the vacuum effect with 0.1 *N* HCl as the release medium (3, 4).

The results of these studies are presented in Fig. 6. It is noted that the release rates are increased with either treatment. Since the surfactant effect provided the faster release rates and therefore the more ideal system, the matrices containing both salicylic and benzoic acid were similarly studied.

The salicylic acid–benzoic acid mixture ratios of 1:6 and 3:1 were selected for these studies to provide examples of both drugs functioning as the faster moving drug boundary. Salicylic acid functions as

[1] Marketed as Aerosol OT by the American Cyanamid Co., Wayne, N. J.

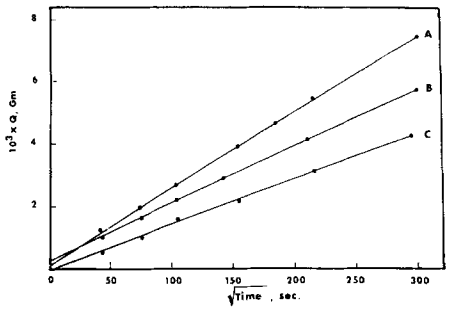

Fig. 6—Salicylic acid release experiments with matrices containing only 20% salicylic acid showing the influence of surfactant and of vacuum treatment. Key: A, *0.1* N *HCl/0.1% AOT as the release medium;* B, *0.1* N *HCl as the release medium and with vacuum treatment (4);* C, *0.1* N *HCl as the release medium.*

the faster moving boundary in the 1:6 mixture ratio but as the slower moving boundary in the 3:1 mixture ratio.

The results of these studies are reported in Table III. The presence of surfactant, as anticipated, increased all release rates. In addition the tortuosity values for region 1 were significantly decreased and remained constant for the four ratios of salicylic acid-to-benzoic acid mixtures indicating that its dependence on which drug was the slower moving boundary disappeared when surfactant was incorporated in the release medium.

The tortuosities of region 2 were also calculated using the surfactant release data for both mixture ratios by substituting the above tortuosities for region 1 in Eq. 2. They are listed in Table III and reveal that the tortuosities for region 2 are significantly decreased for the 1:6 and 3:1 mixture ratios from 34.4 and 10.0 to 6.1 and 8.4, respectively. Very significantly the tortuosity dependence on which drug is slower moving has again disappeared when surfactant is used as the two values are reasonably close.

As with previous studies the tortuosities of region 2 were confirmed by determining the release profile of a matrix containing the 1:6 salicylic acid–benzoic acid mixture into a saturated solution of benzoic acid containing 0.1 *N* HCl and 0.1% AOT. Using Eq. 1, the tortuosity was found to be 7.4 which agrees very well with the tortuosity previously calculated for region 2.

Previous work (3) has shown that the polyethylene matrix generally provides significantly higher drug release rates if surfactant is incorporated in the release medium. This was not true for the PVC matrices (4); the PVC, on the other hand, exhibited a more marked effect than the polyethylene matrix when pretreated by the vacuum technique.

Since the PVC–polyethylene matrix utilized in these studies demonstrated a markedly higher increase in the release rates when surfactant was included in the release medium as compared to the studies utilizing the vacuum treatment, it is strongly suggested that the polyethylene plastic is the controlling plastic in this matrix.

This indicates that in the compressional process the polyethylene plastic predominantly flows around the PVC plastic resulting in a discontinuous phase of PVC plastic surrounded by a continuous phase of polyethylene plastic. Comparison of the physical properties of both plastics indicates that the above is reasonable as polyethylene appears to be more plastic in nature and less rigid than the PVC plastic. In addition, the lower tortuosity values generally obtained in previous studies with the PVC matrices suggested that PVC existed as spheres in the compressed matrices, whereas polyethylene yielded higher tortuosity values in general indicating that it underwent considerable distortion with compression.

The results also indicate that salicylic acid is also predominantly surrounded by the polyethylene plastic under compression as indicated by the much higher tortuosity values obtained for region 2 when it participates as the faster moving drug in nonsurfactant solutions but exhibits a slightly lower tortuosity in the presence of surfactant. In addition, the tortuosity values of region 1 are higher when it participates as the slower moving drug in nonsurfactant solutions but not in the presence of surfactant. Finally the tortuosity of a matrix containing only salicylic acid showed a tortuosity of 12 in nonsurfactant solution but only 4.3 in the presence of surfactant.

Benzoic acid, on the other hand, exhibited consistently low tortuosities in all of the above situations, and this indicates that it is sufficiently distorted under compression to prevent it from being effectively coated in a similar manner.

APPENDIX

Derivation of Eq. 2—This derivation is based on the model previously described in Fig. 1. The equation for continuity at the boundary, s_1, is given by:

$$D_B \frac{\epsilon_1}{\tau_1}\left[\frac{dB}{dx}\right]_1 = D_B \frac{\epsilon_2}{\tau_2}\left[\frac{dB}{dx}\right]_2 \qquad \text{(Eq. 1}a\text{)}$$

Using the concentration gradients obtained from Fig. 1(c), the above yields:

TABLE III—CALCULATION OF THE TORTUOSITY OF REGION 1 AND REGION 2 USING SURFACTANT AS THE RELEASE MEDIUM

SA/BA Ratio	$10^5 Q_{SA}/t^{1/2}$ Solid Release	$10^5 Q_{BA}/t^{1/2}$ Solid Release	τ_{1SA}	τ_{1BA}	$10^5 Q_{SA}/t^{1/2}$[a]	τ_2 Exptl.	τ_2 Theoret.
Pure BA	...	3.90	...	3.2	...	...	...
1:6	0.70	3.10	...	4.0	0.27	7.4	6.1
3:1	2.07	1.16	4.5	...	0.50	...	8.4
Pure SA	2.45	...	4.3	...	...	...	...

[a] Slope of the release study when the salicylic acid–benzoic acid mixture tablet is released into saturated benzoic acid solution.

$$D_B \frac{\epsilon_1}{\tau_1} \frac{C_B'}{s_1} = D_B \frac{\epsilon_2}{\tau_2} \frac{(C_B^s - C_B')}{(s_2 - s_1)} \quad \text{(Eq. 2a)}$$

The concentration of drug B at the boundary s_1 can be obtained by rearrangement of Eq. 2a to give:

$$C_B' = \frac{\frac{\epsilon_2}{\tau_2} C_B^s}{\frac{\epsilon_2}{\tau_2} + \frac{\epsilon_1}{\tau_1} \frac{(s_2 - s_1)}{s_1}} \quad \text{(Eq. 3a)}$$

The equation of continuity dictates that the rate of release of drug B, G_B, must be equal to its rate of diffusion in region 1, *i.e.*,

$$G_B = \frac{dQ_B}{dt} = D_B \frac{\epsilon_1}{\tau_1} \frac{C_B'}{s_1} \quad \text{(Eq. 4a)}$$

Rearrangement of Eq. 4a yields a second relationship for the concentration for drug B at the boundary s_1:

$$C_B' = \frac{G_B \tau_1 s_1}{D_B \epsilon_1} \quad \text{(Eq. 5a)}$$

By equating Eqs. 3a and 5a one obtains:

$$G_B = \frac{dQ_B}{dt} = D_B \frac{\epsilon_2}{\tau_1 s_1} \left[\frac{\frac{\epsilon_2}{\tau_2} C_B^s}{\frac{\epsilon_2}{\tau_2} + \frac{\epsilon_1}{\tau_1} \frac{(s_2 - s_1)}{s_1}} \right] \quad \text{(Eq. 6a)}$$

Since s_1 and s_2 do not readily lend themselves to experimental determination, it would be advantageous to replace them with functions of measurable variables.

If the amount of dissolved drug remaining in the pores is assumed negligible, as compared to the amount of drug released (true for poorly soluble drugs), then:

$$Q_A = s_1 \cdot A_A$$

For the slower moving drug, Q_A is also given by Eq. 1, and its substitution in the above equation yields the following:

$$s_1 = \frac{K_A}{A_A} t^{1/2} \quad \text{(Eq. 7a)}$$

where K_A is given by the slope of the corresponding Q_A *versus* $t^{1/2}$ plot. It would be desirable if a similar relationship could be obtained for s_2 which would then permit the integration of Eq. 6a as all parameters could then be experimentally determined.

Equation 1 cannot be used to obtain a similar expression for s_2 as it involves both regions 1 and 2 as shown in Fig. 1. It can be shown, however, that Q_B is also proportional to $t^{1/2}$. If the ratio of $(s_2 - s_1)/s_1$ is invariant and Eq. 7a is used to substitute for s_1, Eq. 6a shows that G_B is inversely proportional to the square root of time, and therefore Q_B is directly proportional to the square root of time, *i.e.*,

$$Q_B = K_1 t^{1/2} \quad \text{(Eq. 8a)}$$

where K_1 is the integration constant.

The ratio of $(s_2 - s_1)/s_1$ can be shown to be invariant by the following analysis. The equation of continuity, Eq. 2a, dictates that:

$$\frac{C_B^s - C_B'}{s_2 - s_1} = K_2 \frac{C_B'}{s_1} \quad \text{(Eq. 9a)}$$

where K_2 is a function of the porosities and tortuosities of the two regions. s_1 describes the position of the slower moving drug boundary as it moves from the matrix surface and must increase with time. Since s_2 describes the position of the faster moving drug boundary, the value of $s_2 - s_1$ must also increase with time. If C_B' increases, $C_B^s - C_B'$ would correspondingly decrease. Equation 9a, however, would then require $s_2 - s_1$ to correspondingly decrease relative to s_1 and would require the impossible situation of the supposedly slower moving boundary to be the faster one. C_B', on the other hand, cannot decrease as $C_B^s - C_B'$ would correspondingly increase and would require a positive acceleration of the movement of s_2 relative to s_1; yet its maximum acceleration must occur at $t = 0$. Since C_B' must be invariant, rearrangement of Eq. 9a shows that the ratio of $(s_2 - s_1)/s_1$ is indeed invariant and that Eq. 8a is therefore valid.

Its substitution in $Q_B = s_2 A_B$ shows that s_2 is directly proportional to the square root of time and is equal to $K_B t^{1/2}/A_B$ where K_B is the slope of the corresponding Q_B *versus* $t^{1/2}$ plot.

Substituting for s_1 and s_2 into Eq. 6a yields the desired relationship:

$$\frac{dQ_B}{d(t^{1/2})} = 2\left[\frac{dQ_B}{dt}\right] t^{1/2} = \frac{2 D_B \epsilon_1}{\tau_1 \frac{K_A}{A_A}} \times \left[\frac{\frac{\epsilon_2}{\tau_2} C_B^s}{\frac{\epsilon_2}{\tau_2} + \frac{\epsilon_1}{\tau_1} \left(\frac{K_B/A_B - K_A/A_A}{K_A/A_A} \right)} \right] \quad \text{(Eq. 10a)}$$

REFERENCES

(1) Desai, S. J., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **54**, 1459(1965).
(2) Desai, S. J., Singh, P., Simonelli, A. P., and Higuchi, W. I., *ibid.*, **55**, 1224(1966).
(3) *Ibid.*, **55**, 1230(1966).
(4) *Ibid.*, **55**, 1235(1966).
(5) Higuchi, T., *ibid.*, **52**, 1145(1963).

Release Rates of Solid Drug Mixtures Dispersed in Inert Matrices II

Mutually Interacting Drug Mixtures

By PARVINDER SINGH*, SAURABH J. DESAI*, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

The equations developed to describe the release from a mixture of two noninteracting drugs was further extended to cover the case where the two drugs mutually interact with each other. All the parameters were independently determined and evaluated as before. Complexation studies were carried out and their effects on the solubilities and diffusion coefficients were also considered in the theoretical equations. The different regions existing in the tablet matrix were independently analyzed. The theoretical equations for the drug release rates were seen to predict the experimental data extremely well.

In a previous communication, the release behavior of a mixture of two noninteracting drugs dispersed in an inert, insoluble matrix has been discussed (1). As a continuation of the same work, it was decided to study the somewhat more complex system where the two drugs incorporated in the matrix mutually interact. The benzocaine–caffeine mixture provided an interesting system since solution interactions between the two drugs lead to a mutual increase of their solubilities.

The diffusion-controlled model that will be utilized is essentially similar to the one used in the salicylic acid–benzoic acid mixture study. In this system, however, solution interactions of the two drugs require equations of a different form.

The model is schematically shown in Fig. 1. Figure 1(a) shows that benzocaine and caffeine are transported both as free drugs and as complex species through region 1. In region 2, however, the slower moving drug, caffeine, is transported solely as complexes, as the entire region is saturated with respect to free caffeine. Figures 1(b) and (c) show the appropriate boundary conditions at s_1 and s_2 and the resulting concentration gradients which are fully discussed under the *Appendix*.

Mutual interactions were included in the model by utilizing the appropriate stability expressions for the three complexes, B·C, B·C$_2$, and B·C$_3$. An adequate discussion of the release parameters, tortuosity, τ, and porosity, ϵ, was given in the previous paper (1) and the same terminology is applicable here.

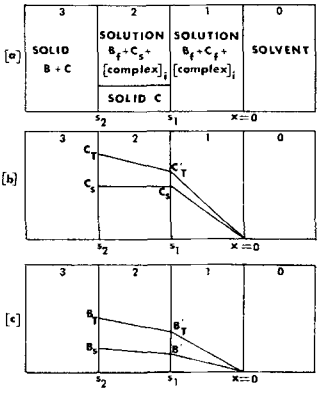

Fig. 1—The physical model that describes the simultaneous release of a mixture of two interacting drugs from an inert matrix. Key: (a) *conditions existing at finite time,* t; (b) *concentration gradients for the drug having the slower moving boundary;* (c) *concentration gradients for the drug having the faster moving boundary.*

The resulting equations are:

$$Q_B = A_B \cdot \gamma t^{1/2} \qquad \text{(Eq. 1)}$$

and

$$Q_C = \left[A_C \cdot \beta + A_B(\gamma - \beta)\left(\frac{\delta}{\alpha}\right)\right] t^{1/2} \qquad \text{(Eq. 2)}$$

where

$$\gamma = \frac{2\epsilon_1\epsilon_2 B_s \alpha}{A_B(\tau_2\epsilon_1\gamma - \tau_2\epsilon_1\beta + \tau_1\epsilon_2\beta)}$$

$$\beta = \left[\frac{2\epsilon_1 D_C C_s \alpha}{\tau_1(A_C\alpha - A_B\delta)}\right]^{1/2}$$

$$\alpha = D_B + D_{BC}K_1C_s + D_{BC_2}K_1K_2C_s^2 + D_{BC_3}K_1K_2K_3C_s^3$$

$$\delta = D_{BC}K_1C_s + 2D_{BC_2}K_1K_2C_s^2 + 3D_{BC_3}K_1K_2K_3C_s^3$$

A_i = concentration of drug i in the matrix,

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication July 17, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This research project was supported in part by grants from the Ciba Pharmaceutical Co., Summit, N. J., and Parke-Davis Co., Detroit, Mich.
* Present address: Ranbaxy Laboratories Ltd., Okhla, New Delhi, India.

Q_i = total amount of drug i released per unit area,
D_i = diffusion coefficient of drug i,
K_i = stability constant of complex i,
C_s = solubility of uncomplexed caffeine,
B_s = solubility of uncomplexed benzocaine,
ϵ_j = porosity of region j,
τ_j = tortuosity of region j.

It should be emphasized that these equations are restricted to a system in which benzocaine exhibits the faster moving boundary. In this system the concentrations of all species are directly proportional to the concentration of free benzocaine in solution. In the reverse situation, however, the concentrations of all species must be expressed in terms of a polynomial involving the free caffeine solution concentration. This requires the solution of a cubic equation and results in a very lengthy and much more complicated set of equations.

EXPERIMENTAL

The matrix system used in these studies was a mixture of polyvinylchloride (PVC) and polyethylene in a 7:3 weight-to-weight ratio. This matrix was chosen for the study of drug release from mixtures of two drugs for reasons described previously (1).

A direct assay in the ultraviolet region for the simultaneous release of both drugs in the presence of each other was not utilized because of the strong mutual absorption at their respective maximum absorption wavelengths. Benzocaine was independently assayed using a colorimetric assay procedure involving a diazotization reaction (2), and recording its absorption at 550 mμ. The total absorption, however, due to both caffeine and benzocaine was determined at 273 mμ. The contribution from benzocaine adsorption at 273 mμ was then accounted for, allowing the determination of the caffeine concentration.

The diffusion coefficients of the pure drugs were determined experimentally by the method described earlier (3), modified by the use of a Gelman[1] Versapor filter in place of a sintered-glass disk.

Two kinds of interaction studies were carried out. In the first case, an excess amount of benzocaine (0.4 Gm.) was weighed into a series of Teflon-stoppered culture tubes and known but varying amounts of caffeine were added to them. Then, 20 ml. of distilled water was added to each tube. The samples were equilibrated in a constant-temperature tumbling water bath for 24 hr. They were then filtered through a Millipore[2] filter (0.45-μ pore size) and the samples prepared for spectrophotometric assay. In the other study, the reverse experiment was carried out where an excess amount of caffeine (1.6 Gm.) was weighed into each culture tube and known but varying amounts of benzocaine added. The samples were then equilibrated, filtered, and assayed.

RESULTS AND DISCUSSON

Interaction Studies—The results of the complexation-solubility experiments are presented in Figs. 2 and 3. As can be seen, when caffeine was added to excess solid benzocaine the total solubility of benzocaine, B_T, increased more rapidly than linearly with added caffeine (Fig. 2). In those experiments where benzocaine was added to excess solid caffeine, the total solubility of caffeine, C_T, was found to be essentially linear with total benzocaine added.

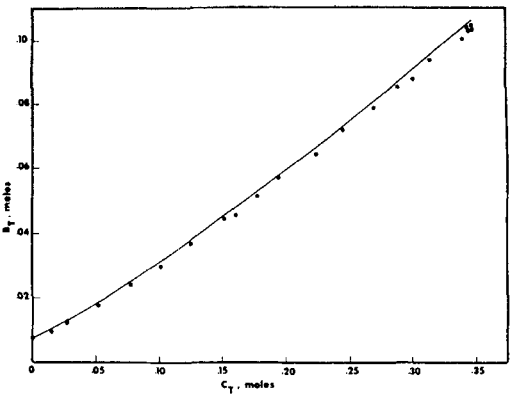

Fig. 2—Interaction study between benzocaine and caffeine using an excess of solid benzocaine and varying amounts of caffeine. The smooth curve represents the theoretical curve based on Eqs. 6 and 7 and the points represent experimental data. (See text for discussion.)

These data indicate that (*a*) for the Fig. 2 experiments, while only the 1:1 complex is important at low caffeine concentrations, there are other complexes contributing significantly to the B_T at the higher caffeine concentrations, and (*b*) in all of the complexes benzocaine probably occurs singly, *i.e.*, BC, BC_2, BC_3, BC_4, *etc.*, as suggested by the linear relationship (Fig. 3). In addition, the average stoichiometry of the complexes at the invariant point (saturation point for both phases) was found to be 2.17 with both sets of experiments. This showed that at least three complexes, the 1:1, the 1:2, and the 1:3 benzocaine–caffeine should be considered in a thermodynamic treatment of all of the data.

Thus, it was assumed that the following solution equilibria were present:[3]

$$B + C \overset{K_1}{\rightleftarrows} B\cdot C \qquad \text{(Eq. 3)}$$

$$B\cdot C + C \overset{K_2}{\rightleftarrows} B\cdot C_2 \qquad \text{(Eq. 4)}$$

$$B\cdot C_2 + C \overset{K_3}{\rightleftarrows} B\cdot C_3 \qquad \text{(Eq. 5)}$$

The stability constants were then evaluated in the following manner.

For the saturated-benzocaine experiment (Fig. 2), the total concentration of caffeine and benzocaine can be given by:

$$C_T = C_{\text{free}} + C_{\text{complexes}}$$

$$= C_i + K_1 B_s C_i + 2K_1K_2 B_s C_i^2 + 3K_1K_2K_3 B_s C_i^3 \qquad \text{(Eq. 6)}$$

and

[1] Gelman Instrument Co., Ann Arbor, Mich.
[2] Millipore Filter Corp., Bedford, Mass.

[3] The possibility is ignored that caffeine may self-associate at high caffeine concentrations as is suggested by the data of Guttman and Higuchi (4).

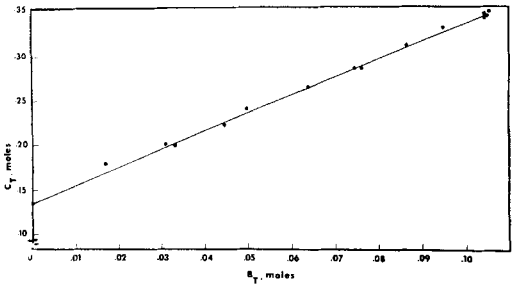

Fig. 3—Interaction study between benzocaine and caffeine using an excess of solid caffeine and varying amounts of benzocaine. The smooth curve represents the theoretical curve based on Eqs. 8 and 9 and the points represent experimental data. (See text for discussion.)

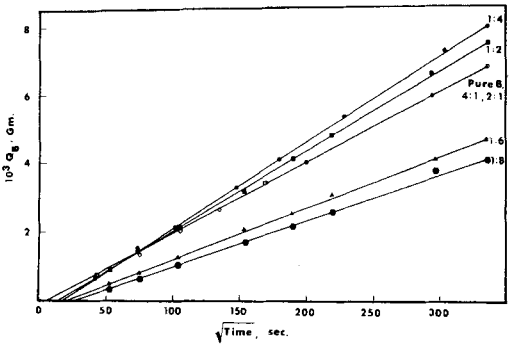

Fig. 4—Release af benzocaine from matrices containing different ratios of benzocaine to caffeine to water. The total amount of drug in all cases was 20% w/w, and the numbers indicate benzocaine-to-caffeine ratios.

$$B_T = B_{\text{free}} + B_{\text{complexes}}$$

$$= B_s + K_1B_sC_i + K_1K_2B_sC_i^2 + K_1K_2K_3B_sC_i^3 \qquad \text{(Eq. 7)}$$

For the saturated-caffeine experiments (Fig. 3) the corresponding expressions will be:

$$C_T = C_s + K_1B_iC_s + 2K_1K_2B_iC_s^2 + 3K_1K_2K_3B_iC_s^3 \qquad \text{(Eq. 8)}$$

and

$$B_T = B_i + K_1B_iC_s + K_1K_2B_iC_s^2 + K_1K_2K_3B_iC_s^3 \qquad \text{(Eq. 9)}$$

For the saturated-caffeine experiments (Fig. 3) where a straight line is obtained we have:

$$C_T = \text{slope} \times (B_T) + \text{intercept}$$

Then, using Eq. 8 and Eq. 9, we obtain:

$$K_2K_3 = \frac{2 + K_1C_s}{K_1C_s^3} \qquad \text{(Eq. 10)}$$

Now K_1 may be determined from the initial slope of the Fig. 2 data. Then K_2 and K_3 can be solved by using Eq. 6 and Eq. 7. The calculated values for K_1, K_2, and K_3 were found to be 34.4, 3.2, and 24.1, respectively.

Using these calculated values for K_1, K_2, and K_3, both curves were reproduced theoretically and these results are given in Figs. 2 and 3 as the smooth curves. It can be seen that these calculated values for the stability constants give an extremely close fit to the experimental data and thereby confirm our hypothesis that, in the benzocaine–caffeine system, a consideration of these three complexes may adequately describe the system.

It should be pointed out that only this set of K_1, K_2, and K_3 values gave a good fit to the data. Thus, ignoring the terms involving K_3 did not give a satisfactory fit of the theory to the data.

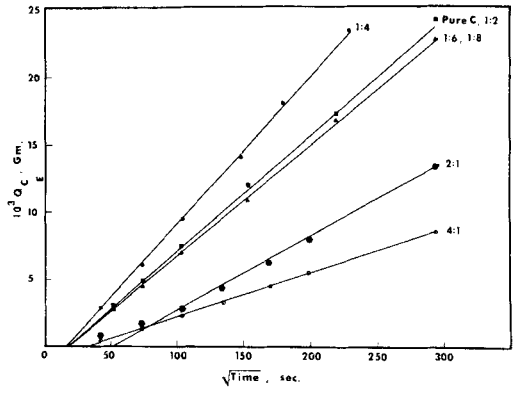

Fig. 5—Data showing caffeine release in the same experiments as in Fig. 4.

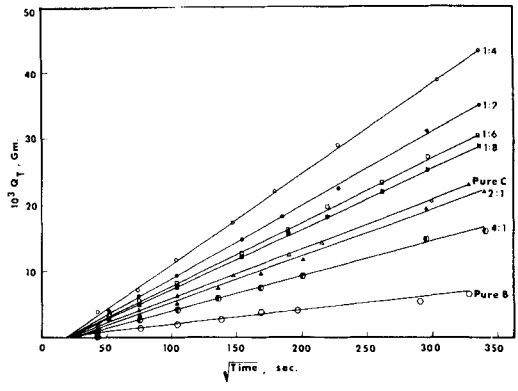

Fig. 6—The sum of the benzocaine and caffeine release in the same experiments as in Figs. 4 and 5.

Release Studies—The release profiles of benzocaine and caffeine as they are simultaneously released in water are presented in Figs. 4 and 5 for various weight-weight ratios of the two drugs in the tablet. Figure 6 shows the sum of benzocaine and caffeine release as a function of the square root of time. It is seen that the square root of time dependence, as predicted by the T. Higuchi equation (5), is observed in all cases. The data also show that for the total release as well as for the release of the individual drugs the 1:4 mixture ratio yields the highest release rate.

This 1:4 critical mixture ratio is in accordance with that predicted by the following relationship which is analogous to Eq. 3 of the previous com-

munication (1), and describes the relative rates of boundary movement for benzocaine and caffeine:

$$\frac{\text{boundary movement for caffeine}}{\text{boundary movement for benzocaine}} = \frac{Q_C/A_C}{Q_B/A_B} = \left[\frac{D_{C(eff.)}C_T A_B}{D_{B(eff.)}B_T A_C}\right]^{1/2}$$

assuming all other factors constant for both systems. Here, $D_{B(eff.)}$ and $D_{C(eff.)}$ are the effective diffusion coefficients for the total transport of benzocaine and caffeine, respectively, when diffusion of both solutes takes place from the solution saturated with respect to both drugs. Thus, when the two boundaries move at the same rate, the lefthand side of the above equation will be unity, and we have:

$$\frac{A_C}{A_B} = \frac{D_{C(eff.)}C_T}{D_{B(eff.)}B_T} = \frac{(6.94 \times 10^{-6})}{(7.08 \times 10^{-6})} \cdot \frac{(6.71 \times 10^{-2})}{(1.73 \times 10^{-2})} = 3.8$$

which is in close agreement with the experimental 1:4 ratio.

The values for the effective diffusion coefficients $D_{B(eff.)}$ and $D_{C(eff.)}$ were estimated from the following equations:

$$D_{B(eff.)} = \frac{D_B(B_s) + D_{BC}(K_1B_sC_s) + D_{BC_2}(K_1K_2B_sC_s^2) + D_{BC_3}(K_1K_2K_3B_sC_s^3)}{B_s + K_1B_sC_s + K_1K_2B_sC_s^2 + K_1K_2K_3B_sC_s^3}$$

and

$$D_{C(eff.)} = \frac{D_C(C_s) + D_{BC}(K_1B_sC_s) + 2D_{BC_2}(K_1K_2B_sC_s^2) + 3D_{BC_3}(K_1K_2K_3B_sC_s^3)}{C_s + K_1B_sC_s + 2K_1K_2B_sC_s^2 + 3K_1K_2K_3B_sC_s^3}$$

The diffusion coefficients for benzocaine and caffeine and the stability constants were determined experimentally, and the diffusion coefficients for the complexes were estimated from the Stokes-Einstein relation (6). That the Stokes-Einstein estimations were reasonable was verified by an experimental determination of the effective diffusion coefficient for benzocaine which was done by diffusing a solution saturated with both caffeine and benzocaine into a solution saturated only with caffeine. (See Table I.)

Analysis of Region 1 Parameters—To test the proposed model, both region 1 and region 2 need to be independently analyzed. The tortuosity value of region 1 cannot be obtained from the experiments as was done in the salicylic acid–benzoic acid problem using the T. Higuchi equation (5) since the total solution concentrations of benzocaine and caffeine at the boundary s_1 are unknowns in this problem. For this reason, a different approach was used. Equation 8*a* (from the *Appendix*) is:

$$G_C = \frac{\epsilon_1}{\tau_1 s_1} D_C \cdot C_s + G_B \frac{\delta}{\alpha} \qquad \text{(Eq. 8a)}$$

s_1 can be substituted for by Eqs. 21*a* and 22*a*, G by $dQ/2t^{1/2}d(t^{1/2})$, and after rearrangement, τ_1 can be expressed as:

$$\tau_1 = \frac{2\alpha\epsilon_1 D_C C_s}{\beta[(Q_C/t^{1/2})\alpha - (Q_B/t^{1/2})\delta]} \qquad \text{(Eq. 11)}$$

Since this model only describes the case where caffeine has the slower moving boundary, the tortuosity values that can be obtained by this method are restricted to the data for benzocaine–caffeine ratios of 1:4, 1:6, and 1:8. These are presented in Table II.

Tortuosity values for region 1 have also been determined independently by liquid leaching experiments (7) and are listed in Table II. The agreement between the two sets of tortuosity values obtained by these separate experiments is generally good. These findings alone lend strong support to the validity of the proposed model.

Analysis of Region 2 Parameters—In order to evaluate the tortuosity factor in region 2, benzocaine release data (see Fig. 7) were obtained with the tablets in media saturated with caffeine, a procedure employed previously (1). The data then may be evaluated directly by means of the following equation:

$$Q_B = \left[\frac{2\epsilon_2 A_B}{\tau_2}(D_B B_s + D_{BC}K_1C_sB_s + D_{BC_2}K_1K_2B_sC_s^2 + D_{BC}K_1K_2K_3B_sC_s^3)t\right]^{1/2} \qquad \text{(Eq. 12)}$$

which was derived using Eq. 9*a* with the boundary conditions that $s_1 = 0$ for all t, and B = 0 and C = C_s at $x = 0$ for all t. The ϵ_2 entering in Eq. 12 is given by:

$$\epsilon_2 = \frac{A_B}{\rho_B} + A_B\frac{G_C'}{G_B\rho_C} + \epsilon_{air} \qquad \text{(Eq. 13)}$$

where ρ_B and ρ_C are the densities of benzocaine and caffeine and ϵ_{air} is the porosity contribution due to air. The second term of Eq. 13 represents the porosity contribution due to the caffeine loss from the tablet resulting from the diffusion of the complexes through the matrix and out of the tablet (see Eq. 10*a* under *Appendix*).

Table III gives the calculated ϵ's and τ_2 values obtained from the experiments (Fig. 7) in saturated caffeine solutions using Eqs. 12 and 13. The stability constants determined in the solubility studies and the diffusion coefficients given in Table I were used.

Test of the Model Using All Parameters—Having obtained all of the matrix parameters (ϵ_1, ϵ_2, τ_1, τ_2) and the interaction parameters (K_1, K_2, K_3, C_T, B_T, and the diffusion coefficients), it was then possible to carry out a composite test of the model proposed

Table I—Diffusion Coefficients of the Different Benzocaine and Caffeine Species Involved in This Study

Drug Species	Diff. Coeff. × 10^6 cm.²/sec.
Benzocaine	9.86[a]
Caffeine	7.53[a]
[B·C]	7.84[b]
[B·C_2]	6.85[b]
[B·C_3]	6.20[b]
Total benzocaine species $D_{B(eff.)}$	= 5.94[a]
$D_{BB(eff.)}$	= 7.08[b]
Total caffeine species $D_{C(eff.)}$	= 6.94[b]

[a] Experimental values. [b] Calculated values, based on the Stokes-Einstein relation.

TABLE II—PARAMETERS INVOLVED IN THE DETERMINATION OF TORTUOSITY OF THE OUTER REGION 1

B:C Ratio	ϵ_1[a]	$10^5 Q_B/t^{1/2}$ Solid Release	$10^5 Q_C/t^{1/2}$ Solid Release	$10^5 Q/t^{1/2}$ Liquid Release[b]	τ_1 from Solid Release (Eq. 11)	τ_1[b] from Liquid Release
Pure B	0.313	1.85	...	1.52	3.2	2.0
4:1	0.312	2.08	3.50	1.38	...	2.2
2:1	0.311	2.20	5.50	1.39	...	2.1
1:2	0.299	2.30	8.70	1.26	...	3.0
1:4	0.295	2.53	11.0	1.15	2.5	2.8
1:6	0.283	1.49	8.0	1.13	3.3	2.6
1:8	0.278	1.34	7.8	1.04	3.8	3.0
Pure C	0.289	...	8.25	1.04	3.0	3.2

$\epsilon_1 = \epsilon_{total} = \epsilon_{benz.} + \epsilon_{caff.} + \epsilon_{air}$. [b] For these experiments, $C_0 = 2.6\%$ caffeine solution (see *Reference 7*).

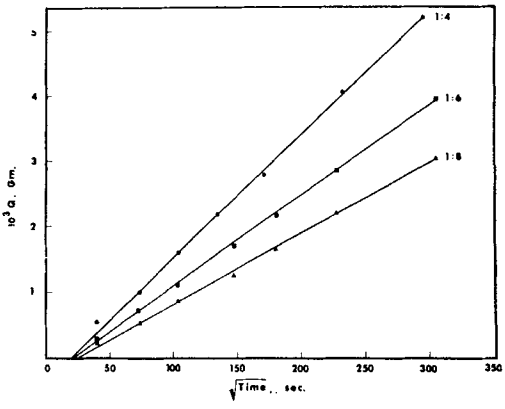

Fig. 7—Release of benzocaine from matrices containing different ratios of benzocaine to caffeine into saturated caffeine solutions.

tion of these equations is based on the model presented in Fig. 1 for the case when benzocaine has the faster moving solid–liquid drug boundary. Since the benzocaine–caffeine system forms more than one complex at stoichiometrically different ratios, the reverse situation of benzocaine having the slower boundary would require different equations.

It has been shown that benzocaine and caffeine interact to form three soluble complexes as represented below:

$$B + C \rightleftharpoons B\cdot C$$
$$B\cdot C + C \rightleftharpoons B\cdot C_2$$
$$B\cdot C_2 + C \rightleftharpoons B\cdot C_3$$

The corresponding stability constants are expressed as:

$$K_1 = \frac{(B\cdot C)}{(B)\,(C)}$$

TABLE III—PARAMETERS INVOLVED IN THE DETERMINATION OF TORTUOSITY OF THE INNER REGION 2

B:C Ratio	$\epsilon_{benz.}$	$\epsilon_{caff.}$[a]	ϵ_{air}	ϵ_2 (Eq. 13)	$10^5 Q/t^{1/2}$	τ_2
1:4	0.037	0.061	0.136	0.234	1.07	7.7
1:6	0.027	0.044	0.121	0.192	1.40	6.6
1:8	0.021	0.035	0.118	0.174	1.88	6.2

[a] $\epsilon_{caff.}$ value for the second term in Eq. 13.

TABLE IV—COMPARISON OF THE EXPERIMENTAL AND THEORETICAL RELEASE RATES

B:C Ratio	Exptl. $10^5 Q/t^{1/2}$ B	Exptl. $10^5 Q/t^{1/2}$ C	Theoret. $10^5 Q/t^{1/2}$ B	Theoret. $10^5 Q/t^{1/2}$ C
1:4	2.53	11.0	2.83	10.8
1:6	1.49	8.00	2.00	10.37
1:8	1.34	7.80	1.45	9.34

in this paper. This was done by substituting the values for all of these parameters into Eqs. 1 and 2, calculating the theoretical rates of drug release for both benzocaine and caffeine, and then comparing these to the experimental values.

Table IV shows the close agreement of experiment with theory for both drugs and for the three mixture ratios.

APPENDIX

Derivation of Theoretical Equations When Benzocaine is the Faster Moving Boundary—The derivation of these equations is based on the model presented in Fig. 1 for the case when benzocaine has the faster moving solid–liquid drug boundary. Since the benzocaine–caffeine system forms more than one complex at stoichiometrically different ratios, the reverse situation of benzocaine having the slower boundary would require different equations.

It has been shown that benzocaine and caffeine interact to form three soluble complexes as represented below:

$$B + C \rightleftharpoons B\cdot C$$
$$B\cdot C + C \rightleftharpoons B\cdot C_2$$
$$B\cdot C_2 + C \rightleftharpoons B\cdot C_3$$

The corresponding stability constants are expressed as:

$$K_1 = \frac{(B\cdot C)}{(B)\,(C)}$$

$$K_2 = \frac{(B\cdot C_2)}{(B\cdot C)\;(C)}$$

$$K_3 = \frac{(B\cdot C_3)}{(B\cdot C_2)\,(C)}$$

The boundary conditions for the two drugs at s_1 and s_2 can be given in terms of K_1, K_2, K_3, and uncomplexed drug concentrations.

At the boundary s_1, the concentration expressions are:

$$(C) = C_s$$
$$(B) = B'$$
$$(B\cdot C) = K_1 B' C_s$$
$$(B\cdot C_2) = K_1 K_2 B' C_s^2$$
$$(B\cdot C_3) = K_1 K_2 K_3 B' C_s^3$$

and at the boundary s_2, they are:

$$(C) = C_s$$
$$(B) = B_s$$

$$(B\cdot C) = K_1 B_s C_s$$
$$(B\cdot C_2) = K_1 K_2 B_s C_s^2$$
$$(B\cdot C_3) = K_1 K_2 K_3 B_s C_s^3$$

where B' is the concentration of benzocaine at the boundary s_1. The other parameters have been defined earlier.

The equations of continuity for region 1 yield the following relationships for the net movement of all species:

$$G_B = \frac{\epsilon_1}{\tau_1}\left[D_B\frac{d(B)}{dx} + D_{BC}\frac{d(BC)}{dx} + D_{BC_2}\frac{d(BC_2)}{dx} + D_{BC_3}\frac{d(BC_3)}{dx}\right] \quad \text{(Eq. 1a)}$$

and

$$G_C = \frac{\epsilon_1}{\tau_1}\left[D_C\frac{d(C)}{dx} + D_{BC}\frac{d(BC)}{dx} + 2D_{BC_2}\frac{d(BC_2)}{dx} + 3D_{BC_3}\frac{d(BC_3)}{dx}\right] \quad \text{(Eq. 2a)}$$

where G_i is the rate of movement of species i, D_i is the diffusion coefficient of species i, and di/dx is the concentration gradient for species i.

Integration of Eq. 1a from $x = 0$ to $x = s_1$ yields:

$$G_B\cdot s_1 = \frac{\epsilon_1}{\tau_1}B'\alpha \quad \text{(Eq. 3a)}$$

where

$$\alpha = (D_B + D_{BC}K_1C_s + D_{BC_2}K_1K_2C_s^2 + D_{BC_3}K_1K_2K_3C_s^3) \quad \text{(Eq. 4a)}$$

Rearrangement of Eq. 3a gives:

$$B' = \frac{G_B s_1 \tau_1}{\epsilon_1 \alpha} \quad \text{(Eq. 5a)}$$

Integration of Eq. 2a similarly yields:

$$G_C s_1 = \frac{\epsilon_1}{\tau_1}(D_C\cdot C_s) + \frac{\epsilon_1}{\tau_1}B'\delta \quad \text{(Eq. 6a)}$$

where

$$\delta = (D_{BC}K_1C_s + 2D_{BC_2}K_1K_2C_s^2 + 3D_{BC_3}K_1K_2K_3C_s^3) \quad \text{(Eq. 7a)}$$

On substituting for B' from Eq. 5a, Eq. 6a then becomes:

$$G_C = \frac{\epsilon_1}{\tau_1\cdot s_1}(D_C\cdot C_s) + G_B\frac{\delta}{\alpha} \quad \text{(Eq. 8a)}$$

The equations of continuity for region 2, on the other hand, are:

$$G_B = \frac{\epsilon_2}{\tau_2}\left[D_B\frac{d(B)}{dx} + D_{BC}\frac{d(BC)}{dx} + D_{BC_2}\frac{d(BC_2)}{dx} + D_{BC_3}\frac{d(BC_3)}{dx}\right] \quad \text{(Eq. 9a)}$$

and

$$G_C' = \frac{\epsilon_2}{\tau_1}\left[D_C\frac{d(C)}{dx} + D_{BC}\frac{d(BC)}{dx} + 2D_{BC_2}\frac{d(BC_2)}{dx} + 3D_{BC_3}\frac{d(BC_3)}{dx}\right] \quad \text{(Eq. 10a)}$$

It is to be noted that G_C' is equal to the rate of transport of total caffeine from region 2. Since region 2 contains a saturated solution with respect to free caffeine, this rate must only involve the movement of caffeine complexes and therefore is different from G_C which includes the diffusion of free caffeine as well as the complexes.

On integrating Eq. 9a from $x = s_1$ to $x = s_2$ one obtains:

$$G_B(s_2 - s_1) = \frac{\epsilon_2}{\tau_2}(B_s - B')\alpha \quad \text{(Eq. 11a)}$$

again, where α is given by Eq. 4a. Equation 5a is substituted for B', yielding:

$$G_B = \frac{\epsilon_2\alpha}{\tau_2(s_2 - s_1)}\left[B_s - \frac{G_B s_1 \tau_1}{\propto \epsilon_1}\right] \quad \text{(Eq. 12a)}$$

Integration of Eq. 10a, on the other hand, gives:

$$G_C' = \frac{\epsilon_2\cdot\delta}{\tau_2(s_2 - s_1)}(B_s - B') \quad \text{(Eq. 13a)}$$

where δ is given by Eq. 7a.

The above equations cannot be solved as there are more variables than equations. Another set of equations, however, can be obtained by considering the amount of each drug released, determined on the basis of the amount incorporated in the tablet matrix and both drug boundary movements. In this way equivalent expressions for G_B and G_C can be obtained. Examination of Fig. 1 shows that the total amount of drug B released, Q_B, is a function of the distance its boundary has moved out, and therefore,

$$Q_B = s_2\cdot A_B \quad \text{(Eq. 14a)}$$

neglecting the amount of drug remaining as solution in the matrix. This condition is met when $C_s <<< A$. Then, differentiating Eq. 14a,

$$G_B = \frac{dQ_B}{dt} = A_B\frac{ds_2}{dt} \quad \text{(Eq. 15a)}$$

The total amount of drug C released, Q_C, on the other hand, is a function of the distance both boundaries have moved and can be expressed as:

$$Q_C = s_1A_C + (s_2 - s_1)A_B\frac{G_C'}{G_B} \quad \text{(Eq. 16a)}$$

where s_1A_C describes the total amount of caffeine released from region 1, and the second term describes the total amount released from region 2. Since caffeine is released solely in the form of complexes from region 2, its amount will be directly proportional to the amount of benzocaine released from region 2 $[(s_2 - s_1)A_B]$. The proportionality constant obviously is given by the ratio of the caffeine and benzocaine rates of release from that region.

The ratio of Eqs. 11a and 13a yield:

$$\frac{G'_C}{G_B} = \frac{\delta}{\alpha}$$

Substitution of the above in Eq. 16a and differentiating provides another expression for G_C:

$$G_C = A_C\frac{ds_1}{dt} + A_B\frac{\delta}{\alpha}\left[\frac{ds_2}{dt} - \frac{ds_1}{dt}\right] \quad \text{(Eq. 17a)}$$

Equating the expressions for G_C, given by Eqs. 8a and 17a:

$$A_C\frac{ds_1}{dt} + A_B\frac{\delta}{\alpha}\left[\frac{ds_2}{dt} - \frac{ds_1}{dt}\right] = \frac{\epsilon_1}{\tau_1 s_1}(D_C \cdot C_s) + G_B\frac{\delta}{\alpha} \quad \text{(Eq. 18a)}$$

Substituting for G_B from Eq. 15a gives:

$$A_C\frac{ds_1}{dt} - A_B\frac{\delta}{\alpha}\frac{ds_1}{dt} = \frac{\epsilon_1 D_C C_s}{\tau_1 s_1} \quad \text{(Eq. 19a)}$$

or

$$s_1\frac{ds_1}{dt} = \frac{\epsilon_1 \cdot D_C \cdot C_s}{\tau_1(A_C - \frac{\delta}{\alpha}A_B)} \quad \text{(Eq. 20a)}$$

Equation 20a can be integrated to yield:

$$s_1 = \beta t^{1/2} \quad \text{(Eq. 21a)}$$

where

$$\beta = \left[\frac{2\epsilon_1 D_C C_s \alpha}{\tau_1(A_C\alpha - A_B\delta)}\right]^{1/2} \quad \text{(Eq. 22a)}$$

As expected, the movement of boundary s_1 has a square root of time dependence.

Similarly, Eqs. 12a and 15a for G_B can be equated. After rearranging, the following expression is obtained:

$$G_B = A_B\frac{ds_2}{dt} = \frac{\epsilon_1\epsilon_2 B_s \alpha}{\tau_2\epsilon_1(s_2 - s_1) + \epsilon_2 s_1 \tau_1}$$

and

$$\frac{ds_2}{dt} = \frac{\epsilon_1\epsilon_2 B_s \alpha}{A_B s_1\left[\tau_2\epsilon_1\frac{(s_2 - s_1)}{s_1} + \epsilon_2\tau_1\right]} \quad \text{(Eq. 23a)}$$

The ratio of $(s_2 - s_1)/s_1$ has been shown to be invariant (1). Equation 21a can be substituted for s_1. Integration of Eq. 23a then shows that:

$$s_2 = \gamma\, t^{1/2} \quad \text{(Eq. 24a)}$$

$$\gamma = \frac{2\epsilon_1\epsilon_2 B_s \alpha}{A_B(\tau_2\epsilon_1\gamma - \tau_2\epsilon_1\beta + \tau_1\epsilon_2\beta)} \quad \text{(Eq. 25b)}$$

Equations When Both Boundaries Move at the Same Rate—It can be seen from Eqs. 3a and 6a that when both boundaries recede at the same rate, we may write:

$$G_B \cdot s_1 = \frac{\epsilon_1}{\tau_1}B_s\alpha \quad \text{(Eq. 26a)}$$

and

$$G_C \cdot s_1 = \frac{\epsilon_1}{\tau_1}D_C \cdot C_s + \frac{\epsilon_1}{\tau_1}B_s\delta \quad \text{(Eq. 27a)}$$

As, for this case, $s_1 = s_2$:

$$G_B = A_B\frac{ds_1}{dt} \quad \text{(Eq. 28a)}$$

and

$$G_C = A_C\frac{ds_1}{dt} \quad \text{(Eq. 29a)}$$

Then these equations may be solved as before to give:

$$Q_B = \left[\frac{2\epsilon_1 A_B}{\tau_1}(D_B B_s + D_{BC}K_1 C_s B_s + D_{BC_2}K_1K_2B_sC_s^2 + D_{BC_3}K_1K_2K_3B_sC_s^3)t\right]^{1/2} \quad \text{(Eq. 30a)}$$

and

$$Q_C = \left[\frac{2\epsilon_1}{\tau_1}A_C(D_C C_s + D_{BC}K_1C_sB_s + 2D_{BC_2}K_1K_2B_sC_s^2 + 3D_{BC_3}K_1K_2K_3B_sC_s^3)t\right]^{1/2} \quad \text{(Eq. 31a)}$$

These equations may also be written as:

$$Q_B = \left[\frac{2\epsilon_1 A_B D_{B(\text{eff.})} B_T t}{\tau_1}\right]^{1/2} \quad \text{(Eq. 32a)}$$

and

$$Q_C = \left[\frac{2\epsilon_1 A_C D_{C(\text{eff.})} C_T t}{\tau_1}\right]^{1/2} \quad \text{(Eq. 33a)}$$

where $D_{B(\text{eff.})}$ and $D_{C(\text{eff.})}$ are the effective diffusion coefficients for the total transport rates of benzocaine and caffeine, respectively.

Equations When Caffeine Is the Faster Moving Boundary—As was done for the first case and considering the continuity relations at the boundaries we obtain the following equations that are analogous to Eqs. 3a, 6a, 11a, and 13a:

For region 1,

$$G_B s_1 = \frac{\epsilon_1}{\tau_1}[D_B B_s + D_{BC}K_1B_sC' + D_{BC_2}K_1K_2B_s(C')^2 + D_{BC_3}K_1K_2K_3B_s(C')^3] \quad \text{(Eq. 34a)}$$

and

$$G_C s_1 = \frac{\epsilon_1}{\tau_1}[D_C C_s + D_{BC}K_1B_sC' + 2D_{BC_2}K_1K_2B_s(C')^2 + 3D_{BC_3}K_1K_2K_3B_s(C')^3] \quad \text{(Eq. 35a)}$$

and, for region 2,

$$G_B'(s_2 - s_1) = \frac{\epsilon_2}{\tau_2}D_{BC}K_1B_s(C_s - C') + D_{BC_2}K_1K_2B_s[C_s^2 - (C')^2] + D_{BC_3}K_1K_2K_3B_s[C_s^3 - (C')^3] \quad \text{(Eq. 36a)}$$

and

$$G_C(s_2 - s_1) = \frac{\epsilon_2}{\tau_2}D_C(C_s - C') + D_{BC}K_1B_s(C_s - C') + 2D_{BC_2}K_1K_2B_s[C_s^2 - (C')^2] + 3D_{BC_3}K_1K_2K_3B_s[C_s^3 - (C')^3] \quad \text{(Eq. 37a)}$$

Here C' is the caffeine concentration and s_1 and G_B' represent the rate of benzocaine movement in region 2. All other symbols are the same as before.

In addition to these equations we have the relations,

$$G_C = \frac{dQ_C}{dt} = A_C\frac{ds_2}{dt} \quad \text{(Eq. 38a)}$$

and

$$G_B = \frac{dQ_B}{dt} = A_B\frac{ds_1}{dt} + A_C\frac{G_B'}{G_C}\left[\frac{ds_2}{dt} - \frac{ds_1}{dt}\right] \quad \text{(Eq. 39a)}$$

As in the first case we now have six equations in six unknowns—G_B', G_B, G_C, C', s_1, and s_2. While in principle the problem can therefore be solved, one finds that the algebra becomes extremely complicated due to the occurrence of an equation cubic in C'.

REFERENCES

(1) Singh, P., Desai, S. J., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **56**, 1542(1967).

(2) Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, **128**, 537(1939).

(3) Desai, S. J., Singh, P., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **55**, 1224(1966).

(4) Guttman, D., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 4(1957).

(5) Higuchi, T., *J. Pharm. Sci.*, **52**, 1145(1963).

(6) Martin, A. N., "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 525.

(7) Desai, S. J., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **54**, 1459(1965).

Penicillin G Interactions with Deoxycholic Acid Polymer-Like Structures

By F. ALHAIQUE, C. BOTRÉ, G. LIONETTI, M. MARCHETTI, and F. M. RICCIERI

Conductivity, activity coefficient, and ultraviolet absorption data have shown that an interaction occurs between deoxycholate helical complexes (DCA) and sodium benzylpenicillin (P). The mechanism of this interaction is still unknown, but a merely electrostatic interpretation can be ruled out. Experimental data seem to be consistent with the stereospecific nature of this interaction. The presence of DCA helical complexes increases benzylpenicillin resistance to the hydrolytic activity of penicillinase. No interaction and therefore no decrease in the hydrolytic activity of penicillinase has been found with different derivatives, *i.e.*, dimethoxyphenyl- and α-aminobenzylpenicillin. At the same time dehydrocholic acid sodium salt (NaDHCA), which in no case forms helical complexes, shows no effect upon P, whereas sodium salt of cholic acid (NaCA) still exerts a protective action upon P but to a lesser degree.

THE FORMATION of a helical complex of macromolecular dimension from sodium deoxycholate has been previously described by Rich and Blow (1, 2). Under appropriate conditions sodium deoxycholate aggregates in solution to form a gel which behaves in many ways like a polymer of high molecular weight. The fibers, which can be drawn from the solutions, showed an X-ray diffraction pattern which is characteristic of a helical aggregate (2). In a previous work (3) the influence of cation dimensions, hydration, and hydrogen bonds on the thermal stability of deoxycholic acid (DCA) "polymer-like" structure was discussed. It was shown that the thermal stability of deoxycholate aggregates is cationic species dependent.

In this paper the possibility of an interaction between DCA or related molecules and antibiotics has been studied. Even if the micelle-forming properties of deoxycholate could account for some of the observed phenomena, the formation of huge micelles with a regular or "crystalline" (2) internal structure seems to be responsible for effects which occur only when a specific system of hydrogen bonding is established within the micelle.

Conductivity, activity coefficient, and ultraviolet absorption data gave evidence that a specific interaction takes place between DCA polymer-like structure and benzylpenicillin (P). The mechanism of this interaction is still unknown, but an interpretation of the experimental data on merely electrostatic grounds can be ruled out.

It seemed sound to suppose that this interaction should somehow affect the biological activity of P, namely, its resistance to the penicillinase hydrolytic activity.

An inclusion, already demonstrated in the case of fatty acids (4, 5), could be proposed as an explanation for the observed decrease of penicillinase activity which has been chosen as reference. At the same time it could be suggested that the other molecular species present in the solution during the formation of the complex, could be attached to the outside of the helical steroid core, as are amino acids and peptides (2).

It cannot be overlooked that the study of this kind of interaction can lead to a more detailed knowledge of the mechanism of action of this class of antibiotics at the level of biomembranes.

Received March 29, 1967, from the Centro Nazionale di Chimica del Farmaco del C.N.R., I Sez. I Rep., Istituto Chimico Farmaceutico, Universita di Roma, Rome, Italy.

Accepted for publication August 2, 1967.

The authors express their gratitude to Professor Giordano Giacomello for his helpful suggestions, stimulating discussions, and critical reading of the manuscript.

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REFERENCES

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(5) Higuchi, T., *J. Pharm. Sci.*, **52**, 1145(1963).

(6) Martin, A. N., "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 525.

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EXPERIMENTAL

Materials—Deoxycholic acid (DCA), dehydrocholic acid (DHCA), and cholic acid (CA) Merck were further crystallized from alcohol before use. Different DCA salts were prepared by neutralizing DCA with solutions of different hydroxides. The DCA salt solutions were then lyophilized. The same procedure was used in preparing DHCA and CA salts.

Quaternary nitrogen bases were prepared by exchanging the corresponding iodides through an ionic exchanger, *i.e.*, Ionenaustauscher III Merck. The solution was then titrated with a standard HCl solution. The absence of halogens from these solutions was checked. A penicillin G sodium salt (P) sample, whose purity was determined by a spectrophotometer (Hitachi Perkin-Elmer model 139) according to a method described elsewhere (6), was used. Penicillinase (Baltimore Biological Laboratory) was a sample containing 2×10^6 units/ml.

All reagents used were of analytical grade.

Procedure—The different DCA salts were prepared by dissolving DCA with an amount of the corresponding hydroxide solution slightly less than that required. The excess of acid was then removed by filtration. The solution was then lyophilized and the DCA salt recovered. Conductivity measurements were carried out in a thermostatic bath, regulated within 0.01°. The complete filling and closing of the cell avoided the evaporation of the solution. A WTW bridge (model LF3) was employed for such measurements.

The pH values were controlled by means of a pH meter (Radiometer pH M4). The activity coefficients were determined by potentiometric titration carried out by means of a coupled permselective membrane electrode system extensively described elsewhere (7). The activity of sodium ions was calculated by means of a calibration curve, which allowed conversion of the recorded E.M.F. values into the activities. This was done stepwise by diluting a sodium chloride solution, recording the E.M.F. at each step, and plotting it against the logarithm of Na^+ ion activity. A linear plot was obtained. The a_{Na^+} values of the solution were calculated from the measured E.M.F. by interpolating the calibration curve. This was done assuming that $\gamma_{Na^+} = \gamma_{\pm NaCl}$ in the calibration solution (8). The penicillinase activity on P was evaluated with the manometric method described by Henry and Housewright (9) and modified by Pollock (10). Each time 3 ml. of a diluted penicillinase solution, corresponding to 300,000 units, was added to 0.5 ml. of different solutions of P in 0.0177 *N* $NaHCO_3$ as a buffer. The temperature was 30° ± 0.01° and the reading of CO_2 development was done every 2 min. All determinations were checked against a blank containing penicillinase inactivated with boiling water.

The ultraviolet absorption measurements were performed by means of a Hitachi Perkin-Elmer apparatus (model 139).

RESULTS AND DISCUSSION

In order to prove that a real interaction occurs between DCA and P, three sets of measurements were carried out, *i.e.*, conductivity, activity coefficient, and ultraviolet absorption. In Fig. 1, specific conductivity χ is plotted against the concentration of the different compounds considered.

Plot A refers to a solution of NaDCA, plot B to a solution of tetrabutylammonium deoxycholate (TBADCA), and plot C to a solution of P. The trend of the resulting plots is linear. In order to correlate the prerequisite of a polymer-like structure of DCA, TBADCA has been chosen because this DCA salt has proved to form no helical complexes in the range of temperatures of the experiments (3).

In Fig. 2 specific conductivity χ is plotted against the concentration of DCA salts in the presence of P (molar ratio 1:1). Plot A refers to a solution containing NaDCA + P in a molar ratio 1:1. Plot B refers to a solution containing TBADCA + P in a molar ratio 1:1. In both sets of measurements the temperature was 25° ± 0.01°.

Plot A of Fig. 2 shows a break point. It is to be noted that even if DCA concentration is the same in both plots, the stability of aggregates is cationic species dependent, as already discussed (3). In order to prove that this discontinuity is due to a

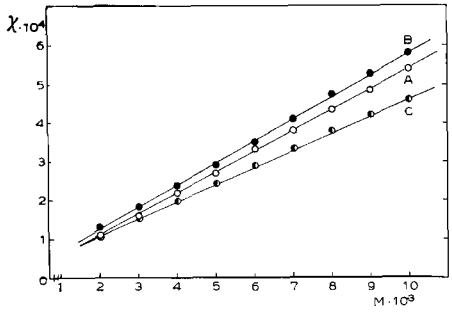

Fig. 1—No discontinuity is detected by conductivity measurements in solutions of different DCA salts. Specific conductivity χ is plotted as a function of the molar concentration of different deoxycholates. Key: A, *a solution of NaDCA;* B, *a solution of TBADCA;* C, *a solution of P.*

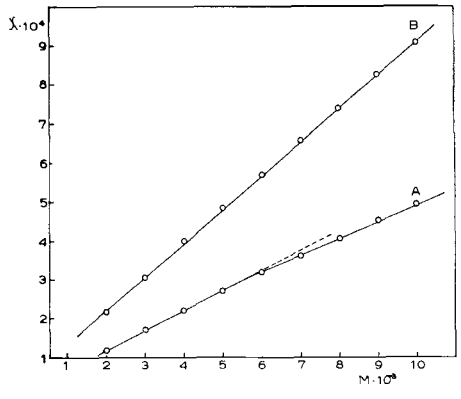

Fig. 2—DCA polymer-like structure interacts with P. No discontinuity is detected when polymer-like structures cannot take place. Specific conductivity χ is plotted against the concentration of DCA salts in the presence of P. Key: A, *NaDCA + P (molar ratio 1:1);* B, *TBADCA + P (molar ratio 1:1). The temperature was 25° ± 0.01°.*

real interaction between P and NaDCA, the activity coefficient of cations in solution was measured through a permselective membrane electrode system described elsewhere (7). The resulting plots are shown in Fig. 3, where plot A refers to a P solution, plot B to a NaDCA solution, and plot C to a NaDCA + P (molar ratio 1:1) solution.

Even in this case the trend of the plot shows a critical point which falls at the same concentration and proves interaction between NaDCA and P.

The increase in cationic activity shown by the complex NaDCA + P would tend to suggest that P interferes with sites of the helical complex, where electrostatic interactions with sodium ions take place. Possibly due to the conformation of the structure there must be a specific nonelectrostatic interaction which results in an increase of cationic activity if compared with that shown by P and DCA, respectively.

Further evidence of a specific interaction was found in extinction coefficient data at a wavelength of 264 mμ. The resulting data are reported in Fig. 4.

As might be expected, the trend of the plots of P and NaDCA taken singularly are linear, whereas

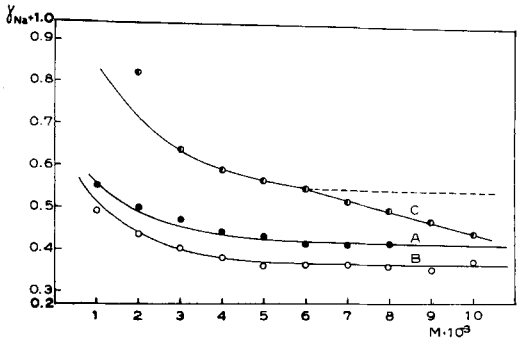

Fig. 3—Activity coefficient measurements show that a real interaction occurs. Activity coefficient γ_{Na^+} is plotted as a function of the molar concentration of a P solution (A), *a NaDCA solution* (B), *and a NaDCA + P solution in a molar ratio 1:1* (C). *The temperature was 25° ± 0.01°.*

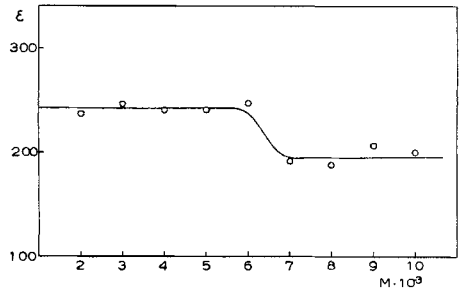

Fig. 4—Ultraviolet absorption data give evidence that DCA polymer-like structure interacts with P at the same value of NaDCA concentration where the transition is observed in both conductivity and activity coefficient measurements. Molar extinction coefficient ε at the wavelength of 264 mμ is plotted as a function of the concentration of a NaDCA + P solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

the plot referring to NaDCA + P shows a "jump" at the same value of NaDCA concentration where the transition was observed in both conductivity and activity coefficient measurements. The hyperchromic effect is such that the ε value of a diluted solution of NaDCA + P is nearly coincident to that of a penicillin solution when DCA concentration is lower than the transitional value (see Fig. 2). It can then be assumed that the structural changes which occur in deoxycholate complexes are such that no more interaction takes place between NaDCA and P. The same measurements were repeated with different DCA salts. Only the data referring to TBADCA have been reported, but constantly whenever temperature, pH, and ionic strength were such that no helical complex could be established (1–3) no singular points were detected by means of activity coefficient, conductivity, and spectrophotometric measurements. When DCA is replaced with DHCA (dehydrocholic acid), no interaction occurs in a number of concentrations ranging from those of DCA (see Fig. 5). At the same time CA (cholic acid) has the same effects as those for DCA, but the transition occurs at much higher values of concentration. (NaCA concentration was about 2×10^{-2} *M*.) In the attempt to elucidate the mechanism of this interaction, the same kind of measurements were carried out on some derivatives of 6-aminopenicillanic acid. On the assumption that the mechanism would be mainly of a steric nature, a molecule whose structure would ensure a higher degree of rigidity was chosen. In this sense the well known 2,6-dimethoxyphenylpenicillin seemed to be suitable. At the same time in order to find out in which way the total absence of a side chain or the presence of a polar group in it would influence the interaction, the authors took into consideration the α-aminophenylacetyl derivative (ampicillin) and the 6-aminopenicillanic acid itself.

All these compounds show the same behavior, and in the case of ampicillin the resulting data are given in Fig. 6. The plots prove that no interaction takes place. It seems reasonable then to suppose that the interaction between DCA and P is probably of a steric nature.

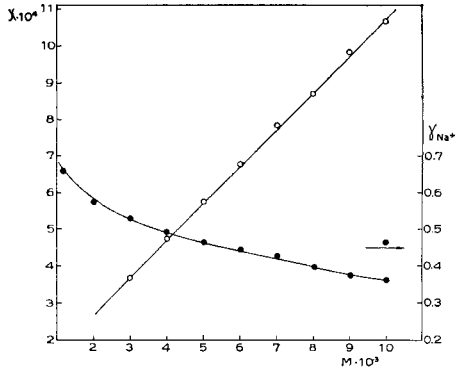

Fig. 5—No interaction occurs in the case of a NaDHCA + P solution. Activity coefficient γ_{Na^+} (●, right ordinate) and specific conductivity χ (○, left ordinate) are plotted against the molar concentration of a NaDHCA + P solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

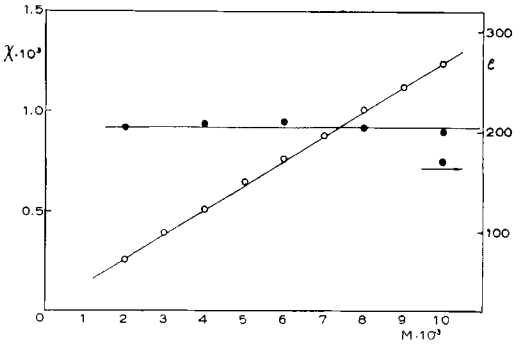

Fig. 6—No singular point can be detected by means of specific conductivity and ultraviolet absorption measurements when benzylpenicillin is replaced with ampicillin. Specific conductivity χ (○, left ordinate) and molar extinction coefficient ε (●, right ordinate) are plotted as a function of the molar concentration of a NaDCA + ampicillin solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

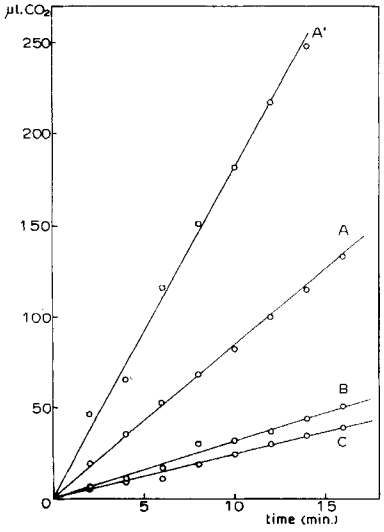

Fig. 7—A protecting action upon benzylpenicillin against hydrolytic activity of penicillinase is shown by DCA polymer-like structures and to a lesser extent by CA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of P 1.20 × 10^{-2} M (A), for a solution of NaCA + P 1.20 × 10^{-2} M, respectively (B), for a solution of NaDCA + P 1.20 × 10^{-2} M, respectively (C), and for a solution of NaDHCA + P 1.20 × 10^{-2} M, respectively (A'). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

The above described interaction between DCA polymer-like structure and P results in a decrease of penicillinase hydrolytic activity. If the interaction can take place only when DCA has acquired its polymer-like structure, it can be foreseen that there will be a decrease of penicillinase hydrolytic activity only when experimental conditions are such that the formation of a helical complex of macromolecular dimension is possible.

In Fig. 7 the amount of carbon dioxide (μl.) liberated by penicillinase is plotted as a function of time. Plot A refers to a solution of P 1.2 × 10^{-2} *M*. Plot B refers to a solution of NaCA + P 1.2 × 10^{-2} *M*, respectively. Plot C refers to a solution of NaDCA + P both at a concentration of 1.2 × 10^{-2} *M*. In each measurement the temperature was 30° ± 0.01° and the pH = 8.5. The volume of CO_2 developed in the case of NaDCA + P solution is much smaller than for a solution of P. NaCA still exerts a protective action upon P but to a lesser degree. On the other hand, if experimental conditions are such that no interaction can occur, there is no decrease of penicillinase activity as shown in Fig. 8. Here plot C refers to a solution of NaDCA + P both at a concentration of 2.37 × 10^{-3} *M*. Plot B refers to a solution of NaCA + P both at a concentration of 2.37 × 10^{-3} *M* and plot A refers to a solution of P 2.37 × 10^{-3} *M*.

Even if the micelle-forming properties of bile

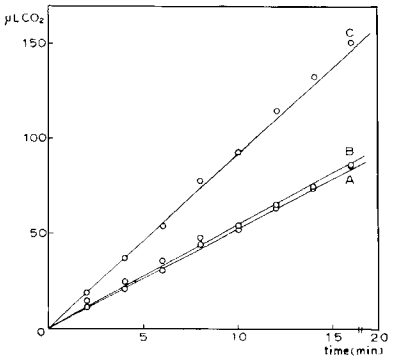

Fig. 8—No protecting action is shown when DCA concentration is lower than the transitional value or when DCA is replaced with CA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of P 2.37 × 10^{-3} M (A), for a solution of NaCA + P 2.37 × 10^{-3} M, respectively (B), and for a solution of NaDCA + P 2.37 × 10^{-3} M, respectively (C). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

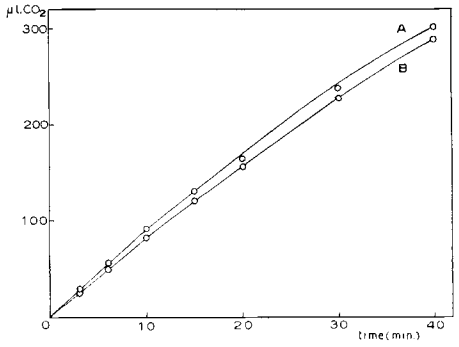

Fig. 9—No variation in penicillinase hydrolytic activity is shown when benzylpenicillin is replaced with ampicillin in a solution of DCA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of ampicillin 1.20 × 10^{-2} M (A) and for a solution of ampicillin + NaDCA 1.20 × 10^{-2} M, respectively (B). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

salts can account for the observed phenomena (11), nevertheless the ability of deoxycholate to give huge micelles with a regular or crystalline internal structure (2) seems to be responsible for effects which differ in their entity and specificity.

The formation of helical complexes of macromolecular dimension produces a large increase in viscosity which is the outstanding feature of this phenomenon. In the study of changes in viscosity of deoxycholate solutions at different concentrations, Ekwall (12) and Rich and Blow (2) found a number of concentration ranges separated by discontinuities in various properties. These discontinuities are ascribed to changes in the type of micelles. The assumption can be made that the type of DCA structure which corresponds to the range of concentrations indicated by conductivity, UV absorption, and ionic activity measurements interacts with penicillin G. In this respect CA and DCA seem to have almost the same effect upon penicillinase activity (see Fig. 7, plots B and C), but experimental conditions, namely pH = 8.5, suggest that DCA is probably hindered in forming its characteristic complexes which would give a more enhanced effect. In the case of CA, micelle formation and inclusion can be considered as being responsible for the data. This interpretation is consistent with the experimental results which showed that CA interacts with P in a range of concentrations much higher than those of DCA.

At the same time a comparison of the hydrolytic activity of penicillinase upon ampicillin in the presence or absence of DCA helical complexes shows (Fig. 9) that the lack of interaction brings no decrease in the development of CO_2.

In Fig. 8, in the case of a solution of NaDCA + P, where NaDCA concentration is lower than the transitional value, there is an increase in the quantity of CO_2 development if compared with that of a P solution at the same concentration.

We assume that, where no helical complex occurs, the presence of DCA results in an activation of the enzyme, possibly due to Van der Waals interactions with hydrophobic sites of the protein. This effect, of course, is always present, but when helical complexes take place, steric interactions become prevalent. This is further confirmed by the plot A′ of Fig. 7 where NaDHCA, which in no case forms helical complexes (2), shows the same effect upon penicillinase. A solution containing DCA helical complexes can be related on electrostatic grounds to a solution containing chain molecules with high densities of ionized groups.

According to Morawetz (13–15), the electrostatic potentials in the neighborhood of the polyion can be such to induce wide variation in the local concentration of mobile ions. If this is the case, then the presence of DCA helical complexes should have an effect exactly opposite to the one experimentally found on P. Therefore, the polymer-like structure must interact with P molecules through a mechanism which, still unknown, must not be of a merely electrostatic nature.

In conclusion if we choose those experimental conditions defined by that part of the plot of Fig. 2 which is above the break point, we will constantly have a decrease of penicillinase activity and therefore a protective action upon benzylpenicillin.

REFERENCES

(1) Rich, A., and Blow, D. M., *Nature*, **182**, 423(1958).
(2) Rich, A., and Blow, D. M., *J. Am. Chem. Soc.*, **82**, 3566(1960).
(3) Botrè, C., Cicconetti, P. A., Lionetti, G., and Marchetti, M., *J. Pharm. Sci.*, **56**, 1035(1967).
(4) Kratky, O., and Giacomello, G., *Monatsh.*, **69**, 427 (1936).
(5) Giacomello, G., and Romeo, M., *Gazz. Chim. Ital.*, **73**, 285(1943).
(6) Higuchi, T., and Connors, K. A., in "Pharmaceutical Analysis," Higuchi, T., and Brochmann-Hanssen, E., eds., Interscience Publishers, Inc., New York, N. Y., 1961, p. 602.
(7) Botrè, C., Borghi, S., and Marchetti, M., *Biochim. Biophys. Acta*, **135**, 208(1967).
(8) Botrè, C., De Martiis, F., and Solinas, M., *J. Phys. Chem.*, **68**, 3624(1964).
(9) Henry, R. J., and Housewright, R. D., *J. Biol. Chem.*, **167**, 558(1947).
(10) Pollock, N. R., *Brit. Exptl. Pathol.*, **31**, 739(1950).
(11) Bates, T. R., Gibaldi, M., and Kanig, J. L., *J. Pharm. Sci.*, **55**, 191(1966).
(12) Ekwall, P., *J. Colloid Sci.*, Suppl. 1, 66(1944); Ekwall, P., *Konikl. Wetenschap. Coloq.*, **1953**, 103.
(13) Morawetz, H., *J. Polymer Sci.*, **42**, 125(1960).
(14) Morawetz, H., and Shafer, J. A., *J. Phys. Chem.*, **67**, 1293(1963).
(15) Morawetz, H., and Shafer, J. A., *Biopolymers*, **1**, 71(1963).

Spectrophotometric Analyses of Ribose and 2-Deoxy-D-ribose Alone and in Mixtures

By EDWARD R. GARRETT, JAIME BLANCH, and JOACHIM K. SEYDEL*

The rates of production of the chromophore for ribose (R) and the maximum absorbance of the chromophore for 2-deoxy-D-ribose (DR), produced under thermal and acidic conditions are directly related to their sugar concentrations. These factors are the bases for qualitative and quantitative analysis of these sugars. The $\lambda_{max.}$ 261 mμ chromophore of degraded DR is stable for at least 15 hr. after 5 hr. of treatment at 80.0° with 1.0 *M* HCl. The $\lambda_{max.}$ 277 mμ chromophore of degraded R increases continuously during the same period at an apparent zero-order rate. These phenomena serve as bases for a sensitive spectrophotometric assay of both sugars, alone and in mixtures. These acid-generated chromophores when made alkaline generate other absorption bands with specific properties which permit additional methods of differentiation between the sugars.

Analyses of sugars in mixtures constitute a broad field of investigation. Sugar mixtures have been separated by paper or column chromatography with further analyses of the effluents (1–3). Methods of separation by gas chromatography of methylated sugars are available (4, 5). Differences in color development after reaction with specific reagents have been another approach (6). Dialysis based on differential kinetics (7) and combinations of different methods (8) have been proposed. However, some of these techniques are nonselective and are insensitive to small sugar concentrations.

The phenomenon of chromophore production when sugars are heated with mineral acids is well known. Correlations between absorbances of those chromophores and the sugar concentration have served as bases for sugar analyses (9–11). Limited attempts have been made to analyze sugars in mixtures by these procedures. The extremely strong conditions in acid concentration and temperature which have been used sometimes do not permit the attainment of reproducible results and are not specific for individual sugars.

If a sugar is subjected to well-defined and mild conditions of acid concentration, temperature, and time, definite chromophores with well-defined properties can result (12–14). For example, when 2-deoxy-D-ribose (DR) is heated 5 hr. in 1.0 *M* HCl at 80.0°, a chromophore $\lambda_{max.}$ 261 mμ (12) is produced as shown in Fig. 1 and is stable under those conditions for at least 15 hr. (Fig. 2). Ribose (R) under the same conditions generates a chromophore $\lambda_{max.}$ 277 mμ (Fig. 1) which increases continuously during the same period at an apparent zero-order rate (Fig. 2). Such differences can provide sensitive assay methods for these sugars, alone and in mixtures. The properties of these acid-generated chromophores were also investigated.

EXPERIMENTAL

Materials and Equipment—D-Ribose (R) and 2-deoxy-D-ribose (DR) were obtained from Calbiochem (analytical reagent grade) and furfural from the Fisher Scientific Co. (reagent grade). All other chemicals used were of analytical reagent grade. A Beckman model DU spectrophotometer, slit width 0.1 mm., was used. Complete absorption spectra were obtained on a Cary model 15 spectrophotometer.

Spectrophotometric Procedures for Calibration Curves of 2-Deoxy-D-ribose (DR)—A calibration curve for DR was prepared using concentrations between $1 \times 10^{-4}M$ and $7 \times 10^{-4}M$ in 1.0 *M* HCl.

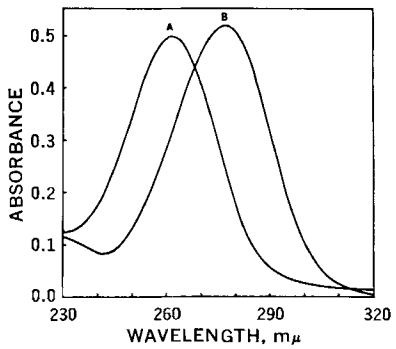

Fig. 1—Ultraviolet spectrum of the products of sugar degradation in 1.0 M HCl at 80.0°. Curve A is for 3.75 × 10⁻⁴ M 2-deoxyribose after 5 hr. of degradation. Curve B is for 8.00 × 10⁻⁴ M ribose after 6.5 hr. of degradation and is also the spectrum of furfural in 1.0 M HCl.

Received June 19, 1967, from the College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication August 7, 1967.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by an institutional grant to the University of Florida by the American Cancer Society, and in part by grant GM-09864-03,04,05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Abstracted in part from a thesis submitted by Jaime Blanch to the Graduate School, University of Florida, Gainesville, in partial fulfillment of Master of Science degree requirements.

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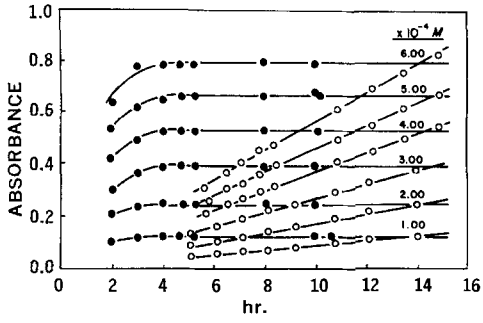

Fig. 2—Effect of time on the absorbances of (○) *1.0* M *HCl acid-degraded ribose measured at 277 mμ, and* (●) *deoxyribose measured at 261 mμ, at 80.0°.*

The samples were maintained at 80.0° in a thermostatted bath. Aliquots were taken between 5 and 20 hr. of degradation. On cooling, the absorbances were read at $\lambda_{max.}$ 261 mμ. A plot of these absorbances against the respective sugar concentration resulted in the calibration curve for DR as shown in Fig. 3.

Calibration Curves of D-Ribose (R)—Samples of R between $1 \times 10^{-4}M$ and $6 \times 10^{-4}M$ were degraded under the same conditions as specified for DR. Samples were taken between 5 and 20 hr. of degradation. The cooled aliquots were read at 277 mμ against a blank solution of 1.0 *M* HCl. The plots of absorbances against time for each R concentration are given in Fig. 2. The apparent zero-order rate constants, the slopes of such lines, are plotted against the respective R concentration in the calibration curve for R (Fig. 3).

Assay of Ribose and 2-Deoxy-D-ribose—Solutions of the sugars were prepared so that the absorbance between 200 and 400 mμ did not exceed 1.50 after heating at 80.0° for 20 hr. in 1.0 *M* HCl. For greater absorbances the solution may be diluted with known volumes of 1.0 *M* HCl. To assay DR, the absorbance at 261 mμ was measured between 5 and 20 hr. of degradation. This value was used to obtain the DR concentration from Fig. 3, the calibration curve. To assay R, samples between 5 and 20 hr. were removed from the reaction flask, and the absorbances of the cooled aliquots were measured at 277 mμ. A plot of absorbance against time was made and the apparent rate constant was measured. This value was used to obtain the R concentration from Fig. 3, the calibration curve. Seven or eight aliquots at 1.0 or 1.5 hr. intervals will provide an accurate value for the rate constant. However, two measurements at 5 and 20 hr. are sufficient to estimate the apparent zero-order rate constant for any R concentration.

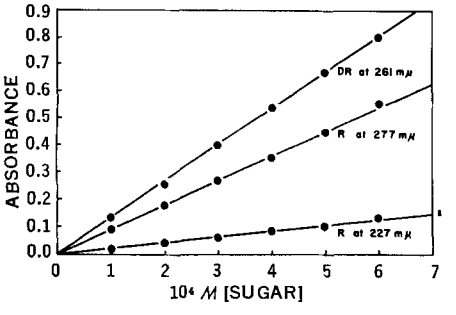

Fig. 3—Calibration curves for the spectrophotometric analyses of acid-degraded ribose and deoxyribose. The ordinate represents the absorbance after 5 hr. for deoxyribose (DR) and the rate constants in 10 × absorbance units/hr. at 277 mμ for ribose (R) as obtained at 80.0° in 1.0 M *HCl.*

Anaylsis of Ribose and 2-Deoxy-D-ribose in Mixtures—Solutions containing the mixture were degraded in 1.0 *M* HCl at 80.0°. Samples were taken between 5 and 20 hr. of degradation. The absorbances at 261 mμ and 277 mμ were measured and plotted against time. In the plot at 277 mμ, the slope was determined and the R concentration obtained from the calibration curve, Fig. 3. The intercept of the plot of absorbances of the mixtures at 261 mμ against time corresponds to the absorbance due to degraded DR. The pertinent DR concentration was read from its calibration curve, Fig. 3.

Investigation of Properties of Furfural—The spectrum of furfural was obtained in 1.0 *M* HCl and followed as a function of time in 0.25 *M* NaOH at 45.0°. The extraction of furfural and the chromophore of acid-degraded ribose from 1.0 *M* HCl by equal volumes of chloroform was followed by observing the decreases in the $\lambda_{max.}$ 277 mμ absorbance in the aqueous phases.

RESULTS AND DISCUSSION

Kinetic Studies—When 2-deoxy-D-ribose (DR) is degraded in 1.0 *M* HCl at 80.0° a chromophore at 225 mμ is generated by an apparent first-order process. The apparent first-order rate constants are proportional to the hydrogen ion activity, a_{H^+}, at a given temperature (12). The compound with this chromophore is the precursor of 5-methyl-3(2*H*)-furanone (13) with a $\lambda_{max.}$ 261 mμ (Fig. 1) which is also generated by an apparent first-order process. The 261 mμ absorbance reaches a constant value A_∞ after 5 hr. of heating in 1.0 *M* HCl, which is maintained for at least 15 hr. (Fig. 2). The A_∞ is directly proportional to DR concentration, and the apparent first-order rate constants are also proportional to the hydrogen ion activity. A complete discussion of the properties of acid-degraded DR, 5-methyl-3(2*H*)-furanone and the chemical nature of the degraded products has been reported (12, 13).

When ribose (R) is degraded under the same conditions, a chromophore at $\lambda_{max.}$ 277 mμ is generated (Fig. 1). After a slight induction period, this chromophore appears at apparent zero-order rates between 5 and 25 hr. after initiation of the degradation (Fig. 1). However, the over-all reaction may not be strictly a zero-order process. As the degradation proceeds, the rate of appearance of the 277 mμ absorbance band decreases, but this deviation from zero order is not significant until after 25 hr. A final absorbance $\lambda_{max.}$ 277 mμ with an apparent molar absorptivity of 7,160 based on the initial ribose concentrations is obtained after 200 hr. at 80.0° in 1.0 *M* HCl.

Concomitant with the appearance of the 277 mμ absorbance band, another at 227 mμ $\lambda_{max.}$ is also observed to arise by an apparent zero-order rate process. The 227 mμ chromophore is not preferred for analytical purposes due to its lessened absorptivity.

The effect of the R concentration on the rate of generation of the chromophores was studied. Concentrations of R ranging from $1 \times 10^{-4}M$ to $6 \times 10^{-4}M$ were degraded in 1.0 M HCl at 80.0°. The resultant absorbances, A, were measured at 227 and 277 mμ and plotted against time, t, as shown in Fig. 2 for the 277 mμ values. The values for the slopes, k_o, of the lines between 5 and 20 hr. of degradation were obtained (Fig. 1) in accordance with the equation:

$$A = k_o t \qquad \text{(Eq. 1)}$$

The apparent intercepts are not significantly different from zero for ribose concentrations less than $6 \times 10^{-4}M$. The k_o values are directly related to the ribose concentration as shown in Fig. 3 where:

$$k_{277} = \alpha_{277}\,\mathrm{R};\ \alpha_{277} = 2.49 \times 10^{-2}\ \text{absorbance units} \times \text{L./mole/sec.} \qquad \text{(Eq. 2)}$$

where the percent of standard deviation of α_{277} is 1.8% and the standard deviation of k_{277} about regression on R is 1.87×10^{-7}, so that at $3.5 \times 10^{-4}M$ ribose the percent of standard deviation of k_{277} is 2.1%. The percent of standard deviation is often referred to as the coefficient of variation Also,

$$k_{227} = \alpha_{227}[\mathrm{R}];\ \alpha_{227} = 5.92 \times 10^{-3}\ \text{absorbance units} \times \text{L./mole/sec.} \qquad \text{(Eq. 3)}$$

The dependency of the apparent zero-order rate constant, k_{277}, at 277 mμ on the HCl concentration was also studied for $1 \times 10^{-3}M$ ribose solution degraded at 80.0°. The values of k_{277} were determined (Table I) and plotted against the respective hydrogen ion activity, $a_{H+} = f[\mathrm{HCl}]$, where f is the activity coefficient obtained from the literature (15). From the plot of k_{277} against a_{H+} (Fig. 4), at 80.0° at $10^{-3}M$ ribose:

$$k_{277} = k_{H+}a_{H+};\ k_{H+} = 3.2 \times 10^{-5}\ \text{absorbance units} \times \text{L./mole/sec.} \qquad \text{(Eq. 4)}$$

Alternatively, in terms of HCl concentration:

$$k_{277} = k_{\mathrm{HCl}}[\mathrm{HCl}];\ k_{\mathrm{HCl}} = 2.3 \times 10^{-5}\ \text{absorbance units} \times \text{L./mole/sec.} \qquad \text{(Eq. 5)}$$

Thus, at 80.0°:

$$\alpha_{277} = [\beta_{277}]_{H+}a_{H+} = (k_{277}/[\mathrm{R}])\,a_{H+};\ [\beta_{277}]_{H+} = 3.2 \times 10^{-2}\ \text{absorbance units} \times \text{L.}^2/\text{mole}^2/\text{sec.} \qquad \text{(Eq. 6)}$$

$$\alpha_{277} = [\beta_{277}]_{\mathrm{HCl}}[\mathrm{HCl}] = (k_{277}/[\mathrm{R}])[\mathrm{HCl}];\ [\beta_{277}]_{\mathrm{HCl}} = 2.3 \times 10^{-2}\ \text{absorbance units} \times \text{L.}^2/\text{mole}^2/\text{sec.} \qquad \text{(Eq. 7)}$$

For a given hydrogen ion activity or HCl concentration, the appropriate value at 80.0° can be determined for the 277 mμ absorbance of degraded ribose (Eqs. 6 and 7). Knowledge of the experimental zero-order rate constant at that acid concentration or hydrogen ion activity permits calculation of the ribose concentration from the rearranged Eq. 2.

The rate dependency on the absolute temperature, °C. + 273, was determined from the appropriate plot (Fig. 5, curve A) of the Arrhenius equation from data obtained at several temperatures (Table I) for $10^{-3}M$ ribose in 1.0 M HCl:

$$\log k_{277} = -(\Delta H_a/2.303\ \mathrm{R})\,1/T + \log P \qquad \text{(Eq. 8)}$$

The apparent heat of activation calculated from the slope of this plot was $\Delta H_a = 28.6$ Kcal./mole, and the log P value was 13.1. Since $k_{\mathrm{HCl}} = k_{277}$ at 1.0 M HCl (Eq. 5), the Arrhenius parameters are the same for log k_{HCl} at 277 mμ. Also, the Arrhenius parameters for log $[\beta_{277}]_{\mathrm{HCl}}$ are $\Delta H_a = 28.6$ Kcal./mole and log $P = 10.1$ so that $[\beta_{277}]_{\mathrm{HCl}}$ may be calculated for various temperatures from the Arrhenius equation (Eq. 8). Substitution of this value and a given HCl concentration in Eq. 7 determine the α_{277} value to be used in Eq. 2 for determining ribose concentration.

Assay of Ribose and 2-Deoxy-D-ribose in Mixtures—The 261 mμ absorbance of degraded DR increases to a stable value after 4 hr., whereas the 277 mμ absorbance of degraded R continues to increase linearly with time (Fig. 6). When a mixture of R and DR is subjected to thermal acid degradation the apparent zero-order rates of increase of the absorbances in the region of 277 mμ after 5 hr. are identical with the rates for the same concentration of ribose alone under the same conditions of acid and temperature (Figs. 2, 3, and

TABLE I—APPARENT ZERO-ORDER RATE CONSTANTS[a] FOR THE APPEARANCE OF THE 227 AND 277 mμ ABSORBANCES FROM ACID-DEGRADED RIBOSE AT 80°

°C.	[HCl]	10^4 [Ribose]	$10^7 k_{227}$	$10^6 k_{277}$
80.0	1.00	1.00	3.89	2.47
80.0	1.00	2.00	11.4	4.86
80.0	1.00	3.00	16.1	7.50
80.0	1.00	4.00	22.8	9.80
80.0	1.00	5.00	28.6	12.5
80.0	1.00	6.00	37.2	15.4
80.0	1.00	10.00	...	25.7
80.0	0.75	10.00	...	18.9
80.0	0.50	10.00	...	10.7
80.0	0.13	10.00	...	2.47
70.0	1.00	10.00	...	7.31
60.0	1.00	10.00	...	2.11

[a] In absorbance units/second and obtained between 5 and 20 hr. after initiation of reaction.

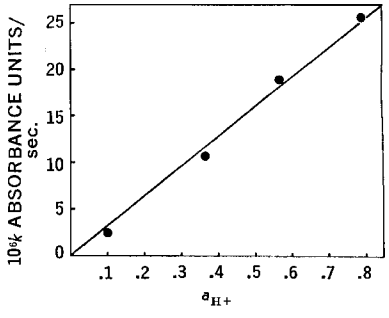

Fig. 4—Dependence of the apparent zero-order rate constant, k, in absorbance units/sec., on the hydrogen ion activity, a_{H+}, for the degradation of 1.0 × 10^{-3} M ribose at 80.0° as determined from the rate o appearance of the 277 mμ chromophore.

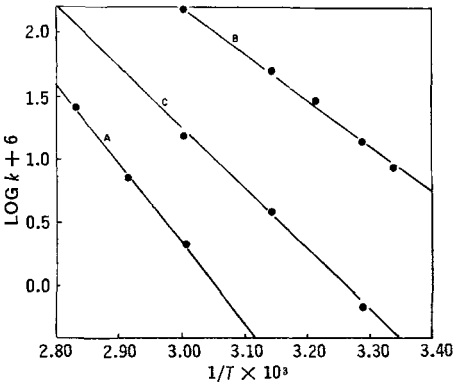

Fig. 5—Arrhenius plots for the apparent zero-order degradation of ribose in 1.0 M HCl (k *in absorbance units/sec.) as measured by the appearance of a* $\lambda_{max.}$ *277 mμ absorbance* (A) *and for the 0.20 M NaOH degradation of acid-degraded ribose as measured by the apparent first-order disappearance of* $\lambda_{max.}$ *277 mμ* (B) *and appearance of* $\lambda_{max.}$ *324 mμ* (C), k *in sec.*$^{-1}$.

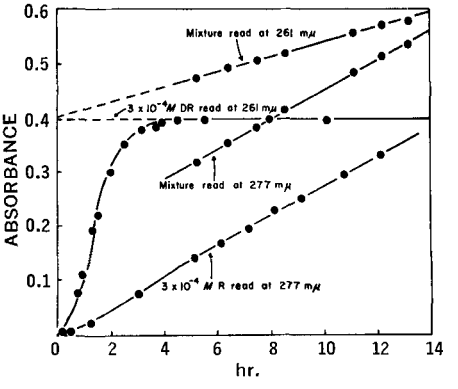

Fig. 6—Examples of analyses of mixtures of ribose (3×10^{-4} M) *and deoxyribose* (3×10^{-4} M) *degraded in 1.0 M HCl at 80.0°. The combined absorbances are read at 261 mμ to determine the intercept value related to the deoxyribose concentration which is obtained by extrapolation of the linearity observed after 5 hr. The combined absorbances are read at 277 mμ to determine the linear slope after 5 hr. which is related to the ribose concentration. The absorbances with time at these* $\lambda_{max.}$ *values are also given for the acid degradation of the separate sugars.*

6). The slope of the absorbance *versus* time plot after 5 hr. for such a mixture is a function only of the R concentration which can be obtained from the calibration curve for R (Fig. 3). The zero time intercept for this linear segment after 5 hr. when absorbance at 261 mμ is plotted against time is only due to the absorbance of the degraded DR (Fig. 6). This intercept is related to the concentration of DR present in the mixture by the appropriate calibration curve (Fig. 3).

This technique was applied to the resolution of several mixtures of both sugars in the concentration ranges from $1 \times 10^{-4}M$ to $6 \times 10^{-4}M$. The results are given in Table II. The estimated percent of standard deviations of each assay in such mixtures is 1.87% for deoxyribose and 2.93% for ribose.

TABLE II—RESULTS OF THE ASSAY OF MIXTURES OF RIBOSE (R) AND 2-DEOXYRIBOSE (DR)

Comp. of the Mixtures		Found by Assay	
10^{-4} M (DR)	10^{-4} M (R)	10^{-4} M (DR)	10^{-4} M (R)
1.00	1.00	1.00	1.15
2.00	1.00	2.00	0.78
3.00	1.00	3.00	1.00
4.00	1.00	3.91	1.00
1.00	2.00	1.00	1.96
2.00	2.00	2.02	2.02
3.00	2.00	3.03	2.02
4.00	2.00	4.03	1.94
1.00	3.00	1.03	2.96
2.00	3.00	2.06	3.03
3.00	3.00	3.03	3.06
4.00	3.00	4.10	2.92
5.00	3.00	5.15	3.08
1.00	4.00	1.02	4.05
2.00	4.00	2.00	3.98
3.00	4.00	3.03	4.00
4.00	4.00	3.95	3.95
6.00	4.00	6.10	4.25
1.00	5.00	1.00	5.00
2.00	5.00	1.90	5.00
3.00	5.00	3.03	4.97
4.00	5.00	4.03	4.93
5.00	5.00	5.00	4.85
1.00	6.00	1.00	6.00
2.00	6.00	1.98	5.97
3.00	6.00	3.12	6.25
4.00	6.00	4.00	5.88
5.00	6.00	5.20	5.92

Alkaline Degradation of the Acid-Generated Chromophore of Ribose—The 261 mμ chromophore of acid-degraded DR can be destroyed in mildly alkaline solutions. The 261 mμ absorbance upon alkalinization shifts to a $\lambda_{max.}$ 293 mμ, which absorbance band disappears rapidly by a first-order process (12). When acid-degraded ribose is made alkaline by addition of NaOH, the $\lambda_{max.}$ 277 mμ absorbance is lost by a first-order process to a small residual absorbance (Fig. 7). The loss in absorbance is accompanied by a gradual hypsochromic shift to a residual absorbance with a $\lambda_{max.}$ about 260 mμ. Concomitant with the 277 mμ absorbance disappearance, a band $\lambda_{max.}$ 324 mμ arises by an apparent first-order process and approaches an asymptotic value. This band subsequently disappears by an apparent first-order process (Fig. 7). The initial isosbestic point at 302 mμ indicates a 1:1 transformation for the first reaction in the apparent sequence, *i.e.*, $A \rightarrow B$, with an apparent first-order rate constant k'_{277}. The subsequent slower transformation, $B \rightarrow C$, of an apparent first-order rate constant k_{324} is accompanied by a loss of the isosbestic point. The respective k'_{277} and k_{324} values at various temperatures and concentrations of alkali are given in Table III. These apparent rate constants at 45.0° when plotted against the respective NaOH concentrations show a linear dependency (Fig. 8) and:

$$k_i = k_{NaOH}[NaOH] \qquad \text{(Eq. 11)}$$

where k_{NaOH} for the disappearance of the 277 mμ chromophore and appearance of the 324 mμ chromophore at 45.0° is 2.58×10^{-4} L./mole/sec., and the k_{NaOH} for the disappearance of the 324 mμ chromo-

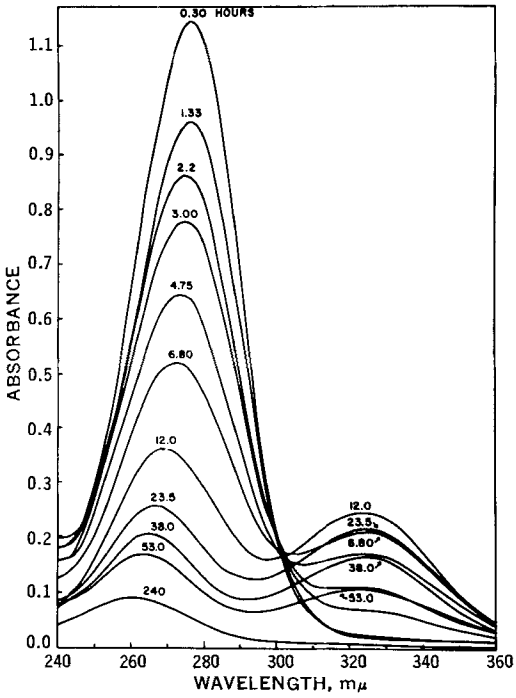

Fig. 7—Ultraviolet spectra of acid-degraded ribose and furfural at various times in 0.26 M NaOH at 45.0°.

TABLE III—APPARENT FIRST-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE IN ALKALI OF THE 277 mμ CHROMOPHORE OF DEGRADED RIBOSE (FURFURAL), k'_{277} IN SEC.$^{-1}$, AND FOR THE SLOWER DISAPPEARANCE OF A RESULTANT 324 mμ CHROMOPHORE, k_{324}

°C.	[NaOH]	$10^5 k'_{277}$	$10^6 k_{324}$
60.0	0.20	15.4	15.3
45.0	0.26	7.09	4.92
45.0	0.20	5.15	3.83
45.0	0.13	3.41	2.47
45.0	0.066	1.42	1.36
38.0	0.20	2.95	...
31.0	0.20	1.40	0.666
25.0	0.20	0.852	...

phore is 1.92×10^{-5} L./mole/sec. The Arrhenius parameters, (Eq. 8) ΔH_a and log P, for the apparent first-order rate constants for the transformation of the acid-degraded ribose chromophores in 0.20 *M* NaOH were 16.5 Kcal./mole and 7.0 for the loss of the $\lambda_{max.}$ 277 mμ and 21.3 Kcal./mole and 9.1 for the loss of the resultant $\lambda_{max.}$ 324 mμ.

Correlation of the Chromophore of Acid-Degraded Ribose with Furfural—It is well-known that furfural is the major product of acid degradation of ribose (16). The ultraviolet spectrum of furfural in 1.0 *M* HCl is exactly that of acid-degraded ribose (Fig. 1). The bands at $\lambda_{max.}$ of 277 mμ and 227 mμ have a ratio of absorbances for furfural A_{277}/A_{227} = 3.78, whereas the spectrum of acid acid-degraded ribose of an equivalent absorbance, $A_{277} = 1.5$, has a ratio of absorbances of 3.68. Furfural in 0.28 *M* NaOH at 45.0° loses its chromophore at 277 mμ with a slight hypsochromic shift. Concomitantly a less intense band at $\lambda_{max.}$ 324 mμ arises with a clear isosbestic at 301 mμ. These are exactly the properties of the chromophore of acid-degraded ribose (Fig. 7). The molar absorptivity of furfural ϵ_f at 277 mμ is 14,962 in agreement with the literature value of 14,800 (17). Thus the ultimate yield of furfural after 200 hr. from acid degradation of ribose at 80.0° in 1.0 *M* HCl is 7,160/14,962 = 47.8%.

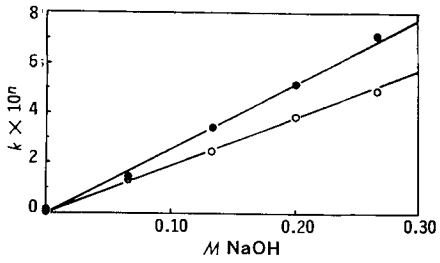

Fig. 8—Dependence of the apparent first-order rate constant, k in sec.$^{-1}$, on the NaOH concentration for the disappearance of the chromophores of the products of acid-degraded ribose at 45.0° where n = 5 for $\lambda_{max.}$ 277 mμ (●) and n = 6 for $\lambda_{max.}$ 324 mμ (○).

Both furfural and the chromophore of acid-degraded ribose are readily extracted from 1.0 *M* HCl by chloroform. When equal volumes of chloroform and 1.0 *M* HCl were used, 94.4% of the 277 mμ absorbance was extracted from the aqueous phase as compared to 94.5% of the 277 mμ absorbance of acid-degraded ribose.

REFERENCES

(1) Hough, L., "Methods of Biochemical Analysis," Glick, I. D., ed., Interscience Publishers, Inc., New York, N. Y., 1945, p. 205.
(2) Walborg, E. F., Jr., Christensson, L., and Gardell, S., *Anal. Biochem.*, **13**, 177(1965).
(3) *Ibid.*, **13**, 186(1965).
(4) Sweeley, C. C., Bentley, R., Mikita, M., and Wells, W. W., *J. Am. Chem. Soc.*, **85**, 2497(1963).
(5) Brower, H. E., Jeffery, J. E., and Folsom, M. W., *Anal. Chem.*, **38**, 362(1966).
(6) Dische, Z., "Methods in Carbohydrate Chemistry," Whistler, R. L., and Wolfrom, M. L., eds., vol. 1, Academic Press, Inc., New York, N. Y., 1962, p. 477.
(7) Siggia, S., Hanna, J. G., and Serencha, N. M., *Anal Chem.*, **36**, 638(1964).
(8) Webb, J. M., and Levy, H. B., *J. Biol. Chem.*, **213**, 107(1955).
(9) Ikawa, M., and Niemann, C., *ibid.*, **180**, 923(1949).
(10) Love, R. M., *Biochem. J.*, **55**, 126(1953).
(11) Rice, F. A. H., and Fishbein, L., *J. Am. Chem. Soc.*, **78**, 1005(1956).
(12) Seydel, J. K., and Garrett, E. R., *Anal. Chem.*, **37**, 271(1965).
(13) Seydel, J. K., Garrett, E. R., Diller, W., and Schaper, K. J., *J. Pharm. Sci.*, **56**, 858(1967).
(14) Garrett, E. R., and Blanch, J., *Anal. Chem.*, **39**, 1109(1967).
(15) Harned, H. S., and Owen, B. B., "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold Publishing Corp., New York, N. Y., 1958.
(16) Rice, F. A. H., and Johnson, A. R., *J. Org. Chem.*, **23**, 1966(1958).
(17) Gillam, A. E., and Stern, E. S., "Electronic Absorption Spectroscopy," 2nd ed., E. Arnold, London, England, 1957, p. 152.

Solubility Profiles for Antipyrine and Aminopyrine in Hydroalcoholic Solutions

By ANTHONY N. PARUTA

The solubilities of antipyrine and aminopyrine have been determined in binary mixtures of ethanol and water of known dielectric constants. The solubility curves produced in this solvent system were compared with the solubility curves found in dioxane–water mixtures. The dielectric requirements (DR's) are apparently not correlatable in these two solvent systems, and the constancy of DR's may be questioned. The solubility ratios of these 2 solutes in the binary mixtures studied is also discussed. In addition, the dielectric constants of the saturated solutions were determined and compared to the dielectric constants of the binary mixtures. It is felt that these data may lead to a consideration of "solute" or "inherent" polarity for these materials.

IT HAD BEEN shown previously (1) that antipyrine and aminopyrine produced rather unique solubility curves in dioxane–water mixtures. The highly water-soluble antipyrine was characterized by a solubility curve running asymptotically to pure water, whereas aminopyrine was characteristic of an ideal semipolar solute having only one maximum in the solvent mixture. The attributed variation in polarity between antipyrine and aminopyrine was felt to be due to the dimethylamino group in aminopyrine causing both a decrease in the magnitude of solubility and a substantially lower DR, or a shift to lower polarity. This rather large difference, while perhaps not too surprising, was somewhat perplexing. Thus, it was felt judicious that these two solutes should be tested in another solvent system, and in this case, ethanol–water mixtures were chosen. It was also expected that the DR's should also correlate and that the nature of the curves should be essentially similar.

Since it had also been observed (2) that the highly water-soluble antipyrine caused substantial dielectric constant changes in syrup vehicles, the dielectric constants of the saturated solutions were also determined. It is hoped that some useful information might be gained from a knowledge of these values.

EXPERIMENTAL

Materials—The solutes used in this study were aminopyrine USP[1] and antipyrine USP.[2] The solvents used were freshly boiled demineralized distilled water and absolute ethanol USP–NF.[3]

Methods—The protocol used in the analysis of solubility data has already been described (1). No pretreatment of the solutes was considered necessary, and they were used directly. The reported results are the averages of at least 4 runs on each material. Each run was internally averaged for both gravimetric and spectrophotometric procedures. Dielectric constant values were determined from calibration curves as previously described (2).

Equipment—A WTW DK-06 multidekameter was used for dielectric constant determinations, a Bantam demineralizer, model BD-1 with mixed resin bed; Bausch & Lomb Spectronic 505, and a Mettler automatic balance, type H 6T, heated vacuum desiccator.[4] A water bath and attendant temperature controls were used for equilibration media.

RESULTS AND DISCUSSION

Solubility—In Fig. 1, the solubility of antipyrine in mg./ml. is plotted *versus* the dielectric constants of the binary mixture indicated. The solubility of aminopyrine in ethanol–water and dioxane–water mixtures (1) is also shown in Fig. 1.

The data for ethanol–water mixtures are from the present work, whereas that for the dioxane–water system has been taken from a previous communication (1). The dielectric requirement (DR) is seen to have a value of about 55 for antipyrine and about 35 for aminopyrine in ethanol–water mixtures. In dioxane–water mixtures, there may be a slight "shouldering" at a dielectric constant value of about 55 for antipyrine.

It was also shown in another communication (3) that changes, *i.e.*, "appearance" of shoulders, could occur upon changing the composition scale coordinate to a dielectric constant scale coordinate. Although this appearance allows for the correlation of the binary systems in the case of antipyrine, this correlation is considered to be vague. This is especially notable in the case of antipyrine in ethanol–water mixtures where a change in the plotting coordinate does not effect a change in the DR value for this solute.

The solubility curve for aminopyrine in dioxane–water mixtures does not indicate the occurrence of a shoulder, although one can "force" this occurrence, it is deemed as noncorrelation of the DR in these solvent mixtures for this solute.

Since the dielectric constants of the saturated solutions in ethanol–water mixtures were deter-

Received March 13, 1967, from the College of Pharmacy, University of Rhode Island, Kingston, RI 02881
Accepted for publication August 15, 1967.

[1] Penick Chemical Division, No. 77414.
[2] Penick Chemical Division, No. 78093.
[3] U. S. Industrial Chemical Co.
[4] Precision Scientific Co.

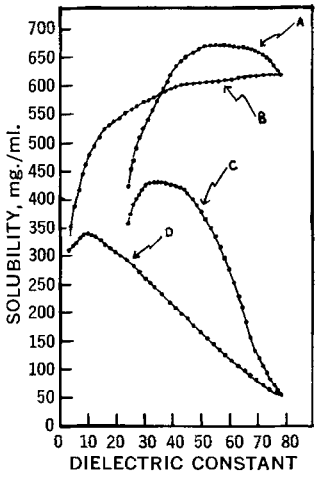

Fig. 1—A plot of the solubilities of antipyrine and aminopyrine in mg./ml. at 25° versus *the dielectric constants of the solvent mixtures. Curves* A *and* C *are for antipyrine and aminopyrine in ethanol–water mixtures. Curves* B *and* D *are for antipyrine and aminopyrine in dioxane–water mixtures.*

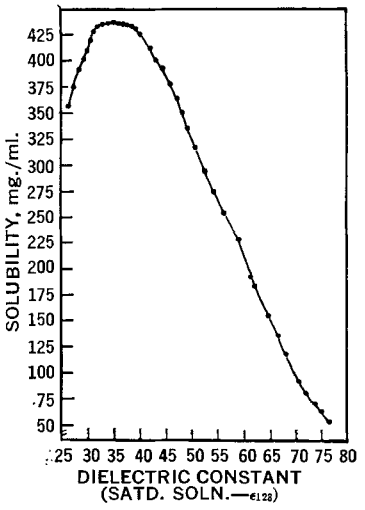

Fig. 3—The solubility of aminopyrine in mg./ml. at 25° versus *the dielectric constant values of the respective saturated solutions.*

mined, it was felt instructive that the solubility curves be plotted *versus* these values. As expected, the solubility curves obtained from this treatment were essentially the same as the curves produced when only the dielectric constants of the solvent mixtures (ϵ_{12}) were used. The solubilities of antipyrine and aminopyrine are plotted *versus* the dielectric constants of the saturated solutions (ϵ_{123}) and are shown in Figs. 2 and 3.

If it were found that the ϵ_{123} and the ϵ_{12} curves were plotted as a function of concentration and these dielectric curves intersected, it would imply that the dielectric constant of the solute might occur at this point of intersection. In other words, the addition of solute to a given composition does not change the dielectric constant of the solvent mixture. Furthermore, were the point of intersection to lie close to the dielectric requirement, where maximum solubility occurs, it would further indicate that solute polarity and solvent polarity were equal in magnitude. This would probably indicate the essential embodiment of either the solubility parameter or dielectric requirement approaches since solubility is maximized when there is no difference in the polarity of the solute and solvent mixture. This is seen to be the case for antipyrine (Fig. 4) and for aminopyrine (Fig. 5) having points of intersection for antipyrine and aminopyrine at 51–53 and 35, respectively, whereas the dielectric requirements were 53–55 and 35, respectively. At any point away from the intersection, one observes either an increase or decrease in the dielectric constant of the saturated solution relative to the solvent system.

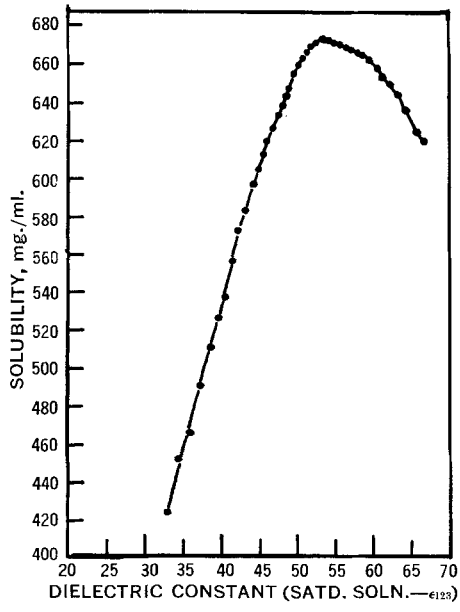

Fig. 2—The solubility of antipyrine in mg./ml. at 25° versus *the dielectric constant values of the respective saturated solutions.*

Since the dielectric constants and solubilities were known, it would then be possible to calculate the approximate change in the dielectric constant of the solvent system/mg. of solute added. Although it should be realized that the addition of 1 unit or mg. of a solute to a given solvent composition may not change the dielectric increment in a linear fashion when more units or mg. are added up to saturation, it may be assumed that on the average this linearity may be approximately correct.

In Tables I and II, the values for the dielectric constants of the binary solvent system and the saturated solutions are listed in columns 2 and 3. The dielectric increment, $\Delta\epsilon$, the solubilities, and the dielectric constant change/mg. of solute are listed in columns 4, 5, and 6.

In Table I, the data for antipyrine are given and several interesting observations can be made. Whereas antipyrine in pure water at a concentration of 620 mg./ml. decreases the dielectric constant substantially (11.8 units), the same concentration

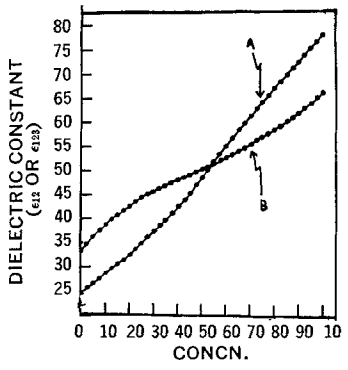

Fig. 4—A plot of the dielectric constants of the solvent mixture (curve A) and saturated solutions (curve B) of antipyrine versus the composition of the solvent mixtures.

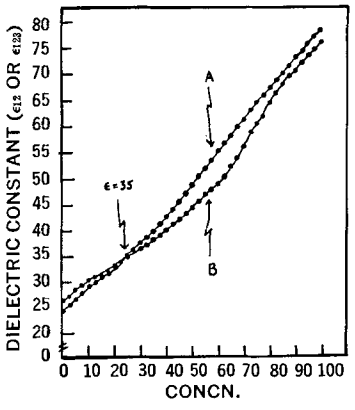

Fig. 5—A plot of the dielectric constants of the solvent mixture (curve A) and saturated solutions (curve B) of aminopyrine versus the composition of the solvent mixtures.

in 70% w/w ethanol–water raises the dielectric constant by 8.5 units.

This may indicate that at these high concentrations of solute, antipyrine and its inherent polarity dominates, and the effect would be one of altering or changing its environment to conform more closely to its own nature.

The dielectric constant increment/mg. of solute has been plotted *versus* the dielectric constant of the solvent system and is shown in Fig. 6 for antipyrine (A) and aminopyrine (B).

In Table II, the data for aminopyrine are given in the same fashion as above, and again there are several interesting observations. It can be seen that although the dielectric increment caused by aminopyrine in pure water is only −2.2 units, the dielectric increment/mg. is 0.042 units which is greater than twice the dielectric increment/mg. for antipyrine which has a value of only 0.019 units. One can also observe that the magnitude of solubility for antipyrine is almost 12 times that of aminopyrine, and thus, the effective dielectric increment of aminopyrine is about 24 times that of antipyrine. The dimethylamino group present in aminopyrine, while causing a diminution of solubility, may also,

TABLE I—SUMMARY OF THE DIELECTRIC CONSTANTS OF SATURATED SOLUTIONS OF ANTIPYRINE, THE DIELECTRIC INCREMENT (Δε), THE RESPECTIVE SOLUBILITY, AND THE DIELECTRIC INCREMENT/mg. SOLUTE

% w/w Ethanol	ϵ_{12}	ϵ_{123}	Δε	Sol., mg./ml.	Δε/mg.
0	78.5	66.7	−11.8	620	−0.019
10	73.2	62.3	−10.9	650	−0.017
20	67.5	58.7	−8.8	665	−0.013
30	61.7	55.8	−6.9	670	−0.010
40	55.4	53.1	−2.3	671	−0.003
50	49.0	50.4	+1.4	660	+0.002
60	42.8	48.1	+5.3	638	+0.008
70	37.5	46.0	+8.5	620	+0.014
80	32.7	42.4	+9.4	573	+0.016
90	29.0	38.7	+9.7	510	+0.019
100	24.3	33.0	+8.7	425	+0.021

TABLE II—SUMMARY OF THE DIELECTRIC CONSTANTS OF SATURATED SOLUTIONS OF AMINOPYRINE, THE DIELECTRIC INCREMENT (Δε), THE RESPECTIVE SOLUBILITY, AND THE DIELECTRIC INCREMENT/mg. OF SOLUTE

% w/w Ethanol	ϵ_{12}	ϵ_{123}	Δε	Sol., mg./ml.	Δε/mg.
0	78.5	76.3	−2.2	53	−0.042
10	73.2	70.7	−2.5	92	−0.027
20	67.5	63.9	−3.6	157	−0.023
30	61.7	56.2	−5.5	253	−0.022
40	55.4	49.2	−6.2	335	−0.019
50	49.0	44.5	−4.5	389	−0.012
60	42.8	40.9	−2.7	425	−0.006
70	37.5	36.6	−0.9	435	−0.002
80	32.7	33.2	+0.5	435	+0.001
90	29.0	29.8	+0.8	409	+0.002
100	24.3	26.4	+2.1	357	+0.006

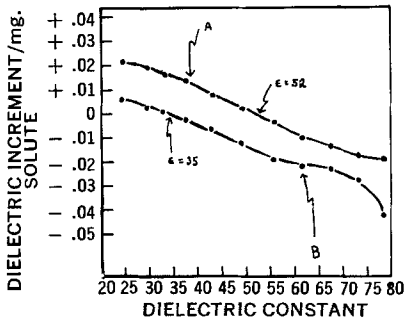

Fig. 6—A plot of the dielectric increment/mg. for antipyrine (curve A) and aminopyrine (curve B) versus the dielectric constant value for the solvent mixture.

because of spacial requirements "thrust" into the hydrogen bonded structure of water more effectively and cause the larger decrease in the dielectic constant. From a mechanistic point of view, one can conceive of the solute "melting," and the liquid produced being miscible with water to a certain extent. Thus, this second "liquid" should lower the dielectric constant of water depending upon its own magnitude. If it is assumed that aminopyrine as the second liquid has an approximate polarity value or dielectric constant of about 35, it should have a

more decided effect of lowering the dielectric constant of water relative to antipyrine which has a value of about 53–55. This would be analogous to the case of adding 2 solvents of differing polarity or dielectric constants. Therefore, methanol should be more effective in lowering the dielectric constant of water, having a dielectric constant value of 33, than glycerin with a value of about 43 at the same concentration. It can be presumed that aminopyrine may be operating through two mechanisms: the first by having an inherent polarity lower than antipyrine, and the second by the side chain thrust into the bonded structure of water.

Although antipyrine is more effective in reducing the dielectric constant of water and ethanol–water mixtures up to about 40% w/w ethanol, antipyrine is also far more effective in increasing the polarity or dielectric constant of ethanol and water–ethanol mixtures up to about 20% w/w water. The dielectric increment/mg. of solute has been summarized in Table III. In this case, the composition for the binary mixtures is given in the first column. The dielectric increment/mg. solute and the ratios of the increments, where their value is greater than unity, are also shown. It can then be seen that the polarity adjustment for any given solvent composition by a given solute probably depends on the inherent polarity of that solute.

Table III—Summary of the Dielectric Increments/mg. of Solute for Aminopyrine and Antipyrine, and the Ratios of Dielectric Increments/mg. in the Respective Solvent Mixtures

% w/w Ethanol	$\Delta\epsilon_1$ Aminopyrine	$\Delta\epsilon_2$ Antipyrine	$\frac{\Delta\epsilon_1}{\Delta\epsilon_2}$	$\frac{\Delta\epsilon_2}{\Delta\epsilon_1}$
0	−0.042	−0.019	2.2	...
10	−0.027	−0.017	1.6	...
20	−0.023	−0.013	1.8	...
30	−0.022	−0.010	2.2	...
40	−0.019	−0.003	6.3	...
50	−0.012	+0.002	...	...
60	−0.006	+0.008	...	...
70	−0.002	+0.014	...	...
80	+0.001	+0.016	...	16
90	+0.002	+0.019	...	9.5
100	+0.006	+0.021	...	3.5

Magnitude of Solubility—There are several ways in which the solubilities of these compounds in 2 solvent mixtures can be viewed. The solubility of antipyrine and aminopyrine in each solvent mixture at common dielectric constant values is shown in Tables IV and V. In this case, the solubilities of the solutes in mg./ml. for dioxane–water and ethanol–water is given in the first 2 columns. The last column shows the solubility ratio, the solubility of the solute in ethanol–water mixtures relative to dioxane–water mixtures.

In Table IV, the data for antipyrine illustrate that ethanol–water mixtures over the dielectric constant range of 35–75 are more efficient cosolvents but only to the extent of 5–10%. Furthermore, at dielectric constant values below 35, dioxane–water mixtures are more efficient cosolvents.

However, in Table V, the aminopyrine data show that ethanol–water mixtures possess far greater cosolvency than dioxane–water mixtures. This greater cosolvency is also seen to occur over the given range of dielectric constant values, 25–75. The increase in solubility caused by the ethanol–water cosolvents is seen to span a range of 22–230%.

This would then lead to the consideration of the ratio of solubility of antipyrine relative to aminopyrine in the 2 binary solvent systems studied. These data are shown in Table VI, where at various dielectric constant values the ratio of the solubility of antipyrine relative to aminopyrine is given for dioxane–water and ethanol–water mixtures. The

Table IV—Summary of the Solubility of Antipyrine in Dioxane–Water and Ethanol–Water Mixtures at Specified Dielectric Constant Values and the Ratio of Solubility in the Noted Solvent Mixtures

Dielectric Constant	Sol., mg./ml. Dioxane–Water (*A*)	Sol., mg./ml. Ethanol–Water (*B*)	Ratio *B/A*
24.3 (Ethanol)	556	424	0.76
30	573	537	0.94
35	583	610	1.05
40	592	633	1.07
45	603	645	1.07
50	605	662	1.09
55	606	667	1.10
60	610	671	1.10
65	613	666	1.09
70	616	658	1.07
75	619	641	1.04
78.5 (Water)	620	620	1.00

Table V—Summary of the Solubility of Aminopyrine in Dioxane–Water and Ethanol–Water Mixtures at Specified Dielectric Constant Values and the Ratio of Solubility in the Noted Solvent Mixtures

Dielectric Constant	Sol., mg./ml. Dioxane–Water (*A*)	Sol., mg./ml. Ethanol–Water (*B*)	Ratio *B/A*
24.3 (Ethanol)	287	370	1.29
27.5	276	401	1.45
30	264	428	1.62
35	242	435	1.80
40	216	434	2.01
45	191	413	2.16
50	165	378	2.30
55	141	333	2.36
60	120	277	2.31
65	100	195	1.95
70	80	119	1.49
75	63	77	1.22
78.5 (Water)	50	50	1.00

Table VI—Summary of the Solubility Ratio (Antipyrine/Aminopyrine) in Dioxane–Water and Ethanol–Water Mixtures

ϵ_{12}[a]	Solubility Ratio (Antipyrine/Aminopyrine) Dioxane–Water	Solubility Ratio (Antipyrine/Aminopyrine) Ethanol–Water
78.5	12.4	12.4
69.7	7.8	4.8
60.8	5.5	2.3
51.9	3.9	1.8
43.0	3.0	1.5
34.3	2.4	1.4
25.9	2.0	1.2

[a] Values of dielectric constants used from *Reference 1.*

far greater cosolvency effect of ethanol–water on aminopyrine is seen in the smaller ratios in this solvent mixture.

SUMMARY

It can be observed from Fig. 1 that in both cases the polarity of antipyrine is greater than aminopyrine by virtue of possessing both higher DR's and magnitude to solubility. Furthermore, the solubility curve for aminopyrine moves closer with respect to both DR and magnitude in the solvent mixture of higher polarity. It would be interesting to note how these differences in DR and magnitude would change by the use of more polar binaries such as methanol–water mixtures and less polar binaries such as propanol–water mixtures.

It may be assumed for this particular pair of solutes in the systems studied that from the differences in the position of the DR's and the concomitant magnitude that antipyrine is inherently more polar than aminopyrine. This is further substantiated by the characterization of solute polarity from dielectric constant studies of saturated solutions.

The rather obvious noncorrelation of DR in the 2 solvent systems for aminopyrine and the vague correlation in the case of antipyrine may imply that the concept of DR agreement, irrespective of the solvent system, is suspect.

Although previous publications (4, 5) had assumed that this concept was true, and at times tenuously true, it is now reasonable to postulate that the solubility profiles and respective DR(s) may be considered unique unto themselves in any given solvent system, a situation previously reported (6–9).

It is felt that this does not obviate either the usefulness or application of this knowledge, and that the cosolvency phenomena relative to dielectric constants has been amply illustrated. It is hoped that continuing studies on solutes of pharmaceutical interest relative to the above points will allow amplification and reinterpretation of past experiences in this area.

Presently, the author's research interests are concerned with the parabens and barbiturates, and these will be the subject of future communications.

REFERENCES

(1) Paruta, A. N., and Irani, S. A., *J. Pharm. Sci.*, **54**, 1334(1965).
(2) Paruta, A. N., and Sheth, B. B., *ibid.*, **55**, 896(1966).
(3) Paruta, A. N., *Am. J. Pharm.*, **137**, 138(1966).
(4) Paruta, A. N., Sciarrone, B. J., and Lordi, N. G., *J. Pharm. Sci.*, **53**, 1349(1964).
(5) *Ibid.*, **54**, 1325(1965).
(6) Krause, G. M., and Cross, J. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 137(1951).
(7) Peterson, C., and Hopponen, R. E., *ibid.*, **42**, 541 (1953).
(8) Moore, W. E., *ibid.*, **47**, 855(1958).
(9) Gorman, W. G., and Hall, G. D., *J. Pharm. Sci.*, **53**, 1017(1964).

Pharmaceutical Heterogeneous Systems I

Hydrolysis of Aspirin in Combination with Tablet Lubricants in an Aqueous Suspension

By S. S. KORNBLUM and M. A. ZOGLIO

The chemical mechanism that explains the accelerated hydrolysis demonstrated by aspirin when in combination with an alkali stearate, *i.e.*, calcium stearate, in a solid dosage form has been elucidated by employing a suspension technique. The mechanism primarily involves a reaction of the alkali cation with aspirin in a solution to form a salt of aspirin which, in the presence of solvated aspirin, comprises a buffer system at a pH detrimental to the stability of aspirin. Using the kinetic data obtained from the suspension study, an attempt was made to predict aspirin stability in a solid dosage form. There is evidence presented to support the concept that stearic acid will serve as a medium for aspirin degradation at temperatures slightly above room temperature.

PREVIOUS RESEARCHERS (1–4) studying the hydrolysis of aspirin in either a homogeneous or heterogeneous aqueous system have demonstrated that decomposition is a function of water content, pH, and temperature. An aspirin tablet can be identified as a heterogeneous system. One may compare an aspirin tablet with an aqueous aspirin suspension in order to facilitate an explicit definition of the hydrolysis phenomena. The effect of antacids in mixtures with aspirin has been studied in the presence of moisture and has been demonstrated to increase decomposition rate (5, 6). Some substances employed as tablet lubricants can effectively act as antacids, *e.g.*, stearate salts. The purpose of this study was to evaluate the commonly used tablet lu-

Received April 14, 1967, from the Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936
Accepted for publication August 8, 1967.
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(5) *Ibid.*, **54**, 1325(1965).
(6) Krause, G. M., and Cross, J. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 137(1951).
(7) Peterson, C., and Hopponen, R. E., *ibid.*, **42**, 541 (1953).
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bricants as to their effect on the stability of aspirin and define the chemical mechanisms that lead to an accelerated hydrolysis in mixtures of aspirin and alkali stearates.

EXPERIMENTAL

Aqueous Aspirin Suspensions—Mixtures of equal quantities (200 mg.) of aspirin crystals (USP) and various tablet lubricants: talc, stearic acid, hydrogenated vegetable oil,[1] calcium stearate, magnesium stearate, and aluminum stearate were prepared. Excess lubricant was employed in an attempt to insure saturation throughout the experiment. The weighed powders were transferred into glass vials of 18.5 ml. capacity and exactly 10 ml. distilled water was added to form a suspension. To prevent leakage the vials were sealed with aluminum foil before securing with Bakelite caps. The vials were then attached to a rotating circular disk in a constant-temperature bath which permitted continuous agitation at 30°. Appropriate aliquots were withdrawn at various time intervals for pH and free salicylic acid determinations. The amount of free salicylic acid formed was determined by adding to an exact volume of the suspension filtrate 4 ml. of 2% ferric chloride solution with subsequent dilution to 100 ml. with distilled water. Exactly 3 min. was permitted for the color development before reading at 540 mμ employing the Perkin-Elmer 202 spectrophotometer. The validity of the salicylic acid assay was demonstrated by adherence to the Beer-Lambert law over the concentration range studied. pH values were obtained by using a Metrohm E 300.

Determination of Aspirin Species in Suspension Filtrate—Total aspirin content was determined by withdrawing a 1-ml. aliquot of the suspension filtrate and diluting to 100 ml. with a 0.2 *M* sodium acetate–acetic acid buffer solution (pH 6.09). The spectrophotometric reading was taken at 268 mμ.

Calcium aspirin content was determined by withdrawing a 5-ml. aliquot of the suspension filtrate and diluting with distilled water to 150 ml. The pH of this solution was adjusted to 12.0–12.5 using 1 *N* sodium hydroxide solution. Hydroxynaphthol blue A.R. was employed as the indicator in a 0.2–0.3 Gm. quantity. The solution was titrated with 0.05 *M* disodium EDTA solution to a clear blue color. Each milliliter of 0.05 *M* disodium EDTA solution is equivalent to 2.0064 mg. of calcium ion.

Free aspirin contained in the suspension filtrate was calculated from the following equation:

$$\text{free aspirin} = \text{total aspirin} - \text{aspirin as calcium aspirin}$$

Effect of Reducing the Water Content in an Aqueous Aspirin–Calcium Stearate Suspension—A study was performed in order to establish the effect of decreasing the water content of the suspension from 10 to 0.1 ml. Below 1 ml. glass beads were placed in the reaction vials to insure uniform agitation. When less than 5 ml. was employed, the suspension was diluted at the time of withdrawal for analysis to contain 10 ml. of water.

Effect of Stearic Acid on Aspirin Degradation in a Solid Mixture Study—Aspirin crystals were dissolved in molten stearic acid USP in a 1:80 ratio at 75°. Equal quantities of the melt were transferred into glass vials and tightly sealed. The melt was quickly cooled by immersion in an ice bath. Samples were then kept in an oven at 40 ± 0.5° and routinely assayed after exact weighing of approximately 800 mg. of the solidified melt. The solidified melt was dissolved and diluted with chloroform to a volume of 200 ml. The solution was spectrophotometrically read in the UV at 310 mμ.

Aspirin crystals containing approximately the same amount of moisture as the stearic acid melt were stored at 40° and used as a control. Moisture present in the samples was determined by Karl Fischer titration. The analytical method employed for the aspirin crystals is outlined in the next section.

Aspirin–Stearic Acid Capsule Study—The following samples were prepared and stored at room temperature, 40°, and 50°: (*a*) aspirin–stearic acid powder blend (20:1 ratio), aspirin–stearic acid capsules[2] (20:1 ratio), (*c*) aspirin crystals, 40 mesh, (*d*) aspirin capsules.[2]

The samples were assayed for salicylic acid after dissolving a quantity equivalent to 200 mg. of aspirin in 10 ml. of chloroform. The solution was transferred to a diatomaceous earth[3] column which had been previously moistened with 2% ferric chloride solution. The column was washed with chloroform until the purple zone migrated to the lower portion of the column. These washings were discarded. Subsequently the column was washed with 10 ml. of 10% acetic acid–chloroform followed by 1% acetic acid–chloroform until the purple zone was eluted. These washings were concentrated to 50 ml. and read spectrophotometrically at 310 mμ. Moisture content of the samples studied was determined by Karl Fischer titration.

Aspirin Degradation in Aspirin–Calcium Stearate Tablets—Tablets were manufactured containing aspirin crystals and calcium stearate (1:1 ratio). The tablets were stored at 30° for 60 days. At the time of assay the tablets were comminuted and a quantity of 400 mg. of the powder was dissolved in 20 ml. chloroform and mixed for less than 1 min. This was rapidly passed through a Millipore membrane into a separator and aqueous washings followed to remove any calcium aspirin. The chloroform solution was passed through a diatomaceous earth column. From this point the analysis proceeds as described under *Aspirin–Stearic Acid Capsule Study*.

RESULTS AND DISCUSSION

The kinetics observed for the hydrolysis of aspirin in the presence of tablet lubricants is depicted in Fig. 1. The reaction rates appear to follow a zero-order reaction law with the exception of the initial rates for the aspirin–calcium stearate and the aspirin–magnesium stearate suspensions. The linear portion of the decomposition curves for each of the aspirin–lubricant combinations was accompanied by a relatively static hydrogen ion concentration for the 30-hr. period studied. A summary of pH values and reaction rate constants for the apparent zero-

[1] Sterotex. Capitol City Products, Columbus, Ohio.

[2] No. 3 Parke-Davis pink capsules were used.

[3] Celite 545. Johns-Manville Corp., New York, N. Y.

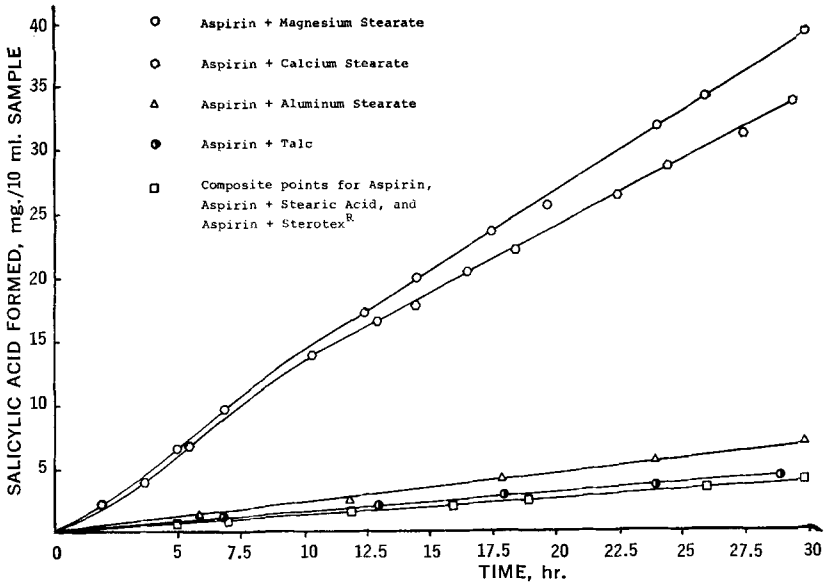


Fig. 1—Aspirin hydrolysis at 30° in aqueous suspensions containing tablet lubricants.

order degradation is given in Table I. The order of magnitude of the reaction rates for aspirin–calcium stearate and aspirin–magnesium stearate suspensions suggested that something other than saturation solubility had manifested the relatively high pH during decomposition. The possibility existed that perhaps a reaction between aspirin and the stearate salt had taken place. If so, then a high concentration of magnesium or calcium aspirin could be expected in solution. The aspirin salt would exert a buffering effect in combination with aspirin in the solvate portion of the suspension. In order that this theory could be verified the quantity of calcium transferring into solution was followed as a function of time during the 30-hr. reaction period. Figure 2 demonstrates that the quantity of calcium ion in solution very quickly exceeds its aqueous saturation solubility (0.02 mg./ml.) in the presence of aspirin and rises radically during the initial 3-hr. period. The period of constant pH is established

TABLE I—PERTINENT DATA FOR ASPIRIN SUSPENSIONS STUDIED[a]

Compn.	Constant pH (Arithmetic Mean)[b]	Apparent Zero-Order Rate Constant, mg. of Free Salicylic Acid/hr.
Aspirin	2.60	0.123
Aspirin + stearic acid	2.62	0.133
Aspirin + hydrogenated vegetable oil	2.68	0.123
Aspirin + talc	2.71	0.133
Aspirin + aluminum stearate	3.16	0.281
Aspirin + calcium stearate	3.75	0.986
Aspirin + magnesium stearate	4.14	1.314

[a] All aspirin suspensions contained exactly 10 ml. distilled water. [b] The arithmetic mean has been determined from a minimum of 6 pH values.

during the first 10 hr. in the aspirin–calcium stearate suspension studied and is thereafter followed by a relatively unchanging calcium ion concentration. The information obtained supports the existence of a buffer system composed of calcium aspirin and aspirin which appears to be controlling the pH from the 10th to 30th hr. of the reaction. Figure 3 establishes the fact that there does exist approximately a 2:1 ratio of calcium aspirin to aspirin in the buffer system. The amount of aspirin shown in Fig. 3 at the 5th hr. is representative of the saturation solubility for aspirin at 30° in the suspension system. This concentration of aspirin is henceforth maintained throughout the 30-hr. time interval studied.

The initial S-shaped portion of Fig. 1 for the aspirin–calcium stearate and aspirin–magnesium stearate systems can be explained by analyzing the data for pH (Fig. 4) and species of aspirin in solution (Fig. 3) *versus* time. Figure 4 depicts a sharp increase in pH for the aspirin–calcium stearate system which then rises to a peak after about 3 hr. and then reverses to establish a constant pH. Observation of Fig. 3 makes it possible to explain the initial rise in pH as a result of the rapid formation of the calcium salt of aspirin. However, the dissolution rate of aspirin falls short of maintaining the saturation of aspirin during the initial stage of the salt-forming reaction. After 1.5 hr. the concentration of aspirin diminishes thus allowing the pH of the solution to be further elevated by the predominance of calcium aspirin. When the reaction has progressed for 3.5 hr., the dissolution rate of aspirin catches up with the utilization of aspirin in the salt formation. By virtue of the lower saturation pH of aspirin in solution (pH = 2.66), as compared with calcium aspirin in solution (pH = 4.2), the system stabilized to a buffer pH at 3.75 and remained constant over the 30-hr. period studied.

Experiments were performed where the amount of water employed in the suspensions was reduced in order to approach that found in a solid dosage

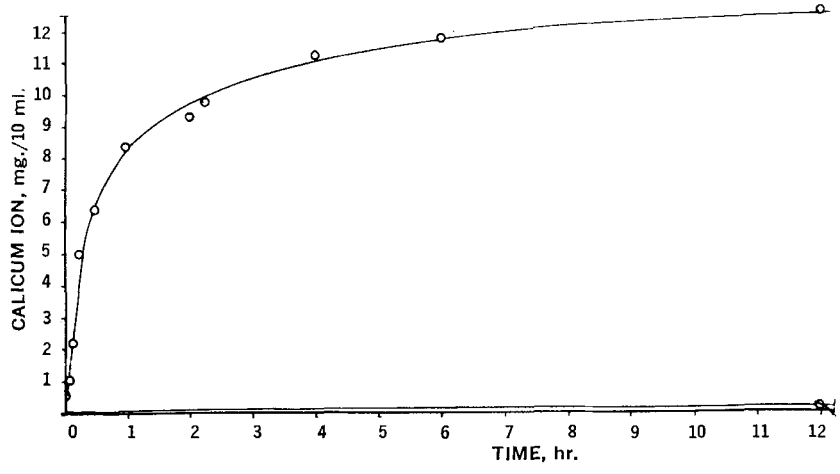


Fig. 2—Calcium ion concentration in solution during the initial segment of the aspirin–calcium stearate suspension study.

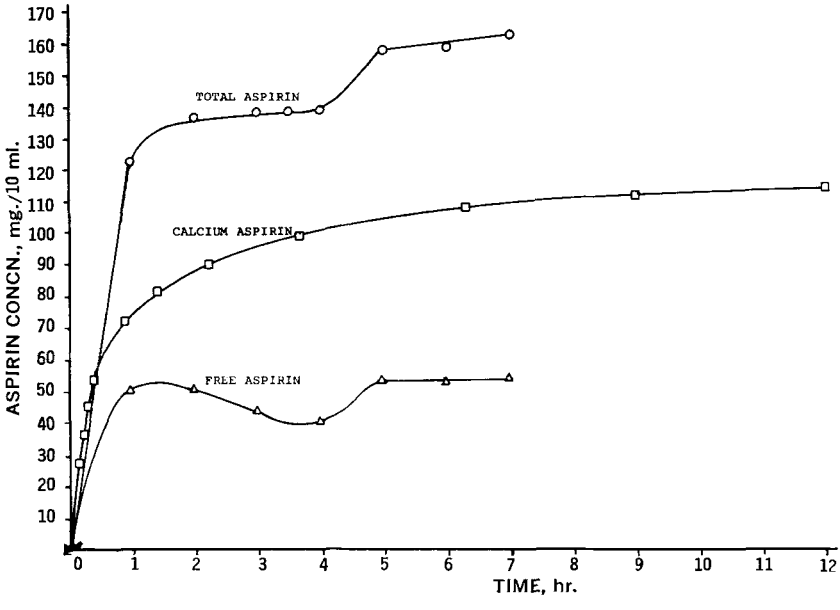


Fig. 3—Species of aspirin in solution during the initial segment of the aspirin–calcium stearate suspension study.

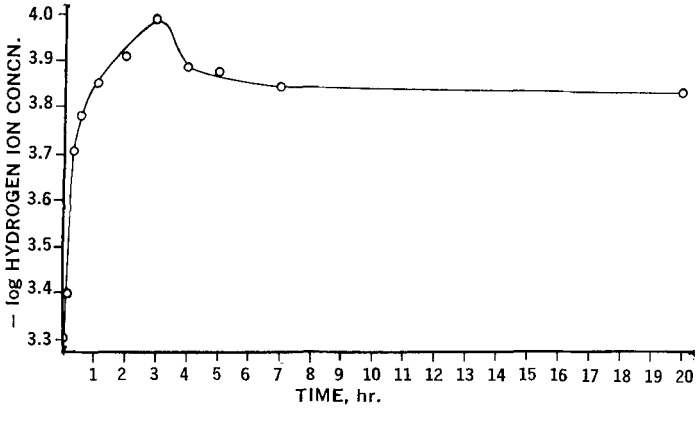


Fig. 4—pH changes occurring during the aspirin–calcium stearate suspension study (each sample contained 10 ml. water).

form. The ultimate aim was the achievement of data that would permit subsequent extrapolation to the tablet or capsule dosage form. Zero-order rate constants for the aspirin-calcium stearate aqueous suspensions were determined as a function of water content, and the data have been plotted in Fig. 5. The curve fortunately appears to be linear in the region of low water content enabling

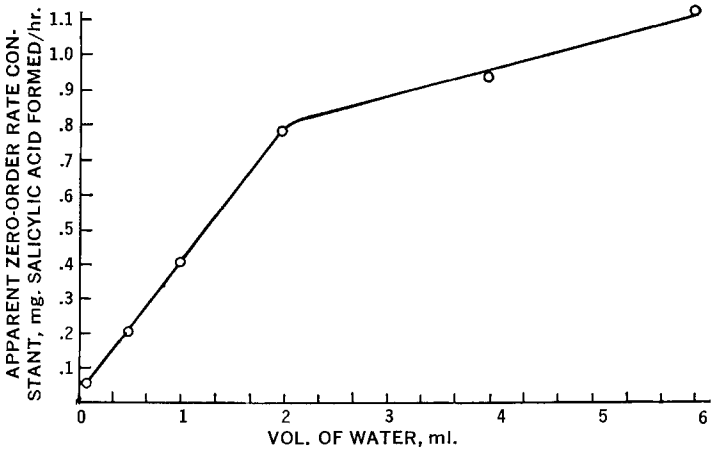


Fig. 5—Relationship of the rate of salicylic acid formation to the volume of water contained in the aspirin–calcium stearate suspension

a correlation with the solid dosage form data. The change of slope above 2 ml. is most likely due to insufficient amounts of calcium aspirin in the system to accomplish saturation. When the aqueous solubility of calcium–aspirin (0.165 Gm./ml.) is used to estimate the volume of water at this change in slope, 0.8 ml. would be indicated. The higher value in Fig. 5 indicates the solubility of calcium aspirin to be considerably lower in the system studied. Table II shows that the pH of the suspensions possessing minimal water content ascends until the pH reaches the saturation pH of calcium aspirin which is 4.2 at 2 ml. It is assumed that the linearity experienced at volumes less than 2 ml. is a result of the saturation of the water with calcium aspirin and the maintenance of the pH observed at 2 ml. The predictive aspects of the results are limited, since the reactions were only studied for a 30-hr. period. From observing Fig. 6, one can see that the pH for a period longer than 30 hr. is not a constant as was true in the suspensions studied. As the degradation products accumulate, the pH declines, until after 8 days a constant pH does exist. The data from Fig. 5 prove valuable in predicting maximum decomposition to be expected in a solid dosage form. This is true even though the reaction rate studies with the suspensions were conducted at pH values more detrimental to aspirin stability than those observed with an extended study (Fig. 6). Aspirin–calcium stearate (1:1 ratio) powder blend and tablets of the same composition were prepared. Both were stored for a 2-month period at 30°.

TABLE II—CONSTANT pH ESTABLISHED FOR ASPIRIN SUSPENSION CONTAINING CALCIUM STEARATE WHEN REDUCING THE WATER CONTENT

Compn.	Water Content, ml.	Constant pH (Arithmetic Mean)
Aspirin + calcium stearate (200 mg. each)	10	3.75
	6	3.91
	4	4.04
	2	4.19

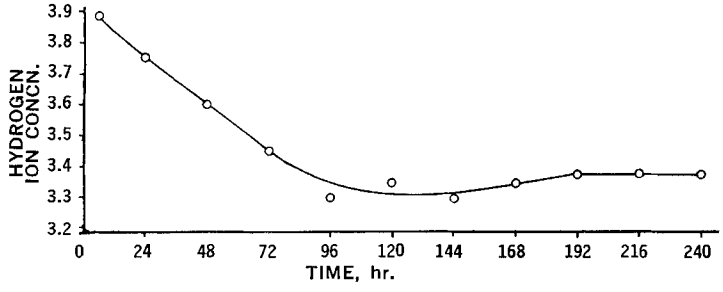


Fig. 6—pH changes occurring during an extended aspirin–calcium stearate suspension study (each sample contained 10 ml. water).

TABLE III—ASPIRIN DEGRADATION IN ASPIRIN–CALCIUM STEARATE TABLETS

Sample	Actual Deg.	Deg. Predicted from Extrapolation of Results from Fig. 5
Aspirin–calcium stearate tablet, 1:1 wt. ratio (0.0843% water)	2.25 mg. of salicylic acid formed/ 200 mg. of aspirin	1.94 mg. of salicylic acid formed/ 200 mg. of aspirin
Aspirin–calcium stearate powder mixture, 1:1 wt. ratio (1.05% water)	2.49 mg. of salicylic acid formed/ 200 mg. of aspirin	2.42 mg. of salicylic acid formed/ 200 mg. of aspirin

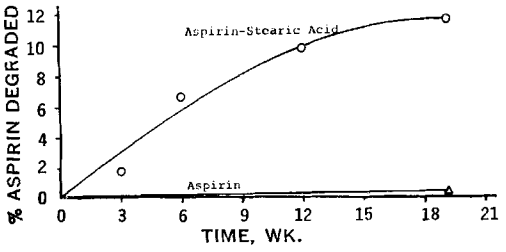


Fig. 7—Degradation of aspirin–stearic acid. Solid mixture (1:80 ratio) at 40°.

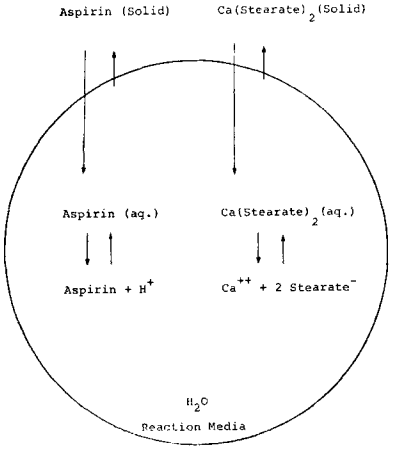


Fig. 8—Solution of aspirin and stearate salt.

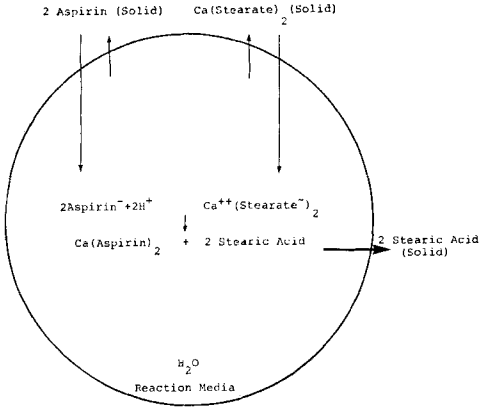


Fig. 9—Calcium aspirin formation.

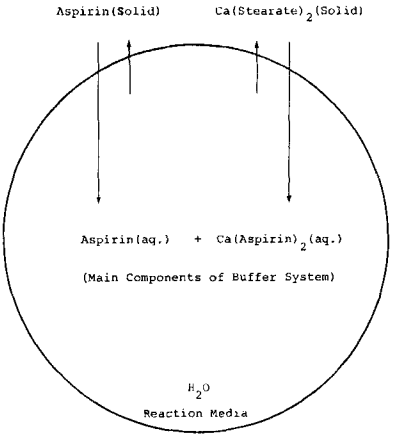


Fig. 10—Buffer system.

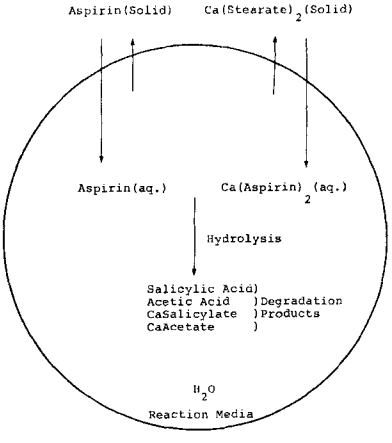


Fig. 11—Aspirin hydrolysis.

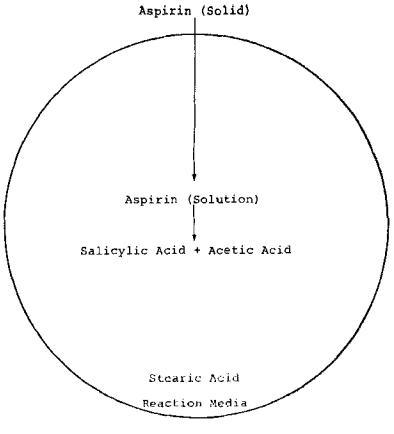


Fig. 12—Anhydrous aspirin degradation in the presence of stearic acid at 40°.

Table III reveals that data obtained from suspension studies can be satisfactorily compared with solid state information, thus enabling one to predict the extent of decomposition in the solid state from suspension data.

The data provided in Figs. 1 through 6 and Tables I and II substantiate the explanation, (Figs. 8–11), of the mechanism involved for the aspirin–calcium stearate system. The presence of moisture in the dosage form results in a solution of the aspirin and the stearate salt as illustrated in Fig. 8 (Figs. 2 and 3). In Fig. 9 calcium stearate and aspirin are shown reacting to form calcium aspirin and stearic acid (Figs. 2 and 3). Initially the buffer system (Fig. 10)

TABLE IV—ASPIRIN–STEARIC ACID CAPSULE STUDY

Aspirin + Stearic Acid (20:1) Moisture Content: 0.10%					
Powder			Capsules		
Free Salicylic Acid Content			Free Salicylic Acid Content		
22°C.	0.092%	(83 days)	22°C.	0.093%	(83 days)
40°C.	0.130%	(30 days)	40°C.	0.170%	(30 days)
50°C.	0.409%	(30 days)	50°C.	1.035%	(30 days)
Aspirin (Pure) Moisture Content: 0.10%					
Powder			Capsules		
Free Acid Salicylic Content			Free Salicylic Acid Content		
22°C.	0.082%	(86 days)	22°C.	0.092%	(86 days)
40°C.	0.068%	(30 days)	40°C.	0.158%	(30 days)
50°C.	0.160%	(30 days)	50°C.	0.200%	(30 days)

is mainly composed of calcium aspirin and aspirin which establishes a pH value detrimental to aspirin stability. In Fig. 11 the calcium–aspirin and aspirin in solution are shown undergoing hydrolysis (Fig. 1). The decomposition products being more acidic in nature than the aspirin–calcium aspirin system eventually overcome the capacity of the buffer system and lower the pH (Fig. 6). Again the pH levels at a lower value than the initial buffer pH but is still detrimental to aspirin stability.

The evaluation of the lubricants from the results in Fig. 1 indicate stearic acid and hydrogenated vegetable oil to be suitable for use in aspirin tablets. Although stearic acid does not demonstrate a pH effect, it apparently can serve as a medium for aspirin decomposition (Fig. 12). This can be demonstrated by comparing the stability of aspirin with aspirin–stearic acid in powder and capsule forms that have been stored at 22°, 40°, and 50° (Table IV).

Free salicylic acid content is significantly higher in the capsules than in the powder, most likely as a result of the water content in the gelatin. Figure 7 shows the degradation effect more dramatically when aspirin is suspended in molten stearic acid and stored at 40°.

CONCLUSION

From the kinetic data obtained with the aspirin–lubricant combinations it appears feasible that the suspension technique employed may be useful in solid dosage form stability predictions, lubricant evaluation, and as a basic method for elucidation of the chemical mechanism for other solid state decomposition.

Figure 1 illustrates the relative acceptability from a stability standpoint of tablet lubricants for combination with aspirin. The order is as follows: hydrogenated vegetable oil, stearic acid, talc, and aluminum stearate. This study has revealed that stearate salts should be avoided as tablet lubricants, if the active component is subject to hydroxyl-ion catalyzed degradation and if there is contained in the dosage form an ingredient which can react with the stearate salt to form a soluble basic species. Aluminum stearate appears to be the most suitable of the alkali stearate salts for combination with aspirin. This seems to be a result of the poor water solubility of aluminum aspirin thus limiting the total aspirin present in solution. Aside from the pH effect, the major factor influencing degradation when aspirin is combined with calcium or magnesium stearate in suspension is the high solubility of calcium aspirin and magnesium aspirin in an aqueous medium.

Talc possesses trace amounts of oxide impurities that affect the pH of the suspension initially, thus causing a slight increase in the amount of salicylic acid formed. However, this does not influence the over-all reaction rate (Table I). Talc can be used with mixtures of stearic acid and hydrogenated vegetable oil, but is not usually employed solely as a tablet lubricant due to its low ratio of efficiency.

Stearic acid appears to be suitable from the suspension studies. However, it has been noted that stearic acid may function as a medium for aspirin degradation at temperatures slightly above room temperature.

REFERENCES

(1) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723(1950).
(2) Leeson, L. J., and Mattocks, A. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 329(1958).
(3) Kubo, F., Imaoka, K., and Kaneko, A., *Kyoritsu Yakka Daragaku Nempo*, **617**, 1(1961–1962).
(4) Lippman, I., and Mattocks, A. M., Reprint of Symposium Papers, APHA, Las Vegas meeting, 1962.
(5) Nazareth, M., and Huyck, C., *J. Pharm. Sci.*, **50**, 608(1961).
(6) Kral, J., Breleszova, H., and Drozkova, Z., *Farm. Obzor.*, **29**, 298(1960).

Kinetics and Mechanisms of Action of Drugs on Microorganisms VII

Quantitative Adherence of Sulfonamide Action on Microbial Growth to a Receptor-Site Model

By EDWARD R. GARRETT and OLGA K. WRIGHT

The lag time between sulfonamide addition to a balanced growth culture of *Escherichia coli* B/r and the observation of action on microbial growth is 5.5 ± 0.5 generations. It is independent of temperature and the degree of growth inhibition by simultaneously added chloramphenicol. The apparent first-order growth rate constants of *E. coli* in the subsequent drug-affected steady state can be rigorously defined as $k_{app.} = k_0 - k_0K_1K_2S/(1 + K_1K_2S)$ where k_0 is the rate constant in the absence of a sulfonamide concentration, S; K_1 is the steady-state equilibration of sulfonamide between the nutrient medium and the biophase; K_2 is the drug-receptor site association constant; and k_0 and K_1K_2 have been defined as functions of absolute temperature. The death rate of completely sulfonamide-inhibited organisms is independent of sulfonamide concentrations. The ΔH values of $1/K_1K_2$ differ for sulfisoxazole (15.9 Kcal./mole), sulfathiazole (4.3 Kcal./mole), and from the k_0 (24 Kcal./mole). The techniques provide sensitive and reliable estimates of differences in sulfonamide activities. The derived $1/K_1K_2$ values for sulfisoxazole are directly proportional to the growth rate constant in its absence but in the presence of rate-inhibiting chloramphenicol. This indicates that the numbers of receptor sites available for sulfonamide action are directly proportional to the microbial growth rate. The apparent first-order growth rate constant of *E. coli* in the presence of simultaneously added sulfonamide (S) and chloramphenicol (C) concentrations can be rigorously defined as $k_{app.} = [k_0' - k_cC][1 + K_1K_2(S - 1)]/(1 + K_1K_2S)$, where k_0' is the growth rate constant in the absence of chloramphenicol and sulfonamide and k_c is the determined inhibitory constant for chloramphenicol. Variations of sulfonamide concentrations after the lag phase demonstrate that reversibility exists and the ultimate $k_{app.}$ is in accordance with the cited predictive equation. Dilution of organisms but maintenance of sulfonamide concentrations maintains the steady-state growth rate. Addition or dilution of sulfonamide concentrations show a time-dependent re-equilibration. These facts are consistent with a competitive receptor site model for sulfonamide and a generation rate-dependent metabolite which produces modified metabolites that are stored during drug-free balanced growth.

THE VARIATION of the time-course of drug distribution in a complex organism when considered with the concomitant time-course of pharmacological activity can give insight into the properties and nature of the biophase in contact with the drug receptors. Operational models of the complex biological organism are needed to correlate drug–receptor interaction and availability at sites of action. Multicompartmental models can be established to consider and quantitatively define the kinetics of drug absorption, distribution, and biological effectiveness (1). If dosage regimens are varied, deduced compartmental capacities and rate-limiting parameters of the drug distributive processes that result in observed biological activities may functionally define the transport processes and the affinity constants of drug–receptor interaction. Unfortunately, valid quantitative and continuous measurements of pharmacological activity in a complex organism are difficult to obtain.

This paper describes the pharmacokinetic approach to the study of the action of sulfonamides on microbial growth rates in a highly reliable and reproducible system. The kinetic dependencies will be defined as functions of sulfonamide concentrations, variations in such concentrations, temperatures, and numbers of organisms, and will be related to receptor site concepts. Similar studies have been given previously with respect to the action of the chloramphenicols and the tetracyclines on microbial growth rates (2–8).

Kohn and Harris (9, 10) initiated some studies of a kinetic nature on sulfonamide action on microbial growth and made many astute observations from technologically limited data. The variability of pH observed was undesirable since sulfonamide transport and microbial growth rates are claimed to be functions of pH (11, 12).

Received April 6, 1967, from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication August 3, 1967.
Presented in part to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The technical assistance of George L. Perry and rewarding discussions with Professor Arthur L. Koch are acknowledged.

It is not clear from their cited evidence (9, 10) if the inoculum was in balanced growth (13, 14) so that the organisms would be statistically distributed among the phases of the growth cycle. If this were not so, it is difficult to dissociate sulfonamide action on microbial growth from accelerating rates of microbial growth in the transition from the induction period to the logarithmic growth phase (2). A rigorous rate constant dependence on sulfonamide concentration was not demonstrated (9, 10).

Modern technological advances with the Coulter counter permit rigorous kinetic analyses of large amounts of data of high reliability and the obtaining of numbers of total intact organisms, live and dead. These can be compared with the numbers of viable organisms obtained by colony count techniques. Coincidence of such numbers of total and viable counts during antibacterial action clearly defines inhibition (5–8).

MATERIALS AND METHODS

The *Escherichia coli* B/r strain was used as the test organism throughout the investigation. The sodium sulfathiazole (Nutritional Biochemicals Corp., Cleveland, Ohio) and sulfisoxazole (Hoffmann-LaRoche Laboratories, Nutley, N. J.) were the drugs studied.

Media—The culture media (15) consisted of 7.00 Gm. K_2HPO_4, 3.00 Gm. KH_2PO_4, 2.0 Gm. glucose, 1.0 Gm. $(NH_4)_2SO_4$, 0.50 Gm. sodium citrate, 0.10 Gm. $MgSO_4 \cdot 7H_2O$, 10.0 Gm. vitamin-free casamino acids (Difco Laboratories, Detroit, Mich.), and 1 L. of distilled deionized water. This medium was filtered twice through a 0.45-mm. Millipore filter and autoclaved at 15 lb. of pressure for 20 min. The pH was 6.90 and did not change throughout the studies. This medium was optimum for the studies in that the time for each study was minimized and the pH maintained constancy throughout the measured growth range. When an inorganic salt–glucose medium was used, the rates of microbial growth were too slow for convenient kinetic studies.

Growth—Replicate slants were prepared from a single colony of *E. coli*, strain B/r, that was isolated from a culture on an agar plate. These slants were frozen and a separate one was used for each experiment. A broth culture was allowed to grow at 37.5° for 16 hr. A sample was then diluted tenfold in fresh broth and the growth was followed by the Coulter counter, model B, (Coulter Electronics Co., Hialeah, Fla.) until it reached an organism concentration of 1×10^8 organisms/ml. in the logarithmic growth phase. Samples were diluted into 5 or 6 replicate 50-ml. volumes of fresh broth in 125-ml. loosely-capped conical flasks to achieve the desired organism concentration. The replicate cultures were maintained at a constant temperature in a 50-gal. constant-temperature water bath equipped with a shaker.

Total Count Method—Appropriate small volume aliquots of each culture were transferred at 40-min. intervals to sterilized vials containing 1 drop of formaldehyde to kill the organisms (5). The samples were appropriately diluted with properly filtered and sterilized 0.9% saline solution so that the total organism count per 50 μl. would not exceed 35,000. The coincidence error was thus less than 0.5% on the Coulter counter model B equipped with a 30-μ orifice. The mean of 3 total counts was obtained on each sample (5–7).

The Coulter counter with this orifice size is able to count particles above 0.5 μ in diameter without significant interference from background noise (Coulter Counter Manual, model B, 4th ed., August 1966). There are negligible numbers of *E. coli* with a volume less than 0.5 μ^3 (14) or a diameter less than 1 μ (16) at our generation rates. Operational counting of *E. coli* has been clearly shown to be effective (5, 14) and in agreement with colony counts. This was also true in the many instances where only bacterial growth inhibition was manifested (5–8).

Viable Count Method—The growing culture, 0.5 ml., was diluted appropriately with 0.9% saline so that 60 to 100 colonies per plate would result. One milliliter of this solution was pipeted onto each of 5 replicate agar plates. The plates were incubated for 48 hr. at 37.5° and the colonies were counted (3–5). Bacto antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) was used for broth cultures and plate counts.

Effect of Drug Concentration on Growth Rates—Six 49-ml. volumes of fresh broth in the constant-temperature baths at 37.5° were inoculated with 1-ml. aliquots of appropriately diluted culture growing in the logarithmic growth phase. The organism population was 10^4 organisms/ml. The cultures were allowed to grow for 80 min. to a population of 10^5 organism/ml. At 80 min., 0.5 ml. of appropriately diluted drug was added to the cultures to produce final concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, and 20.0, 50.0, 75.0, and 100.0 mcg./ml. Samples

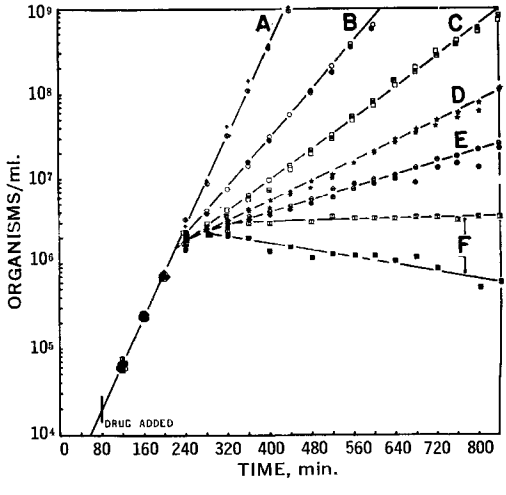


Fig. 1—Typical generation rate curves of E. coli *B/r at 37.5° in the presence of various concentrations of sulfisoxazole. The curves and mcg./ml. concentrations of sulfisoxazole were, respectively:* A, *0.0;* B, *1.0;* C, *2.0;* D, *3.0;* E, *4.0;* F, *20.0. Both viable and total counts were coincident except for the curves* F *where the viable counts (lower curve,* F*) diverged from the total counts (upper curve,* F*).*

were taken every 40 min. and were counted by the Coulter counter method as well as by the method of colony counts (Fig. 1). Sulfisoxazole was tested by both methods. Sodium sulfathiazole was tested only by total counts. A control culture without added drug was also studied to determine the growth rate in the absence of drug.

Effect of Organism Population on Drug-Affected Growth Rates—A series of experiments was conducted at 37.5° in order to check the effect of organism population at the time of drug addition on the bacteriostatic action of sodium sulfathiazole. For each experiment 6 replicate 49-ml. volumes of broth were inoculated with 1 ml. of appropriately diluted culture growing in its logarithmic phase. Experiments were run where the zero time populations were 10^2, 10^3, 10^4, and 5×10^4 organisms/ml. At 80 min. after inoculation, sodium sulfathiazole (0.5 ml. of appropriately diluted master solution) was added to 50 ml. of the cultures to achieve final concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mcg./ml. One of the 6 replicates had no drug. The organism populations at the time of drug addition were, 10^3, 10^4, 10^5, and 5×10^5 organisms/ml. Samples were taken every 40 min. and the total count per milliliter was obtained on the Coulter counter.

Reversibility of Drug Action—A 100-ml. volume of broth in a 125-ml. conical flask was inoculated with *E. coli* to a concentration of 10^3 organisms/ml. (curve A in Fig. 2). The culture grew logarithmically at 37.5° for 80 min. to a population of about 10^4 organisms/ml. For this and the subsequent variations unless specified otherwise, samples were analyzed for total Coulter count every 20 min. At 80 min. a 1.0-ml. aliquot of an appropriately diluted solution (in fresh broth) of sulfisoxazole was added to the culture to give 1.0 mcg./ml. (curve B) and 5.0 mcg./ml. (curve C). At 340 min., after the culture of curve B (Fig. 2) had well-settled to a new logarithmic growth, 25 ml. of this culture was transferred to a sterile 125-ml. conical flask containing enough sulfisoxazole to increase the drug concentration to 5 mcg./ml. (curve D). The growth of this new culture (curve D, Fig. 2) was followed by total Coulter counts every 40 min. for 960 min.

At 280 min., when the culture of curve C (Fig. 2) growth curve was emerging from the lag period and had not yet reached the new steady state of logarithmic growth, a 5-ml. aliquot of the culture was diluted with 20 ml. of fresh broth in a 125-ml. flask (curve E, Fig. 2).

At 600 min. two 5-ml. aliquots of the culture treated with 5 mcg./ml. sulfisoxazole at 80 min. (curve C, Fig. 2) were diluted with 20 ml. of fresh broth. The final drug concentration of one of the new cultures (curve F, Fig. 2) was thus 1 mcg./ml. Enough drug was added to the second culture to maintain the 5 mcg./ml. drug concentration (curve G, Fig. 2).

Effect of Temperature on Drug-Affected Growth Rates—The inhibitory actions of sodium sulfathiazole and sulfisoxazole on the population growth of *E. coli* were studied at 24.7°, 26.0°, 29.3°, 32.0°, 34.7°, 37.5°, and 39.5°. Logarithmic-phase cultures at 4 concentrations of each drug of 1.0, 2.0, 3.0, and 4.0 mcg./ml., and one culture without drug, were studied by the Coulter count method at each temperature.

Combined Action of Sulfa Drugs and Chloramphenicol—Replicate 50-ml. *E. coli* cultures in the logarithmic growth phase at 37.5° were treated with all combinations of 3 concentrations of chloramphenicol and 4 concentrations of sulfisoxazole. The chloramphenicol concentrations were 0.33, 0.67, and 1.00 mcg./ml. The sulfisoxazole concentrations were 1.0, 2.0, 3.0, and 4.0 mcg./ml.. The drugs were added 80 min. after inoculation of the cultures in the logarithmic growth phase. The Coulter count method was used.

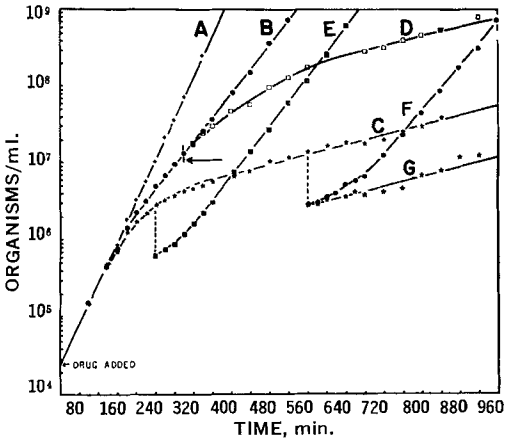


Fig. 2—Generation rate curves of E. coli *B/r at 37.5° on addition and dilution of sulfisoxazole. The population on initial drug addition was 1.6 × 10^4 organisms/ml. The curves, final mcg./ml. concentrations of sulfisoxazole, and apparent steady-state growth rate constants, $k_{app.}$, in sec.$^{-1}$, were:* A, *0.0, 50.2 × 10^{-5};* B, *1.0, 30.2 × 10^{-5};* C, *5.0, 6.29 × 10^{-5};* D, *5.0, 6.00 × 10^{-5};* E, *1.0, 30.9 × 10^{-5};* F, *1.0, 27.6 × 10^{-5};* G, *5.0, 6.28 × 10^{-5}. Arrow on curve* B *denotes more drug added to total 5 mcg./ml.*

RESULTS

Effect of Drug Concentration on Growth Rates—The number of organisms per ml. is plotted against time on semilogarithmic paper for various amounts of sulfa drugs. The example given in Fig. 1 is for sulfisoxazole. A lag period of approximately 120 min. existed before there was any significant effect of any concentration of drug on the population growth of *E. coli* in the logarithmic growth phase at 37.5°. The lag time was the same for both sulfisoxazole and sodium sulfathiazole. Subsequent to the deviation of drug-affected population growth from the control curve, a new linearity in logarithmic growth was attained. At a dosage of 20.0 mcg./ml. that produced complete inhibition of microbial population growth (line F, Fig. 1) and apparent death (line F, Fig. 1) there was an intermediate period of 40–60 min. duration before the new steady-state drug-affected growth curve was established.

At higher dosages of 50.0, 75.0, and 100.0 mcg./ml. the growth curves were superimposable and independent of the dose. Subsequent to the lag period for these higher dosages, the rate of growth decreased, and the maximum value in the number of organisms was obtained 50 min. after the deviation from the initial growth period. A slowly accelerating decay rate in viable organisms was then ob-

TABLE I—EFFECT OF SODIUM SULFATHIAZOLE CONCENTRATIONS, S, ON STEADY-STATE POPULATION GROWTH RATE CONSTANTS, $k_{app.}$ IN SEC.$^{-1}$ FOR VARIOUS POPULATION SIZES OF *E. coli.*, *N*, AT TIME OF ADDITION AT 37.5°

S, mcg./ml.	*N*: 1 × 10³	1.2 × 10⁴	1 × 10⁵	6 × 10⁵
	$k_{app.}$			
0.0	$k_0 = 45.7 \times 10^{-5}$	49.1×10^{-5}	48.0×10^{-5}	48.7×10^{-5}
0.5	36.1×10^{-5}	...	...	...
1.0	...	27.7×10^{-5}	29.2×10^{-5}	29.3×10^{-5}
2.0	18.0×10^{-5}	16.5×10^{-5}	23.5×10^{-5}	21.6×10^{-5}
3.0	10.4×10^{-5}	9.9×10^{-5}	14.9×10^{-5}	12.4×10^{-5}
4.0	7.1×10^{-5}	7.0×10^{-5}	9.7×10^{-5}	10.8×10^{-5}
5.0	4.6×10^{-5}	4.4×10^{-5}	6.2×10^{-5}	...
k_b/k_a[a]	2.3×10^3	1.9×10^3	1.7×10^3	1.8×10^3
$1/k_a$[a]	3.4×10^3	3.1×10^3	3.6×10^3	3.4×10^3

[a] These values were obtained from the slope and intercept of the plot $S/(k_0 - k_{app.})$ *versus* S in accordance with the expression $S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)\,S$.

TABLE II—EFFECT OF SULFISOXAZOLE CONCENTRATION, S, ON STEADY-STATE POPULATION GROWTH RATE CONSTANTS, $k_{app.}$ IN SEC.$^{-1}$, FOR SEVERAL TEMPERATURES[a]

S, mcg./ml.	Temp.: 24.7°C.	26.0°C.	29.3°C.	32.0°C.	34.7°C.	37.5°C.	39.5°C.
				$k_{app.}$			
0.0	$k_0 = 2.13 \times 10^{-4}$	2.34×10^{-4}	3.18×10^{-4}	3.99×10^{-4}	4.28×10^{-4}	5.03×10^{-4}	5.31×10^{-4}
1.0	8.00×10^{-5}	9.02×10^{-5}	1.40×10^{-4}	2.09×10^{-4}	2.56×10^{-4}	3.12×10^{-4}	3.33×10^{-4}
2.0	3.79×10^{-5}	4.67×10^{-5}	7.08×10^{-5}	1.16×10^{-4}	1.75×10^{-4}	1.74×10^{-4}	2.22×10^{-4}
3.0	2.68×10^{-5}	2.26×10^{-5}	4.87×10^{-5}	8.05×10^{-5}	1.34×10^{-4}	1.44×10^{-4}	1.59×10^{-4}
4.0	1.53×10^{-5}	1.38×10^{-5}	2.99×10^{-5}	6.40×10^{-5}	9.00×10^{-5}	1.08×10^{-4}	1.10×10^{-4}
k_b/k_a[b]	4.23×10^3	3.71×10^3	2.97×10^3	2.27×10^3	2.04×10^3	1.73×10^3	1.39×10^3
$1/k_a$[b]	2.99×10^3	3.21×10^3	3.43×10^3	3.10×10^3	3.72×10^3	3.47×10^3	3.92×10^3
$k_a/k_b = qk_m$[c]	2.37×10^{-4}	2.70×10^{-4}	3.37×10^{-4}	4.41×10^{-4}	4.91×10^{-4}	5.77×10^{-4}	7.18×10^{-4}
$k_b = K_1K_2$[d]	1.42	1.16	0.865	0.780	0.550	0.499	0.355

[a] Sulfisoxazole added to 10^5 organisms/ml. [b] These values were obtained from the slope and intercept of the plot $S/(k_0 - k_{app.})$ *versus* S in accordance with the expression $S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)\,S$. [c] Reciprocal of slope of such a plot. [d] Ratio of slope to intercept of such a plot.

TABLE III—EFFECT OF SODIUM SULFATHIAZOLE CONCENTRATION, S, ON STEADY-STATE POPULATION GROWTH RATE CONSTANTS, $k_{app.}$ IN SEC.$^{-1}$, FOR SEVERAL TEMPERATURES[a]

S, mcg./ml.	Temp.: 24.7°C.	26.0°C.	29.3°C.	32.0°C.[b]	34.7°C.	37.5°C.	39.5°C.
				$k_{app.}$			
0.0	$k_0 = 2.13 \times 10^{-4}$	2.35×10^{-4}	3.14×10^{-4}	3.95×10^{-4}	4.36×10^{-4}	4.76×10^{-4}	5.31×10^{-4}
1.0	1.18×10^{-4}	1.24×10^{-4}	1.65×10^{-4}	2.15×10^{-4}	2.67×10^{-4}	2.92×10^{-4}	3.25×10^{-4}
2.0	6.80×10^{-5}	8.54×10^{-5}	1.11×10^{-4}	1.49×10^{-4}	2.04×10^{-4}	2.35×10^{-4}	2.27×10^{-4}
3.0	3.67×10^{-5}	4.94×10^{-5}	6.95×10^{-5}	1.05×10^{-4}	1.60×10^{-4}	1.49×10^{-4}	1.70×10^{-4}
4.0	3.15×10^{-5}	2.98×10^{-5}	5.47×10^{-5}	8.43×10^{-5}	7.85×10^{-5}	9.71×10^{-5}	1.28×10^{-4}
k_b/k_a[c]	3.77×10^3	3.41×10^3	2.85×10^3	2.21×10^3	1.90×10^3	1.66×10^3	1.50×10^3
$1/k_a$[c]	5.96×10^3	5.46×10^3	5.23×10^3	4.04×10^3	4.07×10^3	3.56×10^3	3.16×10^3
$k_a/k_b = qk_m$[d]	2.66×10^{-4}	2.93×10^{-4}	3.51×10^{-4}	4.53×10^{-4}	5.27×10^{-4}	6.02×10^{-4}	6.67×10^{-4}
$k_b = K_1K_2$[e]	0.632	0.626	0.544	0.547	0.466	0.466	0.476

[a] Sodium sulfathiazole added to 10^5 organisms/ml. [b] $k_{app.} = 6.22 \times 10^{-5}$ sec.$^{-1}$ at 5.0 mcg./ml. of sodium sulfathiazole. [c] These values were obtained from the slope and intercept of the plot $S/(k_0 - k_{app.})$ *versus* S in accordance with the expression $S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)\,S$. [d] Reciprocal of slope of such a plot. [e] Ratio of slope to intercept of such a plot.

TABLE IV—EFFECT OF SULFISOXAZOLE CONCENTRATION, S, ON STEADY-STATE POPULATION GROWTH RATE CONSTANTS, $k_{app.}$ IN SEC.$^{-1}$, FOR SEVERAL CHLORAMPHENICOL CONCENTRATIONS, C, AT 37.5°C.[a]

S, mcg./ml.	C, mcg./ml.[b]: 0.33	0.67	1.00
		$k_{app.}$	
0.00	$k_0 = 4.08 \times 10^{-4}$	3.41×10^{-4}	2.38×10^{-4}
1.00	2.44×10^{-4}	2.07×10^{-4}	1.22×10^{-4}
2.00	1.63×10^{-4}	1.45×10^{-4}	0.95×10^{-4}
3.00	1.02×10^{-4}	1.13×10^{-4}	0.58×10^{-4}
4.00	0.86×10^{-4}	0.85×10^{-4}	0.47×10^{-4}
k_b/k_a[c]	2.23×10^3	2.96×10^3	4.22×10^3
$1/k_a$[c]	4.10×10^3	4.40×10^3	4.40×10^3
$k_a/k_b = qk_m$[d]	4.48×10^{-4}	3.38×10^{-4}	2.37×10^{-4}
$k_b = K_1K_2$[e]	0.544	0.673	0.960
$1/k_b$	1.84	1.49	1.04

[a] Sulfisoxazole and chloramphenicol added to 10^5 organisms/ml. [b] The $k_{app.}$ values in sec.$^{-1}$ prior to the addition of chloramphenicol 0.33, 0.67, and 1.00 mcg./ml. were 5.10, 5.12, and 5.17 × 10^{-4}, respectively. [c] These values were obtained from the slope and intercept of the plot $S/(k_0 - k_{app.})$ *versus* S in accordance with the expression: $S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)\,S$. [d] Reciprocal of slope of such a plot. [e] Ratio of slope to intercept of such a plot.

served for 120 min. Subsequently, the logarithmic decay of the numbers of viable organisms was constant with time for these doses with an apparent first-order rate constant of 9.75×10^{-5} sec.$^{-1}$.

The total count technique showed a decreasing rate of increase in total numbers over 120 min. after the deviation from the initial growth curve. At that time and for 400 subsequent min., there was a negligible change in the total number of organisms.

Apparent population growth rate constants, $k_{app.}$ in sec.$^{-1}$, were obtained from the slopes of these new linear logarithmic growth curves in accordance with the apparent first-order expression:

$$\log N = (k_{app.}/2.303)t + \text{constant} \quad \text{(Eq. 1)}$$

where t is time and N is the number of organisms/ml. The $k_{app.}$ values for various concentrations of sodium sulfathiazole and sulfisoxazole under varied conditions are given in Tables I–IV.

When the $k_{app.}$ values are plotted against the concentrations of the sulfa drugs, the curves are nonlinear and demonstrate that the $k_{app.}$ is not a linear function of the concentrations of the sulfa drugs. However, when the reciprocal of the differences between the population growth rate constant in the absence of drug, k_0, and the $k_{app.}$ values for population growth in the presence of drug are plotted against the reciprocal of the concentration of the sulfa drug, S in mcg./ml., excellent linearity is obtained (Fig. 3 for sulfisoxazole) in accordance with the expression:

$$1/(k_0 - k_{app.}) = (1/k_a)\,(1/S) + k_b/k_a \quad \text{(Eq. 2)}$$

where k_a and k_b are constants.

Similarly, when Eq. 2 is multiplied through by the concentration of the sulfa drug, this concentration divided by the difference between the population growth rate constants in the presence and absence of sulfa drug is a linear function of sulfa drug concentration, S, as in Fig. 4 for sulfathiazole:

$$S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)S \quad \text{(Eq. 3)}$$

The intercepts, $1/k_a$, and slopes, k_b/k_a, derived from such plots are also given in Tables I–IV.

Effect of Temperature on Drug-Affected Growth Rates—The data for the effect of sulfonamide drugs on the steady-state growth of *E. coli* (Tables II and III) were analyzed by application of Eqs. 2 and 3 for each temperature studied, and the parameters k_a/k_b and k_b were determined from the slopes and intercepts of the plots in accordance with these equations (Figs. 3 and 4). The k_a and k_b parameters reasonably fit the Arrhenius or Van't Hoff equation for the dependence of reaction rate or equilibrium constants upon temperature (Fig. 5):

$$\log k = \log P - (\Delta H/2.303\,R)\,(1/T) \quad \text{(Eq. 4)}$$

where R is 1.987 Kcal./mole and T is in degrees Kelvin. The apparent ΔH values for k_b are −15.9 Kcal./mole for sulfisoxazole and −4.3 Kcal./mole for sodium sulfathiazole. For both sulfonamide studies, the apparent heat of activation for the derived k_a/k_b was 24 Kcal./mole. Similar plots for the k_0 values, *i.e.*, the population growth rate constants at the various temperatures in the absence of any sulfonamide were parallel to the plots for the k_a/k_b values and thus of the same apparent ΔH values.

The interval from the time of addition of sulfonamide to the time when the population growth rate

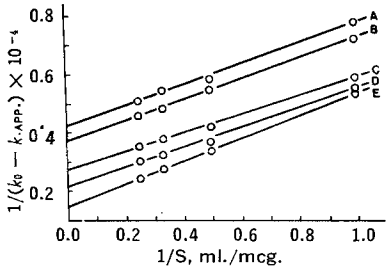


Fig. 3—Examples of quantitative relations between apparent E. coli *growth rate constants,* $k_{app.}$, *and sulfisoxazole concentrations,* S, *at various temperatures. The curves are plotted in accordance with the expression* $1/(k_0 - k_{app.}) = (1/k_a)(1/S) + k_b/k_a$ *where the growth rate constant in the absence of sulfonamide,* k_0, *and* $k_{app.}$ *are in sec.*$^{-1}$ *and S is in mcg./ml. The curves and respective temperatures are:* A, *24.7°;* B, *26.0°;* C, *29.3°;* D, *32.0°;* E, *39.5°.*

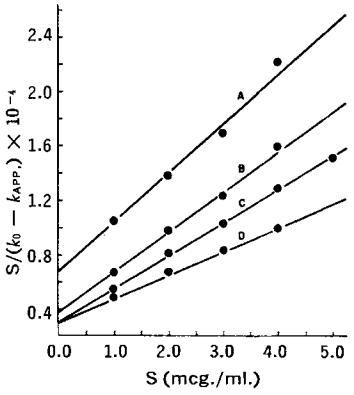


Fig. 4—Examples of quantitative relations between apparent E. coli *growth rate constants,* $k_{app.}$, *and sodium sulfathiazole concentrations, S, at various temperatures. The curves are plotted in accordance with the expression* $S/(k_0 - k_{app.}) = 1/k_a + (k_b/k_a)S$ *where the growth rate constant in the absence of sulfonamide,* k_0, *and* $k_{app.}$ *are in sec.*$^{-1}$ *and S is in mcg./ml. The curves and respective temperatures are:* A, *24.7°;* B, *29.3°;* C, *32.0°;* E, *39.5°.*

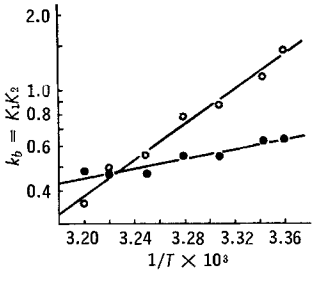


Fig. 5—The variation of a derived parameter, $k_b = K_1K_2$, *of sulfonamide action on* E. coli *microbial growth as a function of temperature. The curves for sulfisoxazole* (○) *and sulfathiazole* (●) *are plotted in accordance with the expression: log* $k_b = -\Delta H/2.3RT + \log P$ *where T is the absolute temperature.*

demonstrates a deviation to a drug-affected rate is termed the lag time (Fig. 1). This lag time is inversely proportional to the growth rate constant in the absence of sulfonamides for all temperatures studied. The linearity of the plot of lag time *versus* $1/k_0$ is demonstrated in Fig. 6. At any temperature, this lag time corresponded to 5.5 ± 0.5 generations.

Effect of Chloramphenicol on Sulfonamide-Affected Growth Rate—Chloramphenicol and sulfisoxazole were added simultaneously to a growing culture of *E. coli* at 10^6/ml. The chloramphenicol caused an almost instantaneous change in the rate of growth, but the sulfonamide effect was not observed until after a definitive lag time. Subsequent to this lag time, a drug-equilibrated growth rate was observed which was an apparent function of sulfisoxazole concentration. A typical semilogarithmic plot of such growth curves is given in Fig. 7 for 0.666 mcg./ml. chloramphenicol and 0.0 to 4.0 mcg./ml. sulfisoxazole.

The lag time from the time of addition of both drugs to the time when sulfonamide action was demonstrated was inversely proportional to the growth rate constant in the absence of sulfonamide (Fig. 6). At any chloramphenicol concentration used this lag time corresponded to 5.5 ± 0.5 generations.

The data for the effect of combined sulfonamide and chloramphenicol drugs on the steady-state growth rate constants for *E. coli* after the lag time are given in Table IV. These data were consistent with Eqs. 2 and 3 as demonstrated in the plot given in Fig. 8. The k_0' values chosen were those values in the presence of chloramphenicol but in the absence of sulfisoxazole. The slopes and intercepts of the curves are also given in Table IV.

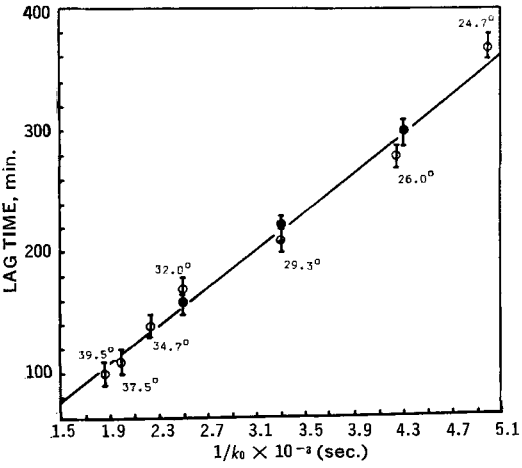


Fig. 6—Lag-time before sulfonamide affects E. coli *growth as a function of the generation time, $1/k_0$, at the time of sulfonamide addition. Key: ○, estimated lag times for sulfathiazole and sulfisoxazole-affected microbial cultures at the various cited temperatures; ●, lag times for microbial cultures simultaneously treated with sulfonamide and various concentrations of chloramphenicol at 37.5°. The vertical lines through the points represent ranges of the estimated values. The k_0 values are the observed growth rate constants in sec.$^{-1}$.*

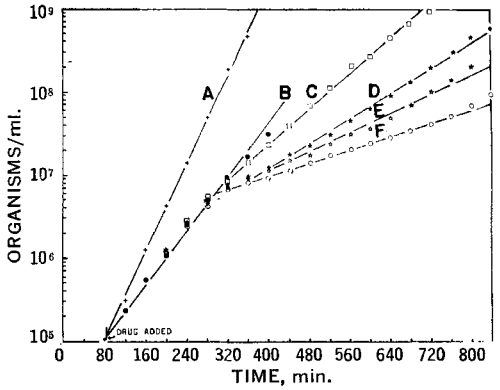


Fig. 7—Typical generation rate curves of E. coli *B/r at 37.5° in the presence of chloramphenicol and various concentrations of sulfisoxazole. The population on the addition of both drugs was 10^5 organisms/ml. Curve* A *is for the growth rate in the absence of both drugs and curve* B *is for 0.666 mcg./ml. of chloramphenicol. The other curves were for 0.666 mcg./ml. of chloramphenicol and the following mcg./ml. of sulfisoxazole:* B, *0.0;* C, *1.0;* D, *2.0;* E, *3.0;* F, *4.0.*

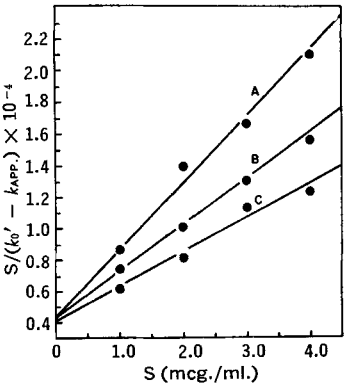


Fig. 8—Quantitative relations between apparent E. coli *growth rate constants, $k_{app.}$, and sulfisoxazole concentrations, S, at various chloramphenicol concentrations at 37.5°. The curves are plotted in accordance with the expression $S/(k'_0 - k_{app.}) = 1/k_a + (k_b/k_a)S$ where the growth rate constant in the absence of chloramphenicol and sulfisoxazole, k_0, is related to the growth rate constant, k_0', in the presence of chloramphenicol by $k_0' = k_0 + k_cC$ where C is the concentration of chloramphenicol in mcg./ml. and $k_c = 2.68 \times 10^{-4}$ ml./mcg./sec. The curves and respective concentrations in mcg./ml. of chloramphenicol are:* A, *1.00;* B, *0.67;* C, *0.33.*

Effect of Organism Population on Drug-Affected Growth Rates—The apparent growth rate constants for *E. coli*, $k_{app.}$, obtained in the presence of various concentrations of sodium sulfathiazole at various inoculum sizes at time of drug addition are listed in Table I. The values were obtained from the slopes of the curves of the logarithm of the total organism count *versus* time after the steady state attributed to sulfa drug effects had resulted as demonstrated in Fig. 1. There were no large differences in the $k_{app.}$ values obtained for the various organism population sizes on addition of a given concentration of sodium sulfathiazole for the particular *E. coli* culture used

(Table I). The variations were greatest for higher organism and sulfa drug concentrations. These facts imply that it is best to do comparative studies of sulfonamide action on *E. coli* growth at as constant a population as is possible. For this reason, sulfonamide was added at about 10^6 organisms/ml. Similar studies were also performed with different *E. coli* B/r cultures. In many of these cases, organism population had a greater effect on drug-equilibrated growth rates than with the specific culture used throughout these experiments.

Reversibility of Drug Action—As previously stated, the addition of sulfonamide to an inoculum demonstrated no immediate effect on the microbial growth rate (curves A and B, Fig. 2). It takes 5.5 generations for a drug effect on the microbial growth rate to be observed. At the lower concentrations of drug (curve B, Fig. 2), the transition period to the new steady-state growth rate can be considered as small and is 20–30 min. for 1.0 mcg./ml. of sulfisoxazole. At the higher concentrations of drug (curve C, Fig. 2), the transition period to the new steady-state growth rate is larger and is 140–160 min. for 5.0-mcg./ml.-affected growth rate (curve D). The ultimate steady-state growth rate achieved is independent of whether the total sulfisoxazole is added to the initial culture (curve C), or added stepwise (curves B and D), or the drug-affected culture is diluted and sufficient drug is added to maintain the same concentration (curves C and G). The growth rate constant, $k_{app.}$, was the same in all these instances when steady state was attained.

When the culture in the drug-affected growth period is diluted so that the sulfisoxazole concentration is diminished, the growth rate increases (curve C to E and to F) to achieve a new steady-state rate of growth. The increase in rate appears to occur immediately after dilution, but 100–130 min. must elapse before the new steady-state rate of growth is achieved. The ultimate growth rate is equivalent to that which would have been ultimately achieved with the same concentration of drug added to fresh inoculum.

DISCUSSION

The action of sulfonamide on drug-equilibrated microbial growth in the individual cell may be explained by the simple model:

$$S \underset{K_1}{\overset{}{\rightleftharpoons}} S' + R \underset{\frac{k_2}{K_2}}{\overset{k_1}{\rightleftharpoons}} (S'R) \quad \text{(Eq. 5)}$$

This model assumes an equilibrated partitioning of the sulfonamide concentration S in the media with the concentration S′ in the cell which is available to the unreacted receptor sites, R. The fact that the growth rate is constant for a given sulfonamide in the drug-equilibrated steady state implies that the two equilibria characterized by K_1 and K_2 are achieved in that state.

If θ is the fraction of receptor sites reacted with drug, the metabolic rate, $d\,M/dt$, may be proportional to the fraction, $1 - \theta$, of receptor sites that are unreacted with drug:

$$d\,M/dt = k_m\,(1 - \theta) \quad \text{(Eq. 6)}$$

An implicit assumption in this postulate is that the number of receptor sites in a single bacterium are constant and independent of the mass of the cell or time. Possible cases where this was not assumed have been discussed previously (7).

The equilibrium constant, K_2, for the drug–site interaction of Eq. 5 may be defined by:

$$K_2 = k_2/k_1 = (S'R)/[R_t - (S'R)]\,(S') = (S'R)/(S')(R) \quad \text{(Eq. 7)}$$

where $(S'R)$ is the number of sulfonamide-reacted sites, R is the number of unreacted sites, and R_t is the total number of sites whether reacted or unreacted. The fraction θ of sites reacted with drug may be obtained from rearrangement of Eq. 7:

$$\theta = (S'R)/R_t = K_2S'/(1 + K_2S') \quad \text{(Eq. 8)}$$

Thus, Eq. 6 on substitution becomes:

$$d\,M/dt = k_m - k_mK_2S'/(1 + K_2S') \quad \text{(Eq. 9)}$$

where k_m is the steady-state rate of metabolism in the absence of sulfonamide.

The concentration of sulfonamide, S′, in the cell is related to the concentration, S, in the media by:

$$S' = K_1S \quad \text{(Eq. 10)}$$

It follows that the rate of metabolism in the steady state of a single bacterium is related to the sulfonamide concentration, S, in the media by:

$$d\,M/dt = k_m - k_mK_1K_2S/(1 + K_1K_2S) \quad \text{(Eq. 11)}$$

If it is postulated that the rate of population increase of N organisms is proportional to this rate of metabolism and to the number of organisms in the balanced growth culture, it follows that:

$$d\,N/dt = q(dM/dt) \times N \quad \text{(Eq. 12}a\text{)}$$

$$d\,N/dt = [qk_m - qk_mK_1K_2S/(1 + K_1K_2S)]N \quad \text{(Eq. 12}b\text{)}$$

$$d\,N/dt = [k_0 - k_aS/(1 + k_bS)] \times N \quad \text{(Eq. 12}b\text{)}$$

$$d\,N/dt = k_{app.} \times N \quad \text{(Eq. 12}c\text{)}$$

where $k_0 = qk_m = k_a/k_b$, $k_a = qk_mK_1K_2$, $k_b = K_1K_2$ and:

$$k_{app.} = k_0 - k_aS/(1 + k_bS) \quad \text{(Eq. 13)}$$

where $k_{app.}$ is the apparent first-order steady-state growth rate constant in the presence of a constant sulfonamide concentration, S.

On integration, Eq. 12c becomes Eq. 1, and the $k_{app.}$ values were obtained from the slopes of semilogarithmic plots of drug-equilibrated growth (Figs. 1, 2, and 7; Tables I–IV).

The Eq. 13 can be rearranged into the forms of Eqs. 2 and 3, from which by appropriate plotting (Figs. 3, 4, and 8) the k_a and k_b values and related parameters were obtained (Tables I–IV).

The adherence of the data to Eqs. 2 and 3 demonstrates the validity of the model of Eq. 5 to explain the action of sulfonamide on microbial growth. The metabolic rate in the drug-affected steady state that results in microbial growth is proportional to the number of receptor sites unreacted with or unbound by sulfonamide. These receptor sites may be enzymes that are needed in the metabolic pathway (17–22) or an actual and constant amount of

substrate needed in the metabolic sequence that is inactivated by the sulfonamide (23, 24).

It has been argued (17–24) that certain sulfonamide antagonists such as *p*-aminobenzoic acid (PABA) and the sulfonamide compete for such receptor sites, and PABA reaction is necessary in the metabolic sequence. If this is true, the experimental confirmation of Eq. 5 demonstrates that all available receptor sites are occupied either by sulfonamide or PABA and the relative degree of occupation is determined competitively.

It is implied by the experimental verification of Eqs. 5–13 that total reaction of sulfonamide with the total number of receptor sites, R_t, is necessary for complete cessation of microbial growth. This should mean that the obtained $k_a/k_b = gk_m$ (Eqs. 2, 3, 12, and 13) should have the same magnitude as k_0, the apparent growth rate constant in the absence of sulfonamide. This is not exactly true as is evidenced by the values given in Tables II and III. For sulfisoxazole, $k_0 = 0.90\ k_a/k_b$, and for sodium sulfathiazole, $k_0 = 0.82\ k_a/k_b$, with average deviations of 0.03. These facts imply that it is only necessary to affect 82% or 90% of the available receptor sites to obtain the asymptotic cessation of microbial growth by these sulfonamides. The detailed analysis of this concept has been given previously (7).

Although the major action of sulfonamides is inhibitory, microorganism death is observed at high sulfonamide concentrations. Viable and total organism counts are coincident for 0.0 to 4.0 mcg./ml. of sulfisoxazole and demonstrate that microbial growth inhibition is the effective process in this range (Fig. 1). However, at much higher doses of sulfisoxazole, which included 20.0, 50.0, 75.0, and 100.0 mcg./ml., the constancy of the drug-equilibrated total microbial count with time (curve F, Fig. 1) implied completely inhibited growth. The loss of viables by a first-order process of dying must be assigned to the death of growth-inhibited microorganisms. In studies with 50.0, 75.0, and 100.0 mcg./ml., the viable count with time did not vary among these sulfisoxazole concentrations and went through a maximum after the lag period. The same ultimate, first-order kill rate was observed at 37.5° for all these concentrations. These observations imply that the nongrowing, sulfonamide-affected microorganism does not lose its viability as a function of sulfonamide concentration. It is probable that the metabolic limitations placed on the organisms by saturation of receptor sites with sulfonamide results in a statistical probability of death demonstrated by a first-order rate of dying.

It has been shown previously (7) that a plot of the logarithm of the microbial growth rate constant against the reciprocal of the absolute temperature is reasonably linear for drug-free *E. coli* cultures in the temperature range 25° to 39° and that ΔH values can be determined from the slopes of such plots in accordance with Eq. 4. This reasonable adherence to the Arrhenius or Van't Hoff Eq. 4 implies that one step, or several steps with similar temperature dependencies, in the metabolic processes of the organism may be rate limiting in this temperature range. The coincidence of the apparent ΔH values, 24 Kcal./mole for the derived k_a/k_b values from the sulfonamide studies (Tables II and III) and the observed k_0 values in the absence of sulfonamide indicate that these constants represent the same processes.

The derived parameter most descriptive of the quantitative action of a sulfonamide on microbial growth is $k_b = K_1K_2$ (Tables II–IV). Unfortunately, this product cannot be kinetically dissociated into the partition coefficient or transfer process for drug across the cell membrane, K_1, and the affinity constant, K_2, for sulfonamide reaction with receptor site. The product of these two equilibrium constants is temperature dependent (Fig. 5) and is reasonably in accordance with the Arrhenius or Van't Hoff Eq. 4 over the temperature range studied. The ΔH from this equation is not the same for sulfisoxazole and sulfathiazole. In terms of dissociation constants, $1/K_2$, and partition coefficient for the ratio of concentrations in the media to that within the cell, *i.e.*, $1/K_1$, the ΔH of $1/K_1K_2$ is 15.9 Kcal./mole for sulfisoxazole and 4.3 Kcal./mole for sulfathiazole. These values are significantly different from the ΔH of 24 Kcal./mole for microbial growth in the absence of sulfonamide, and are significantly different from each other. These facts imply that a fundamental difference exists between the reactivities and/or transport of these two sulfonamides into the cell.

Heats of diffusion are generally small, about 2–5 Kcal./mole. Thus, if partition or transport of the sulfonamide into the cell is a diffusion-controlled passive process, large ΔH values would indicate that definitive heats of reaction may be assigned to drug–receptor site interaction since:

$$\ln K_1K_2 = \ln P_{K_1K_2} - \Delta H_{K_1K_2}/RT = \ln K_1 + \ln K_2 = \ln P_{K_1}P_{K_2} - (\Delta H_{K_1} + \Delta H_{K_2})/RT \quad \text{(Eq. 14)}$$

so that:

$$\Delta H_{K_1K_2} = \Delta H_{K_1} + \Delta H_{K_2} \quad \text{(Eq. 15)}$$

For example, the $\Delta H_{K_1K_2}$ values for these two sulfonamides may imply that the receptor site–drug interaction or active transport processes have greater significance in the sulfisoxazole case than in the sulfathiazole case.

Several mechanisms of sulfonamide action have been proposed. One that has had wide acceptance is that these drugs act competitively with essential metabolites such as *p*-aminobenzoic acid, PABA, in a process which is necessary for growth (17–24). This can be competition for an enzyme in a vital enzymatic transformation (17–22), or is actually a competitive chemical reaction involving a vital metabolite in a necessary metabolic sequence (23, 24). To be consistent with our experimentally verified Eqs. 13 and 14, acceptance of these concepts implies that all receptor sites in Eq. 5 (whether enzymatic *or* chemical) are occupied by or reacted with either the sulfonamide or the available PABA in the drug-affected steady state. The number of sulfonamide-unreacted sites or substrates, *i.e.*, R are really PABA-reacted sites or PABA-reacted substrates and the rate of metabolism linked to growth is proportional to this R.

The lag time is the interval from the time of addition of sulfonamide to the time when the population growth rate demonstrates a deviation to a drug-affected rate (9, 25). This lag time is now shown to be inversely proportional to the growth

rate constant before sulfonamide affects the growth rate for all temperatures studied, and when chloramphenicol was added concomitantly with the sulfonamide (Fig. 6). At any chloramphenicol concentration and for any temperature, this lag time corresponded to 5.5 ± 0.5 generations. The 5.5 ± 0.5 generations after sulfonamide addition and before an observed deviation in microbial growth rate imply that cellular division is primarily responsible for the conditions necessary for sulfonamide action or microbial growth to be observed. This could be assigned to the depletion of PABA-derived metabolite [or folic acid precursor (22, 26–28)] storage in a single cell, in agreement with the arguments of Kohn and Harris (9, 10) and Rose and Fox (29). As long as these compounds are in excess, the metabolic pipeline can be maintained full and the rate of microbial growth dependent on a delivered quantity of metabolite per unit of time is invariant. The limiting factor in microbial growth may be the size of this pipeline. In more conventional terms, this would be equivalent to the saturation value of a compartment, tissue, enzyme, or process. It also follows that a standard excess of PABA metabolite and PABA is produced in the balanced growth culture.

Evidence for this is provided by the variation in the dose-response of sulfonamide action on drug-affected steady-state microbial growth as a function of inoculum size. Although this was of little significance in the inoculum ranges with the particular culture used in this paper, many other cultures (30–32), including other strains of *E. coli* B/r that were investigated, showed this effect in varying degrees. In general, for these other strains the greater the number of organisms on drug addition, the more sulfonamide was needed to give the same inhibition of microbial growth, or the greater the antagonism to sulfonamide action. If each organism in a balanced growth culture produced an excess of PABA or PABA-derived metabolites which "leaked" into the nutrient media, the response to sulfonamide would obviously be a function of the numbers of such organisms prior to the inhibition of production of PABA or PABA-derived metabolite.

Ultimately, a situation results where the amounts of stored PABA metabolites per unit cell are insufficient to saturate the pipeline and a gradual decrease in rate of microbial growth occurs to the new drug-affected steady state. This gradual change in rate also may be attributed in part to cellular division and thus to depletion of PABA metabolites in a single cell. The new drug-affected steady state then may be assigned to the competition of PABA from its new steady-state production and the cellular sulfonamide concentration. They compete for all available receptor sites and the production of PABA metabolites depends on the results of this competition.

An alternative or concomitant possibility is the diminution of available receptor sites (or enzymes) in the new steady state (27). Since depletion of stored PABA metabolites explains the lag period (9, 10, 29), lowered rates of PABA production and/or decreased numbers of receptor sites due to the feedback from metabolites' inhibition by sulfonamide are plausible. As first approximations, the rate of PABA production and/or the numbers of available receptor sites may be assumed to be proportional to the microbial growth rate.

The greater the sulfonamide concentration of the balanced growth culture (Fig. 1), the longer the time to achieve the new drug-affected steady state. This may imply time-dependent processes either for equilibration of sulfonamide in the culture media with the biophase concentration, or for diminution of rates of PABA production, or for transfer of PABA into the biophase.

The sulfonamide concentration was reduced by dilution of the culture with nutrient media or was increased by adding more sulfonamide (Fig. 2). This was done after the lag phase, both before and during the drug-affected steady-state growth process. There were gradual transitions to the new steady-state microbial growth rate characteristic of the new sulfonamide concentration. Since PABA-derived metabolites were already depleted, this may be assigned to either one or a combination of the following: (*a*) a time interval is necessary to rejuvenate or inhibit PABA production to its new steady state; (*b*) a finite time interval is necessary to equilibrate receptor sites with sulfonamide and/or PABA; (*c*) the equilibration between sulfonamide in the biophase surrounding the receptor sites and the sulfonamide in the culture media is a time-dependent process; (*d*) the biophase compartment is removed from the PABA generation site and the distributive processes for PABA are time-dependent; (*e*) there is a lag in transfer of the PABA-derived metabolite to or in the metabolic pathway that results in metabolism and growth.

The authors have previously made the statement that, as first approximations, the rate of PABA production and/or the numbers of receptor sites (*e.g.*, folic acid synthesizing enzymes) may be assumed to be proportional to the microbial growth rate. These postulates would explain the appearance of a new drug-affected steady state of microbial growth where the new rate of PABA production and the sulfonamide in the cell would compete for available receptor sites. Thus, the numbers of sulfonamide-unaffected sites, R in Eq. 5, that give rise to metabolism and growth, are PABA-reacted and would be direct functions of PABA production. It follows from Eq. 7 that the sulfonamide–receptor site dissociation constant, $1/K_2$ is directly proportional to this number of receptor sites, R, and thus should be directly proportional to the microbial growth rate. The same reasoning results if a feedback mechanism is operative (27) and the numbers of folic acid synthesizing enzymes are reduced with a reduced microbial growth rate.

Evidence in support of these statements is provided by the studies of microbial growth on simultaneous addition of chloramphenicol and sulfisoxazole (Table IV, Fig. 7). Confirmation of the adherence of the data to Eqs. 2, 3, 12, and 13 is demonstrated by the plots of Fig. 8. The derived parameters are listed in Table IV. It is apparent that the obtained K_1K_2 values, all at 37.5°, vary as a function of the chloramphenicol concentration, C, as do the k_0 values. Consistent with previous studies (3–8) the data of Table IV show that:

$$k_0 = k_0' - k_c\mathrm{C} \qquad \text{(Eq. 16)}$$

where $k_0' = 5.10 \times 10^{-4}$ sec.$^{-1}$ is the first-order growth rate constant in the absence of chloramphenicol and $k_c = 2.68 \times 10^{-4}$ ml./mcg./sec. is the inhibitory constant where C is in mcg./ml.

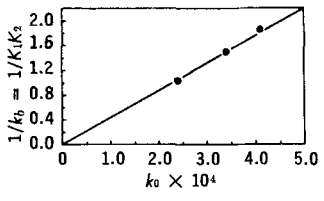


Fig. 9—The product ($1/K_1K_2$) of sulfonamide partition constant ($1/K_1$) and the sulfonamide-receptor site dissociation constant ($1/K_2$) as a function of the growth rate constant k_0 in the presence of chloramphenicol at 37.5°.

The action of chloramphenicol on microbial growth has been assigned to the inhibition of the function of messenger RNA in protein synthesis by blocking its attachment to ribosomes through competition for ribosomal binding sites (33). The differences in the kinetics in regard to lag period, reversibility, and concentration dependence are indicative that chloramphenicol and sulfonamide mechanisms of action differ.

Since $1/K_2$ is directly proportional to R and if the numbers of receptor sites, R, are a function of the microbial growth rate, it follows that $1/K_1K_2$ should be a linear function of k_0, the growth rate constant in the presence of chloramphenicol and in the absence of sulfisoxazole. Since the data (Table IV) are at constant temperature, the partition constant, K_1, should be invariant.

The plot of the data in Fig. 9 confirms this reasoning since it demonstrates that a linear relation with an intercept of zero exists and:

$$1/K_1K_2 = mk_0 \qquad \text{(Eq. 17)}$$

where $m = 0.440$.

For predictive purposes, we can describe the number of microorganisms at any time, t, when chloramphenicol and sulfonamide are administered simultaneously as:

$$N = N_1 e^{k_{app.}(t - t_1)} \qquad \text{(Eq. 18)}$$

where N_1 is the number of *E. coli* in balanced growth at time t_1, where within the first 5.5 generations $k_{app.}$ is defined by k_0 in Eq. 16. When the new sulfonamide-affected steady state is achieved, $k_{app.}$ is defined from consideration of Eqs. 12, 13, and 16 as:

$$k_{app.} = \frac{[k_0' - k_cC][1 + K_1K_2(S - 1)]}{1 + K_1K_2 S} \qquad \text{(Eq. 19)}$$

where k_0, k_c, K_1, and K_2 have been shown in this paper and others (7, 8) to conform to the Arrhenius–Van't Hoff relation over a considerable temperature range:

$$k = P_e - \Delta H/RT \qquad \text{(Eq. 20)}$$

For a given temperature where m in Eq. 17 is known, Eq. 19 can be written as:

$$k_{app.} = k_0 - 1/(m + S/k_0) \qquad \text{(Eq. 21)}$$

REFERENCES

(1) Garrett, E. R., Ågren, A. J., and Lambert, H. J., *Intern. J. Clin. Pharm.*, **1**, 1(1967).
(2) Garrett, E. R., *Antibiot. Chemotherapy*, **8**, 8(1958).
(3) Garrett, E. R., and Brown, M. R. W., *J. Pharm. Pharmacol.*, **15**, 185T(1963).
(4) Brown, M. R. W., and Garrett, E. R., *J. Pharm. Sci.*, **53**, 179(1964).
(5) Garrett, E. R., and Miller, G. H., *ibid.*, **54**, 427(1965).
(6) Garrett, E. R., Wright, O. K., Miller, G. H., and Smith, K. L., *J. Med. Chem.*, **9**, 203(1966).
(7) Garrett, E. R., Miller, G. H., and Brown, M. R. W., *J. Pharm. Sci.*, **55**, 593(1966).
(8) Garrett, E. R., *Arzneimittel-Forsch.*, **16**, 1364(1966).
(9) Kohn, H. I., and Harris, J. S., *J. Pharmacol. Exptl. Therap.*, **73**, 343(1941).
(10) Harris, J. S., and Kohn, H. I., *ibid.*, **73**, 383(1941).
(11) Cowles, P. B., *Yale J. Biol. Med.*, **14**, 599(1942).
(12) Brueckner, A. H., *ibid.*, **15**, 813(1943).
(13) Campbell, A., *Bacteriol. Rev.*, **21**, 263(1957).
(14) Harvey, R. J., Marr, A. G., and Painter, P. R., *J. Bacteriol.*, **93**, 605(1967).
(15) Anton, A. H., *J. Pharmacol. Exptl. Therap.*, **129**, 282 (1960).
(16) Powell, E. O., and Errington, F. P., *J. Roy. Microscop. Soc.*, **82**, 39(1965).
(17) Northey, E. H., "The Sulfonamides and Allied Compounds," Reinhold Publishing Co., New York, N. Y., 1948.
(18) Woods, D. D., *Brit. J. Exptl. Pathol.*, **21**, 74(1940).
(19) Woods, D. D., and Fildes, P., *Chem. Ind. (London)*, **59**, 133(1940).
(20) Fildes, P., *Lancet*, **I**, 955(1940).
(21) Woods, D. D., *Ann. N. Y. Acad. Sci.*, **52**, 1197(1950).
(22) Brown, G. M., *J. Biol. Chem.*, **237**, 536(1962).
(23) Seydel, J. K., Krüger-Thiemer, E., and Wempe, E., *Tuberk.-Forschungsinst. Borstel, Jahresber.*, **5**, 651(1961).
(24) Seydel, J. K., *Antibiot. Chemotherapia*, **12**, 135(1964); Seydel, J. K., and Wempe, E., *Arzneimittel-Forsch.*, **14**, 705 (1964).
(25) Muir, R. D., Shaleffer, V. J., and Jones, L. R., *J. Bacteriol.*, **44**, 95(1951).
(26) Nimms-Smith, R. H., Lascelles, J., and Woods, D. D., *Brit. J. Exptl. Pathol.*, **29**, 264(1948).
(27) Lascelles, J., and Woods, D. D., *ibid.*, **33**, 288(1952).
(28) White, P. J., and Woods, D. D., *J. Gen. Microbiol.*, **40**, 243(1965).
(29) Rose, H. M., and Fox, C. L., *Science*, **95**, 412(1942).
(30) MacLeod, C. M., *J. Exptl. Med.*, **72**, 217(1940).
(31) Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, **97**, 265(1943).
(32) White, P. J., and Woods, D. D., *J. Gen. Microbiol.*, **40**, 243(1965).
(33) Weisberger, A. S., and Wolfe, S., *Federation Proc.*, **23**, 976(1964).

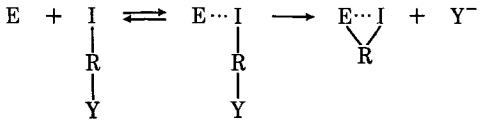
Enzyme Inhibitors XXI

Hydrophobic and Hydroxylic Binding Sites of Adenosine Deaminase

By HOWARD J. SCHAEFFER and CHARLES F. SCHWENDER

Recent studies have shown that the *ortho* and *para*-isomers of 9-(bromoacetamidobenzyl)adenine are good irreversible inhibitors of adenosine deaminase. In an attempt to prepare other types of irreversible inhibitors of this enzyme, a series of 9-(ω-bromoacetoxyalkyl)adenines were synthesized. However, none of these compounds were active as irreversible inhibitors although they were reversible inhibitors of this enzyme. Thus, it appears that the alkyl chain of the 9-(ω-bromoacetoxyalkyl)adenines does not complex with the enzyme in a manner identical to the 9-substituent of 9-(*p*- or *o*-bromoacetamidobenzyl)adenine. When the 9-(ω-hydroxyalkyl)adenines were evaluated as reversible inhibitors of adenosine deaminase, it was found that as the alkyl chain was lengthened, the compounds became initially more potent inhibitors, then less potent, and finally more potent again as the alkyl chain was lengthened to octyl. These results can be rationalized by assuming two different modes of binding. The short chain compounds bind to a hydroxyl binding site whereas the long chain compounds bind to a hydrophobic region which based on other data appears to extend approximately seven or eight carbons from the 9-position of adenine.

One of the prime requirements of an active-site-directed irreversible inhibitor is that the enzyme and inhibitor (1) form a reversible complex prior to the reaction in which the irreversible inactivation occurs (see Scheme I).

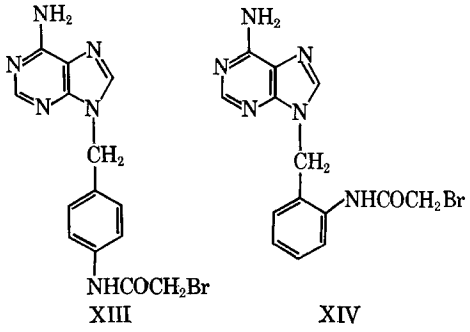


Scheme I

Thus, knowledge concerning types of binding sites on the enzyme required for reversible complexation are essential for the design of active-site-directed irreversible inhibitors. It has previously been shown that adenosine deaminase has both a polar and a nonpolar area that are important in binding certain substituents of a 9-substituted adenine (2). A successful attempt to utilize the hydrophobic region of adenosine deaminase for the preparation of active-site-directed irreversible inhibitors resulted in the synthesis of some 9-benzyl- and 9-phenethyladenines in which the benzene ring carried an alkylating agent (3–6). In order to determine if certain 9-alkyladenines bearing an alkylating group on the alkyl substituent could irreversibly inhibit adenosine deaminase, the authors decided to prepare some 9-(ω-bromoacetoxyalkyl)adenines where the alkyl group varies from ethyl to octyl. The present paper describes the synthesis and enzymic evaluation of these compounds as well as a study of the hydrophobic and hydroxyl binding sites of adenosine deaminase with some 9-(ω-hydroxyalkyl)adenines.

DISCUSSION

An examination of Table I reveals that the compounds prepared for this study were capable of causing reversible inhibition of adenosine deaminase. However, when the potential irreversible inhibitors (VII–XI) were incubated with the enzyme for periods of 5 hr. at 37°, no irreversible inactivation was observed. The incubations were performed with concentrations of inhibitors such that the amount of the reversible E· · ·I complex was at least 0.5 E_t. Under similar conditions, 9-(*p*-bromoacetamidobenzyl)adenine caused extensive irreversible inactivation of adenosine deaminase (3, 4). Comparison of the rates of reaction of VII–XI with iodoacetamide using 4-(*p*-nitrobenzyl)pyridine as the nucleophilic reagent revealed that VII–XI were approximately 4–5 times more reactive than iodoacetamide. Thus, VII–XI exhibit approximately the same rates of reaction as do 9-(*p*- and *o*-bromoacetamidobenzyl)adenines (XIII and XIV) with 4-(*p*-nitrobenzyl)pyridine (3–5).



XIII XIV

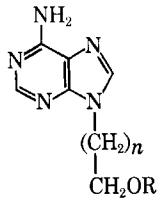
Received April 14, 1967, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 19, 1967.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by grant T-337A from the American Cancer Society, by a research grant 5-RO1-GM-09775-05 from the U. S. Public Health Service, by research career program award 5-K3-CA-18718-05 from the National Cancer Institute, and training grant 5-T1-GM-555-05 from the Division of Medical Sciences, U. S. Public Health Service, Bethesda, Md.

TABLE I—INHIBITION OF ADENOSINE DEAMINASE BY



Compd.[a]	n	mM Concn. for 50% Inhibition[b]	$(I/S)_{0.5}$[b]	Relative Reactivity
R = H				
I	1	0.070 ± 0.004[c]	1.1 ± 0.05[c,d]	...
II	2	0.046 ± 0.005	0.70 ± 0.08[d]	...
III	3	0.128 ± 0.009	1.94 ± 0.14[d]	...
IV	4	0.200 ± 0.007	3.0 ± 0.12[d]	...
V	5	0.112 ± 0.010	1.7 ± 0.14	...
VI	7	0.042 ± 0.001	0.64 ± 0.02	...
R = $-COCH_2Br$				
VII	1	0.122 ± 0.006	1.8 ± 0.09	4.0
VIII	2	0.106 ± 0.002	1.6 ± 0.04	4.2
IX	3	0.060 ± 0.004	0.90 ± 0.05	4.1
X	5	0.046 ± 0.002	0.71 ± 0.04	4.8
XI	7	0.026 ± 0.002	0.39 ± 0.02	4.8

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 mM. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 mM. In those cases where a higher concentration is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/V *versus* (*I*). [c] Average deviation. [d] Data taken from *Reference 2*. [e] Ratio of the rates of reaction of the various 9-(ω-bromoacetoxyalkyl)adenines to iodoacetamide with 4-(*p*-nitrobenzyl)pyridine.

The fact that VII–XI form reversible complexes with adenosine deaminase and possess good alkylating activity but nevertheless are not irreversible inhibitors of the enzyme is evidence that the 9-substituent of these compounds does not complex with the enzyme in a manner identical to the 9-substituent of XIII and XIV. In addition, it was found that 9-(2-bromoacetoxypropyl)adenine was a poor reversible inhibitor and did not cause irreversible inactivation of adenosine deaminase. Moreover, when the stability of the bromoesters to phosphate buffer was evaluated, it was found that less than 10% of the alkylating ability of these compounds was lost during a 2-hr. incubation at 37°. The lack of irreversible inactivation of adenosine deaminase by VII–XI substantiates the selectivity that one may obtain with active-site-directed irreversible inhibitors (1). An examination of molecular models reveals that VII–XI may assume several conformations identical with XIII or XIV. Even though all of these compounds form reversible complexes with the enzyme and have nearly equal alkylating activity, only 9-(*o*- and *p*-bromoacetamidobenzyl)adenines and 9-(*m*-bromoacetamidophenethyl)adenine are good irreversible inhibitors of adenosine deaminase.

An interesting phenomenon was observed when a comparison was made of the reversible inhibition of adenosine deaminase by some 9-(ω-hydroxyalkyl)adenines and some 9-alkyladenines (see Fig. 1). As the alkyl chain of the 9-alkyladenines is lengthened, the ability of the inhibitor to form a complex with the enzyme is increased. It was on these data that we previously concluded that the hydrophobic area of adenosine deaminase extends from approximately three to seven carbon atoms (2). The authors have recently prepared 9-nonyl- and 9-decyladenines which unfortunately were not

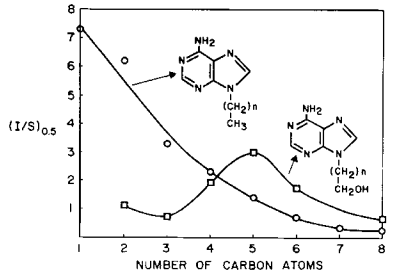


Fig. 1—A plot of the index of inhibition vs. *the number of carbons in the 9-substituent of some 9-(alkyl)- and 9-(hydroxyalkyl)adenines.*

sufficiently water-soluble to allow an accurate measurement of their inhibition index. Nevertheless, when these compounds were evaluated as inhibitors of adenosine deaminase in phosphate buffer containing 40% dimethylsulfoxide, they were weaker inhibitors than was 9-octyladenine evaluated under the same conditions. From these data, it would appear that the hydrophobic area on adenosine deaminase does terminate at a length of approximately seven or eight carbon atoms. However, an examination of a plot of $(I/S)_{0.5}$ *versus* chain length for a number of 9-(ω-hydroxyalkyl)adenines reveals that as the alkyl group is lengthened, the inhibitors become more potent, then less potent, and finally more potent again. The authors believe that these data conclusively demonstrate that two types of forces are involved in the formation of a complex with the 9-substituent of 9-(ω-hydroxyalkyl)adenines. When the 9-position of adenine bears either a 2-hydroxyethyl or a 3-hydroxypropyl group, both

the hydroxyl site and the hydrophobic region on the enzyme are making a contribution to binding. It has previously been shown that the hydroxyl site and the large hydrophobic site are not related to each other in an unbranched manner; *i.e.*, a straight carbon chain compound, such as 2-hydroxyheptyl, cannot bridge to both sites (7). This observation is further supported by the study of the compound with a 4-hydroxybutyl group at the 9-position of adenine. In this case, the 9-substituent cannot bind to both areas on the enzyme at the same time. When the butyl group binds to the hydrophobic region of the enzyme, there is some repulsion caused by proximity of the terminal hydroxyl group to the nonpolar area. However, by extensive folding of the butyl chain which is energetically unfavorable, it is possible for the alcohol function to bind to its hydroxyl binding site. The repulsive factor on the one hand is almost equally balanced by the second binding site so that the inhibition caused by III is almost equal to that caused by 9-butyladenine. The substituent of 5-hydroxypentyl represents a case where the hydroxyl group apparently does not make a contribution to binding. In fact, its major effect is a repulsion from the hydrophobic region. Finally, in the case of the 6-hydroxyhexyl and especially in the case of 8-hydroxyoctyl, the hydroxy group has essentially lost its repulsive effect. This occurs because the hydrophobic region has a length of only seven or eight carbon atoms. Thus, when the octyl group of 9-(8-hydroxyoctyl)adenine is complexed with the enzyme, the hydroxyl group extends beyond the hydrophobic region and is no longer repelled. Consequently, 9-octyladenine and 9-(8-hydroxyoctyl)-adenine approach each other in their inhibitory properties. Finally, a plot of $(I/S)_{0.5}$ *versus* the number of carbons in the alkyl chain of the 9-(ω-bromoacetoxyalkyl)adenines reveals a reasonably smooth curve with the inhibition becoming greater as the chain is lengthened. We interpret these data to mean that there is only one type of interaction important in binding the 9-substituent of the 9-(ω-bromoacetoxyalkyl)adenines and that this type of interaction is mainly hydrophobic.

CHEMISTRY

The synthesis of 9-(6-hydroxyhexyl)adenine was accomplished by a modification of a procedure (8) in which adenine was alkylated with 6-chloro-1-hexanol in dimethylacetamide in the presence of potassium carbonate. 9-(8-Hydroxyoctyl)adenine was synthesized by condensing 8-hydroxy-1-octanol with 5-amino-4,6-dichloropyrimidine. The resultant substituted pyrimidine was cyclized with trimethyl orthoformate to the desired 6-chloro-9-(8-hydroxyoctyl)purine which on treatment with methanolic ammonia gave 9-(8-hydroxyoctyl)adenine.

Several procedures were investigated to prepare the 9-(ω-bromoacetoxyalkyl)adenines using bromoacetyl bromide and bromoacetic anhydride, but the yields were consistently low. Finally, it was found that the bromoacetoxy derivatives could best be prepared by simple esterification of the hydroxyl group with bromoacetic acid catalyzed by ethanesulfonic acid. In this way 9-(ω-bromoacetoxyalkyl)adenines were prepared where the alkyl group was ethyl, propyl, butyl, hexyl, and octyl. In addition 9-(2-bromoacetoxypropyl)adenine was prepared by this general procedure.

EXPERIMENTAL[1]

9-(6-Hydroxyhexyl)adenine (V)—A mixture of 9.92 Gm. (58.0 mmoles) of adenine dihydrate, 15.8 Gm. (116 mmoles) of 6-chloro-1-hexanol, and 8.01 Gm. (58.0 mmoles) of anhydrous potassium carbonate in 40 ml. of dimethylacetamide was heated at 125° for 43 hr. under an atmosphere of nitrogen. The reaction mixture was filtered, and the filtrate was evaporated *in vacuo* to a residual solid, 6.01 Gm., which was placed on a column (35 × 900 mm.) of 450 Gm. of neutral alumina and eluted with a methanol–chloroform mixture collected in 100-ml. fractions. The desired material was obtained from fractions 51–66 (4% methanol in chloroform). Removal of the volatile material *in vacuo* gave a crude solid, 3.64 Gm. (37.4%), m.p. 184–187°, which was shown by TLC on silica gel to be one component, R_f 0.67 (20% methanol in chloroform).

8-Amino-1-octanol—To a stirred suspension of 19.9 Gm. (125 mmoles) of 8-aminooctanoic acid in 250 ml. of absolute ethanol at 0°, was slowly added 16.7 Gm. (140 mmoles) of thionyl chloride. The mixture was then refluxed for 8 hr. The reaction mixture was evaporated *in vacuo* and gave crude solid ethyl 8-aminooctanoate hydrochloride in a quantitative yield. The crude material was dissolved in 150 ml. of chloroform and washed with cold 5% sodium hydroxide solution (3 × 10 ml.). After the chloroform solution had been dried with anhydrous magnesium sulfate, the chloroform was evaporated *in vacuo* and gave the crude ethyl 8-aminooctanoate, 16.5 Gm. (71.2%). To a suspension of 10.6 Gm. (280 mmoles) of lithium aluminum hydride in 1 L. of anhydrous ether was added 16.5 Gm. of crude ethyl 8-aminooctanoate in 1 L. of ether. The reaction mixture was refluxed for 6 hr. and then water (45 ml.) was added to the cooled mixture. The resulting suspension was filtered, and the residual solid was washed with chloroform (500 ml.). The filtrates were combined and evaporated *in vacuo;* yield, 13.4 Gm. of crude 8-amino-1-octanol, m.p. 54–57°. Recrystallization of the material from benzene–hexane gave the pure white product; yield, 11.6 Gm. (63.8%), m.p. 54–59°. ν in cm.$^{-1}$ (KBr): 3450 (OH); 3300, 1550 (NH).

Anal.[2]—Calcd. for $C_8H_{19}NO$: C, 66.17; H, 13.19; N, 9.64. Found: C, 66.26; H, 13.11; N, 9.87.

This compound has previously been prepared by a different procedure and a m.p. 45° was reported (9).

5-Amino-4-chloro-6-(8-hydroxyoctylamino)pyrimidine—A mixture of 692 mg. (4.23 mmoles) of 5-amino-4,6-dichloropyrimidine, 644 mg. (4.44 mmoles) of 8-amino-1-octanol, and 504 mg. (5.00 mmoles) of triethylamine in 35 ml. of 1-butanol was heated at reflux for 23.5 hr. under nitrogen. The mixture was evaporated *in vacuo* and gave a residual syrup which was crystallized from a mixture of ethanol and water; yield, 940 mg. (81.0%), m.p. 80–85°. Recrystallization of the material from

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 202 spectrophotometer; the enzyme studies were done on a Gilford instrument model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

TABLE II—PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR SOME PURINES AND THEIR INTERMEDIATES

Compd.	Recrystn. Solvent[a]	M.p., °C.	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Hal. % Calcd.	Hal. % Found	Cosolvent	Ultraviolet $\lambda_{max.}$ ($\epsilon \times 10^{-4}$) 0.1 *N* HCl	H_2O	0.1 *N* NaOH
V	*A*	190–192	37.4	$C_{11}H_{17}N_5O$	56.10	56.01	7.28	7.48	29.77	29.60			10% EtOH	261 (1.24)	263 (1.27)	262 (1.24)
VI	*B*	207–210	59.2	$C_{13}H_{22}ClN_5O$	52.08	51.81	7.37	7.42	23.36	23.29	11.83[b]	11.66	5% EtOH	260 (1.48)	262 (1.42)	262 (1.42)
VII	*C*	190–192 dec.	18.7	$C_9H_{10}BrN_5O_2$	36.01	35.82	3.34	3.40	...	...	26.63[c]	26.54		261 (1.28)	263 (1.28)	263 (1.26)
VIII	*C*	182–184 dec.	56.5	$C_{10}H_{12}BrN_5O_2$	38.22	38.54	3.86	3.96	...	...	25.43[c]	25.29	10% EtOH	262 (1.22)	264 (1.30)	263 (1.50)
IX	*D*	139 dec.	31.0	$C_{11}H_{14}BrN_5O_2$	40.25	40.40	4.30	4.35	...	...	24.35[c]	24.60	7.5% EtOH	262 (1.28)	264 (1.35)	263 (1.48)
X	*C*	173–178 dec.	32.7	$C_{13}H_{18}BrN_5O_2$	43.86	43.62	5.08	5.09	19.66	19.90	22.44[c]	22.50	1% EtOH	262 (1.31)	263 (1.37)	263 (1.31)
XI	*E*	152–155 dec.	48.2	$C_{17}H_{24}BrN_5O_6$[d]	43.01	43.00	5.10	5.14	14.76	14.71	16.85[c]	16.90	12% EtOH	262 (1.42)	262 (1.45)	262 (1.39)
XII[e]	*C*	203 dec.	68.9	$C_{10}H_{12}BrN_5O_2$	38.22	38.42	3.81	3.99	22.29	22.53	25.47[c]	25.62	8% EtOH	258 (1.24)	261 (1.18)	261 (1.23)

A, ethanol; *B*, methanol–ether; *C*, acetone–hexane; *D*, ethyl acetate–hexane; *E*, 1% oxalic acid in methanol. [b] Chlorine analyzed. [c] Bromine analyzed. [d] Isolated as oxalate salt. [e] Compound XII is 9-(2-bromoacetoxypropyl)adenine.

ethanol–water gave the analytical product; yield, 855 mg. (73.7%), m.p. 84–87°. ν in cm.$^{-1}$ (KBr): 3400 (OH); 3300 (shoulder), 1640 (NH); 1580 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 12% EtOH/0.1 *N* HCl, 305 (1.20); 12% EtOH/H_2O, 265 (1.13), and 287 (1.08); 12% EtOH/0.1 *N* NaOH, 265 (0.982) and 295 (1.00).

Anal.—Calcd. for $C_{12}H_{21}ClN_4O$: C, 52.83; H, 7.75; Cl, 13.00; N, 20.54. Found: C, 52.42; H, 8.00; Cl, 12.72; N, 20.59.

9-(8-Hydroxyoctyl)adenine Hydrochloride (VI)—A mixture of 5.34 Gm. (19.6 mmoles) of 5-amino-4-chloro-6-(8-hydroxyoctylamino)pyrimidine, 268 mg. (2.43 mmoles) of ethanesulfonic acid, and 25 ml. of trimethyl orthoformate was stirred at room temperature for 23.5 hr. The reaction mixture was evaporated *in vacuo* to give a syrup which would not crystallize. The crude 6-chloro-9-(8-hydroxyoctyl)purine (17.6 mmoles) was heated at 80° with 60 ml. of methanolic ammonia solution in a steel bomb for 66 hr. The reaction mixture was cooled and a solid which precipitated was collected by filtration; yield, 3.68 Gm. (79.7%), m.p. 186–192°. For purification the product was converted into the hydrochloride salt which was recrystallized from methanol–ether.

9-(ω-Bromoacetoxyalkyl)adenines—These materials were prepared by the same general procedure. Therefore, only one procedure will be presented. The physical constants and analytical data for these compounds are given in Table II.

9-(6-Bromoacetoxyhexyl)adenine (X)—A mixture of 258 mg. (1.10 mmoles) of 9-(6-hydroxyhexyl)-adenine, 3.06 Gm. (22.0 mmoles) of bromoacetic acid, and 138 mg. (1.25 mmoles) of ethanesulfonic acid in 20 ml. of chloroform was heated at reflux for 23 hr. The chloroform volume was increased to 40 ml. and then washed with a saturated sodium bicarbonate solution (8 × 1 ml.). The chloroform phase was dried with anhydrous magnesium sulfate and evaporated *in vacuo* to a residual solid. The crude material after recrystallization from acetone–hexane gave 322 mg. (82.3%) of crude X, m.p. 174–177° dec. Further recrystallizations from acetone–hexane gave the analytical product; yield, 128 mg (32.7%), m.p. 173–178° dec.

CHEMICAL REACTIVITY OF THE ALKYLATING AGENTS

The procedure has previously been described (5) and is a modification of a literature procedure (10–12).

ENZYME ASSAY AND REAGENTS

Adenosine deaminase (type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The assay procedure for reversible inhibitors is a modification of the method developed by Kaplan (13).

The method used to study the irreversible inactivation of adenosine deaminase at 37° has been described (5) and is a modification of the general procedure of Baker (14).

REFERENCES

(1) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(2) Schaeffer, H. J., and Vogel, D., *J. Med. Chem.*, **8**, 507(1965).

(3) Schaeffer, H. J., and Odin, E., *J. Pharm. Sci.*, **54**, 1223(1965).
(4) Schaeffer, H. J., and Odin, E., *J. Med. Chem.*, **9**, 576(1966).
(5) *Ibid.*, **10**, 181(1967).
(6) Schaeffer, H. J., and Johnson, R. N., *ibid.*, to be published.
(7) Schaeffer, H. J., and Schwender, C. F., *J. Pharm. Sci.*, **56**, 207(1967).
(8) Montgomery, J. A., and Thomas, H. J., *J. Heterocyclic Chem.*, **1**, 115(1964).
(9) Ruzicka, L., Goldberg, M. W., Hurbin, M., and Boekenoogen, H. A., *Helv. Chim. Acta*, **16**, 1323(1933).
(10) Epstein, J., Rosenthal, R. W., and Ess, R. J., *Anal. Chem.*, **27**, 1435(1955).
(11) Bardos, T. J., Datta-Gupta, N., Hebborn, P., and Triggle, D. J., *J. Med. Chem.*, **8**, 167(1965).
(12) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(13) Kaplan, N. O., *Methods Enzymol.*, **2**, 473(1955).
(14) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).

Modified Hofmann Degradation for the Analysis of *n*-Alkylbenzyldimethylammonium Chlorides by Gas Chromatography I

C_{14}- to C_{18}-Alkyl Compounds

By E. C. JENNINGS, JR., and H. MITCHNER*

The Hofmann degradation of quaternary ammonium compounds has been studied for *n*-alkylbenzyldimethylammonium chlorides. Gas chromatography of the reaction products provides resolution of the benzyldimethylamine and the corresponding alkene. Quantitative analysis of quaternary content and qualitative evaluation of impurities have been obtained for a number of commercial products.

Of the methods for splitting of C—N bonds in amines, such as the von Braun with cyanogen bromide (1), the Hofmann degradation by pyrolysis of quaternary ammonium hydroxides (1), the Emde reduction of quaternary ammonium salts with sodium amalgam (2), and catalytic hydrogenation (2, 3), only the Hofmann and catalytic hydrogenation methods lend themselves readily to the analysis of *n*-alkylbenzyldimethylammonium chlorides. Warrington (3) has determined the alkyl distribution in benzalkonium chloride by the gas chromatographic analysis of the amines produced by catalytic hydrogenation. The determination of impurities was not feasible by this method, and the quantitative assay of the benzalkonium chloride was not afforded. Metcalfe (4) attempted the analysis of long chain quaternary ammonium compounds by a Hofmann degradation with direct injection on an alkaline treated gas chromatographic column. The reaction did not follow the expected course, and the results were not sufficiently precise for quantitative assay. Changing of the column with use also altered the alkyl distributions.

The Hofmann degradation for the analysis of *n*-alkyltrimethylammonium halides (5), and the catalytic hydrogenation of *n*-alkylbenzyldimethylammonium chloride (6, 7) have been reported in connection with gas chromatography for the determination of the long chain alkyl group. It has been suggested that in the Hofmann degradation (5) a disproportion reaction results, giving rise to both alkenes-1 and the corresponding alkanols, although this was not established by quantitative data.

Catalytic hydrogenation requires special equipment and does not afford a ready means of quantitatively determining volatile impurities. The Hofmann reaction suggested a simpler analytical approach. This work was directed toward determination of optimum reaction conditions with subsequent gas chromatography analysis of the reaction products. Assay of volatile impurities prior to Hofmann degradation combined with analysis of the products of the reaction provided a means of complete assay of commercial C_{14} to C_{18} alkylbenzyldimethylammonium chlorides.

APPARATUS AND REAGENTS

An F&M 810R-12 gas chromatograph equipped with a linear temperature programmer and dual hydrogen flame ionization detectors was employed with dual columns, 5 ft. × 1/8 in. (0.101 in. i.d.),

Received March 15, 1967, from the Control Laboratory, Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, CA 94086
Accepted for publication August 3, 1967.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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(3) Schaeffer, H. J., and Odin, E., *J. Pharm. Sci.*, **54**, 1223(1965).
(4) Schaeffer, H. J., and Odin, E., *J. Med. Chem.*, **9**, 576(1966).
(5) *Ibid.*, **10**, 181(1967).
(6) Schaeffer, H. J., and Johnson, R. N., *ibid.*, to be published.
(7) Schaeffer, H. J., and Schwender, C. F., *J. Pharm. Sci.*, **56**, 207(1967).
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(9) Ruzicka, L., Goldberg, M. W., Hurbin, M., and Boekenoogen, H. A., *Helv. Chim. Acta*, **16**, 1323(1933).
(10) Epstein, J., Rosenthal, R. W., and Ess, R. J., *Anal. Chem.*, **27**, 1435(1955).
(11) Bardos, T. J., Datta-Gupta, N., Hebborn, P., and Triggle, D. J., *J. Med. Chem.*, **8**, 167(1965).
(12) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 21(1965).
(13) Kaplan, N. O., *Methods Enzymol.*, **2**, 473(1955).
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APPARATUS AND REAGENTS

An F&M 810R-12 gas chromatograph equipped with a linear temperature programmer and dual hydrogen flame ionization detectors was employed with dual columns, 5 ft. × 1/8 in. (0.101 in. i.d.),

Received March 15, 1967, from the Control Laboratory, Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, CA 94086
Accepted for publication August 3, 1967.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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TABLE I—RELATIVE RETENTION DATA FOR CALIBRATION COMPOUNDS

Carbon No.	n-Alkanes		1-Alkenes		n-Alkanols	
	Rel. Tr[a]	RRT[b]	Rel. Tr	RRT	Rel. Tr	RRT
12	0.85	0.58	0.89	0.69	1.50	2.5
14	1.02	1.10	1.08	1.22	1.67	3.0
16	1.20	1.64	1.26	1.77	1.78	3.4
18	1.39	2.16	1.43	2.28		3.8

[a] With respect to BDMA = 1.00, Tr = 135°. [b] With respect to BDMA = 1.00, tr = 7.4 min. Benzylmethylamine gave a rel. Tr = 1.15 and RRT = 1.44. Benzylamine gave a rel. Tr = 1.60 and RRT = 2.8.

316 stainless steel, packed with 20% Carbowax 20M on polyvinylpyrrolidone (1%) coated support (Chromosorb W, 60/80 mesh). A total packing of 3.06 to 3.08 Gm. was obtained using only moderate vibration from a hand vibrator.[1] Hydrogen was supplied with an Elhygen generator[2] and tank nitrogen was used as carrier gas. Air for combustion was taken from the laboratory supply line after regulation to 23 psig and filtration through 13X molecular sieve. Samples were injected with a 10-μl. syringe (Hamilton 701 NW/G). A glass insert was placed within the injector chamber to confine the caustic solutions used to the area of this disposable glass liner and to protect the syringe needle from damage by striking any metal tubing. These glass liners were prepared from 3 mm. × 11 cm. glass tubing and were given a slight flare at the septum end. The liners were slotted 1.5 cm. from the flared end to allow entry of carrier gas. An emery disk of a Dremel Moto-Tool[3] was used for slotting.

The chromatograms were recorded with a Honeywell, model 16, recorder equipped with a Disc integrator, model 227.[4]

Even numbered compounds from C_{12} to C_{18} inclusive were obtained in 99% purity from Matheson, Coleman, and Bell for the homologous series of alkanes, alkenes-1, and alkanols and used without further purification.

Benzyldimethylamine was fractionally redistilled before use. Benzylmethylamine and benzylamine were used without further treatment. Reagent grade silver oxide and potassium hydroxide were used as received.

Glass ampuls (5 ml.), Pasteur disposable pipets, 5-ml. gas tight syringe (Hamilton 1005), and an ampul sealing torch (Matheson Scientific) were the only other items that may not be standard supplies in all laboratories.

PROCEDURE

Parameters for Programmed Temperature Gas Chromatography—The injection port temperature was set at 180°, and the oven was given a lower limit of 100°. A post-injection dwell time of 1 min. was used. The temperature was programmed to an upper limit of 240°, at a rate of 6.0°/min. An upper limit dwell time of 8 min. was held prior to the automatic recycling and resetting of initial conditions. All compounds of interest for the determination of the alkyl group distribution were eluted within 20 min. The program provided sufficient time to ensure that higher boiling impurities found in most quaternary ammonium salts had been eluted prior to the next run.

The hydrogen pressure was set at 13–15 psig which gave a flow of 40 to 48 ml./min. The nitrogen pressure was set at 60 psig, and flow through each column was adjusted at the upper temperature limit to balance the columns for column bleed. The measuring column had a flow of 25 ml./min., and the reference column a flow of 20 ml./min. The air pressure to the detector was set at 23 psig which gave a flow of 340 ml./min.

Gas Chromatography of Reference Compounds—With 5.0-μl. injections of solutions containing 10 mg./ml. of each reference compound a range of 100 and an attenuation of 128 were used. The relative retention data of all compounds were taken with respect to benzyldimethylamine (BDMA). The detector responses in micrograms per disk unit of integration (mcg./DU) were calculated for a range of 10 and an attenuation of 1×. (Table I.)

Determination of Optimum Hofmann Reaction Conditions—Initially the Hofmann degradation with silver oxide was studied. A modification of the Hofmann degradation, using 4 *N* KOH, was used for a replicate set of experiments. Equal volumes of 8 *N* KOH and aqueous quaternary ammonium salt solution were pipeted into ampuls to provide a final concentration of 4 *N* KOH and 25 mg./ml. of the quaternary ammonium chloride. Ampuls were heated to 110°, and samples were analyzed at 1, 2, and 4 hr.

Determination of Impurities—Injections of the aqueous solutions of the quaternary ammonium compounds were made prior to running Hofmann reactions. Clean glass injection port liners were used in each run to obviate residual alkali from previous experiments that could give rise to a Hofmann degradation. The injector port temperature was reduced to a practical minimum of 180° to minimize thermal pyrolysis of the quaternary ammonium chlorides. Three alkylbenzyldimethylammonium chlorides, C_{14}-alkyl, C_{16}-alkyl, and C_{18}-alkyl, were studied.

From gas chromatographic runs the major volatile impurities were isolated using an effluent splitter at a ratio of 1:1. The impurities were collected directly on a 13-mm. Millipore filter disk, type TH (8). Infrared spectra of isolates were obtained by scanning the Millipore disks on a Beckman IR-8 spectrophotometer. A clean disk was used in the reference beam.

Isolation of impurities was also made by processing a chloroform solution of the quaternary ammonium chloride on a chromatographic column of silicic acid (19 mm. × 100 mm.). The total loading of the column was 2.0 Gm. of quaternary ammonium

[1] Vibro Graver, model V-73, Burgess Vibrocrafters, Inc., Grayslake, Ill.
[2] Milton Roy Co., St. Petersburg, Fla.
[3] Cole-Parmer Instrument and Equipment Co., Chicago, Ill.
[4] Disc Instruments, Inc.

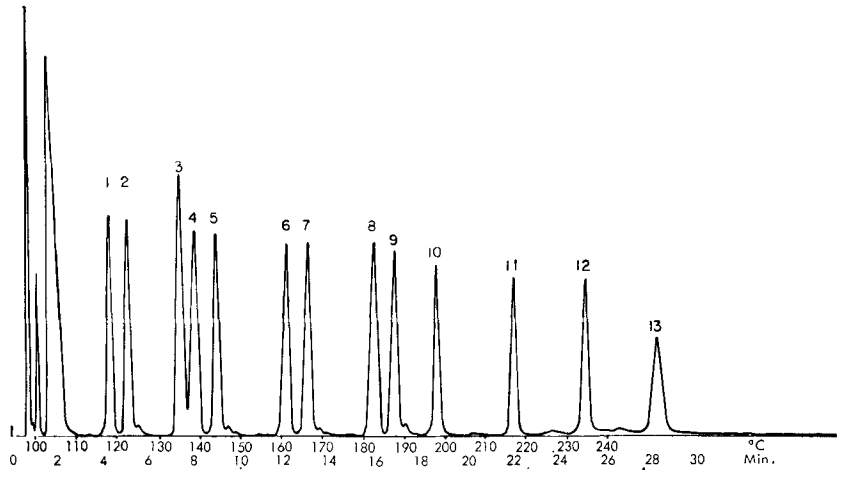

Fig. 1—Calibration run with temperature program ming from 100° to 240°. Key: 1, n-$C_{12}H_{26}$; 2, *1*-$C_{12}HO_{24}$; 3, *BDMA;* 4, n-$C_{14}H_{30}$; 5, *1*-$C_{14}H_{28}$; 6, n-$C_{16}H_{34}$; 7, *1*-$C_{16}H_{32}$; 8, n-$C_{18}H_{38}$; 9, *1*-$C_{18}H_{36}$; 10, n-$C_{12}H_{25}OH$; 11, n-$C_{14}H_{29}OH$; 12, n-$C_{16}H_{33}OH$; 13, n-$C_{18}H_{37}OH$.

TABLE II—HOFMANN DEGRADATION OF C_{16}-ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDE (PERCENT ALKYL DISTRIBUTION WITH DIFFERENT BASES)

	4 *N* KOH				Silver Oxide			
	Reaction Time, hr.							
1-Alkene	3/4	1.0	2.0	4.0	3/4	1.0	2.0	4.0
C_{12}	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C_{14}	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C_{16}	95.5	96.2	96.3	95.0	96.4	95.5	93.0	78.2
C_{18}	4.5	3.8	3.7	5.0	3.6	4.5	7.0	21.8
Total alkene, μm.	0.142	0.152	0.140	0.142	0.265	0.348	0.315	0.485
BDMA, μm.	0.182	0.197	0.183	0.185	0.359	0.347	0.246	0.188
Ratio: total alkene / BDMA	0.78	0.77	0.78	0.77	0.74	1.0	1.3	2.6

chloride. Development of the column was made with chloroform. During the washing of the column with solvent, the eluate was observed in ultraviolet light (366 mμ), and fraction cuts were made where it appeared that a change in fluorescence could be seen. Finally the column was washed with methanol to remove the quaternary ammonium chloride when the column fluorescence was not further reduced by the chloroform washing. The solvent from each fraction collected was evaporated and the residues were examined (*a*) by microscopy at 100× in polarized light, (*b*) by IR spectroscopy, and (*c*) by gas chromatography of a chloroform solution of the residue. The residues were compared by these methods to known compounds. In the case of the C_{18}-alkyl compound crystals of a major impurity separated from the initial aqueous solution used for the Hofmann reaction. These were subjected also to the foregoing methods of identification.

RESULTS AND DISCUSSION

Preliminary experiments with Hofmann degradation and gas chromatographic studies of the reaction products indicated the possible presence of alkanes and alcohols, as well as the expected alkenes and

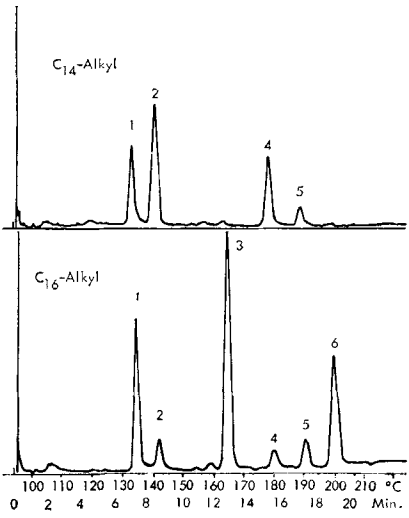

Fig. 2—Hofmann degradation in 4 N *KOH, 1 hr. 105°; 5-µl. injection, range 10, attenuation 32×. Key:* 1, *BDMA;* 2, *1*-$C_{14}H_{28}$; 3, *1*-$C_{16}H_{32}$; 4, n-$C_{18}H_{38}$; 5, *1*-$C_{18}H_{36}$; 6, n-$C_{12}H_{25}OH$.

benzyldimethylamine. To identify the products and to ascertain that the materials were being resolved, reference compounds of C_{12} to C_{18} straight chain alkanes, alcohols, and alkenes were obtained and synthetic mixtures chromatographed (Fig. 1). Relative retention data to benzyldimethylamine was used for comparison and identification. (Table I.) Retention data were also obtained for benzylmethylamine and benzylamine considered as possible impurities. Programmed chromatograms resolved all compounds.

For determinations of optimum conditions to conduct the Hofmann degradation, a C_{16}-alkylbenzyldimethylammonium chloride was reacted at several time periods in the presence of silver oxide. With increasing reaction time the composition of products changed. When 4 *N* KOH was substituted for silver oxide, constant composition results were obtained as shown in Table II. All subsequent studies used 4 *N* KOH with 1.0 hr. reaction time in sealed ampuls. Chromatograms for the C_{14}- and C_{16}-alkyl quaternaries after Hofmann degradation are shown in Fig. 2.

The relative retention data for the major impurities in the C_{16}-alkyl compound were consistent with that for the C_{12} and C_{16} straight chain alcohols and the C_{16}-alkane. Some monomethylbenzylamine was present, as was some C_{18}-alkene, the latter attributable to the presence of some C_{18} quaternary in the original product. The C_{12} and C_{16} alcohols from the C_{16}-alkyl compound were also isolated by column chromatography on silicic acid and positively identified. The composition of the volatile components from the gas chromatograms were calculated from the detector sensitivities of the individual components of reference compounds (Table III). The alkyl distribution and quaternary assay for the C_{16}-alkylbenzyldimethylammonium chloride is given in Table IV. The material was approximately 50% quaternary ammonium compounds and 50% impurities, the latter primarily lauryl and cetyl alcohols. A C_{14}-alkyl compound, a second C_{16}-alkyl compound, and a C_{18}-alkyl compound were also subjected to a purity study. The data for these materials are shown in Table V. Based on the total material injected, the total recovery of volatile materials was about 90%, and the total quaternary ammonium content ranged from 59% to 67%. About 10% of the material injected was of a non-

TABLE III—DETECTOR RESPONSE FOR CALIBRATION COMPOUNDS

Compd.	Response (mcg./DU) at Range 10, 1×
n-Alkanes ($C_{12}-C_{18}$)	$44 \pm 1 \times 10^{-6}$
1-Alkenes ($C_{12}-C_{18}$)	$45 \pm 1 \times 10^{-6}$
n-Alkanols ($C_{12}-C_{18}$)	$55 \pm 3 \times 10^{-6}$
BDMA, BMA, and BA	$66 \pm 1 \times 10^{-6}$

TABLE IV—ASSAY OF C_{16}-ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDE BY THE MODIFIED HOFMANN REACTION WITH 4 *N* KOH[a]

Compd.	RRT	Av. Peak Area, Range 100, 64×	% Alkyl Distribution		mcg.
BDMA	1.00	604		Total quat.[b]	74.5
MBA	1.48	43		Total impurity	72.3
n-$C_{16}H_{34}$	1.61	63		Total vol. material	146.8
1-$C_{16}H_{32}$	1.72	1070	95.6	% Quat.	50.8
1-$C_{18}H_{36}$	2.31	48	4.4	% Impurity	49.2
n-$C_{12}H_{25}OH$	2.53	1960			100.0
n-$C_{16}H_{33}OH$	3.40				

[a] Average of data used for Table II. [b] Based on the micromole of BDMA found and the distributed mol. wt. of 397 from the corresponding alkyl distribution.

TABLE V—ASSAY OF THREE COMMERCIALLY AVAILABLE ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDES

	C_{14}-Alkyl	C_{16}-Alkyl	C_{18}-Alkyl
Concn.	2.50 mg./ml.	2.53 mg./ml.	2.79 mg./ml.
Vol. injected	5 µl.	5 µl.	5 µl.
Total material	12.5 mcg.	12.7 mcg.	14.0 mcg.
Alkyl Group	Distrib.	Distrib.	Distrib.
C_{12}	4.7	0	0
C_{14}	90.0	16.8	6.2
C_{16}	5.3	83.2	9.4
C_{18}	0	0	84.4
Total	100.0%	100.0%	100.0%
Distributed mol. wt.	369	392	417
BDMA, µm.	0.0227	0.0201	0.0200
Total quat.[c]	8.38 mcg.	7.87 mcg.	8.33 mcg.
Total vol. impurities[a]	4.60 mcg.	3.46 mcg.	4.31 mcg.
Total recovery	12.98 mcg.	11.33 mcg.	12.64 mcg.
% Recovery	103.8	89.2	89.5
% Quat.[b]	67.0	62.0	59.4
% Impurity[b]	33.0	38.0	40.6

[a] Methylbenzylamine, hexadecane, octadecane, lauryl, myristyl, cetyl, and stearyl alcohols. [b] Based on total material injected. [c] Total quaternary compound is based on distributed mol. wt. and BDMA found.

TABLE VI—ASSAY OF PURIFIED C_{14}-ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDE[a]

Compd.	Peak Area, R10, 1×	Factor	μm.	% Alkyl Distrib.
1-$C_{14}H_{28}$	401920 DU	45.4×10^{-6} mcg./DU	0.09300	90.20
1-$C_{16}H_{32}$	8960		0.00182	1.87
1-$C_{18}H_{36}$	45440		0.00818	7.93
		Total alkenes	0.10300	100.00
BDMA	232960 DU	66×10^{-6} mcg./DU	0.1132	

Impurities	Peak Area	Factor	Amt.
$C_{12}H_{25}OH$	5120 DU		
$C_{16}H_{33}OH$	47360		
Total alcohol	52480 DU	55×10^{-6} mcg./DU	2.87 mcg.
$C_{12}H_{26}$	12800 DU		
$C_{18}H_{38}$	220160		
Total alkane	232960 DU	44×10^{-6} mcg./DU	10.18 mcg.
		Total impurities	13.05 mcg.

[a] Total material injected = (5.0 μl.) (10.31 mcg./μl.) = 51.55 mcg. Total material injected less impurities (total quat.) = 38.5 mcg. Total quat. micromoles (based on distributed mol. wt.) = 0.1145. Total quat. based on BDMA and distributed molecular wt. = 0.1132. Percent yield = 99%.

volatile nature and probably represented inorganic salts that occur in such commercial materials.

It has been reported (5) that a Hofmann reaction can result in a disproportionation reaction with both the alkene and its corresponding alcohol appearing as reaction products. The C_{14}-alkylbenzyldimethylammonium chloride, purified by silicic acid absorption chromatography, was used to study this possibility. Pre-Hofmann chromatograms showed that C_{14} alcohol was absent in the purified material. Following degradation in 4 *N* KOH no C_{14} alcohol was produced. Results are shown in Table VI.

SUMMARY

It has been shown that the various commercial *n*-alkylbenzyldimethylammonium chlorides are mixtures of two or more quaternary ammonium compounds and that impurities of higher alkanes, C_{16} to C_{18}, and higher alcohols, C_{12} to C_{18}, are significant. The compounds investigated contained from 50% to 70% quaternary ammonium chlorides.

The modified Hofmann degradation is a relatively simple procedure and provides for the determination of alkyl distribution, quaternary ammonium salt content, and impurities. Standard deviations obtained show good precision for this type of procedure. Quantitative assay is dependent upon the amount of BDMA formed by the reaction and is directly proportional to the total quaternary ammonium chloride present.

REFERENCES

(1) "Organic Chemistry," vol. I, Gilman, H., ed., John Wiley & Sons, New York, N. Y., 1938, p. 713.
(2) *Ibid.*, vol. II, p. 1025.
(3) Warrington, H. P., Jr., *Anal. Chem.*, **33**, 1898(1961).
(4) Metcalfe, L. D., *J. Am. Oil Chemists' Soc.*, **40**, 25(1963).
(5) Van Senden, K. G., *Rec. Trav. Chim.*, **84**, 1459(1965).
(6) Southworth, B. C., *Anal. Chem.*, **28**, 1611(1956).
(7) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 66.
(8) "Application Data Manual," ADM-70, Millipore Filter Corp., Bedford, Mass., 1963, p. 30.

Modified Hofmann Degradation for the Analysis of *n*-Alkylbenzyldimethylammonium Chlorides by Gas Chromatography II

Benzalkonium Chloride

By H. MITCHNER* and E. C. JENNINGS, JR.

The modified Hofmann degradation with gas chromatography of degradation products has been applied to a study of benzalkonium chloride. Quantitation is possible to 0.05 percent and the determination of alkyl group distribution is simultaneously possible. A good correlation was found between the Hofmann degradation and the USP catalytic hydrogenation for determination of alkyl ratios.

BENZALKONIUM CHLORIDE is a germicide and preservative which has found increasingly greater use. Its germicidal effectiveness is based on its content of a mixture of *n*-alkyl dimethylbenzylammonium chlorides, the most important of which are the C_{12} and C_{14}. The United States Pharmacopeia requires compendia labeled material to contain not less than 40% of the C_{12}-alkyl, and that C_{14} be not less than 20% of the total alkyldimethylammonium chloride content with the sum of the C_{12} and C_{14}-alkyls not less than 70% of the total (1).

The analysis of the alkyl substituent in benzalkonium chloride poses a difficult analytical problem. For the official analysis the USP applies a gas chromatographic assay following an Emde degradation. This allows a determination of alkyl group ratios but is not quantitative and requires specialized hydrogenation equipment. A new gas chromatographic approach to the analysis of *n*-alkyl dimethylbenzalkonium chlorides employing a modified Hofmann degradation (2) was found applicable to the analysis of benzalkonium chloride.

The modified Hofmann degradation was applied directly to several commercial benzalkonium chloride preparations. The catalytic hydrogenation procedure utilized in the benzalkonium chloride monograph of the USP (1), which is essentially the procedure of Warrington (3) and the special apparatus of Southworth (4), was compared with the Hofmann degradation procedure with similar results.

Received March 15, 1967, from the Control Laboratory, Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, CA 94086
Accepted for publication August 3, 1967.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Previous paper: Jennings, E. C., Jr., and Mitchner, H., *J. Pharm. Sci.*, **56**, 1590(1967).
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APPARATUS AND REAGENTS

In addition to the apparatus employed previously in this study (2), special adaptation of the Parr hydrogenation apparatus for microsamples was used.

For further calibration of the F&M 810R-12 gas chromatograph, pure alkyldimethylamines with even numbered alkyl groups C_8 to C_{18} were obtained from Matheson, Coleman, and Bell. These compounds were used as received and corrections for minor alkyldimethylamines were made, based on gas chromatograms of these materials.

Samples of benzalkonium chloride[1] from several manufacturers and a standard benzalkonium chloride from the USP reference standards,[2] as distributed by the United States Pharmacopeial Convention, Inc., were obtained.

PROCEDURE

Based on the label assay of the commercial benzalkonium chloride preparations, aqueous concentrations of 100 mg./ml. were prepared. For the modified Hofmann degradation, 1.8 ml. of the benzalkonium chloride solution was transferred with a Pasteur disposable pipet actuated with a 5-ml. Hamilton syringe. An equal volume of 8 *N* KOH was added to give final concentrations of 50 mg./ml. of benzalkonium chloride in 4 *N* KOH. Ampuls were sealed and heated 1.0 hr. at 110°. After cooling the ampul under tap water and shaking to thoroughly remix any separated phases, the ampul was opened, and 5 μl. was injected into the chromatograph. All conditions of operation were the same as used in the previous study (2).

For the catalytic hydrogenation, 2.0 ml. of the benzalkonium chloride solution was transferred to the micro glass reaction flask and 35 mg. of palladium chloride (10%) on charcoal was added. After initial evacuation of the reaction flask, hydrogen was introduced to a final pressure of 35 psig and the reaction was allowed to proceed until no further pressure drop was obtained. The procedure of the USP (1) was continued for the recovery of the alkyldimethylamines formed.

The calibration runs on the gas chromatograph

[1] These materials were taken from file samples and may not reflect current production material.
[2] USP Reference Standards, New York, N. Y.

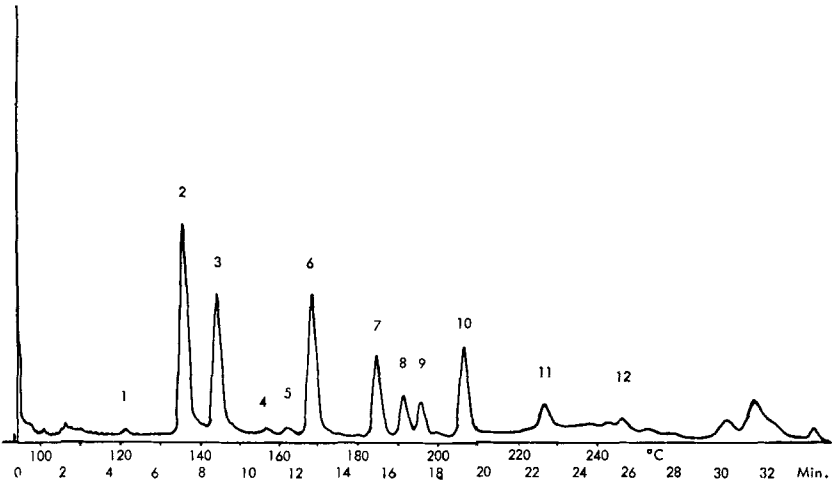

Fig. 1—Synthetic mixture of C_{14}, C_{16}, and C_{18} alkyl benzyldimethylammonium chlorides. Key: 1, *1-*$C_{12}H_{24}$; 2, *BDMA;* 3, *1-*$C_{14}H_{28}$; 4, *BMA;* 5, n-$C_{16}H_{34}$; 6, *1-*$C_{16}H_{32}$; 7, n-$C_{18}H_{38}$; 8, *1-*$C_{18}H_{36}$; 9, n-$C_{12}H_{25}OH$; 10, n-$C_{14}H_{29}OH$; 11, n-$C_{16}H_{33}OH$; 12, n-$C_{18}H_{37}OH$.

TABLE I—RELATIVE ELUTION TEMPERATURE AND DETECTOR RESPONSE FOR AMINE CALIBRATION COMPOUNDS

Carbon No.	*n*-Alkyldimethylamines RTr
C_8	0.38
C_{10}	0.66
C_{12}	1.00
C_{14}	1.34
C_{16}	1.65
C_{18}	1.95
Detector response (mcg./DU) $\times 10^{-6}$	37 ± 1

TABLE II—*n*-ALKYL DISTRIBUTION IN A SYNTHETIC MIXTURE OF C_{14}, C_{16}, AND C_{18}-ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDES

1-Alkene	Synthetic Mixture, 2.3 mg./ml. Sample *1*	Sample *2*	Sample *3*	Av.	Calcd. as Mixed
C_{12}	0.0	0.0	0.0	0.0	0.0
C_{14}	45.6	42.8	42.5	43.7	48.0
C_{16}	43.4	43.2	42.4	43.0	41.6
C_{18}	11.0	14.0	15.0	13.3	10.4
	100.0	100.0	100.0	100.0	100.0

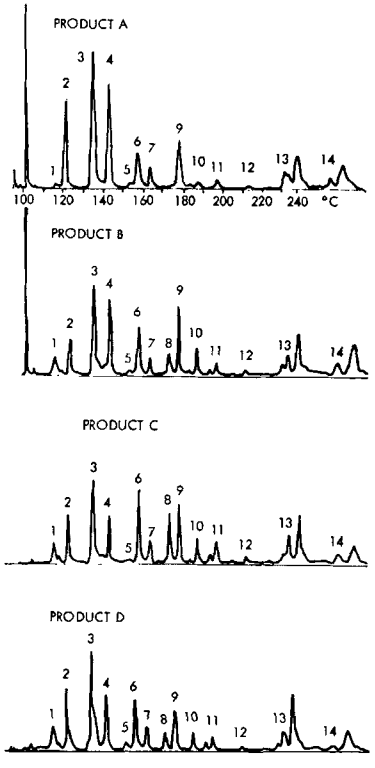

Fig. 2—Hofmann degradation of four commercial benzalkonium chlorides. Key: 1, n-$C_{12}H_{26}$; 2, *1-*$C_{12}H_{24}$; 3, *BDMA;* 4, *1-*$C_{14}H_{28}$; 5, *BMA;* 6, n-$C_{16}H_{34}$; 7, *1-*$C_{16}H_{32}$; 8, n-$C_{18}H_{38}$; 9, $C_{10}H_{21}OH$; 10, *1-*$C_{18}H_{36}$; 11, $C_{12}H_{25}OH$; 12, $C_{14}H_{29}OH$; 13, $C_{16}H_{33}OH$; 14, $C_{18}H_{37}OH$.

were temperature programmed runs (6°/min.) from 100–240° and linear plot of relative elution temperature to benzyldimethylamine for all alkanes, alkenes-1, and alkanols found in the modified Hofmann degradation against carbon number in the various homologous series gave straight line relationships. The same operating conditions were used also for the analysis of the catalytic hydrogenation products.

The relative elution temperature and average detector response in micrograms per disk unit of integration (mcg./DU) for the alkanes and alkanols have been previously reported (2). Table I shows the relative retention characteristics of alkyldimethylamines with respect to lauryldimethylamine under similar conditions.

TABLE III—ALKYL DISTRIBUTION OF FOUR BENZALKONIUM CHLORIDES BY THE MODIFIED HOFMANN DEGRADATION

Alkyl Group	Product *A*	Product *B*	Product *C*	Product *D*[a]
C_{12}	25.8	24.8	34.9	36.1
C_{14}	47.0	47.4	34.1	37.5
C_{16}	15.1	10.4	14.4	14.4
C_{18}	12.1	18.4	16.6	12.0
	100.0	100.0	100.0	100.0

[a] Prepared from the 93.5% concentrate.

RESULTS AND DISCUSSION

Alkyl Distribution Studies—To determine the applicability of the modified Hofmann degradation in conjunction with gas chromatography for the analysis of alkyl distribution of a mixture, such as benzalkonium chloride, a synthetic mixture was prepared from samples of C_{14}-alkyl-, C_{16}-alkyl-, and C_{18}-alkylbenzyldimethylammonium chlorides, previously analyzed by the same technique (2). As shown in Fig. 1, good resolution of all peaks was obtained, and reasonable replication of alkyl distribution between samples was achieved. (Table II.)

TABLE IV—ALKYL DISTRIBUTION OF PRODUCT *E*[a] BY THE MODIFIED HOFMANN DEGRADATION

	Lot No. →	02349	02269	02152	01920	01742	
Alkyl Group	Date Recd. →	3/29/66	11/30/65	8/2/65	12/21/64	7/9/64	Av.
C_{12}		57.2	50.2	52.2	51.2	50.0	52.2
C_{14}		34.9	36.1	35.6	35.4	34.4	35.3
C_{16}		6.7	12.4	10.7	13.1	12.7	11.1
C_{18}		1.2	1.3	1.5	0.3	2.9	1.4
		100.0	100.0	100.0	100.0	100.0	100.0

[a] Prepared from the 50% aqueous solution from samples received over a 2-year period.

TABLE V—COMPARISON OF THE CATALYTIC HYDROGENATION WITH THE MODIFIED HOFMANN DEGRADATION FOR THE ALKYL DISTRIBUTION IN BENZALKONIUM CHLORIDE

	Product *A*, Lot 1470 (50%)		Product *A*, Lot 0508 (50%)	
Alkyl Group	Catalytic Hydrogenation	Hofmann Degradation	Catalytic Hydrogenation	Hofmann Degradation
C_{12}	39.3	39.9	44.3	38.0
C_{14}	46.5	46.2	44.1	48.9
C_{16}	11.2	10.5	8.5	11.1
C_{18}	3.0	3.4	3.1	2.0
	100.0	100.0	100.0	100.0

TABLE VII—COMPARISON OF THE CATALYTIC HYDROGENATION WITH THE MODIFIED HOFMANN DEGRADATION FOR THE ALKYL DISTRIBUTION IN A USP REFERENCE STANDARD BENZALKONIUM CHLORIDE

Alkyl Group	Label Assay	Catalytic Hydrogenation	Hofmann Degradation
C_8	0.4	...	...
C_{10}	0.7	...	...
C_{12}	55.0	62.4	51.2
C_{14}	34.0	29.4	36.7
C_{16}	9.7	8.2	12.1
C_{18}	0.9	...	...
	100.7	100.0	100.0

TABLE VI—MAJOR VOLATILE IMPURITIES PRESENT IN FOUR COMMERCIAL BENZALKONIUM CHLORIDES BASED ON PEAK AREAS

Carbon No.	Product *A*			Product *B*			Product *C*			Product *D*		
	Alkane	Alkanol	BMA	Alkane	Alkanol	BMA	Alkane	Alkanol	BMA	Alkane	Alkanol	BMA
C_{10}	...	825	...	...	875	...	...	765	...	...	515	...
C_{12}	55	170	...	65	200	...	75	275	...	100	195	...
C_{14}	...	60	...	...	100	...	...	90	...	...	65	...
C_{16}	635	255	...	690	310	...	995	1020	...	740	280	...
C_{18}	265	240	...	315	350	...	585	200	...	225	220	...
Total disk units	955	1550	160	1070	1835	115	1655	2350	95	1065	1275	160
mcg.[a]	26.9	54.6	7	30.1	64.6	5	46.6	82.8	4	30	44.9	7
Total impurities, mcg.		88.5			99.7			133.4			81.9	
Total material injected, mcg.		261			252			250			278	
% Impurity		33.9			39.6			53.3			29.5	
% Quat.[b]		57.8			54.4			54.4			49.9	
Total		91.7			94.0			107.7			79.4	

[a] Based on detector responses given in Table I. [b] Based on detector responses given in Table I for BDMA and distributed molecular wt. of quaternary ammonium chlorides.

Four different commercial benzalkonium chlorides were analyzed after Hofmann degradation. Qualitatively similar chromatograms were obtained (Fig. 2), but analysis of peak areas showed considerable variation in the alkyl ratio distribution (Table III). This study was expanded to evaluate possible lot to lot variations in a commercial product used for an extended time and samples of benzalkonium chloride (product *D*) as received over a period of 2 years were analyzed for their alkyl distribution. Constant alkyl ratios were obtained (Table IV). Two lots of product *A* were also analyzed with similar good agreement for alkyl distribution between lots (Table V).

Impurities in Benzalkonium Chlorides—In addition to the expected alkenes, samples of benzalkonium chlorides showed the presence of impurities of alkanols, alkanes, and benzylmethylamine. Many of these impurities have been previously characterized (2) and can be identified in chromatograms by their retention data. Considerable variation in the amounts of the impurities between different benzalkonium chlorides were noted. An analysis of the peak areas of the major impurities in the chromatograms from Fig. 2 is shown in Table VI. The volatile impurities were a significant contribution to the content of the products and were from 30–53%. No evaluation of nonvolatile impurities was made.

Quantitation—The use of the modified Hofmann degradation approach for quantitative results is based on the formation of benzyldimethylamine by any of the quaternaries present, irrespective of the alkyl substituents. The ratios of the alkenes found allows an assignment of the amount of the particular quaternary. Quantitation was possible to a lower limit of 0.05%.

Comparison of Catalytic Hydrogenation and Hofmann Degradation—The official USP method for the determination of alkyl distribution in benzalkonium chloride requires specialized hydrogenation equipment in addition to a temperature programmed gas chromatograph (1). In the chemical work-up after hydrogenation and before analysis, many of the impurities possibly present in the original compounds or created with hydrogenation are eliminated by the USP procedure. The ratio of the amines resulting from the hydrogenation of the benzalkonium chloride are obtained from the chromatograms. A comparison of the USP procedure with the modified Hofmann degradation was made for two lots of product *A* (Table V) and for a USP reference standard (Table VII). The results obtained by both methods showed that the differences between methods were within the limits of variation for either method on a single sample.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 66.
(2) Jennings, E. C., Jr., and Mitchner, H., *J. Pharm. Sci.*, **56**, 1590(1967).
(3) Warrington, H. P., Jr., *Anal. Chem.*, **33**, 1898(1961).
(4) Southworth, B. C., *ibid.*, **28**, 1611(1956).

N-Aralkyl-*N*-methylaminoethyl Carbanilates as Hypocholesteremic Agents

By WILLIAM J. ROST, BLAINE M. SUTTON, BENJAMIN BLANK, FRANCIS R. PFEIFFER, WILLIAM L. HOLMES, NICHOLAS W. DITULLIO, and EDWIN B. INGRAM

A group of aralkylaminoethyl esters and ureas of substituted carbanilic acids was prepared and studied for hypocholesteremic activity in eucholesteremic mice. Biological results indicated that modification of either terminal aromatic ring altered the activity of the resultant compound. The *meta* and *para* methyl substituted carbanilates of aralkylmethylaminoethanol consistently exhibited the most desirable effect. In the dicarbanilate series of aralkyl or aryl substituted iminodiethanols, no appreciable activity was seen.

A previous publication (1) reported on the preparation of a series of *N*-aralkyl-*N*-methylaminoethyl carbanilates and their local anesthetic activity. Since that report, additional biological testing revealed that certain members of the series possessed hypocholesteremic activity in experimental animals. The novelty of this activity as well as the potential value of a safe effective agent exhibiting these properties prompted the study of this type of compound in greater detail. This paper describes preparation of additional examples in this class of compounds, their effect on cholesterol metabolism in the mouse, and chemical structure-biological function relationships in the series.

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This study was supported by grants from Smith Kline & French Laboratories, Philadelphia, Pa.

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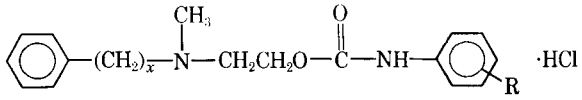
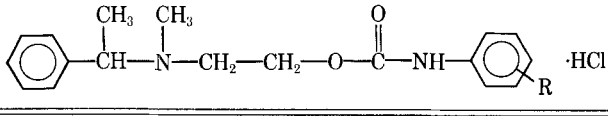
TABLE I—*N*-ARALKYL-*N*-METHYLAMINOETHYL CARBANILATE HYDROCHLORIDES



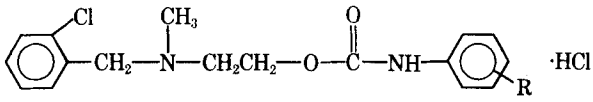
No.	x	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
3	1	*o*-Cl	137–139	$C_{17}H_{20}Cl_2N_2O_2$	C, 57.47 H, 5.67 N, 7.89	57.60 5.71 7.83
6	1	*o*-CH_3	143–145	$C_{18}H_{23}ClN_2O_2$	C, 64.56 H, 6.92 N, 8.37	64.77 6.96 8.28
8	1	*o*-CH_3O	114–116	$C_{18}H_{23}ClN_2O_3$	C, 61.82 H, 6.61 N, 7.99	61.80 6.58 7.99
11	2	*m*-Cl	194–195	$C_{18}H_{22}Cl_2N_2O_2$	C, 58.54 H, 6.00 N, 7.59	58.47 6.15 7.53
12	2	*o*-Cl	165–166	$C_{18}H_{22}Cl_2N_2O_2$	C, 58.54 H, 6.00 N, 7.59	58.79 6.05 7.26
14	2	*m*-CH_3	185–186	$C_{19}H_{25}ClN_2O_2$	C, 65.41 H, 7.22 N, 8.03	65.54 7.18 7.99
15	2	*o*-CH_3	168–170	$C_{19}H_{25}ClN_2O_2$	C, 65.41 H, 7.22 N, 8.03	65.53 7.26 8.22
17	2	*o*-CH_3O	117–119	$C_{19}H_{25}ClN_2O_3$	C, 62.54 H, 6.91 N, 7.68	62.28 6.86 7.83
21	3	*o*-Cl	113–115	$C_{19}H_{24}Cl_2N_2O_2$	C, 59.53 H, 6.31 N, 7.31	59.49 6.27 7.22
24	3	*o*-CH_3	132–134	$C_{20}H_{27}ClN_2O_2$	C, 66.19 H, 7.50 N, 7.72	66.33 7.54 7.69
25	3	*o*-CH_3O	136–138	$C_{20}H_{27}ClN_2O_3$	C, 63.40 H, 7.18 N, 7.39	63.67 7.17 7.45
28	3	*p*-NO_2	177–179	$C_{19}H_{24}ClN_3O_4$	C, 57.94 H, 6.14 N, 10.67	57.63 6.09 10.64
29	3	*p*-NH_2	220–222	$C_{19}H_{27}Cl_2N_3O_2$	C, 57.00 H, 6.80 N, 10.50	57.19 6.88 10.41

TABLE II—*N*-α-METHYLBENZYL-*N*-METHYLAMINOETHYL CARBANILATE HYDROCHLORIDES



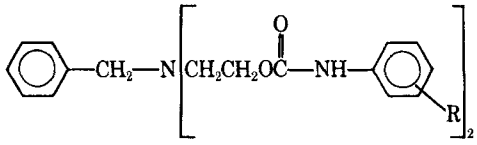
No.	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
30	*p*-CH_3	195–197	$C_{19}H_{25}ClN_2O_2$	C, 65.41 H, 7.22 N, 8.03	65.34 7.07 8.03
31	*m*-CH_3	125–127	$C_{19}H_{25}ClN_2O_2$	C, 65.41 H, 7.22 N, 8.03	65.57 7.19 8.12

TABLE III—*N*-*o*-CHLOROBENZYL-*N*-METHYLAMINOETHYL CARBANILATE HYDROCHLORIDES



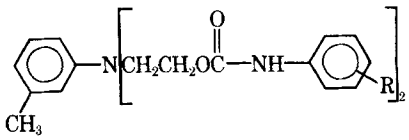
No.	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
32	*p*-Cl	208–210	$C_{17}H_{19}Cl_3N_2O_2$	C, 52.37 H, 4.91	52.41 4.75
33	*m*-Cl	192–193	$C_{17}H_{19}Cl_3N_2O_2$	C, 52.37 H, 4.91	52.26 4.92
34	*p*-CH_3	187–189	$C_{18}H_{22}Cl_2N_2O_2$	C, 58.54 H, 6.00	58.64 6.00
35	*m*-CH_3	203–205	$C_{18}H_{22}Cl_2N_2O_2$	C, 58.54 H, 6.00	58.56 6.00
36	*p*-CH_3O	184–186	$C_{18}H_{22}Cl_2N_2O_3$	C, 56.11 H, 5.75	56.17 5.83

TABLE IV—*N*-BENZYLIMINODIETHYL DICARBANILATES



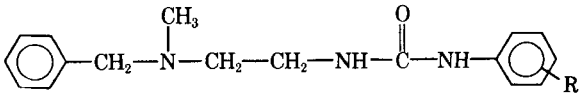
No.	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
37	*p*-Cl	115	$C_{25}H_{25}Cl_2N_3O_4$	C, ... H, ...	... ...
38	*m*-Cl	106	$C_{25}H_{25}Cl_2N_3O_4$	C, 59.76 H, 5.01	60.10 5.10
39	*p*-CH_3	122–123	$C_{27}H_{31}N_3O_4$	C, 70.25 H, 6.76	70.34 6.80
40	*m*-CH_3	74–75	$C_{27}H_{31}N_3O_4$	C, 70.25 H, 6.76	70.29 6.69
41	*p*-CH_3O	100	$C_{27}H_{31}N_3O_6$	C, 65.70 H, 6.33	65.81 6.27

TABLE V—*N*-*m*-TOLYLIMINODIETHYL DICARBANILATES



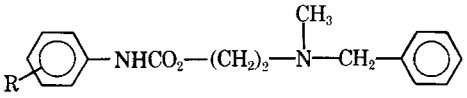
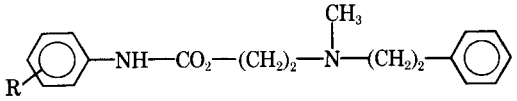
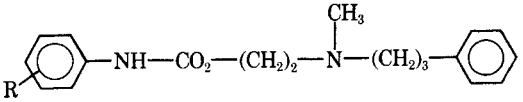
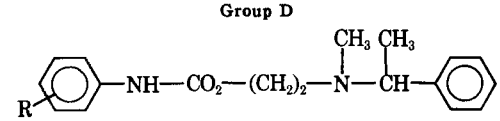
No.	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
42	*p*-Cl	136–137	$C_{25}H_{25}Cl_2N_3O_4$	C, 59.76 H, 5.01	59.88 5.07
43	*m*-Cl	126–127	$C_{25}H_{25}Cl_2N_3O_4$	C, 59.76 H, 5.01	59.89 5.13
44	*p*-CH_3	151	$C_{27}H_{31}N_3O_4$	C, 70.25 H, 6.76	70.41 6.80
45	*m*-CH_3	125–126	$C_{27}H_{31}N_3O_4$	C, 70.25 H, 6.76	70.25 6.75
46	*p*-CH_3O	131	$C_{27}H_{31}N_3O_6$	C, 65.70 H, 6.33	65.84 6.46

TABLE VI—*N*-BENZYL-*N*-METHYLAMINOETHYL-*N*-ARYLUREAS



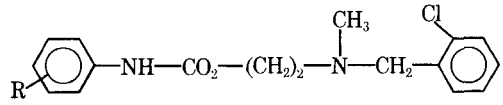
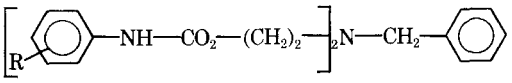
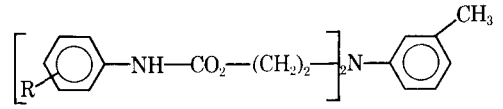
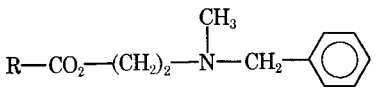
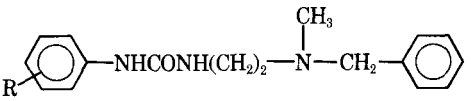
No.	R	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
50	*p*-Cl	118–120	$C_{17}H_{20}ClN_3O$	C, 64.25 H, 6.34	64.39 6.34
51	*m*-Cl	117–119	$C_{17}H_{20}ClN_3O$	C, 64.25 H, 6.34	64.09 6.19
52	*p*-CH_3	116–118	$C_{18}H_{23}N_3O$	C, 72.69 H, 7.80	72.64 7.80
53	*m*-CH_3	127–129	$C_{18}H_{23}N_3O$	C, 72.69 H, 7.80	72.51 7.84
54	*p*-CH_3O	97–98	$C_{18}H_{23}N_3O_2$	C, 68.98 H, 7.40	68.78 7.34

TABLE VII—RESULTS OF BIOLOGICAL ASSAY OF *N*-ARALKYL-*N*-METHYLAMINOETHYL CARBANILATES

No.	R	Plasma Cholesterol, % Change from Control	Liver Cholesterol, % Change from Control	No.	R	Plasma Cholesterol, % Change from Control	Liver Cholesterol, % Change from Control
		Group A					
							
1	*p*-Cl	−13.5	−0.8	*6*	*o*-CH_3	−11.5	+18.0
2	*m*-Cl	−11.3	−0.5	*7*	*p*-CH_3O	−1.4	−13.4
3	*o*-Cl	−7.7	+0.9	*8*	*o*-CH_3O	−20.5	+13.9
4	*p*-CH_3	−27.9	−12.1	*9*	H	−19.2	+18.6
5	*m*-CH_3	−13.9	−18.2				
		Group B					
							
10	*p*-Cl	−21.6	−19.3	*15*	*o*-CH_3	−6.2	+3.7
11	*m*-Cl	−11.5	+10.5	*16*	*p*-CH_3O	−6.3	−15.5
12	*o*-Cl	−26.9	+17.4	*17*	*o*-CH_3O	−20.5	+11.7
13	*p*-CH_3	−11.3	−25.4	*18*	H	+6.7	−13.3
14	*m*-CH_3	−20.4	−16.0				
		Group C					
							
19	*p*-Cl	−14.3	+7.7	*25*	*p*-CH_3O	−7.6	−18.2
20	*m*-Cl	−5.3	−26.4	*26*	*o*-CH_3O	−1.3	+29.6
21	*o*-Cl	+10.0	+18.8	*27*	H	−7.0	−8.4
22	*p*-CH_3	−29.0	−16.9	*28*	*p*-NO_2	−12.7	+4.7
23	*m*-CH_3	−20.9	−25.2	*29*	*p*-NH_2	0.0	+10.3
24	*o*-CH_3	−9.7	−31.3				
		Group D					
							
30	*p*-CH_3	−1.4	+16.9	*31*	*m*-CH_3	−1.3	+18.2

(*Continued on next page.*)

TABLE VII—(*Continued.*)

No.	R	Plasma Cholesterol, % Change from Control	Liver Cholesterol, % Change from Control	No.	R	Plasma Cholesterol, % Change from Control	Liver Cholesterol, % Change from Control
		Group E					
							
32	*p*-Cl	+4.7	+10.6	*35*	*m*-CH_3	+18.0	+19.3
33	*m*-Cl	+25.3	+70.8	*36*	*p*-CH_3O	+3.4	+16.8
34	*p*-CH_3	+4.0	+15.8				
		Group F					
							
37	*p*-Cl	+14.9	+14.8	*40*	*m*-CH_3	−3.8	+27.2
38	*m*-Cl	+14.2	+20.8	*41*	*p*-CH_3O	−4.5	+0.2
39	*p*-CH_3	+18.0	+18.1				
		Group G					
							
42	*p*-Cl	+17.8	−7.1	*45*	*m*-CH_3	+10.9	+6.1
43	*m*-Cl	−5.8	−7.1	*46*	*p*-CH_3O	−1.5	−3.8
44	*p*-CH_3	+1.5	+8.0				
		Group H					
							
47	$(CH_3)_2N$-	−1.6	−21.7	*48*	C_4H_9-NH-	−21.6	−15.7
		Group I					
							
49	*p*-Cl	+4.0	+11.9	*52*	*m*-CH_3	+23.1	−9.0
50	*m*-Cl	+19.5	+44.6	*53*	*p*-CH_3O	−20.1	−11.6
51	*p*-CH_3	−0.6	+8.0				

EXPERIMENTAL

Chemical

Preparation of N-Aralkyl-N-methylaminoethyl Carbanilate Hydrochlorides—The compounds listed in Tables I, II, and III were prepared from the appropriate alcohol and substituted phenyl isocyanate in the manner previously described by Barnes and Rost (1).

Preparation of N-Benzyliminodiethyl Dicarbanilates—The compounds listed in Table IV were prepared from *N*-benzyliminodiethanolamine and substituted phenylisocyanate in the same manner as that described for *N*-benzyliminodiethyl di-*p*-chlorophenylcarbanilate by McKay and Hatton (2).

Preparation of N-m-Tolyliminodiethyl Dicarbanilates—The compounds in Table V were prepared from *m*-tolyliminodiethanol and the appropriate substituted phenyl isocyanate in the same manner as the *N*-benzyliminodiethyl dicarbanilates previously described.

Preparation of N_1-(N-Benzyl-N-methylaminoethyl)-N_3-p-chlorophenylurea—The compound was prepared by dissolving 3.25 Gm. (0.02 mole) of *N*-benzyl-*N*-methylaminoethylamine in 10 ml. of toluene and adding 3.2 Gm. (0.022 mole) of *p*-chlorophenylisocyanate which was dissolved in 10 ml. of toluene. After refluxing 1 hr., the mixture was permitted to stand overnight. The crystals which formed were filtered off and recrystallized from acetone. The 3.4 Gm. yield (53%) melted at 118–120°.

The compounds listed in Table VI were prepared in the same manner as that described for N_1-(*N*-benzyl-*N*-methylaminoethyl)-N_3-*p*-chlorophenylurea.

Preparation of N-Benzyl-N-methylaminoethyl-N'N'-dimethylcarbamate Hydrochloride—A mixture of 15.2 Gm. (0.1 mole) of β-chloroethyl dimethylcarbamate (3) and 24.2 Gm. (0.2 mole) of benzylmethylamine was heated at 100° for 1 hr. After cooling, the solid reaction mixture was extracted with ether, the combined extracts concentrated, and the residue fractionally distilled to give 15.1 Gm. (64%) of ester, b.p. 110–120°/0.25 mm. The ester was dissolved in anhydrous ether and precipitated as the hydrochloride salt by the addition of an ethereal solution of hydrogen chloride. The resultant oil crystallized on standing and was recrystallized from an ether–alcohol mixture. The product melted at 146°.

Anal.—Calcd. for $C_{13}H_{21}ClN_2O_2$: C, 57.24; H, 7.76. Found: C, 56.93; H, 7.19.

Preparation of N-Benzyl-N-methylaminoethyl-N'-butylcarbamate Hydrochloride—The compound was prepared as noted in the carbanilate series (1). The product melted at 122–123° and was obtained in 58.5% yield.

Anal.—Calcd. for $C_{15}H_{25}ClN_2O_2$: C, 59.88; H, 8.38. Found: C, 60.11; H, 8.05.

Preparation of N-α-Methylbenzyl-N-methylaminoethanol—1-Bromoethylbenzene was reacted with *N*-methylaminoethanol as described previously (1) to give *N*-α-methylbenzyl-*N*-methylaminoethanol (67%), b.p. 131–133°/5 mm.

Preparation of N-o-Chlorobenzyl-N-methylaminoethanol—*o*-Chlorobenzyl chloride was reacted with *N*-methylaminoethanol as previously described (1) to give *N*-*o*-chlorobenzyl-*N*-methylaminoethanol (85%), b.p. 112–120°/1.5 mm.

Preparation of N-Benzyl-N-methylaminoethylamine—Benzylmethylamine was reacted with ethyleneimine according to the method of Coleman and Callen (4) to give *N*-benzyl-*N*-methylaminoethylamine (73%), b.p. 80–93°/2 mm.

Biological Assay

Each compound was evaluated for its hypocholesteremic activity in male, Swiss Webster mice. Ten mice, weighing between 28–34 Gm., were used for each test compound. The mice were maintained on a fat-supplemented, cholesterol-free diet.[1] Test compounds were administered orally in a tragacanth suspension at a dose of 25 mg./Kg. b.i.d. At the end of the 11-day treatment period, animals were sacrificed and pooled tissue cholesterols[2] were determined by the Nury-Smith method (5). Percent change from controls was obtained by comparison with values obtained in an untreated control group of animals carried simultaneously in the experiment. Decreases in plasma or liver total cholesterol greater than 20% from control values without an increase of 8% in either end point, or decreases of 12% or greater in both end points were considered desirable biosignificant results.[3]

DISCUSSION

The types of compounds reported here have not been previously described as exhibiting hypocholesteremic activity in experimental animals. In this study their effect on both plasma and liver cholesterol is reported. It is considered desirable to lower cholesterol levels in one or both of these tissues without increasing either before concluding that a material has produced a desirable effect.

It is difficult if not impossible to ascribe the variable activities of the different members of this series to any specific portion of the molecule, but certain observations might be significant. As seen in Table VII, the presence of an aralkyl substitution seems advantageous, but it can be varied considerably. The *N*-benzyl, *N*-phenethyl, and *N*-phenpropyl amines show activity in lowering plasma and liver cholesterol (groups *A*, *B*, and *C*). However, the branching of the aralkyl group (group *D*) and substitution in the aralkyl group (group *E*) appear detrimental.

Substitution in the carbanilate ring markedly affects activity in this series of compounds. *Meta* and *para* substitutions produce compounds with a more consistent desirable activity than *ortho* substitution. Methyl substitution appears more favorable than the chloro or methoxy. Analogous ureas (group *I*) did not exhibit patterns similar to the carbanilate series. Here only the *p*-methoxy compound (53) appeared interesting. Even the aromatic ring was not essential, as seen by the activity of the butylcarbamate (48).

Increasing the bulk of the molecule to compounds of the dicarbanilate type (groups *F* and *G*) did not yield useful agents.

The results of this biological study indicate that both terminal portions in the molecules of the compounds reported contribute to the type of activity produced by them. Although several of these compounds do block *in vitro* biosynthesis of cholesterol from mevalonate (7), their mechanism of hypocholesteremic action *in vivo* is unknown. If they should be acting by inhibiting cholesterol biosynthesis, it is tempting to suggest that the enzymatic site of activity has two or more foci of attachment. Such an implication has been suggested by Kraml *et al.* (8) when describing the hypocholesteremic activity of *N,N'*-dibenzylethylenediamine and related compounds.

[1] Vitamin free casein, 24%; sucrose, 45%; Alphacel, 4%; salt mixture (USP XIV), 4%; dextrin, 20%; vitamin fortification mixture, 2%; ethyl linoleate, 1%.

[2] It was found more convenient to use pooled rather than individual samples in this work because of the large number of assays necessary. The authors have found previously in groups of ten animals that the mean of ten individual assays did not differ measurably from the result obtained with a pooled sample.

[3] Triparanol tested under these conditions decreased serum cholesterol 17.8% and liver cholesterol 37.6%. Gas chromatography of tissue extracts of triparanol treated animals shows accumulation of desmosterol (6). No abnormal sterols were observed in the tissue extracts of the animals treated with the compounds of this report.

REFERENCES

(1) Barnes, R. W., and Rost, W. J., *J. Pharm. Sci.*, **51**, 146(1962).
(2) McKay, A. F., and Hatton, W. G., *Can. J. Chem.*, **31**, 772(1953).
(3) Carter, H. E., Frank, R. L., and Johnson, H. W., "Organic Synthesis," coll. vol. III, John Wiley & Sons, Inc., New York, N. Y., p. 167.
(4) Coleman, G. H., and Callen, R. E., *J. Am. Chem. Soc.*, **68**, 2006(1946).
(5) Nury, F. S., and Smith, E. R. B., *Clin. Chem.*, **3**, 110(1957).
(6) Holmes, W. L., and DiTullio, N. W., *Am. J. Clin. Nutr.*, **10**, 310(1962).
(7) DiTullio, N. W., unpublished data.
(8) Kraml, M., Humber, L. G., Dubuc, J., and Gaudry, R., *J. Med. Chem.*, **7**, 500(1964).

Dermatitic Effect of Nonionic Surfactants II

Changes in Phospholipid and in Deoxyribonucleic Acid Content of Rabbit Epidermis *In Vivo*

By MICHAEL MEZEI* and R. W. SAGER

A hypothesis, namely, that the site of action of surfactants is in the biological membranes, was investigated with an indirect method. One representative member from each of 3 major types of nonionic surfactants was tested. Sorbitan trioleate, polysorbate 85, and polyoxyethylene ether 96 were incorporated, 10 percent in concentration, in white petrolatum and applied to rabbit skin daily. Measurements of lipid phosphorus indicated a considerable increase in the phospholipid content of the 0.1 mm. thick skin samples representing mainly the epidermis. Two reference standards were used: the wet weight and the deoxyribonucleic acid (DNA) content. After 4 days of treatment with polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96 preparations, the increase in phosphorus was found to be 23–56 percent, 28–73 percent, and 46–90 percent, respectively, on the basis of wet weight and 26–53 percent, 27–58 percent, and 47–81 percent, respectively, if DNA content was used as the reference standard. After 10 days of treatment, the increase in phosphorus was considerably smaller if calculated on the basis of DNA present in the sample, but did not differ much if wet weight was used as the reference. A comparison of the two reference standards demonstrated that the DNA content also increased after 10 days of treatment. The smaller spread in results indicated that DNA content may be more reliable as a reference standard, but the wet weight still can be used as a practical and useful standard of reference especially if the DNA content is also influenced by the test.

THE DERMATITIC effects of nonionic surfactants have been the subject of much research (1–7). All of these studies, however, have been limited to surfactants present in household cleaners or in aqueous solutions. Most of these investigations employed *in vitro* and/or patch test techniques, which were based upon experimental procedures that were empirical in nature and arbitrary in evaluation; they relied on gross observations and on microscopic evaluations. These evaluation methods can reveal little or nothing about the biochemical changes at the cellular level which probably initiate the morphological changes observed.

The aim throughout the present investigation has been to design and apply more definitive and rational test procedures. In contrast to the *in vitro* and patch test techniques, attempts were made to reproduce the frequent application of dermatological and cosmetic preparations, which may contain these surfactants, by the general public. Besides macroscopic and microscopic observations, attempts were made to investigate biochemical changes by standard and well-accepted assay methods. Measurements and observations regarding morphological changes (on both the macroscopic and microscopic level) and of oxygen consumption were reported as a first part of the study (8). This presentation is mainly concerned with the measurement of quantitative changes in the phospholipid content of the rabbit epidermis induced by surfactant applied to the skin in the form of an ointment. The DNA content was also determined with the intention of using a more reliable reference standard than the wet weight of sample is believed to be.

MATERIALS AND METHODS

Thirteen New Zealand white rabbits of both sexes, 6–12 weeks old, were used as experimental animals. They were housed in hanging screen-bottom cages and were maintained on Purina rabbit chow and water *ad libitum*. The trunk of each rabbit was clipped free of hair with an electric hair clipper (Oster model A2, size 40), and was divided into 6 areas. Harness-type restrainers (9) were used for restraining rabbits with a minimum amount of stress.

One representative member from each of 3 major types of surfactant was selected. Sorbitan trioleate,[1] polysorbate 85,[2] and polyoxyethylene ether 96[3] were incorporated in white petrolatum USP in 10% concentration and were applied to the center of the designated areas of rabbit skin daily. The other three areas on the rabbits' back were reserved for controls: (*a*) untreated skin *A*, (*b*) untreated skin *B*, and (*c*) skin treated with the ointment base only. Untreated skin *B* was used as a control to determine

Received April 3, 1967, from the School of Pharmacy, Oregon State University, Corvallis, OR 97331
Accepted for publication August 15, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Previous paper: Mezei, M., Sager, R. W., Stewart, W. D., and deRuyter, A. L., *J. Pharm. Sci.*, **55**, 584(1966).
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[1] Marketed as Span 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Marketed as Tween 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[3] Marketed as Brij 96 by Atlas Chemical Industries, Inc., Wilmington, Del.

whether the surfactant which penetrated into the skin influenced the extraction of lipids and DNA, which could also be a reason for finding different amounts of lipids and perhaps DNA in control and treated skin. To this control area one of the surfactant preparations was applied only once: 15 min. before the sample was taken. This single application provided some surfactant in the skin homogenate, but did not induce any measurable biological changes. The sites of control and treated areas were randomly selected from different animals to exclude any influence of body area. After 4 or 10 days of treatment, the animals were killed by fracturing the neck. The test areas were quickly washed with cotton soaked in ether to remove not only the substances applied previously, but also the surface lipids. Skin samples were taken with the aid of the Castroviejo keratotome (10) set to cut a 0.1-mm. thick skin slice. The samples were immediately dipped in liquid nitrogen, and kept there until the wet weight was determined on an analytical balance. The weight of each sample was around 100 mg. The elapsed time between removing and weighing the samples was not more than 20–30 min. Immediately after weighing, the skin samples were homogenized in a glass homogenizer and extracted with 2.0 ml. of chloroform–methanol (2:1) after the method of Folch *et al.* (11). The residue was set aside for DNA extraction. Nonlipid contaminants were removed from the chloroform–methanol extracts with 0.2 vol. of 0.05% $CaCl_2$ solution. The phospholipid content of the lipid extracts was determined by the method of Bartlett (12). The residue obtained after the extraction of lipids was extracted for the determination of DNA content by the method of Santen and Agranoff (13).

RESULTS AND DISCUSSION

In studies involving skin lipid analyses the greatest problem has been to provide skin samples in chemically unaltered form with well-defined anatomical structure. Skin lipid samples have usually been divided into 3 categories (14): (*a*) epidermal lipid samples; (*b*) lipid samples from various sebaceous type glands, or from the dermis and, (*c*) lipid samples from the skin surface.

There have been only indirect methods reported for the determination of lipid composition of human epidermis (14–18). In these studies the separation of epidermis from the dermis was achieved by chemical and physical methods (19, 20). In the present investigation, in order to avoid any artifact induced by these separation techniques, 0.1 mm. thick skin slices were cut from rabbits. Microscopic examinations revealed that these samples contained mainly the epidermis, and occasionally a trace of dermis (Fig. 1). The surface lipids were extracted with ether before cutting off the skin slices. This technique provided a well-isolated and chemically unaltered epidermis sample for lipid analysis.

The total phospholipid content of control and treated rabbit skin after 4 days of treatment is shown by Tables I and II. Using DNA content as the reference standard, it was found that the phosphorus (P) content of the control skin samples (untreated skin *A* and *B*) was within the range of 7.44–10.84 mcg. P/100 mcg. of DNA. In skin samples which were treated with the ointment base only,

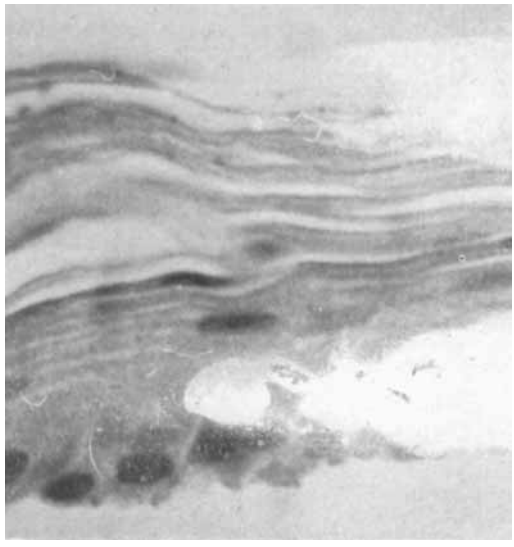

Fig. 1—Photomicrograph of skin sample obtained with the aid of the Castroviejo keratotome set to cut 0.1 mm. thick skin slices.

which were also considered as controls, the range was somewhat higher, 9.26–11.24 mcg. P/100 mcg. DNA. Among the treated skin samples the one which was treated with polyoxyethylene ether 96 (10% in petrolatum) showed the largest increase in phosphorus content; the range was 11.52–17.31 mcg. P/100 mcg. DNA, which represented a 47–81% increase as illustrated by Fig. 2.

Treatment with polysorbate 85 and sorbitan trioleate also produced a considerable increase in phosphorus content, 26–53% and 27–58%, respectively. If the phosphorus content is referred to the wet weight of the sample, it is found that the same pattern exists (see Fig. 3 and Table II). The phosphorus content of treated skin samples also showed an increase, but the spread in the results was somewhat larger. The increase of phosphorus, calculated according to the wet weight for samples treated with petrolatum, polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96, was within the range of 1–15%, 23–56%, 28–73%, and 46–90%, respectively.

Tables III and IV show the results of a similar treatment but for a longer period of time. After 10 days of treatment with polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96, the increase in phosphorus was found to be 19–38%, 18–35%, and 22–33% (Fig. 2), respectively, on the basis of DNA content, and 40–66%, 24–67%, and 46–76%, respectively, on the basis of wet weight of sample (Fig. 3). The increase of phospholipid phosphorus was considerably smaller than that found after 4 days of treatment, if calculated on the basis of DNA present in the sample, but did not differ much if wet weight was used as the reference. A probable explanation could be that the DNA content was also increased; consequently, the reference standard became greater in the case of DNA, and probably changed nothing in case of the wet weight.

The wet weight is generally used with caution as a reference standard in biological studies, especially

TABLE I—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mcg. DNA) AFTER 4 DAYS OF TREATMENT

Substance	Rabbit No. 51	52	53	54	55	56	57	58	59
None (untreated skin *A*)	8.56	8.60	8.83	10.24	9.01	8.45	7.86	7.84	7.44
None (untreated skin *B*)	9.73	8.23	7.62	...	10.84	...	...	...	...
Petrolatum	9.59	9.86	9.26	11.24	9.80	...	...	...	...
Polysorbate 85	10.78	12.07	12.89	14.30	12.64	12.5	11.16	11.52	11.41
Sorbitan trioleate	13.17	13.54	13.97	13.67	13.48	11.71	9.98	11.42	11.20
Polyoxyethylene ether 96	14.54	15.20	15.04	17.31	14.51	15.28	11.52	11.80	11.67

TABLE II—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mg. WET WEIGHT) AFTER 4 DAYS OF TREATMENT

Substance	Rabbit No. 51	52	53	54	55	56	57	58	59
None (untreated skin *A*)	22.46	20.84	20.89	26.20	27.55	26.18	25.74	26.08	23.85
None (untreated skin *B*)	22.26	22.98	18.0	...	27.11	27.98	...	...	...
Petrolatum	25.61	21.13	23.79	27.88	28.98	30.07	...	...	...
Polysorbate 85	32.43	25.67	26.99	34.51	33.71	37.16	40.05	39.67	36.35
Sorbitan trioleate	34.78	28.58	26.86	33.54	46.78	42.41	44.56	35.95	34.74
Polyoxyethylene ether 96	35.51	32.25	30.49	44.86	43.50	49.72	44.20	38.37	36.76

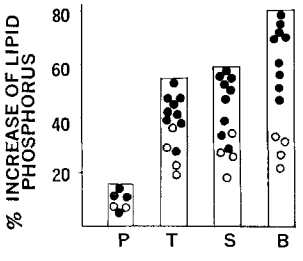

Fig. 2—Percent increase of lipid phosphorus calculated on the basis of DNA content of skin sample. Skin samples treated with P, *petrolatum;* T, *polysorbate 85, 10%;* S, *sorbitan trioleate, 10%;* B, *polyoxyethylene ether 96, 10%;* ●, *after 4 days of treatment;* ○, *after 10 days of treatment. The percent increase of lipid phosphorus was calculated individually for each rabbit. Example: rabbit 51 (Table I)—untreated skin, 8.56 mcg. P/100 mcg. DNA; skin treated with polysorbate 85, 10.78 mcg. P/100 mcg. DNA; increase in lipid phosphorus, 2.22 mcg.; percent increase, 2.22/8.56 × 100 = 25.93%.*

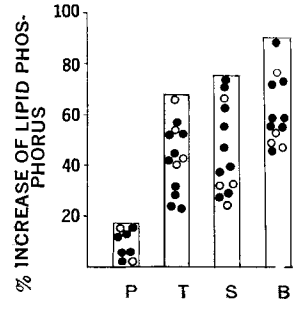

Fig. 3—Percent increase of lipid phosphorus calculated on the basis of wet weight of skin sample. Skin samples treated with P, *petrolatum;* T, *polysorbate 85, 10%;* S, *sorbitan trioleate, 10%;* B, *polyoxyethylene ether 96, 10%;* ●, *after 4 days of treatment;* ○, *after 10 days of treatment.*

TABLE III—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mcg. DNA) AFTER 10 DAYS OF TREATMENT

Substance	Rabbit No. 60	61	62	63
None (untreated skin)	8.24	7.26	8.25	8.25
Petrolatum	8.83	7.85	...	...
Polysorbate 85	10.64	8.6	10.04	11.42
Sorbitan trioleate	11.12	9.30	9.76	10.50
Polyoxyethylene ether 96	10.74	8.88	10.41	11.0

TABLE IV—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mg. WET WEIGHT) AFTER 10 DAYS OF TREATMENT

Substance	Rabbit No. 60	61	62	63
None (untreated skin)	20.37	26.0	34.63	36.50
Petrolatum	23.56	26.54	...	...
Polysorbate 85	33.76	39.40	48.57	51.58
Sorbitan trioleate	25.14	43.34	44.78	47.92
Polyoxyethylene ether 96	35.79	39.77	51.54	53.20

when the tissue studied is heterogeneous in nature. An attempt was made in this study to use samples with uniform structure. Skin samples of 0.1-mm. thick slices could, however, contain different proportions of the four layers of epidermis (stratum germinativum, stratum spinosum, stratum granulosum, and stratum corneum). In order to find a more reliable reference standard than the tissue wet weight is believed to be, the DNA content of each sample was determined. DNA content is used to indicate the number of viable cells. On the basis of DNA content the results of lipid phosphorus determinations show patterns similar to those found when the wet weight was used as a reference. There were, however, 2 distinct differences between the results, calculated on the basis of the two refer-

TABLE V—DNA CONTENT RELATED TO WET WEIGHT (mcg. DNA/100 mg. WET WEIGHT)

Substance	4 Days Treatment						10 Days Treatment					
	Rabbit No.											
	52	53	54	55	56	57	58	59	60	61	62	63
None (untreated skin *A*)	232	236	256	306	309	340	342	325	247	358	420	440
None (untreated skin *B*)	279	241	...	250	244	346	...	...	...	...	...	...
Petrolatum	214	257	248	296	261	...	...	...	291	310	...	...
Polysorbate 85	213	209	241	267	296	359	343	315	317	458	462	473
Sorbitan trioleate	211	192	245	347	362	429	315	310	227	466	459	445
Polyoxyethylene ether 96	212	203	359	427	325	469	325	315	330	452	464	486

ence standards. Using DNA as a reference, the range of these percentages is narrower than if wet weight is used as a reference standard. The second difference is apparent with the results of the 10 day treatment. Here not only the range is narrower but the increase of phosphorus is also smaller when using DNA as a reference. The smaller spread in results indicates that DNA may be more reliable as a reference standard, but the wet weight still can be used as a practical and useful standard of reference, especially if the DNA content is also influenced by the test.

Table V shows the comparison of the 2 reference standards. DNA content, as related to wet weight of the sample, shows an increase after 10 days of treatment. This might be due to irritation of the skin, caused by the surfactant treatment which could initiate a higher metabolic and mitotic activity. An increased rate in cell proliferation, and hyperplasia of the epidermis, are often paralleled with an increased rate of DNA synthesis and an increase in DNA content (21–24).

It was postulated (8) that the effect of nonionic surfactants on the respiratory metabolism of rabbit skin is partly an indirect one and may be due to permeability changes of cell membranes.

The structure of biological membranes is based upon a lipid–protein positional relationship. The classical Danielli-Davson (25) model of the membrane and the more recent "unit membrane" hypothesis of Robertson (26) have suggested a bimolecular phospholipid leaflet as the backbone of membrane structure. Recent reports and reviews (27–32) describe the structure and function of membranes in a more detailed manner. All of these descriptions are highly speculative in nature; there is no proven theory for the exact nature of membranes at the molecular level.

On the basis of these hypotheses it can be stated, however, that a qualitative and/or quantitative change in the lipid composition of a tissue might indicate structural changes in the membranes, especially if these changes were induced by substances which more likely act on the membranes.

Surfactants, due to their hydrophilic–lipophilic character, generally possess a particular affinity for membranous structures. As a result of this affinity, a nonionic surfactant can (*a*) interact by hydrophobic bonding, being a macromolecule itself; (*b*) interact by hydrogen bonding, because of the strong hydrogen bonding affinity of the ether oxygens of the polyoxyethylene chains; (*c*) disturb the micellar structure of phospholipids, forming micelles, or replacing phospholipid molecules in the continuous lipid micelles; and (*d*) interact with water molecules participating in these micelles. These are only a few possibilities which may result in alterations of membrane structure and, consequently, functional changes and metabolic disorders.

On the basis of the postulation that the primary site of action of surfactants is in the biological membranes, it was decided to seek ways to test this possibility. It was apparent that proving this hypothesis with direct methods would be difficult, since there is no way at the present time to study membrane structures at the molecular level. The determination of quantitative changes of phospholipids, an indirect method, was intended to investigate and to support this hypothesis.

The probable reason for the increase of phospholipids is that the surfactants damaged the biological membranes by either rupturing the membranes or replacing certain phospholipid molecules in the continuous phospholipid micelles present in the membranes. To repair this damage, a higher rate in the biosynthesis of certain phospholipids should be expected, in order to regenerate the original membrane structure. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants are measured along with the newly formed molecules during the analysis of skin tissue.

An investigation of the effects of polysorbate 80 on the *in vitro* metabolism of cells, reported by Kay (33), should be mentioned here to support this hypothesis. Treatment with polysorbate 80 induced a marked increase in cell permeability in preparations of Ehrlich-Lettré ascites cells. An extraordinary increase (up to almost 300%) in the incorporation of ^{32}P into phospholipid fractions was also observed in cell preparations containing 1% polysorbate 80.

The above phenomena might also be explained by the assumption that the increase in permeability is due to structural changes (disruption of membranes). The rapid incorporation of ^{32}P into phospholipids after the surfactant treatment suggests a reconstitution of cell membrane material.

A further study of the rate of biosynthesis of phospholipids in rabbit and in human skin treated with surfactant preparations should prove the above hypothesis.

Another well-known phenomenon, namely, that surfactants generally increase percutaneous absorption of various substances (34), could be explained partly by permeability changes, due to structural changes in skin "barrier" membranes.

The results reported here are in accordance with the authors' previous findings regarding gross and microscopic changes in rabbit skin induced by surfactants. It is apparent that the polyoxylene ether-type surfactants have the highest capacity to pro-

duce dermal reactions: severe erythema, hyperkeratinization, and desquamation with marked induration and fissuring on the macroscopic level; inflammation with a number of polymorphonuclear and round cells in the dermis and acanthosis with hyperplasia and various degrees of necrosis in the epidermis on the microscopic level; and the highest increase in lipid phosphorus content.

REFERENCES

(1) Treon, J. F., *Proc. Sci. Sect. Toilet Goods Assoc.*, **40**, 40 (1963).
(2) Choman, B. R., *J. Invest. Dermatol.*, **40**, 117(1963).
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(5) *Ibid.*, **76**, 120(1964).
(6) Chapman, R. E., and Short, B. F., *Australian J. Biol. Sci.*, **18**, 703(1965).
(7) Gisslén, H., and Magnusson, B., *Acta Dermato-Venereol.*, **46**, 269(1966).
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(9) Newmann, E. A., *Lab. Animal Care*, **13**, 207(1963).
(10) Blank, H., Rosenberg, E. W., and Sarkany, I., *J. Invest. Dermatol.*, **36**, 303(1961).
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(12) Bartlett, G. R., *ibid.*, **234**, 466(1959).
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(21) Barka, T., *Exptl. Cell Res.*, **37**, 662(1965).
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(32) Korn, E. D., *Science*, **153**, 1491(1966).
(33) Kay, E. R. M., *Cancer Res.*, **25**, 764(1965).
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Reaction Gas Chromatography III

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EXPERIMENTAL

Apparatus—*Gas Chromatograph*—Burrell Kromotog K-2 equipped with thermal conductivity detector and gas valves for directing the carrier gas to the chromatographic column directly or *via* the reactor, as required.

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duce dermal reactions: severe erythema, hyperkeratinization, and desquamation with marked induration and fissuring on the macroscopic level; inflammation with a number of polymorphonuclear and round cells in the dermis and acanthosis with hyperplasia and various degrees of necrosis in the epidermis on the microscopic level; and the highest increase in lipid phosphorus content.

REFERENCES

(1) Treon, J. F., *Proc. Sci. Sect. Toilet Goods Assoc.*, **40**, 40 (1963).
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EXPERIMENTAL

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Column—Glass tube (225 cm. long, 6 mm. i.d.)

packed with 20% Carbowax 20M on base-washed firebrick and maintained at 140 ± 1°.

Carrier Gas—Helium, 75 ml./min.

Reactor—Construction of the reactor has been described (1). It was filled with 0.7 Gm. of the catalyst and maintained at 280 ± 5° with the help of an external heating jacket. A thin platinum wire was inserted into the tapered end of the reactor leading into the column in order to prevent deposition of less volatile products.

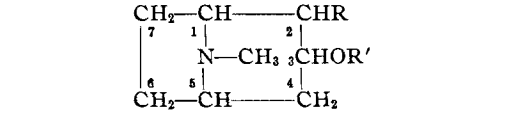
Catalyst—Five percent platinum on base-washed firebrick (30–60 mesh) prepared as described before (1).

Procedure—Ethanolic solutions of the substances were employed. Aliquots containing 1–3 mg. of the samples were used for injections. Substances existing in the liquid state at room temperature were used without solvent. The chromatographic peaks were tentatively identified by comparison of retention times with those of pure reference standard. Identifications of compounds possessing chromophoric groups were confirmed by collecting the corresponding effluent fractions in ethanol and recording the ultraviolet spectra.

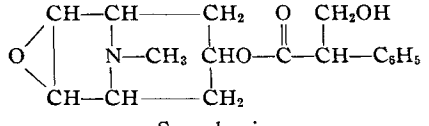
RESULTS AND DISCUSSIONS

Tropane alkaloids are derivatives of 3-tropanol formed by esterification of the hydroxyl group and/or by substitutions on other carbon atoms. The formulas of compounds used in the present investigations are shown in Table I. It may be

TABLE I—TROPANE DERIVATIVES



Compd.	R	R′
3-Tropanol	H	H
Atropine	H	—C(=O)—CH(CH₂OH)—C₆H₅
Homatropine	H	—C(=O)—CH(OH)—C₆H₅
Ecgonine	—COOH	H
Cocaine	—COOCH₃	—C(=O)—C₆H₅



Scopolamine

expected that the products of pyrolytic dehydrogenation of tropanol would be present among the products derived from the alkaloids. It was, therefore, considered worthwhile first to study the degradation of tropanol and then to employ the data for interpretation of results obtained by dehydrogenation gas chromatography of the alkaloids.

Tropanol—The reaction gas chromatogram of tropanol exhibited several peaks (Fig. 1,A). Apparently the fused ring system of tropanol had suffered cracking under the experimental conditions, leading to the formation of smaller molecules. This was confirmed by identification of some of the reaction products. The first step of the reaction may be the dehydration of tropanol to tropidine (3). Degradation of tropidine may then proceed *via* rupture of C—N or C—C bonds as indicated in Scheme I. The rupture of a C—N bond on passage of vapors of tropane over a palladium-asbestos catalyst at 300° was reported by Ehrenstein and Marggraff (4). Under more drastic conditions—*viz.*, fusion with alkali—the nitrogen atom was completely detached in the form of methylamine (3, 5). The rupture of C—C bonds of the tropane skeleton has also been observed. Thus when tropidine was dehydrogenated with bromine, 3,5-dibromopyridine, ethylene dibromide, and methyldibromopyridine were formed (6). Although the presence of pyrrole derivatives among the products of the above degradations was not reported, their formation under conditions of drastic degradation is quite likely to take place since it would involve only rupture of C—C bonds without affecting the N—CH_3 group (4). The rupture of the fused ring system of tropanol may, therefore, give rise to three types of monocyclic systems: a five-membered heterocyclic ring, a six-membered heterocyclic ring, and a seven-membered carbocyclic ring.

As expected, *N*-methylpyrrole, pyridine, and pyrrole were readily identified among the products as peaks No. 4, 5, and 9, respectively. (Table II.) Peak No. 3 was generated by toluene. This aromatic hydrocarbon must have been formed by isomerization of tropilidine produced, presumably, by detachment of the N—CH_3 bridge from the molecule of tropidine (or tropanol). Isomerization of tropilidene to toluene has been the subject of several recent publications (7–12). Under the present experimental conditions hydrogen transfer reactions may also take place. Thus peaks No. 1 and 2 were assigned to pyrrolidine and piperidine. Peaks No. 6 and 7 appeared to be due to partially dehydrogenated piperidines. Peaks at the same retention times were observed along with the peaks of pyridine and unchanged piperidine, when the latter was passed through the reactor under the same experimental conditions. Highly volatile products, *e.g.*, methane, ethylene, methylamine, *etc.*, were presumably eluted within the first 2 min., and their peaks were masked by those generated by ethanol, the solvent employed for injecting the samples.

Atropine and Homatropine—Atropine and homatropine are esters of tropanol with tropic and mandelic acids. Pyrolysis of these substances would be expected to yield tropidine and the corresponding acids, which products would undergo further degradation to simpler molecules. Thus the reaction chromatograms of these alkaloids (Fig. 1,B and C) exhibited all the peaks generated by tropanol. In addition atropine showed a peak (No. 8) which, according to ultraviolet spectral analysis, may be assigned to styrene. This product was also obtained when tropic acid was passed through the reactor under the same experimental

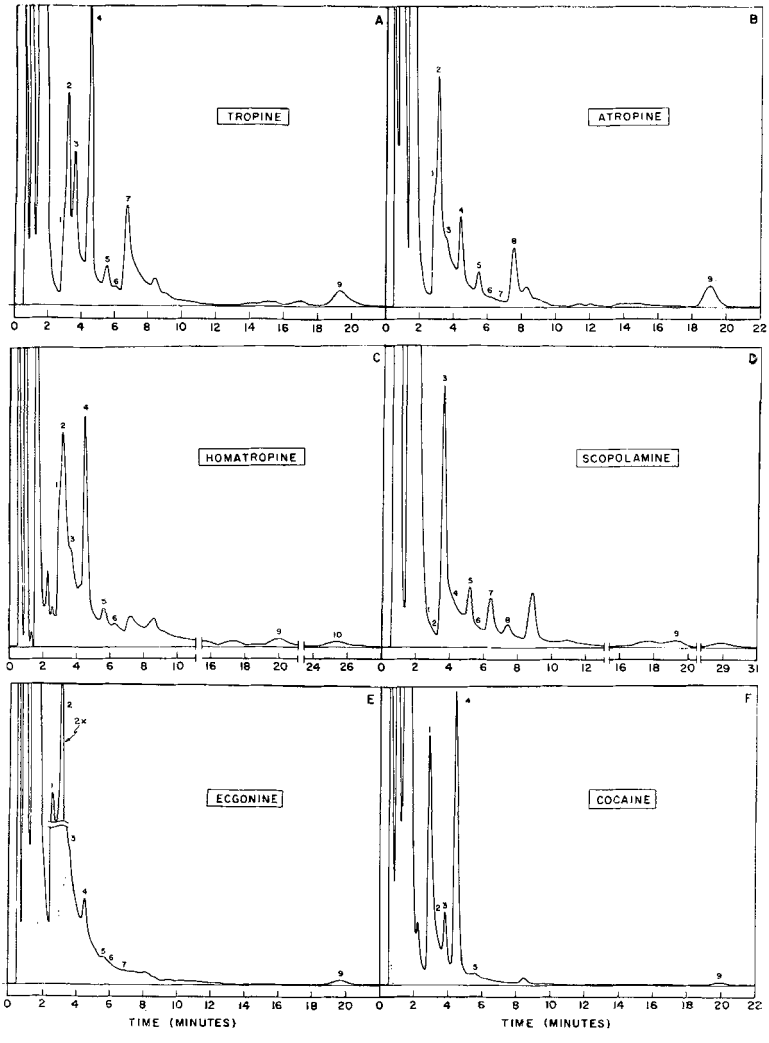
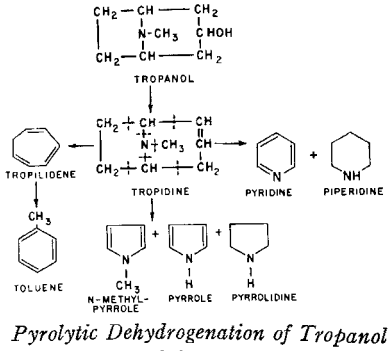


Fig. 1—Reaction chromatograms of tropanol and tropane alkaloids. (See Table II for assignment of peaks.)



Pyrolytic Dehydrogenation of Tropanol
Scheme I

conditions. The reaction chromatogram of homatropine exhibited a peak due to benzaldehyde, formed by degradation of mandelic acid moiety in the alkaloid. This was confirmed by injecting mandelic acid in the reactor, when benzaldehyde was observed to be the main product of reaction.

$$C_6H_5CH(COOH)CH_2OH \rightarrow C_6H_5CH{=}CH_2$$
$$C_6H_5CH(OH)COOH \rightarrow C_6H_5CHO$$

TABLE II—ASSIGNMENT OF PEAKS IN REACTION CHROMATOGRAMS

Peak No.[a]	Relative Retention Time[b]	Assignment
1	0.64	Pyrrolidine
2	0.71	Piperidine
3	0.82	Toluene
4	1.00	*N*-Methylpyrrole
5	1.25	Pyridine
6	1.38	Partially dehydrogenated piperidines
7	1.52	
8	1.68	Styrene
9	4.55	Pyrrole
10	5.83	Benzaldehyde

[a] See Fig. 1. [b] Reference standard: *N*-methylpyrrole.

Scopolamine—Scopolamine is an epoxy-atropine. On pyrolytic dehydrogenation it yielded products many of which were the same as those produced by atropine (see Fig. 1,D). However, the peaks due to *N*-methylpyrrole, pyrrole, and pyrrolidine

were comparatively smaller presumably due to further oxidative degradation of the five-membered heterocyclic ring carrying the epoxy group.

Ecgonine and Cocaine—Ecgonine is a carboxy derivative of tropanol, while cocaine is a carbomethoxy derivative of tropanyl benzoate. Although these compounds would be expected to yield the same products of degradation, the yield of six-membered heterocyclic compounds was in fact, very poor (Fig. 1,E and F). Presumably these compounds do not decarboxylate easily and are retained by the basic support.

CONCLUSIONS

The experimental data reported above demonstrate that the described technique should prove of value in establishing the identity of tropane alkaloids as well as in recognizing the tropane carbon skeleton in alkaloids of unknown structure.

REFERENCES

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(7) Halpher, W. M., Gaertner, G. W., Swift, E. W., and Pollard, G. E., *Ind. Eng. Chem.*, **50**, 1131(1958).
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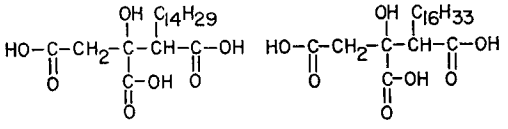
Pharmacologic Study of Norcaperatic and Agaricic Acids

By RICHARD A. CARRANO* and MARVIN H. MALONE

In rats norcaperatic acid produced delayed onset, dose-related effects of mydriasis, skeletal muscle weakness, and central nervous system depression similar to those induced by its homolog, agaricic acid. Agaricic acid was a nonspecific potentiator of furtrethonium in isolated rat jejunum. Both acids produced a slow onset of leiomyotonic effects on isolated guinea pig ileum. This effect could be blocked by papaverine but was not affected by high concentrations of atropine, pyribenzamine, or by a depolarized muscle. Tonic activity could also be produced in a K^+ deficient muscle. Both norcaperatic acid and agaricic acid were competitive inhibitors of the enzyme aconitase with norcaperatic acid being slightly more potent. The potentially toxic properties of *Cantharellus floccosus* due to the presence of norcaperatic acid may be related to the structural chemical relationships of this substance to citric acid.

CANTHARELLUS FLOCCOSUS Schw. has been reported to cause serious delayed gastrointestinal disturbances in individuals (1). Miyata *et al.* (2) isolated and characterized the active principle as norcaperatic or α-tetradecylcitric acid ($C_{20}H_{36}O_7$). Agaricic acid ($C_{22}H_{40}O_7$), an isolate from the mushroom *Polyporus officinalis* Fr., is a homolog of norcaperatic acid and has been included in this study (I and II).



NORCAPERATIC ACID I

AGARICIC ACID II

The isolation of norcaperatic acid from *C. floccosus* marked its first reported occurrence in nature, although agaricic acid had been isolated and studied by various investigators and classified as an anhydrotic and parasympatholytic agent (3–5). The literature reports do not satisfactorily settle whether the mechanism of action on smooth muscle is musculotropic or neurotropic. This study hoped to clarify this problem and to study the possible mechanisms by which these agents could induce mushroom poisoning.

Received June 9, 1967, from the Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs, CT 06268
Accepted for publication August 10, 1967.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Furtrethonium was provided by courtesy of Smith Kline & French Laboratories, Philadelphia, Pa.
This work was supported in part by University of Connecticut Research Foundation grant 400-5-5J, research grant GM-08239 from the National Institutes of Health, and Atlas Chemical Industries, Inc., Wilmington, Del.
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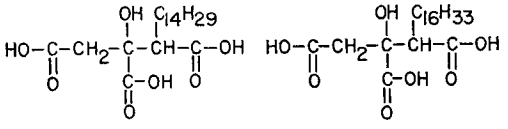

NORCAPERATIC ACID I

AGARICIC ACID II

The isolation of norcaperatic acid from *C. floccosus* marked its first reported occurrence in nature, although agaricic acid had been isolated and studied by various investigators and classified as an anhydrotic and parasympatholytic agent (3–5). The literature reports do not satisfactorily settle whether the mechanism of action on smooth muscle is musculotropic or neurotropic. This study hoped to clarify this problem and to study the possible mechanisms by which these agents could induce mushroom poisoning.

Received June 9, 1967, from the Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs, CT 06268
Accepted for publication August 10, 1967.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Furtrethonium was provided by courtesy of Smith Kline & French Laboratories, Philadelphia, Pa.
This work was supported in part by University of Connecticut Research Foundation grant 400-5-5J, research grant GM-08239 from the National Institutes of Health, and Atlas Chemical Industries, Inc., Wilmington, Del.
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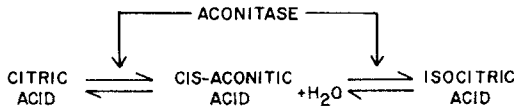
EXPERIMENTAL

In Vivo Hippocratic Screening—Nonfasted albino rats of either sex (Wistar strain, 150–250 Gm.) were injected intraperitoneally with various log-doses of the test drugs suspended in 0.25% agar according to the gross observational method of Malone and Robichaud (6). All required observations were made using the standard worksheet at +5, 10, 15, 30, and 60 min. postinjection, +2, 4, and 24 hr. postinjection, and +2, 4, and 7 days postinjection.

Isolated Rat Jejunum—Using the methods of Ariëns (7), van Rossum (8), and van Rossum and van den Brink (9), cumulative concentration-response curves were recorded with furtrethonium used as the reference agonist. Tyrode's solution oxygenated with 95% oxygen and 5% carbon dioxide and containing the calcium disodium salt of ethylenediaminetetraacetic acid (1×10^{-5} Gm./ml.) was the perfusion media. The rat jejunum section was mounted in a 50-ml. bath (37.5°) using a modified Magnus technique. All drug concentrations were calculated in terms of drug base.

Isolated Guinea Pig Ileum—The ileum wa mounted in the bath (37.5°) using a modified Magnus technique. In the protective studies a submaximal concentration of reference agonist was followed by: a specified concentration of test drug, a specified incubation period, and a repeat of the same concentration of reference agonist. In the antagonism studies a specified dose of experimental drug producing a suitable response was followed by: a specified concentration of prototype antagonist, a specified incubation period, and a repeat of the same concentration of experimental drug. In the K^+ deficient studies, KCl was omitted from the Tyrode's solution and replaced with an equivalent weight of NaCl. In the K^+ excess studies, KCl was added to the Tyrode's solution until the final concentration of potassium ion was 47 m*M*.

Enzymatic Studies—Aconitase was prepared from fresh pig hearts and diluted to a concentration giving workable activity (10). The reaction was studied in a Beckman DU spectrophotometer by following the change in light absorption (240 mμ and 25°) caused by the appearance or disappearance of *cis*-aconitic acid (Scheme I). The reaction was



Scheme I

started by the addition of substrate. Total cell volume was kept at 3.0 ml. by varying the amount of 0.05 *M* phosphate buffer. To assure initial reaction rates, the changes in extinction values (240 mμ at 25°) were recorded at intervals between start and +45 sec., checked for proper kinetics, and calculated as change in absorbance units/min. Experimental drugs were incubated with the enzyme preparation for 3–5 min. prior to activation with substrate. A control rate for a specific concentration of substrate was determined prior to testing the inhibitor's effect on that rate. All drug concentrations were calculated in terms of drug base and were made in 0.05 *M* phosphate buffer, pH 7.4 (10–12).

RESULTS AND DISCUSSION

In Vivo Hippocratic Screening—The whole powdered mushroom of *C. floccosus* and the 70% ethanol extractive obtained from the Drug Plant Laboratory, University of Washington, Seattle, were toxic and showed definite dose-related activity beginning at a dose of 50 mg./Kg. Mydriasis, skeletal muscle weakness, and central nervous system depression accompanied by hypothermia were among the symptoms. Although parasympatholytic activity appeared to be indicated, the presence of lacrimation without salivation, a delayed onset of all activity, and a profound and progressive loss in body weight suggested a more complicated mechanism of action. The extract, as expected, was considerably more toxic and showed more pronounced symptoms. The marc was also studied and was shown to contain residual amounts of activity indicating that the 70% ethanol extraction did not entirely exhaust the active components of the mushroom.

The hippocratic profiles for the samples of agaricic acid and norcaperatic acid were qualitatively identical to that of the mushroom preparations (CNS depression, hypothermia, mydriasis, lacrimation, *etc.*). Fine body tremors were also apparent as a delayed effect. In general, these gross *in vivo* studies suggested a parasympatholytic (hyoscine-like) mechanism. However, the complicating symptomatology, the delayed onset of all effects, and the progressive loss of body weight all suggested metabolic blockade.

Isolated Rat Jejunum—Figure 1 shows the cumulative log-concentration curves of furtrethonium obtained in the presence of agaricic acid. In general, a potentiating effect was seen. Longer incubation resulted in a greater effect. When agaricic acid was incubated with the tissue for 30 min. and washed for 10 min., a significant decrease in the intrinsic activity but not in the affinity of the furtrethonium to the receptor was seen. Apparently, while agaricic acid could be washed out, the muscle or one of its components had been physically changed by the drug. Agaricic acid alone increased both the tone and spontaneous activity of the jejunum. After incubation for about 5 min., a small contrac-

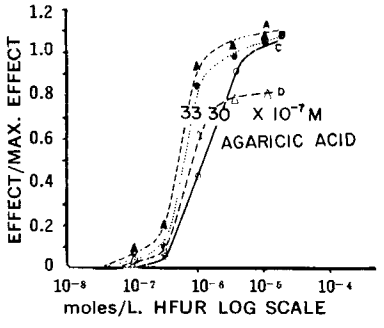


Fig. 1—Cumulative log-concentration curves for the acetylcholinomimetic furtrethonium (HFUR) in the presence of agaricic acid (3×10^{-7} M). Key: A, 30-min. incubation; B, 8-min. incubation; C, control with no agaricic acid; D, 30-min. incubation followed by three washes over a 10-min. period. Rat jejunum.

tion would result. A musculotropic (nonspecific) mechanism of action could explain these results.

Isolated Guinea Pig Ileum—Both agaricic and norcaperatic acids (1–5 × $10^{-5}M$) caused a delayed onset, slow, persistent contraction of the guinea pig ileum when the tissue was attached to a lightly loaded isotonic lever system. This leiomyotonic contraction had musculotropic rather than neurotropic characteristics in that it did not begin immediately upon introduction of the drugs but was seen only after about a 3-min. wait. The effect was also very slow (5–6 min. to reach maximum) and did not fade away as is the case with most neurotropic agents (*e.g.*, acetylcholine). The contraction could not be blocked by atropine (1 × $10^{-5}M$), pyribenzamine (1 × $10^{-5}M$), or by previously depolarizing the muscle by increasing the potassium ion concentration to 47 mM. It was still present in the absence of potassium ion in the perfusate. Papaverine, a nonspecific antagonist, could block the effect. These results indicated that the mechanism of action for the test drugs would have to be musculotropic in nature. Dose-related activity is one of the basic prerequisites for true drug activity. Figure 2 depicts the dose-response curve obtained when agaricic acid was accumulated in the bath.

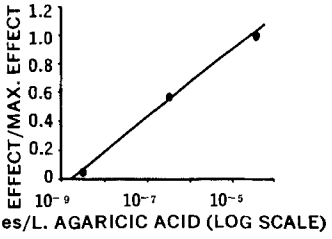


Fig. 2—Cumulative log-concentration curve of agaricic acid. Guinea pig ileum.

Enzymatic Studies—The musculotropic action on smooth muscle when considered along with the noted *in vivo* effects of loss of body weight and delayed onset of toxic activity strengthens the hippocratic diagnosis of metabolic blockade. The structural resemblance of these agents to citric acid initiated a study to determine the effect of these acids on the activity of the enzyme aconitase. Figures 3 and 4 show that agaricic acid and norcaperatic acid (1 × $10^{-4}M$) are competitive inhibitors of aconitase. In these experiments *cis*-aconitic acid was used as the substrate. As substrate concentration was increased, the inhibition caused by the test drugs was overcome. Finally, all of the enzyme surface was occupied by the substrate and the reaction velocity was only dependent on substrate concentration. The K_m for *cis*-aconitic acid was 3.3 × $10^{-4}M$. The K_i for agaricic acid was 4.3 × $10^{-4}M$ and for norcaperatic acid, 3.0 × $10^{-4}M$.

Figures 5 and 6 show the results of a similar study in which *dl* + allo, trisodium isocitric acid was used as the substrate for aconitase. The test agents were confirmed to be competitive inhibitors of the enzyme. The K_m for *dl* + allo, trisodium isocitric acid is 6.9 × $10^{-3}M$, while the K_i for agaricic and nor-

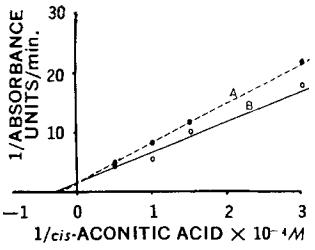


Fig. 3—Lineweaver-Burk plot of the reciprocal of reaction velocity versus *the reciprocal of the* cis-*aconitic acid concentration. Key:* A, *reaction in the presence of 1* × 10^{-4} M *of agaricic acid;* B, *control.*

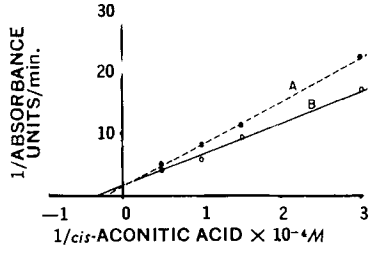


Fig. 4—Plot of the reciprocal of reaction velocity versus *the reciprocal of the* cis-*aconitic acid concentration. Key:* A, *reaction in the presence of 1* × 10^{-4} M *of norcaperatic acid;* B, *control.*

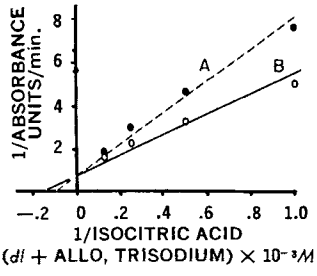


Fig. 5—Lineweaver-Burk plot of the reciprocal of reaction velocity versus *the reciprocal of the* dl + *allo, trisodium isocitric acid concentration. Key:* A, *reaction in the presence of 1* × 10^{-3} M *of agaricic acid;* B, *control.*

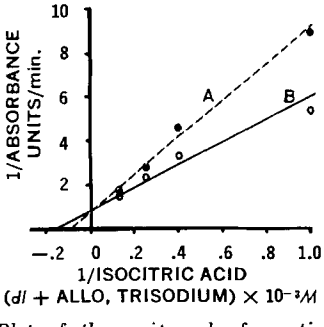


Fig. 6—Plot of the reciprocal of reaction velocity versus *the reciprocal of the* dl + *allo, trisodium isocitric acid concentration. Key:* A, *reaction in the presence of 1* × 10^{-3} M *of norcaperatic acid;* B, *control.*

caperatic acids are 3.0×10^{-3} and $2.4 \times 10^{-3}M$, respectively. Norcaperatic acid seemed to have a greater affinity to the enzyme than either of the substrates or agaricic acid. The order of decreasing affinities to aconitase was: norcaperatic acid, *cis*-aconitic acid, agaricic acid, and *dl* + allo, trisodium isocitric acid.

A chemical-steric relationship exists between the test acids and fluorocitric acid. Peters (13) states that fluorocitric acid is a competitive inhibitor of aconitase. *In vivo* it produces effects similar to those seen with agaricic and norcaperatic acids.

The basic concepts of enzyme action and steric requirements have been discussed by Koshland (14) and Ogston (15, 16) and a three-point attachment of citric acid to aconitase has been postulated. The citric acid is the correct size and shape to bind to the enzyme surface at three points as the conformation of the enzyme changes to accommodate it. In this natural situation, the proper alignment of bonds needed for catalytic activity is formed. It may be that when the bulky molecule of norcaperatic acid (citric acid with a $C_{14}H_{29}$ substituted for a H atom) approaches and attaches to the enzyme surface, it is bound; but because of its size, the proper formation of the catalytic bonds is prevented (steric hindrance). Agaricic acid and fluorocitric acid could act in the same manner since a $C_{16}H_{33}$ group or a F atom is also sterically much larger than a H atom. Moreover norcaperatic, agaricic, and fluorocitric acids all contain two asymmetrical carbon atoms, while citric acid does not. This makes four stereoisomeric forms possible for each structure. These forms would each have varying abilities to form enzyme and/or receptor attachments (affinity). In conclusion, the two naturally occurring acids studied here may be useful biochemical tools—especially for seeking antidotes, since the gross symptoms of certain toxic mushrooms have been shown here to be due, at least in part, to subcellular activity.

REFERENCES

(1) Tyler, V. E., Jr., *Progr. Chem. Toxicol.*, **1**, 339(1963).
(2) Miyata, J. T., Tyler, V. E., Jr., Brady, L. R., and Malone, M. H., *Lloydia*, **29**, 43(1966).
(3) Bergström, E., *Scand. Arch. Physiol.*, **53**, 278(1928).
(4) Sollmann, T., "A Manual of Pharmacology," 8th ed., W. B. Saunders Co., Philadelphia, Pa., 1957, p. 394.
(5) Rodman, M. L., *Federation Proc.*, **9**, 311(1950).
(6) Malone, M. H., and Robichaud, R. C., *Lloydia*, **25**, 320(1962).
(7) "Molecular Pharmacology," Ariëns, E. J., ed., Academic Press Inc., New York, N. Y., 1964.
(8) van Rossum, J. M., *Arch. Intern. Pharmacodyn.*, **140**, 592(1962).
(9) van Rossum, J. M., and van den Brink, F. G., *ibid.*, **143**, 240(1963).
(10) "Methods of Enzymatic Analysis," Bergmeyer, H. U., ed., Academic Press Inc., New York, N. Y., 1963, p. 318.
(11) "Methods in Enzymology," vol. 1, Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1955, p. 695.
(12) Racker, E., *Biochim. Biophys. Acta*, **4**, 211(1950).
(13) Peters, R. A., *Advan. Enzymol.*, **18**, 113(1957).
(14) Koshland, D. E., Jr., *Science*, **142**, 1533(1963).
(15) Ogston, A. G., *Nature*, **162**, 963(1948).
(16) *Ibid.*, **181**, 1462(1958).

Effect of Tris(hydroxymethyl)aminomethane on Removal of Urea by Peritoneal Dialysis

By W. M. MCLEAN, D. M. POLAND, M. S. COHON, S. C. PENZOTTI, and A. M. MATTOCKS*

From earlier *in vitro* studies it was thought that tris(hydroxymethyl)aminomethane (tromethamine) might accelerate diffusion of substances across the peritoneal membrane by a mechanism other than causing ionization of a weak acid in the dialysis fluid. This concept was tested by measuring the rate of dialysis of urea in rabbits, urea being essentially nonionized at the pH of the tromethamine fluid. Blood and dialysate concentrations were measured following intravenous injection of tagged urea and following incorporation of tagged urea in the dialysis fluid (reverse dialysis). Dialysis rates were calculated. It was found that tromethamine increased the dialysis rate two to threefold. It is concluded that tromethamine accelerates dialysis by an unknown mechanism.

SEVERAL WORKERS have reported on the use of tris(hydroxymethyl)aminomethane (tromethamine)[1] in peritoneal dialysis (1–6). These have dealt with the use of tromethamine to remove salicylates, barbiturates, and urate, the theory being that the alkalinity of the dialysis fluid would cause ionization of the drug in the peritoneum and thus maintain a higher concentration gradient of unionized drug across the dialyzing membrane. It is important that the mechanism by which tromethamine exerts its effect on dialysis be understood in order that a search for other more effective substances be facilitated.

In a study conducted in these laboratories, it was found that both ionized and unionized pento-

Received February 13, 1967, from the Pharmacy Research Laboratory, University Hospital, University of Michigan, Ann Arbor, MI 48104, and the * School of Pharmacy, University of North Carolina, Chapel Hill, NC 27515
Accepted for publication August 15, 1967.
This investigation was supported in part by grant 1-R01-Ac-00312 from the Center for Urban and Industrial Health, U. S. Public Health Service, Bethesda, Md.

[1] Tham.

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REFERENCES

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barbital and salicylate diffused rapidly and at almost equal rates across isolated rabbit mesentery (7). This agreed with other work (8–10) which demonstrated the diffusion of rubidium and phosphate ions across the isolated membrane. These findings suggest that the effect of tromethamine in accelerating dialysis might be something other than simple change of pH of the peritoneal fluid. On the other hand, it remains to be shown that the isolated membranes truly represent the diffusion characteristics of the live organ; thus *in vivo* tests are required.

One approach to determine whether tromethamine has an effect other than that of causing ionization of the compound in the peritoneum is to measure its effect on the dialysis of a substance which is not ionized at the pH of the tromethamine dialysis fluid or that of the blood. Urea is such a substance, having a pKa of 0.18, and it was selected for this study.

At the outset it was recognized that the rate of dialysis of a substance at any time would be a function of the blood (and extracellular fluid) concentration at that instant. The blood level, in turn, is affected not only by dialysis of the drug into the peritoneal fluid, but also by excretion and metabolism. Thus evaluation of dialysis rate simply by the amounts in dialysis fluid at intervals of time would be subject to errors resulting from different rates of metabolism and excretion by different animals. To avoid these variations experiments were designed to measure the blood and dialysate levels throughout the procedure. Results could then be calculated in terms of rate constants which are independent of the other variables.

MATERIALS AND METHODS

Healthy, mature male albino rabbits were selected as the experimental animal. No general anesthesia was used. A urinary catheter was inserted and an intravenous injection of 1 Gm. of ^{14}C-tagged urea (200 mg./ml.) was administered. Twenty to thirty minutes were allowed for the distribution of the drug throughout the body fluids before beginning dialysis, then 150 ml. of dialysis fluid was introduced into the peritoneum by means of a pediatric size peritoneal catheter. Blood samples were taken from the marginal ear vein. Dialysate samples were withdrawn through the peritoneal catheter, taking care to return contents of the outlet tubing and catheter after removal of the sample. Samples of blood and peritoneal fluid were removed at 10–15-min. intervals over periods up to 200 min. Urine samples were taken also but are not used in this analysis and are not presented here.

Several tests of "reverse dialysis" were performed, wherein the tagged urea was placed in the dialysis fluid prior to its injection into the peritoneum. In these cases 2 ml. of the urea-^{14}C injection was mixed with 150 ml. of dialysis fluid just prior to injection, and samples of dialysate and blood were taken at 5–8-min. intervals over periods up to 120 min.

The urea injection was prepared by dissolving aseptically 1.2 mg. of urea-^{14}C (specific activity 3.67 mc./mmole) in 50 ml. of a sterile solution containing 200 mg. urea/ml. and 0.9% benzyl alcohol as a preservative. The injection was stored in the refrigerator.

The control dialysis fluid was a standard hospital formula containing: dextrose, 1.5%; sodium chloride, 0.62%; sodium lactate, 0.39%; calcium chloride monohydrate, 0.026%; and magnesium chloride hexahydrate, 0.015%. Tromethamine-I fluid was the same solution to which was added 18.17 Gm. (150 mmoles) tromethamine/liter. This fluid, although equivalent to control in content of dextrose and salts, was more hypertonic, and it is possible that an effect observed might be partially due to withdrawal of fluid by osmosis. Thus, a second fluid, tromethamine-II, was prepared containing 18.17 Gm. tromethamine/L. and sodium chloride 0.40%, no dextrose, and the same quantities of calcium chloride, magnesium chloride, and sodium lactate as the control solution. This solution was measured on an osmometer and found to have the same osmolality as the control (0.351). The dextrose and lactic acid used were USP; all other chemicals were analytical or reagent grade. The reagent grade of tromethamine is of such purity as to be commonly used as a primary standard in acidimetry.

Measurements of concentration in blood and dialysate were made by standard radioactive counting methods. Blood was prepared for counting in two ways, which were shown to give equal values: (*a*) 0.5 ml. of serum was added to 1 ml. of a solubilizer[2] and the mixture was allowed to stand 24 hr. Then 0.1 ml. concentrated hydrochloric acid and 10 ml. of dioxane phosphor counting solution were added. (*b*) Approximately 0.5 ml. of blood was drawn directly into a graduated centrifuge tube containing 1 ml. of 6% trichloroacetic acid; the volume was recorded and the contents shaken thoroughly. The tube was then centrifuged, and an aliquot of the clear colorless supernatant was added to 10 ml. of dioxane counting solution. The latter method was preferred, since no quenching was observed with this procedure. A 0.5-ml. portion was added with dialysate samples directly to the dioxane counting solution. An accurate dilution of the injection was used as a standard, which was counted with each set of samples. This was also used as the internal standard whenever quenching corrections were required. The dioxane counting solution consisted of 7 Gm. PPO (2,5-diphenyloxazole), 0.05 Gm. POPOP [(1,4-bis-2,5-phenyloxazolyl) benzene], and 50 Gm. naphthalene in sufficient spectro quality dioxane to make 1 L. All samples were counted at least 3 times in a Packard 314-X Tri-Carb liquid scintillation counter.

RESULTS AND TREATMENT OF THE DATA

Data on dialysis with control and the 2 tromethamine fluids are presented in Table I. Typical curves for blood and dialysate concentrations are shown in Figs. 1 and 2. As one might anticipate,

[2] Hyamine 10X, Rohm and Haas Co., Philadelphia, Pa.

TABLE I—CONCENTRATIONS OF EXOGENOUS UREA IN BLOOD AND DIALYSATE FOLLOWING INTRAVENOUS INJECTION AND DURING PERITONEAL DIALYSIS

Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.
Rabbit No. *1*, Control Dialysis Started, 20 min.		
10	581	0
29	507	13
40	385	30
50	433	53
62	...	78
72	320	107
83	246	140
94	299	157
104	233	167
114	236	181
125	201	192
134	...	189
145	...	180
Rabbit No. *2*, Control Dialysis Started, 20 min.		
12	498	0
27	449	27
37	439	74
53	464	92
62	436	126
72	...	160
83	415	172
92	443	177
102	461	191
114	462	205
124	435	218
134	...	244
144	425	243
154	442	264
164	409	270
174	444	298
185	406	293
194	433	301
Rabbit No. *3*, Tromethamine-I Dialysis Started, 30 min.		
11	485	0
22	425	0
32	332	34
42	402	87
53	343	142
62	366	228
72	364	253
82	322	270
90	330	269
102	310	290
113	310	273
122	351	282
132	310	259
144	337	268
154	314	270
164	284	302
174	270	267
185	271	272
194	271	251
204	262	260
215	258	263
Rabbit No. *4*, Control Dialysis Started, 30 min.		
15	374	0
30	329	0
40	304	9
50	303	25
60	277	46
70	274	70
80	265	84
90	265	96
100	243	115
110	243	136
120	237	134
130	261	137
140	213	161
150	204	168
160	208	164
170	216	160

Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.
Rabbit No. *5*, Tromethamine-I Dialysis Started, 30 min.		
11	441	0
30	392	0
40	324	23
51	348	104
61	294	120
71	293	142
80	287	156
91	236	206
100	309	224
110	316	237
121	276	262
132	227	266
142	303	268
150	277	278
161	276	291
171	299	287
180	293	283
193	261	293
204	278	290
Rabbit No. *6*, Tromethamine-I Dialysis Started, 20 min.		
10	578	0
24	238	0
35	441	77
45	394	153
55	405	311
65	366	...
75	362	342
85	402	309
97	380	353
106	358	337
117	...	363
125	354	375
135	344	366
145	...	358
155	347	330
166	343	299
177	338	292
187	324	288
197	341	...
207	310	...
Rabbit No. *7*, Control Dialysis Started, 23 min.		
10	535	0
25	426	0
35	406	97
45	397	151
55	377	164
67	398	191
75	367	196
85	321	217
95	349	223
105	330	218
115	329	231
125	369	252
135	352	238
145	327	252
155	299	255
165	249	260
175	...	259
185	253	270
195	294	265
205	276	264
Rabbit No. *8*, Control Dialysis Started, 30 min.		
20	318	0
29	310	0
40	310	14
51	287	46
60	296	75
70	262	89
80	261	106
91	270	118

(Continued on next page.)

TABLE I—(*Continued.*)

Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.
100	...	137
110	240	137
121	229	141
132	247	148
140	224	154
150	246	158
160	...	168
170	...	176
180	231	177
190	227	180
200	213	178
210	227	181
Rabbit No. *9*, Tromethamine-II Dialysis Started, 35 min.		
30	302	0
46	272	21
56	263	54
65	239	99
76	255	133
86	225	181
96	250	198
106	251	199
115	220	203
125	193	211
135	235	223
146	236	228
155	...	224
165	231	234
175	229	230
185	253	244
195	216	236
205	...	237
Rabbit No. *10*, Tromethamine-II Dialysis Started, 30 min.		
46	558	133
56	494	199
68	548	333
78	544	355
85	492	356
98	507	392
105	451	403
117	455	415
125	460	420
137	443	417
147	416	408
155	440	412
166	407	420
176	...	414
186	455	412
196	...	412
206	396	405
216	404	408
Rabbit No. *11*, Tromethamine-II Dialysis Started, 30 min.		
43	657	91
53	755	227
64	470	273
73	410	329
83	394	368
94	450	377
104	515	397
114	397	384
123	405	388
133	378	388
143	...	400
153	378	402
164	353	386
173	328	378
183	319	386
193	324	382
203	...	375
213	325	307
Rabbit No. *12*, Control Dialysis Started, 30 min.		
32	664	0
42	536	85
53	518	128
62	489	171
73	474	188
82	392	203
92	365	219
102	391	230
112	345	239
122	321	254
132	339	253
142	318	264
153	285	266
162	279	271
172	273	264
182	279	264
192	278	260
202	286	260
212	285	263
Rabbit No. *13*, Control Dialysis Started, 30 min.		
33	422	0
43	...	89
54	431	119
64	391	139
73	371	159
83	357	174
94	365	199
103	...	206
113	325	215
123	358	227
133	368	233
143	314	248
153	286	248
163	277	261
173	280	276
183	319	279
193	272	284
205	280	270
213	...	280
Rabbit No. *19*, Tromethamine-II Dialysis Started, 30 min.		
30	376	16
40	313	40
50	292	65
60	290	133
70	272	172
80	250	195
90	277	214
100	270	224
110	262	239
120	277	246
130	253	245
140	279	260
150	294	245
160	304	269
170	302	265
Rabbit No. *20*, Tromethamine-II Dialysis Started, 30 min.		
33	481	28
40	415	59
50	462	110
60	445	187
70	412	227
80	439	266
90	407	303
100	431	329
110	420	341
122	378	347
131	402	355
140	405	361
150	413	372
160	412	381
170	337	387
Rabbit No. *21*, Tromethamine-II Dialysis Started, 58 min.		
60	331	0

(*Continued on next page.*)

TABLE I—(*Continued.*)

Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.	Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.
68	318	50	139	283	257
79	294	93	149	247	266
89	279	135	159	275	269
98	290	180	169	220	265
108	287	216	179	260	263
118	292	240	188	272	270
129	285	241	199	243	290

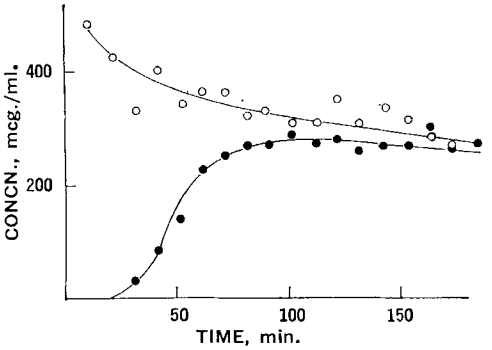


Fig. 1—Blood and dialysate concentrations of exogenous urea, rabbit No. 3, tromethamine-I dialysis fluid. Key: O, blood; ●, dialysate.

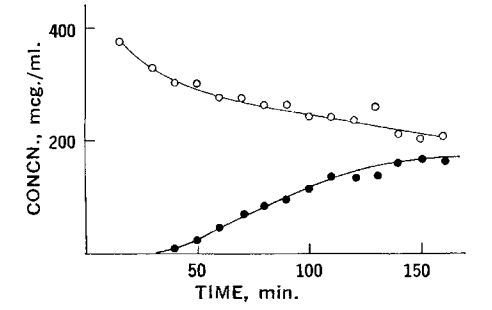


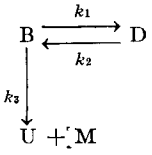
Fig. 2—Blood and dialysate concentrations of exogenous urea, rabbit No. 4, control dialysis fluid. Key: O, blood; ●, dialysate.

the blood levels and dialysate levels varied from one animal to another due to differences in metabolism and excretion rates. On the other hand, the more rapid rate at which the dialysate curves approach the blood curves in the case of tromethamine is obvious in most instances. As has been mentioned, the impression formed from such an observation might be quite misleading, since the reason for blood and dialysate curves approaching one another rapidly may be due to rapid rate of excretion or metabolism rather than rapid dialysis. For this reason it is necessary to compute dialysis rates to make a meaningful comparison.

The data on reverse dialysis are presented in Table II, and curves of dialysate concentrations are shown in Figs. 3 and 4. In Fig. 3 it is seen that the 2 tromethamine fluids lost urea at a consistently faster rate than the control. In Fig. 4 the tromethamine curve is quite close to control, the difference being mainly in curvature. As is shown later, the analysis yields higher rate constants in this case, since the rate constant reflects the differences in curvature between initial and final portions of the curve. The rate is slower in this instance, however, and may not be significant.

Several methods were utilized to analyze the data, all giving approximately the same results. The one judged to be most generally acceptable is presented in this paper. Rather than use equations relating total amounts in compartments which requires estimates of distribution volumes (11, 12), equations relating concentrations in the compartments were used.

The distribution of exogenous urea after an initial period for mixing throughout the body fluids may be depicted as follows:



(Eq. 1)

where B is blood or equivalent body fluid, D is dialysate in the peritoneum, and U and M represent urinary excretion and metabolites. Equations for treating this type of system may be found in Rescigno and Segre (13) and in Rodiguin and Rodiguina (14). The equation for dialysate concentration may be stated as follows:

$$X_2 = \frac{X_0 k_1}{m_2 - m_1}(e^{-m_1 t} - e^{-m_2 t}) = A(e^{-m_1 t} - e^{-m_2 t}) \quad \text{(Eq. 2)}$$

where X_2 is concentration in dialysate, X_0 is concentration in blood at time dialysis is begun, m_1 and m_2 are the roots of the auxiliary equation (13, 15). The equation for the concentration in blood is:

$$X_1 = \frac{X_0}{m_2 - m_1}[(k_2 - m_1)e^{-m_1 t} + (m_2 - k_2)e^{-m_2 t}] = B_0 e^{-m_1 t} + C_0 e^{-m_2 t} \quad \text{(Eq. 3)}$$

where X_1 is the concentration in blood, and other constants are the same as in Eq. 2. If t is zero at the time of starting dialysis, the concentration in blood at that time becomes B_0 plus C_0, and this sum may be taken as X_0 in subsequent calculations.

The dialysate curve is far more sensitive to the magnitude of k_1, since the blood concentration is

TABLE II—CONCENTRATIONS OF EXOGENOUS UREA IN BLOOD AND DIALYSATE DURING REVERSE DIALYSIS

Time, min.	Blood, mcg./ml.	Dialysate, mcg./ml.
Rabbit No. *14*, Control		
0	0	2666
6	11	2446
12	26	2290
17	35	2081
23	45	1915
28	47	1850
35	58	1710
43	56	1613
50	67	1464
58	69	1371
67	74	1253
77	78	1157
85	83	1088
94	81	1046
102	83	957
110	84	889
118	81	821
126	86	788
Rabbit No. *15*, Control		
0	0	2666
7	15	2237
13	33	1975
19	49	1815
24	62	1777
29	70	1587
37	66	1448
46	64	1429
54	114	1293
62	77	1209
70	105	1153
79	93	1063
88	93	981
95	86	924
103	103	851
111	93	786
119	97	739
Rabbit No. *16*, Tromethamine-II		
0	0	2666
5	28	2507
11	63	2299
16	81	2024
21	93	1849
26	95	1611
31	101	1469
36	118	1315
44	110	1147
52	120	1076
60	120	1006
68	122	920
76	133	842
84	133	776
91	127	689
100	118	627
109	136	560
Rabbit No. *17*, Tromethamine-II		
0	0	2666
5	18	2430
10	28	2321
15	42	1990
20	49	1781
25	50	1687
30	61	1496
36	51	1454
41	60	1455
46	64	1443
53	62	1333
61	64	1269
69	70	1147
77	80	1098
85	81	1080
93	80	1022
102	79	1007
Rabbit No. *18*, Tromethamine-II		
0	0	2666
6	40	1898
11	66	1975
16	79	1786
22	74	1576
31	94	1227
36	80	1132
41	95	1081
47	96	1026
52	83	961
58	98	834
67	96	804
75	100	669
85	96	635
94	92	585
103	91	531
112	98	471

affected less than 10% by the amount diffused into dialysate, hence the dialysate curve and Eq. 2 were used to calculate the values of m_1 and m_2, while the blood curve and Eq. 3 were used to obtain values of X_0. Equation 2 was solved on an analog computer, and the values of m_1 and m_2 were put into Eq. 3 on the computer to be certain that they fit the blood curve as well. This is easily and rapidly done with the analog computer and a modern *x-y* plotter. The rate constant was then calculated from an expression derived from Eq. 2:

$$k_1 = \frac{A}{X_0}(m_2 - m_1) \qquad \text{(Eq. 4)}$$

Values obtained for k_1 are presented in Table III.

With reverse dialysis the dialysis curve was again used for evaluation of m_1 and m_2 and for the values X_{20} and D of the following equation, which is the one applicable for the condition where the drug is administered in the dialysis fluid (13):

$$X_2 = \frac{X_{20}}{m_2 - m_1}\left[(D - m_1)e^{-m_1 t} + (m_2 - D)e^{-m_2 t}\right] \qquad \text{(Eq. 5)}$$

where X_2 is concentration in dialysate, X_{20} is its initial value, D is k_1 plus k_3, m_1 plus m_2 equals the sum of k_1, k_2, and k_3, the k's having the same meaning as in Eq. 1. The value of k_2, representing transport from the dialysate to the blood, was calculated as $m_1 m_2 - D$. The values obtained for k_2 are shown in Table III.

It is seen from Table III that an increased rate of dialysis was observed with tromethamine rather consistently. One control was as high as 2 of the tromethamine solutions, but it was high for a control solution. The average rate with tromethamine was more than twice that of controls. Tromethamine-I and tromethamine-II fluids showed no difference, thus all tromethamine values were averaged together.

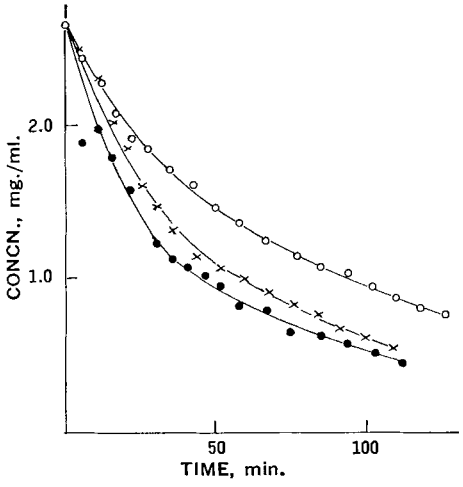


Fig. 3—Dialysate concentrations of exogenous urea during reverse dialysis. Key: O, rabbit No. 14 with control solution; ●, rabbit No. 18 with tromethamine-II solution; ×, rabbit No. 16 with tromethamine-II solution.

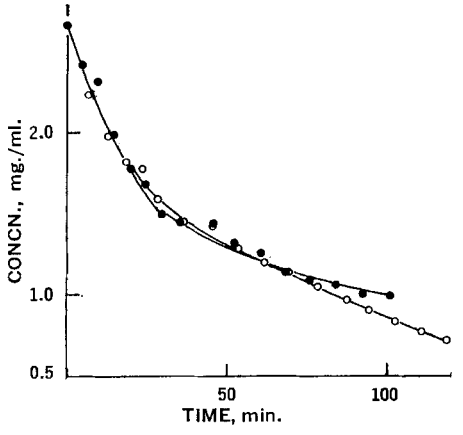


Fig. 4—Dialysate concentrations of exogenous urea during reverse dialysis. Key: O, rabbit No. 15 with control solution; ●, rabbit No. 17 with tromethamine-II solution.

DISCUSSION AND CONCLUSIONS

The blood curves for urea showed greater deviations than one might generally expect for a typical drug or injected substance. These variations were the subject of much laboratory study, and a number of variations in assay were attempted in an effort to obtain better uniformity. It did not appear that the variation was due to assay procedure, or that urea was entrapped in protein precipitates since results were nearly identical when several methods were used, including those not requiring protein precipitation on the same set of samples. It is believed that these variations result from traces of radioactive carbon dioxide present in the blood and tissues following metabolism of urea, removal of this metabolite being a slow and nonuniform process.

TABLE III—RATE CONSTANTS, k_1 AND k_2, FOR DIALYSIS OF EXOGENOUS UREA

Dialysis Fluid	Rabbit No.	Wt., Kg.	k_1 min.$^{-1}$ × 10^2
Control	*1*	3.61	0.8
	2	3.86	0.9
	4	4.45	1.0
	7	3.96	1.9
	8	4.26	1.1
	12	2.09	1.4
	13	1.90	0.9
Av.		3.45	1.2
Tromethamine-I	*3*	4.26	4.3
	5	3.85	1.7
	6	4.00	2.4
Tromethamine-II	*9*	3.96	2.4
	10	2.32	3.4
	11	2.42	3.6
	19	3.98	2.2
	20	2.84	1.9
	21	3.41	2.3
Av.		3.45	2.7
	Reverse Dialysis		k_2 min.$^{-1}$ × 10^2
Control	*14*	3.73	1.6
	15	3.37	1.9
Tromethamine-II	*16*	2.94	4.1
	17	3.69	2.9
	18	3.96	4.1

The fitting of the dialysate curve to the data points is reasonably precise. A change in values of curve parameters of 5% or more will not yield a satisfactory fit. Furthermore, a variation in one constant tends to be corrected for in another; for example, if one uses too large a value for m_1 a correspondingly higher value for m_2 is required, making the difference $m_2 - m_1$ about the same. Thus, the values for A, m_1, and m_2 appear to be within 5%. The greatest error in calculating k_1 results from the estimation of X_0, initial blood level. This value is restricted, however, by the requirement that m_1 and m_2 of the dialysis curve must also apply to the blood curve.

Reverse dialysis was run with the aim of determining whether tromethamine might have a uni-directional effect on dialysis. One possible mechanism for its action might be the formation of a tromethamine–urea complex which is nondiffusible or slowly diffusible, thus increasing the effective blood to dialysate concentration gradient. If this were the mechanism, then in reverse dialysis the presence of tromethamine in the peritoneum would cause a much slower transport from fluid to blood than would be observed in controls. Since the tromethamine actually accelerated transport in 2 of 3 cases, this hypothesis for tromethamine action was rejected. The reason for a less significant effect in one case of reverse dialysis (Fig. 4) is not clear, but since the failure to retard dialysis answered the purpose of the experiment, this was not investigated further.

The values of dialysis rate constants, which are independent of individual variations in blood levels, excretion, and metabolism rates, clearly demonstrate that the diffusion of exogenous urea across the peritoneal membrane is accelerated by the presence of tromethamine. In these experiments the pH of the

tromethamine dialysate was found to change to a variable degree. The original fluid was pH 9.7, and it dropped rapidly after injection to 8.0–8.2, then more slowly as it approached the pH of body fluids. This drop occurs as a result of neutralization in the abdomen and due to absorption of tromethamine (5). One might expect the dialysis rate constant to vary with these changes, but a single constant was found to give acceptable fit to the data throughout the dialysis period.

The rate constants obtained in this work are based only on exogenous urea which may be considered to diffuse from one compartment to another quite independently of all other molecules. The constants themselves should be applicable to any urea present in the animal, but the total amounts of urea in the various compartments in the animals studied cannot be deduced without having measurements of specific activity. Since this work was designed to evaluate the rates for the added urea only, specific activities in the various fluids were not measured. Thus, by following only the exogenous urea the complexities of analysis and errors in measurement of total urea were avoided. If one treats the kinetics of endogenous urea, the biosynthesis of urea must be taken into account, and an equation of form similar to that of Teorell (11) for amount of drug in tissues with 3 exponential terms would be required. Obviously a direct comparison of blood and dialysate concentrations would not reflect the dialysis rates. This greater complexity of the system, coupled with the relatively low urea levels found in normal animals, probably accounts for the failure to detect the effect of tromethamine on urea dialysis by other workers, such as Knochel and Mason (6) where urea diffusion was followed.

The acceleration of urea dialysis by tromethamine cannot be attributed to a pH effect on ionization since urea is essentially nonionized under all conditions of the experiments. The mechanism of the tromethamine effect in this case is unknown, but it is certainly worthy of further investigation, particularly with the aim of finding other more effective substances. Some possible mechanisms are: (*a*) a wetting effect to improve contact between the membrane and dialysis fluid, especially where fatty deposits are present on the membrane, (*b*) a direct chemical effect on the peritoneal membrane to increase membrane permeability, (*c*) improved circulation of the extracellular fluid to the membrane. Some preliminary tests of these possibilities have been made. Measurements of protein content of dialysates showed the tromethamine fluids to have extracted no more protein than the control solutions. Examination of the membranes after dialysis revealed no gross visible changes, except a possibility that the membranes have less fatty film. Knochel *et al.* (1) reported no gross changes in the membrane with tromethamine fluids. Three tests with isolated membrane in the manner used by Shenouda and Mattocks (7) indicated only small increases in permeability with tromethamine. Further studies are in progress to define the nature of the tromethamine effect.

Although this work suggests the possibility of the use of tromethamine in dialysis for uremia, it should be noted that tromethamine is rapidly absorbed from the dialysis fluid (5) and is primarily eliminated *via* the kidneys. In the uremic patient elimination may be extremely slow, resulting in rapid accumulation in the body. Thus, the toxicity in the uremic patient may be much greater than in the normal. Additional studies with nephrectomized animals are needed to define the safe and effective ranges of the procedure before application in the clinic. Such experiments are being undertaken in this laboratory.

REFERENCES

(1) Knochel, J. P., Clayton, L. E., Smith, W. L., and Barry, K. G., *J. Lab. Clin. Med.*, **64**, 257(1964).
(2) Setter, J. G., Freeman, R. B., Maher, J. F., and Schreiner, G. E., *Trans. Am. Soc. Artif. Int. Organs*, **10**, 340 (1964).
(3) Nahas, G. G., Giroux, J. J., Gjessing, J., Verosky, M., and Mark, L. C., *ibid.*, **10**, 345(1964).
(4) Knochel, J. P., and Barry, K. G., *J. Lab. Clin. Med.*, **65**, 361(1965).
(5) Nahas, G. G., Gjessing, J., Giroux, J. J., Verosky, M., and Mark, L. C., *Clin. Pharmacol. Therap.*, **6**, 560(1965).
(6) Knochel, J. P., and Mason, A. D., *Am. J. Physiol.*, **210**, 1160(1966).
(7) Shenouda, L. S., and Mattocks, A. M., *J. Pharm. Sci.*, **56**, 464(1967).
(8) Berndt, W. O., and Gosselin, R. E., *Biochem. Pharmacol.*, **8**, 359(1961).
(9) Berndt, W. O., and Gosselin, R. E., *Am. J. Physiol.*, **200**, 454(1961).
(10) *Ibid.*, **202**, 761(1962).
(11) Teorell, T., *Arch. Intern. Pharmacodyn.*, **57**, 205(1937).
(12) *Ibid.*, **57**, 226(1937).
(13) Rescigno, A., and Segre, G., "Drug and Tracer Kinetics," Blaisdell Publishing Co., Waltham, Mass., 1966, p. 28.
(14) Rodiguin, N. M., and Rodiguina, E. N., "Consecutive Chemical Reactions," D. van Nostrand Co., Princeton, N. J., 1964, p. 26.
(15) Mellor, J. W., "Higher Mathematics," Dover Publications, Inc., New York, N. Y., 1954, p. 400.

Pharmacopeial Standards and Specifications for Bulk Drugs and Solid Oral Dosage Forms

Similarities and Differences

By W. N. FRENCH, F. MATSUI, DENYS COOK, and LEO LEVI

Tests for tablet weight variation, drug content, and disintegration time described in the "United States Pharmacopeia," "The National Formulary," the "British Pharmacopoeia," the "Pharmacopée Française," the "State Pharmacopoeia of the U.S.S.R.," and the "Nordic Pharmacopoea" are compared with regard to methodology, apparatus, scope, and compliance. Similarities and differences characterizing these standards are discussed. Comparable appraisals are made of assays for bulk drugs and compressed tablets included in the United States Pharmacopeia, the National Formulary, the British Pharmacopoeia, and the State Pharmacopoeia of the U.S.S.R. Discrepancies of sufficient magnitude exist between these tests and specifications to warrant closer cooperation among pharmacopeial agencies. Such cooperation should ensure greater uniformity of drug testing, encourage wider drug trade, and promote better public health throughout the world. These objectives are actively pursued by the World Health Organization.

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PHARMACOPEIAL STANDARDS have been developed in many countries. Realizing their value as mutually acceptable criteria of pharmaceutical quality control and their commercial importance, some countries have come to agree on common standards and specifications. The Nordic Pharmacopoea, official in Denmark, Finland, Iceland, Norway, and Sweden, may be cited as an example, and the European Pharmacopoeia—aiming to encourage and facilitate drug trade between countries of the European Common Market—likewise reflects this trend. Yet a great deal of work remains to be done to establish truly international standards as reference criteria, *i.e.*, standards that can be used conveniently anywhere and mean the same thing to analysts working in their national laboratories throughout different parts of the world.

It is the purpose of this paper to point out certain differences and similarities which exist between pharmacopeial tests and specifications applied in the quality control of bulk drugs and

Received May 15, 1967, from the Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication September 1, 1967.
The authors express their appreciation to Dr. Lloyd C. Miller, the United States Pharmacopeia; Dr. Edward G. Feldmann, the National Formulary, Dr. T. C. Denston, British Pharmacopoeia Commission; Professor M. D. Mashkovsky, Ministry of Health, U.S.S.R., and Dr. Kjeld Ilver, Farmakopékommissionens Laboratorium, Copenhagen, Denmark, for courteously reviewing the manuscript. They are also indebted to Dr. D. F. Bray, Statistical Services Division, Food and Drug Directorate, for valuable discussions.

TABLE I—WEIGHT VARIATION TOLERANCES[a]
(USP XVII, NF XII, B.P. 1963)

Av. Wt. of Tablet (mg.) from 20 Determinations			Not More Than Two Tablets Differ from Av. Wt. by More Than	No Tablet Differs from Av. Wt. by More Than
USP XVII	NF XII	B.P. 1963		
	<13[b]		±15%[b]	±30%[b]
<130	13–130	<120	±10%	±20%
130–324	130–324	120–300	±7.5%	±15%
>324	>324	>300	±5%	±10%

[a] Not applicable to sugar-coated, compression-coated, or enteric-coated tablets. [b] Deleted in Second Supplement to NF XII.

TABLE II—WEIGHT VARIATION TOLERANCES[a]
(PHARMACOPÉE FRANÇAISE VIII)

Theoret. Wt. of Tablet, mg.	Tolerance, %
<150	±7.5
>150	±5

[a] Not applicable to coated tablets.

TABLE III—WEIGHT VARIATION TOLERANCES[a]
(STATE PHARMACOPOEIA U.S.S.R. IX)

Av. Wt. of Tablet, mg.	Tolerance from Av. Wt., %
<120	±10
>120	±5

[a] Not applicable to coated tablets.

tablet preparations, and to emphasize the need for greater interpharmacopeial uniformity.

GENERAL STANDARDS APPLICABLE TO SOLID ORAL DOSAGE FORMS

Weight Variation

Pharmacopeial standards and specifications have been established to provide limits for permissible variations in the weights of individual dosage forms, expressed in terms of the allowable deviation from the average weight of a representative sample. Separate procedures and limits are described in most reference compendia for uncoated tablets, capsules, and sterile solids.

United States Pharmacopeia, National Formulary, and British Pharmacopoeia—USP XVII (1), NF XII (2), and B.P. 1963 (3) specify that 20 whole tablets be weighed individually, the average weight calculated, and the variations compared with specifications. Samples meet requirements if weight variations observed are not greater than those shown in Table I.

The British Pharmacopoeia allows performance of this test on 10 tablets also, specifying that in this case not more than *one* tablet may deviate from the average weight by a percentage greater than that shown in the table, and none of the tablets differ from the average by more than double that percentage.

Pharmacopée Française VIII (Codex Médicamentarius Gallicus) (4)—This compendium specifies that 10 tablets be weighed individually from a batch of homogeneous manufacture, the average weight determined, and the variations compared with specifications. Samples meet the requirements if weight variations observed are not greater than those shown in Table II.

State Pharmacopoeia U.S.S.R. IX (5)—This compendium specifies that 10 tablets be weighed collectively and the average weight calculated. Another 10 tablets are to be weighed individually (each to within 10 mg.) and the variations compared with specifications. Samples meet requirements if weight variations observed are not greater than those shown in Table III.

Pharmacopoea Nordica III (6)—This compendium requires that 100 tablets[1] be weighed collectively (to within 1 mg. if the tablet is lighter than 80 mg. and to within 10 mg. if the tablet is heavier than 80 mg.) and the average weight calculated (to within 0.1 mg. if the tablet is lighter than 80 mg. and to within 1 mg. if the tablet is heavier than 80 mg.). Thirty tablets are selected at random from this sample and weighed individually (to within 0.2 mg. if the tablet is lighter than 80 mg. and to within 1 mg. if the tablet is heavier than 80 mg.). Requirements are met if weight variations determined are in accord with specifications shown in Table IV.

Similarities and Differences—Although the tests described are simple, easily carried out, and serve the same purpose—namely, the establishment of the weight uniformity of uncoated, compressed tablets from a given lot—they differ markedly in both methodology and requirements for compliance.

Methodology—The USP and NF tests are based on the use of a representative sample of 20 tablets weighed collectively and individually. Results based on similar examination of only 10 tablets are accepted by the B.P. The French pharmacopeia also specifies that 10 tablets be taken for the test, while the Russian pharmacopeia requires that 10 tablets be weighed collectively to assess the average weight and another 10 be weighed individually to appraise variations from the average weight. The Nordic pharmacopeia generally calls for the weighing of 100 tablets to compute their average weight and the examination of 30 of these tablets to determine individual variations from the average weight.

Compliance—Tolerances are generally a function of average tablet weight. The greater the average tablet weight, the smaller are the weight variations permitted. Yet, the trend lacks uniformity. While the NF recognized four ranges (one range deleted, see *Footnote b*, Table I) of average tablet weights and specified corresponding tolerances, it,

[1] If it is not possible to use 100 tablets, weight determination may be made with a smaller number, but not less than 20,

as well as the B.P. and USP, now cover three such classifications. The French and Russian pharmacopeias both designate relevant parameters for only two categories—limiting tablet weights being 150 mg. and 120 mg., respectively. For uncoated compressed tablets weighing 80 mg. or more, the Nordic pharmacopeia, unlike other compendia, avoids step-wise changes in variability with respect to permitted tablet weight by using the formula: $y = 4 + 0.05x$, where y is the tolerance allowed in mg. (90% of sample), and x is the average weight in mg. In general, therefore, different tolerances are assigned to categories which cannot be readily compared, and products meeting the requirements of one national compendium need not necessarily meet those of another.

Scope—Although many products have been tested in the laboratories of the Food and Drug Directorate following the USP, NF, and B.P. procedure, few ever failed to comply. Pharmacopeial tolerances appear generally to be too wide and unappreciative of advances made during recent years in pharmaceutical manufacturing technology. Solid dosage forms of considerably smaller weight variations than those specified as pharmacopeial standards are produced with modern tableting machines.

Consider, for example, a batch of digoxin tablets, (USP requirements: assay ±8%; assay for content uniformity ±15%) formulated to weigh 100 mg. and contain 0.25 mg. of digoxin each, which had been prepared from a perfectly homogeneous and accurately dosed granulation but, manufactured under adverse conditions of compression, just met USP specifications for weight variation. A cardiac patient, maintained at a 0.25-mg. daily dose of the drug and dispensed 20 such tablets, could conceivably receive only four-fifths of the potent medication one day, and 1.5 times as much the following day (0.2 mg. and 0.3 mg., respectively).

It has been argued that weight variation is not an essential criterion of product quality—what is important is drug content. Little if any significance need be attached to differences in weight between tablets from a given batch or even from batch to batch as long as there is present in each the required amount of active ingredient.[2] Admittedly, the drug content of a tablet cannot be deduced from the weight variation test. It can only be derived from quantitative analyses of individual dosage forms. Such assays have already found recognition as pharmacopeial standards, and as their usefulness through application in pharmaceutical quality control is becoming more apparent, the need for retaining official weight variation tolerances much longer has been questioned.

As a pharmacopeial standard the test has, however, many virtues. Weight variation is easily determined. Requiring only a balance, the test provides a reliable means of gauging tablet uniformity in terms of tablet weight within a given batch as well as from batch to batch. Applied readily to all tablets, large and small, with practically the same degree of accuracy and precision, it is a dependable indicator of good pharmaceutical manufacturing practices and production technology. Uniform specifications of methodology and compliance and more realistic tolerances reflecting the precision with which tablet weight can be controlled by means of modern tableting equipment would greatly enhance its universal value in pharmaceutical quality control.

[2] It has so far not yet been established whether the physiological availability of a medication from a tablet is totally independent of tablet weight for *any* type of formulation. Lozinski, for example, has shown that dicoumarol tablets of identical formulation and drug content, but larger size, displayed markedly reduced therapeutic efficacy. [*Can. Med. Assoc. J.*, **83**, 177(1960).]

TABLE IV—WEIGHT VARIATION TOLERANCES[a] (PHARMACOPOEA NORDICA III)

Av. Tablet Wt., mg.	Tolerances Based on 30 Determinations
<80	27 tablets (90% of sample) may differ from av. wt. by ±10% and 3 tablets (10% of sample) may differ from av. wt. by ±20%[b]
>80	27 tablets (90% of sample) may differ from av. wt. by ± (4 mg. +5% of av. wt.) and 3 tablets (10% of sample) may differ from av. wt. by ± (8 mg. +10% of av. wt.)

[a] Applicable to compressed, uncoated tablets. [b] Tolerance also applicable to coated and uncoated tablets not prepared by compression, regardless of weight.

Drug Content

As a rule, pharmacopeial assays for active ingredients are based on analyses of aliquots obtained from a given number of tablets reduced to a fine powder.

USP XVII and NF XII—*Methods*—(*a*) *Composite Assays*—Ten or, in most instances, 20 tablets are required for physicochemical assays of drug content. They are finely powdered and aliquots of the triturate examined in accordance with the method of analysis specified in the corresponding monograph.

(*b*) *Single Dosage Assays (Content Uniformity)*—A representative sample consisting of 30 tablets is obtained from a given lot, and 10 of these are analyzed individually by the method of assay specified in the relevant monograph. At the analyst's discretion the degree of dilution of solutions and/or the volume of aliquots used may be adjusted so that the concentration of the drug in the final solution will be comparable to that obtained for the assay described in the corresponding monograph.

Compliance—(*a*) *Composite Assays*—Experimental results, indicative of the drug content of an aliquot from a number of tablets, are expressed in terms of the percent of labeled amount of drug claimed to be present in a single tablet. Tolerances are specified in individual monographs and vary depending on the nature of the product examined and the analytical method applied. (See under *Assays of Bulk Drugs and Compressed Tablets.*)

(*b*) *Single Dosage Assays*—Requirements which must be met are shown in Table V.

B.P. 1963—*Method*—It is essentially that adopted for composite assays by the United States Pharmacopeia, with tolerances "framed to allow for all permissible variations including that of the active ingredient itself and that due to the process of manufacturing."

TABLE V—SINGLE DOSAGE ASSAYS FOR CONTENT UNIFORMITY OF TABLETS (USP XVII, NF XII)

No. of Tablets Analyzed (Out of 30)	Requirements I	Requirements II
10	All results must be within 85–115% of av. of tolerances specified in official monograph	If one result exceeds limits specified under I, each of remaining 20 tablets must be within limits specified under I[a]

[a] A rare "flyer" will thus not cause rejection of an entire batch.

Compliance—Experimental results are expressed as previously defined. In circumstances where the required number of tablets cannot be obtained, a smaller number, but not less than five, may be assayed by the official method. To allow for sampling errors in such instances tolerances are widened progressively, as shown in Table VI.

The corrections are to be applied to tablets for which tolerances ranging from 90–110% have been specified. For limits exceeding these values, proportionately larger allowances are to be made. Reasons for extending consistently upper limits more than the corresponding lower ones are not stated.

Pharmacopée Française VIII—Monographs for tablets have not been included in this edition and generally applicable specifications for drug content and content uniformity are not described.

State Pharmacopoeia U.S.S.R. IX—Specimens are prepared by grinding one or more tablets to a fine powder. The amount of sample required for analysis, the assay procedure to be followed, and tolerances permitted are specified in official monographs. Tablets for which such monographs are not given must meet the requirements shown in Table VII.

Pharmacopoea Nordica—The examination of a specified aliquot obtained as a rule from the trituration of at least 10 tablets is required. In general, drug content may vary by not more than ±10% from label claims. The tolerances are considered to take into account variations arising from manufacture and storage as well as analytical methodology.

Canadian Food and Drugs Act and Regulations (7)—*Method*—Schedule B of the Canadian Food and Drugs Act and Regulations lists seven pharmacopeial compendia officially recognized by the Food and Drug Directorate. They include, at present, the Pharmacopoea Internationalis, the British Pharmacopoeia, the United States Pharmacopeia, the Codex Français, the Canadian Formulary, the British Pharmaceutical Codex, and the National Formulary. Methods specified in these reference texts are endorsed by the Food and Drug Directorate as valid standards of pharmaceutical quality control, unless an "official method," *i.e.*, a method of analysis or examination designated as such by the Director-General for use in the administration of the Act, is the method to be applied.

Compliance—Tolerances set forth in any of the pharmaceutical compendia cited above are accepted for products thus identified. For nonofficial drugs "put up in tablet or any other individual dosage or dispensing form other than in ampoules or vials, variations within the limits stated in the following table as determined by an acceptable method" are permitted (Table VIII).

TABLE VII—DRUG CONTENT OF TABLETS (STATE PHARMACOPOEIA U.S.S.R. IX)

Active Ingredient Per Tablet, mg.	Tolerance, %
>100	±5
<100	±10

TABLE VIII—LIMITS OF VARIABILITY FOR NONOFFICIAL SOLID ORAL DOSAGE FORMS (CANADIAN FOOD AND DRUGS ACT AND REGULATIONS)

Amt. of Drug Per Tablet gr.	Amt. of Drug Per Tablet mg.[a]	Limits, %
>5	>324	94–106
0.5 –5	32.4–324	93–107
0.02–0.5	3.24–32.4	92–108
0.01–0.02	0.65–3.24	91–109
<0.01	<0.65	90–110

[a] Equivalents not given in original table.

Two exceptions are made: (*a*) "glyceryl trinitrate shall contain not less than 85% and not more than 115% of the labelled amount," and (*b*) "if the drug consists of several ingredients, the amount of each ingredient so dispensed shall be not less than 90% and not more than 110% of the amount calculated from the label description."

Similarities and Differences—Tablet drug content and content uniformity depend on a number of processes associated with tablet manufacture, *e.g.*, compounding, mixing, drying, slugging, dispersion, compression, *etc.* Pharmacopeial standards have been established to control these processes, permit

TABLE VI—ASSAY TOLERANCES FOR TABLETS INCLUDED IN B.P. 1963 BASED ON ANALYSIS OF LESS THAN 20 SPECIMENS

Wt. of Drug in Tablet, mg.	Tablets Used for Analysis, No. 15 — Extend Limits Specified in Monographs by Following Percentages: Lower	15: Upper	10: Lower	10: Upper	5: Lower	5: Upper
<120	0.2	0.3	0.7	0.8	1.6	1.8
120–300	0.2	0.3	0.5	0.6	1.2	1.5
>300	0.1	0.2	0.2	0.4	0.8	1.0

determination of the amount of active ingredient present in a given product, and gauge the uniformity with which the drug is incorporated into individual dosage units.

Methodology—The USP and NF require the examination of a specified aliquot obtained as a rule from the trituration of 20 tablets. The B.P. accepts assay values derived from the analysis of aliquots from a smaller number of tablets as well, and endorses results obtained with as few as 5 tablets if only that many are available. The French pharmacopeia does not include monographs for solid dosage forms, and guidelines concerning general techniques and methodologies are, likewise, not described.

Assays given in the Russian pharmacopeia are not based on the examination of an aliquot from a definite number of tablets, but on direct analysis of a specified amount of sample material representing a fraction of one or several tablets. The Pharmacopoea Nordica, in general, requires the use of at least 10 tablets. The Canadian Food and Drugs Act and Regulations endorse any acceptable method, *i.e.*, any method of analysis or examination sanctioned by the Director-General for use in the administration of the Act.

It should be emphasized in this connection that different methods of analysis displaying different degrees of selectivity and sensitivity may be specified for the same preparation in different pharmacopeias. Single dosage assays have so far been adopted only by the United States Pharmacopeia[3] and the National Formulary.[4]

Compliance—Tolerances are stated in official monographs and marked variations exist between different pharmacopeial standards (see under *Assays of Bulk Drugs and Compressed Tablets*). Limits are generally a function of the weight of active ingredient claimed to be present in a single dosage unit. The greater the amount of active ingredient per tablet, the smaller the variation permitted. Unlike any other pharmacopeia, the B.P. allows for a further extension of tolerances if assays are based on less than 20 tablets. No reference is made in the USP, B.P., or NF to tolerances for products for which official monographs have not been described. The Russian pharmacopeia, on the other hand, specifies tolerances for such preparations as well. Products containing more than 100 mg. of active ingredient may vary by $\pm 5\%$ and those containing less than this amount by $\pm 10\%$ from label claims. The Canadian Food and Drugs Act and Regulations also cover nonofficial products, specifying five concentration ranges and corresponding tolerances. The classification is an unrealistic one in the light of modern technology, and efforts to revise it are now being made.

Scope—It is generally recognized that tablet weight variation does not necessarily reflect drug content variation. While tablets satisfying pharmacopeial specifications for weight variation are readily made by means of modern machines, it is most difficult to produce truly homogeneous tablet granulations and to feed solid blends continuously into the tableting machine for compaction into truly uniform dosage forms. The smaller the concentration of the active ingredient present, the more difficult it becomes to attain product uniformity. Tablets containing potent drugs, *i.e.*, tablets whose safety and efficacy demand careful control, are, therefore, particularly prone to compositional variations.

Several studies relating tablet weight and drug content have been published during recent years. They covered both practical and theoretical aspects associated with the production of solid dosage forms (8, 10), principles of mixing solids and their application to pharmacopeial standards for content uniformity in the absence of single dosage assays (11, 12), the effect of sampling and bulk mix heterogeneity on tablet variation (13), reproducibilities of assay and drug recovery from dosage forms (14), the nature and scope of sampling techniques (15), the application of automated equipment to single-tablet assays (16), and the effect of tableting technology on the relationship between tablet weight variation and percent composition (17, 18).

Relevant investigation on commercial products were carried out in the laboratories of the Canadian Food and Drug Directorate (19) and are continuing (20, 21). The following experiments may serve to illustrate some of the problems encountered during the course of these studies.

Ten tablets of hydrocortisone (5 mg.)[5] were taken at random from a bottle of 100 and analyzed in accordance with the USP procedure (tolerances allowed 90–110%). They were found to be below labeled strength (87.3%). Another analyst repeated the assay using a second lot of 10 tablets selected, likewise, at random from the same container. His results showed that the product complied (91.8%). Concerned about the discrepancy, a third analyst decided to assay 10 tablets individually. He obtained an average assay value of 100.8% on the basis of results varying from 68.4% to 151.2%. In each case, the 10 tablets used for analysis met perfectly the requirements of the weight variation test.

Because they are based on the examination of sample composites obtained from randomly selected tablets, pharmacopeial assays cannot be relied upon to provide infallible criteria for uniformity of tablet drug content. The weakness inherent in these methods is their inconsistency in relating experimental design to data utilization. They express product dosage on an individual tablet basis but are, themselves, based on sample composites of many tablets. Such analyses may not only average out minor compositional variations between tablets, as originally believed, but also mask major deviations reflecting substandard "pharmaceutical workmanship." The greater the number of tablets used for such analyses, the greater the possibility of masking variation in active ingredient due to imperfections in mixing all components during formulation, which process is considered a most critical one (9, 11, 12). On statistical grounds, the variation in drug content of an individual tablet taken from a number of tablets may

[3] Applicable to tablets of chlorpromazine hydrochloride, digoxin, ergonovine maleate, hydrocortisone, methylergonovine maleate, metyrapone, phenobarbital, prednisolone, prednisone, and prochlorperazine maleate.

[4] Applicable to tablets of amphetamine phosphate, amphetamine sulfate, betamethasone, cortisone acetate, dexamethasone, dextroamphetamine phosphate, methylprednisolone, methyltestosterone, and syrosingopine.

[5] Average weight of tablets 104.4 mg.; maximum deviation from mean 5.4 mg.

be as large as the square root of this number multiplied by the limit set for the composite assay. That is, an individual tablet taken at random from a group of 20 tablets for which drug content limits of 90–110%, *i.e.*, ±10% have been set, may deviate from the standard by as much as $\sqrt{20} \times 10 = 44.7\%$. Conversely, if all tablets should be within the range of 90–110%, *i.e.*, ±10% of label claim, the limit of variability for the composite assay based on 20 tablets should be no more than $10 \div \sqrt{20} = 2.25\%$.

Atropine sulfate tablets B.P., for example, may contain as little as 0.25 mg. of the potent anticholinergic and no less than 80 such tablets are required for the official assay. Yet, theoretically, a sample composite complying with the official B.P. standard of drug content (90–110%) may consist of individual tablets, some of which could contain as little as 10% or as much as 190% of the required amount. More pertinent information concerning the extent of tablet variation can be obtained by carrying out several composite assays and calculating standard deviations of individual tablets from the standard deviations of the composites. However, direct criteria of drug content uniformity are provided only by single-tablet assays, as described in the United States Pharmacopeia and the National Formulary, respectively (see Table V). Admittedly, such a scheme of quality control increases the time and cost of drug analysis, but it permits a more reliable appraisal of true product uniformity and its application to tablets containing potent chemotherapeutic agents should be of major concern to governmental and industrial laboratories alike.

The principle of pharmacopeial standards for monitoring intertablet dosage variation has been favorably received and accepted by the pharmaceutical industry in Canada and the United States.[6] At present, analytical methods for determining content uniformity involve spectrophotometric techniques only, and provided tablet formulations are amenable to such determinations, accurate measurements are readily made. Other equally sensitive methods are being developed in order to obtain single-tablet assays for as many products as possible. It should be in the pharmaceutical manufacturer's interest to produce only simple dosage forms which can readily be subjected to quantitative analysis.

Tablet Disintegration

Tests for gauging the disintegration of tablets under controlled conditions are described in most official compendia. Although not necessarily indicators of therapeutic efficacy, they are widely applied in pharmaceutical quality control.

Apparatus can be obtained commercially, and methodology is simple. Most pharmacopeias require that the tablet be placed in a tube (transparent plastic or glass) of precise dimensions fitted at its lower end with a wire gauze of specified mesh. The tube, suspended in a fluid kept at constant temperature, is raised and lowered at a uniform rate throughout a specified distance for a given period of time.

The tablet is considered disintegrated when, except for fragments of insoluble coating, only a soft mass having no palpable firm core remains above the gauze. The time required to reach this stage is called the disintegration time. Depending on the type of product and the pharmacopeial standard selected, a plastic disk of definite weight, shape, and size may be placed above the tablet in the tube either for the duration or throughout certain phases of the test.

Commercial units meeting official requirements are available and permit testing of as many as 6 tablets at a time.

USP XVII and NF XII—*Apparatus*—Vessel for basket rack assembly: a suitable vessel, preferably a 1-L. beaker. Temperature of medium: 37 ± 2°. Tube dimensions: length, 7.75 ± 0.25 cm.; inside diameter, 21.5 mm.; wall thickness, 2 mm. Wire mesh: nominal width of aperture 0.075 in. (1.90 mm.). Disk: material, transparent plastic, sp. gr. 1.18–1.20; thickness, 9.5 ± 0.15 mm.; diameter, 20.7 ± 0.15 mm.; perforations, five, each 2-mm. wide; notches, four having V-shaped planes. Movement: rate, 30 ± 2 c.p.m.; distance, 5–6 cm. Wire mesh position: high point, not less than 2.5 cm. below surface of fluid; low point, not less than 2.5 cm. from bottom of vessel.

Methodology and Compliance—These are summarized in Table IX.

B.P. 1963—*Apparatus*—Vessel: depth not less than 15 cm. Temperature of medium: 37 ± 2°. Tube dimensions: length, 8–10 cm.; inside diameter, 28 mm.; wall thickness, 2–3 mm.; volume, 200–250 ml. Wire mesh: nominal width of aperture 0.0661 in. (1.68 mm.). Disk: material, plastic; thickness, 2 mm.; diameter, 26 mm.; weight, 1.9–2.1 Gm. Guide ring: 27 mm. o.d. Movement: rate, 30 c.p.m. (by hand or mechanically); distance, 7.5 cm.; high point, wire gauze just breaks surface of medium; low point, upper rim of tube remains clear of medium.

Method—Five tablets are placed into the tube suspended in the required medium. The tube is raised and lowered repeatedly in a uniform manner, by hand or mechanically, through the specified distance and disintegration of the tablets observed continuously or at critical time intervals.

Compliance—(*a*) Tablets which are not enteric coated should disintegrate in water within 15 min. unless otherwise stated in the monograph.

(*b*) Tablets which are sugar coated should disintegrate in water within 1 hr.

If the tablets referred to under (*a*) or (*b*) fail to comply, the test is to be repeated on a further five tablets using the guided disk positioned directly above the tablets—and under these conditions they must meet requirements.

(*c*) Tablets which are enteric coated are tested in acid pepsin solution[7] for a period of 3 hr. (there should be no evidence of disintegration) washed rapidly by immersion in water, and thereafter placed in alkaline pancreatin solution[8] in which

[6] The authors are indebted to Dr. A. E. Slesser, Smith Kline & French Laboratories, Philadelphia, Pa., and the PMA Committee on Inter-Tablet Dosage Variation, Quality Control Section, for access to relevant reports on collaborative studies conducted in a number of industrial laboratories throughout the U. S. during the last 5 years.

[7] Composition: pepsin, 3 Gm.; hydrochloric acid, 6 ml.; water, 1,000 ml.

[8] Composition: pancreatin, 3 Gm.; sodium bicarbonate 15 Gm.; sodium tauroglycocholate, 5 Gm.; water, 1,000 ml

TABLE IX—TABLET DISINTEGRATION (USP XVII, NF XII) METHODOLOGY AND COMPLIANCE

Pretreatment	Disks	Immersion Fluid and Test Conditions	Compliance[a]
		Uncoated (6 Tablets in Test)	
None	+	Water, unless otherwise specified in official monograph	At end of time limit specified in monograph all tablets should have disintegrated. If one or two have not disintegrated, test is to be repeated on 12 additional tablets. Not less than 16 of total of 18 tablets must disintegrate completely
		For example, if disintegration time specified in monograph is 15 min., the time limit is 15 min. in water.	
		Plain Coated[b] (6 Tablets in Test)	
If desired, immerse in water at room temperature for 5 min. to wash off soluble external coating	+	Simulated gastric fluid T.S.[c] for 30 min. If disintegration incomplete at that time, simulated intestinal fluid T.S.[d] for a total period of time, including exposure to water equal to the time specified in monograph plus 30 min.	If at end of test one or two tablets have not disintegrated, repeat on 12 additional samples. Not less than 16 of total of 18 tablets must disintegrate completely
		For example, if disintegration time specified in monograph is 15 min., the time limit is either (*i*) 30 min. in simulated gastric fluid plus 15 min. in simulated intestinal fluid, or (*ii*) 5 min. in water plus 30 min. in simulated gastric fluid plus 10 min. in simulated intestinal fluid.	
		Enteric-Coated (6 Tablets in Test)	
If desired, immerse in water at room temperature for 5 min. to wash off soluble, external coating	−	Simulated gastric fluid T.S. for 1 hr., followed by	No distinct evidence of dissolution or disintegration after gastric fluid treatment
	+	Simulated intestinal fluid T.S. for 2 hr. *plus* time limit specified in individual monograph, or where only an enteric-coated tablet is recognized for only the time limit specified in monograph	If at end of test one or two tablets fail to disintegrate, repeat on 12 additional samples. Not less than 16 of total of 18 tablets should disintegrate completely
		For example, if (*i*) 60 min. is the time specified in the monograph, the time limit is 60 min. in simulated gastric fluid plus 180 min. in simulated intestinal fluid; (*ii*) an enteric-coated tablet only is recognized, and a time of 120 min. is specified, the time limit is 60 min. in simulated gastric fluid plus 120 min. in simulated intestinal fluid	
		Buccal (6 Tablets in Test)	
None	−	Water, unless stated otherwise in monograph	All tablets should have disintegrated after 4 hr.
		Sublingual (6 Tablets in Test)	
None	−	Water, unless stated otherwise in monograph	All tablets must disintegrate within time limit specified in individual monograph[e]

[a] Tablets exempted from these requirements: those exceeding 15 mm. in diameter; those used as troches; those which are to be chewed; those designed to liberate drug content gradually over a period of time (prolonged action); those designed to release the drug over two or more separate periods with a distinct time interval between such release periods (repeat action). [b] Plain coated is any tablet having a nonenteric coating. [c] Composition: sodium chloride 2 Gm., pepsin 3.2 Gm., hydrochloric acid 7.0 ml., water *ad* 1,000 ml. The pH of this solution is approximately 1.2. [d] Composition: potassium phosphate (monobasic) 6.8 Gm. in 250 ml. water, sodium hydroxide (0.2 *N*) 190 ml., water 400 ml., pancreatin 10 Gm., sodium hydroxide (0.2 *N*) to adjust pH (7.5 ± 0.1), water *ad* 1,000 ml. [e] If one or two tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely (First Supplement to USP XVII).

medium disintegration should be complete within 1 hr.

If the tablets fail to disintegrate in the alkaline pancreatin solution as required, the entire test must be repeated with a further five tablets using the guided disk during operation in the alkaline pancreatin solution. The tablets must then comply.

Canadian Food and Drugs Act and Regulations—Although apparatus, methods, and requirements for compliance are similar and in some respects even identical to those specified in the USP, there are some essential differences.

Apparatus—Its size differs considerably. The F.D.D. test is carried out in a vessel of approximately 3-L. capacity containing 2.5 L. of fluid. The USP test is carried out in a 1-L. vessel but does not specify the exact volume of fluid. A specific component of the F.D.D. apparatus is its plunger—a unit of precise dimensions consisting of a stainless steel rod separating two plastic disks. It is placed above the tablet or the slotted perforated disk riding atop the tablet in the tube.

Methodology and Compliance—These are summarized in Table X.

TABLE X—TABLET DISINTEGRATION
(CANADIAN FOOD AND DRUGS ACT AND REGULATIONS) METHODOLOGY AND COMPLIANCE (22)

Disk	Plunger	Immersion Fluid and Test Conditions		Compliance[a]
Uncoated and Plain Coated (6 Tablets in Test)				
+	+	Simulated gastric juice[b] for 30 min. and, after gentle rinse with water, simulated intestinal juice[b] until disintegration is complete	Average disintegration time of tablets should be not more than 60 min. (30-min. testing in simulated gastric juice and remainder of time in simulated intestinal juice)	If 1 of 6 tablets disintegrates in more than 75 min. (30-min. exposure to gastric juice plus more than 45-min. exposure to intestinal juice) the test must be repeated with additional 12 tablets. Average disintegration time of 18 tablets must not be more than 60 min. and not more than one tablet shall disintegrate in more than 75 min. (total time)
Enteric-Coated (6 Tablets in Test)				
−	+	Simulated gastric juice for 60 min.; there should be no distinct evidence of disintegration	Tablets should not disintegrate while tested for 60 min. in gastric juice but their average disintegration time should be not more than 60 min. during subsequent testing in simulated intestinal juice	If 1 of 6 tablets disintegrates in more than 75 min. the test must be repeated with additional 12 tablets. Average disintegration time of 18 tablets must not be more than 60 min. and not more than 1 tablet shall disintegrate in more than 75 min.
+	+	Simulated intestinal juice thereafter until disintegration is complete		
Enteric-Coated Vitamins (6 Tablets in Test)				
Same as for enteric-coated tablets			Tablets should not disintegrate while tested for 60 min. in simulated gastric juice, but average disintegration time should be not more than 30 min. in simulated intestinal juice	If 1 of 6 tablets disintegrates in more than 40 min. the test must be repeated with additional 12 tablets. Average disintegration time of 18 tablets must not be more than 30 min. and not more than 1 tablet shall disintegrate in more than 40 min.

[a] All tablets intended to be swallowed whole are subject to these requirements except those which release their medicaments at time intervals (repeat action) or in sustaining quantities over a specified period of time (prolonged action). [b] Composition identical to that specified in USP XVII (1).

Pharmacopée Française VIII (Codex Médicamentarius Gallicus)—*Apparatus*—Vessel: glass jar (vol. ~400 ml.); i.d. 7.5 cm. Temperature of medium: 37 ± 2°. Petri dish: height, 3 cm.; i.d., 5 cm. Cylindrical screen: height, 9.5 cm.; i.d., 6.5 cm.; wire mesh, nominal width of aperture 0.0787 in. (2 mm.). Movement: rate, 30 c.p.m. for 30 sec. every 5 min.; distance, 5 cm.

Method—The cylindrical screen is placed upon the Petri dish in the vessel containing 350 ml. of water kept at 37 ± 2°. Five tablets are transferred onto the wire mesh of the cylindrical sieve which, after the elapse of 5 min., is moved up and down for 30 sec. completing one cycle each 2 sec. This mode of operation is maintained throughout the duration of the test.

Compliance—(*a*) Uncoated tablets must disintegrate in water within 45 min. (*b*) Coated tablets must disintegrate in water within 2 hr. (*c*) Enteric-coated tablets should not show any evidence of disintegration in 0.6% (v/w) aqueous hydrochloric acid for 1 hr., but disintegrate within 2 hr.

Tablets exempt from these requirements include: (*a*) those intended to dissolve or disintegrate in the mouth; (*b*) those which are designed for implantation; (*c*) those not intended to dissolve in the stomach; (*d*) those designed for prolonged action; and (*e*) those designed to obtain a specific therapeutic action from the active ingredient.

State Pharmacopoeia U.S.S.R. IX—*Apparatus*—Special apparatus is not required.

Method—A tablet is placed into a 100-ml. flask, 50 ml. of water heated to 37° is added, and the flask is gently moved back and forth once or twice per second.

The tablet is considered disintegrated when it has dissolved or been reduced to a powder, small fragments, or loose mass. At least three such tests must be carried out.

Compliance—(*a*) Uncoated tablets must disinte-

grate within 10 min. (*b*) Coated tablets must disintegrate within 30 min. All tablets should be tested annually.

Pharmacopoea Nordica III—*Apparatus*—No special apparatus is required.

Method and Compliance—Three tablets are placed into a conical flask containing 30 ml. of distilled water warmed to 36–40°. The flask is shaken gently while the tablets are being observed for evidence of softening, dissolution, or disintegration. All three tablets must dissolve, become a soft, palpable mass, or disintegrate within 10 min. Coated tablets are subject to the same procedure, but their disintegration time may take 1 hr.

Enteric-coated tablets are tested in 30 ml. of a phosphate buffer solution (pH 7.4) instead of in 30 ml. of distilled water. They must disintegrate in less than 1 hr.

Glyceryl trinitrate tablets must disintegrate in less than 1 min.

Similarities and Differences—Comparison of apparatus, methodology, and tolerances embodied in pharmacopeial standards for the disintegration of tablets reveals that there exist far more differences than similarities. Experimental data obtained in accordance with specifications of one pharmacopeia do not, therefore, indicate compliance with specifications of some other pharmacopeia. The most common requirement is that tests be performed at 37 ± 2°.

A few obvious differences in experimental design and data interpretation may be cited to illustrate the extent of interpharmacopeial discordance. Such a critical parameter of disintegration as the width of the aperture of the wire mesh is specified to be 1.9 mm. in the USP, NF, and the Canadian Food and Drugs Act and Regulations, 2 mm. in the French pharmacopeia, and 1.68 mm. in the B.P. No screen is used in the apparatus specified by the Russian and Nordic pharmacopeias, respectively.

The tubes and tablets are raised and lowered at a rate of about 30 c.p.m. through a distance of 5–6 cm. in accordance with specifications of the USP NF, and the Canadian Food and Drugs Act and Regulations, throughout a distance of 7.5 cm. in accordance with specifications of the B.P. (either by hand or mechanically), and throughout a distance of 5 cm. in accordance with specifications of the French pharmacopeia. No such specifications are given in the Russian or Nordic pharmacopeias. Most compendia require that movement of the tubes be a continuous one—yet the French pharmacopeia specifies that it be a discontinuous operation.

The USP, NF, and the Canadian Food and Drugs Act and Regulations specify that six tablets be tested simultaneously, each in a separate tube, whereas the B.P. requires that five tablets be tested collectively in a single tube. The French pharmacopeia specifies that five tablets be tested together in a moving cylindrical screen, and the Russian pharmacopeia requires that three tablets be examined individually. The Pharmacopoea Nordica specifies that three tablets be tested collectively.

The test medium is, moreover, of variable composition. The British Pharmacopoeia uses water as the immersion fluid for tablets which are not enteric coated, and an acid pepsin plus alkaline pancreatin solution for tablets which are enteric coated. The French pharmacopeia uses water as the immersion fluid for uncoated and plain coated tablets, but dilute hydrochloric acid as the immersion fluid for enteric-coated tablets. The Russian pharmacopeia uses water as the test medium for all types of tablets. Simulated gastric and/or intestinal fluid, different in composition from that specified in the B.P., is required for tests carried out in accordance with methodologies described in the USP, NF, and the Canadian Food and Drugs Act and Regulations. The volumes of the test media and the exposure times of different tablets to these media vary also appreciably.

Differences in apparatus and methodology are reflected in marked differences of compliance as well. The number of tablets required for examination varies considerably (from 3 to 18). The USP, NF, and the Canadian Food and Drugs Act and Regulations allow for distinct deviations of 1 or 2 tablets out of 18 from an average value, whereas the British, French, Nordic, and Russian pharmacopeias require that all tablets tested comply with specifications. Tolerances for buccal and sublingual tablets are given in the USP and NF; the Pharmacopoea Nordica indicates tolerances specifically for only glyceryl trinitrate tablets in this category. Limits, based on data of physiological availability, for the disintegration time of tablets containing vitamins are specified in the Canadian Food and Drugs Act and Regulations.

Scope—The test was developed to assess batch-to-batch disintegration reproducibility of tablet formulations made by definite processes from specific ingredients and subjected to appropriate in-process manufacturing controls. It was also considered to provide information concerning the relative ease with which tablet formulations break up under controlled experimental conditions simulating *in vivo* environment. For over a quarter of a century it has been recognized as a pharmacopeial standard (23).

Far less appreciated than its value in pharmaceutical quality control, however, remained its value in the assessment of drug response. Originally, a tablet disintegrating rapidly was thought to be clinically more effective than one disintegrating over a long period of time and the process of disintegration regarded not only as a tool for ensuring product compliance but also as a means of gauging the physiological availability of medicinal preparations. It is not intended to trace the historical development of the test or present a detailed account of its applications, for several excellent reviews have been written on this subject (24–31). The importance of the disintegration test cannot be denied. Specifying simple sets of conditions under which different types of tablets are examined, it represents a physical method of pharmaceutical quality control which allows a drug manufacturer or distributor to check his products for uniformity of performance from batch to batch without recourse to complex and expensive apparatus or personnel requiring advanced academic training. In this respect the test fulfills and will, no doubt, continue to fulfill a useful function.

As a pharmacopeial standard it postulates minimum and maximum allowable tolerances for tablet formulations most frequently used, but for a fuller appreciation of the significance of the data

it provides, other closely related and important criteria must, likewise, be taken into account. It has been pointed out that it would not be difficult to formulate a compressed tablet meeting pharmacopeial requirements for disintegration time from granules of cement passing through the gastrointestinal tract without change (25). Experiments conducted in these laboratories as well as elsewhere have shown that tablets claimed to contain potent chemotherapeutic agents do in fact so behave because of improper or faulty formulation (32). Such observations emphasize that tablet disintegration is indeed an important criterion of product quality, for no therapeutic effect can be expected from a tablet that fails to disintegrate.

The role of the disintegration test in the assessment of drug response cannot, however, be summarized that categorically. Original studies concerning this aspect of tablet disintegration were first carried out in the research laboratories of the Canadian Food and Drug Directorate under the direction of J. A. Campbell. They showed that tablet disintegration *in vitro* had to occur within a given period of time to ensure physiological availability of drugs administered as compressed or coated solid dosage forms. Data obtained from *in vitro* disintegration tests were correlated with results from *in vivo* measurements based on urinary excretion and/or blood level determinations and meaningful tolerances were thus established. Official specifications embodied in the Canadian Food and Drugs Act and Regulations—made mandatory in 1957—are largely based on this work (33–46). They motivated Canadian manufacturers to modify many of their tablet formulations for the purpose of producing pharmaceutical dosage forms displaying improved physiological availability and therapeutic efficacy.

Related studies published since are in accord with the findings that were made and confirm that results of *in vitro* disintegration tests can be considered as criteria of physiological availability only to the extent they correlate with quantitative *in vivo* data obtained on humans. *In vitro* results alone cannot be relied upon as indices of drug availability. Only through correlation with data reflecting *in vivo* response can they be utilized as parameters of biopharmaceutical quality control and monitors for product reproducibility. This shortcoming of the test is, unfortunately, not referred to in pharmacopeial texts.

What is to be more generally realized is that contrary to concepts originally conceived and accepted without question or scientific inquiry, tablets disintegrating quickly need not necessarily display enhanced clinical effectiveness and tablets disintegrating slowly need not necessarily display reduced therapeutic activity. Lack of knowledge of mechanisms underlying drug absorption and inability to duplicate precisely physiological conditions *in vitro* stand in the way of translating reliably data obtained *via* physical tests to drug response. It has been demonstrated, for instance, that agitation of a tablet during the USP disintegration test is far more intense (possibly more than three times as great) than during its residence time in the human stomach or during intestinal transit (29, 31). In this regard alone, most disintegration tests must be considered physiologically unrealistic. Only through independent and objective appraisal of physiological availability can the therapeutic efficacy of tablet formulations be ascertained. This task should be a most important consideration of the pharmaceutical manufacturer in the development of new drug products.

Although some pharmacopeial commissions have made considerable revisions for tablet disintegration times and specified stricter limits for many dosage forms in succeeding editions of compendia (see Tables XI–XIII, summarizing data extracted from the United States Pharmacopeia and the National Formulary) these standards still provide liberal tolerances for tablet disintegration time, and at the present state of the art the production of solid oral dosage forms meeting official specifications is not likely to impose any hardships on the pharmaceutical industry.

The need to establish—in Canada—tolerances providing for disintegration times longer than those stated in the Food and Drugs Act and Regulations (7) has so far not been demonstrated. Nevertheless, the Food and Drug Directorate allows for the production and sale of compressed as well as enteric-coated tablets which fail to meet official specifications for disintegration time provided experimental evidence demonstrates that all therapeutically active ingredients are physiologically available as claimed. It should be in the manufacturer's interest to keep tablet composition and processing as simple as possible in order to avoid unpredictable effects not only in disintegration time but on the therapeutic efficacy of the product as well (47–52).

Difficulties in establishing accurate methods of analysis should always be regarded as early warning signals for a reassessment of biological response. Concerned about potential health hazards resulting from formulation changes, the Canadian Food and Drug Directorate requires that all manufacturers duly inform the Director-General of any modification in composition and/or processing for any medication accepted on the basis of a preclinical or new drug submission (7, 53, 54). In this connection, the World Health Organization recently drew to the attention of member states pertinent observations concerning the composition of a chloramphenicol palmitate formulation which had failed to produce satisfactory clinical response (55). Dissemination of such information under the auspices of WHO should be encouraged.

During the last few years the inherent weaknesses of the disintegration test have come to light and into sharper focus through the development of and correlation with a companion test appraising drug dissolution. Although a prerequisite to drug response, tablet disintegration is but one of a number of phenomena preceding drug absorption. The rate at which a drug diffuses from a tablet matrix into the surrounding fluids has been shown to represent an even more important process. Studies correlating disintegration time, dissolution rate, and physiological availability have supported this concept (29–31).

Recent investigations in the authors' laboratories have shown that wide variations in rate of drug dissolution may occur not only between products of different manufacturers (different brands containing the same active ingredient), but also between different lots of a given product supplied by the

TABLE XI—TABLET DISINTEGRATION TIMES SPECIFIED IN THE UNITED STATES PHARMACOPEIA

Tablet	Disintegration Time, min. USP XVI (1960)	USP XVII (1965)		Reduction, min.
Acetozolamide	30	30		...
Aminophylline	30	30		...
Aminosalicylic acid	30	30		...
Ammonium chloride (enteric-coated)	150		120	30
Amobarbital	30	30		...
Ascorbic acid	30	30		...
Aspirin (listed as acetylsalicylic acid USP XVI)	5	5		...
Atropine sulfate	30	30		...
Bethanechol chloride	30	30		...
Bishydroxycoumarin	15	15		...
Busulfan	30	30		...
Calcium gluconate	60	30		30
Calcium pantothenate	30	30		...
Carbarsone	30	30		...
Chlorambucil		30		...
Chlorcyclizine hydrochloride	30	30		...
Chloroquine phosphate	60	30		30
Chlorpheniramine maleate	30	30		...
Chlorpromazine hydrochloride	30	30		...
Chlorpropamide		30		...
Codeine phosphate	30	30		...
Colchicine	15	15		...
Cyclizine hydrochloride	10	10		...
Dapsone		30		
Decavitamin	120		60	60
Desoxycorticosterone acetate (buccal)				
Dextroamphetamine sulfate	30	30		...
Dichlorphenamide		30		
Diethylcarbamazine citrate	30	30		...
Diethylstilbestrol	30	30		...
Digitalis	60	30		30
Digitoxin	30	30		...
Digoxin	30	30		...
Diiodohydroxyquin	60		60	...
Dimenhydrinate	30	15		15
Ergonovine maleate	30	30		...
Ergotamine tartrate	30	30		...
Erythromycin	60		60	...
Ethinyl estradiol	30	30		...
Ferrous fumarate		30		...
Ferrous sulfate	60	30		30
Folic acid	30	30		...
Griseofulvin		30		
Guanethidine sulfate		30		
Hydrochlorothiazide		30		
Hydrocortisone	30	30		...
Hydroxychloroquine sulfate	60	30		30
Iopanoic acid	30	30		...
Isoniazid	30	30		...
Isoproterenol hydrochloride (sublingual)		3		
Lucanthone hydrochloride		30		
Magnesium trisilicate		30		
Mecamylamine hydrochloride	30	30		...
Meclizine hydrochloride	30	30		...
Medroxyprogesterone acetate		30		
Menadione	30	30		...
Mercaptopurine	30	30		...
Methadone hydrochloride	30	30		...
Methamphetamine hydrochloride[a]		30		
Methazolamide		30		
Methenamine mandelate	30	30		...
Methimazole	30	30		...
Methylergonovine maleate[a]		30		
Morphine sulfate	30	30		...
Neomycin sulfate	60		60	...
Neostigmine bromide	30	30		...
Niacinamide	30	30		...
Nitrofurantoin	30	30		...
Nitroglycerin (sublingual)		2		
Nystatin (oral)	120		120	...
Nystatin (intravaginal)	60		60	...
Phenacetin		30		
Phenobarbital	30	30		...
Phthalylsulfathiazole	30	30		...
Phytonadione	30	30		...
Piperazine citrate	30	30		...
Potassium chloride	30	30		...
Potassium penicillin G (gastric)			60	
Potassium permanganate				
Potassium phenoxymethyl penicillin (gastric)	60		60	...
Prednisolone	30	30		...
Prednisone	30	30		...
Primaquine phosphate	30	30		...
Primidone	30	30		...
Probenecid	30		60	
Prochlorperazine maleate	30	30		...
Promethazine hydrochloride	30	30		...
Propantheline bromide	30	30		...
Propylthiouracil	30	30		...
Pyridostigmine bromide	30	30		...
Pyridoxine hydrochloride	30	30		...
Pyrimethamine	30	30		...
Pyrvinium pamoate		30		
Quinacrine hydrochloride	30	30		...
Quinidine sulfate	30	30		...
Reserpine	60	30		30
Riboflavin	30	30		...
Sodium aminosalicylate	30	30		...
Sodium bicarbonate (gastric)	30	30		...
Sodium chloride	30	30		...
Sodium levothyroxine	30	30		...
Sodium liothyronine	30	30		...
Sodium salicylate	30	30		...
Sodium sulfoxone (enteric-coated)	150		60	90
Sodium warfarin		30		
Spironolactone		30		
Succinylsulfathiazole		30		
Sulfadiazine	30	30		...
Sulfamethoxypyridazine	30	30		...
Sulfapyridine	30	30		...
Sulfisoxazole	60	30		30
Thiamine hydrochloride	30	30		...
Thyroid	30	30		...
Tolbutamide	30	30		...
Trihexyphenidyl hydrochloride	30	30		...
Tripelennamine hydrochloride	30	30		...
Trisulfapyrimidines	30	30		...

[a] Transferred from NF XI without change in disintegration time.

same manufacturer (56, 57). Related studies have illustrated the effects of dosage form variation on the physiological availability of drugs (58, 59).

So far, pharmacopeial standards for tablet dissolution have not been formulated. The need for such standards will grow since tablet dissolution is of more fundamental importance than tablet disintegration for determining drug availability. The disintegration test merely measures the time required for a tablet to break up into granules smaller than a given size, but it tells nothing about how rapidly the drug is released from these granules into the substrate. Dissolution, on the other hand, has been shown to control the rate of build-up of many drugs in the blood stream and has been correlated more accurately than disintegration time with therapeutic efficacy.

Undoubtedly, dual tests comprising coordinated *in vivo* and *in vitro* evaluation of drug products are becoming the most critical parameters of

TABLE XII—TABLET DISINTEGRATION TIMES SPECIFIED IN THE NATIONAL FORMULARY

Tablet	Disintegration Time, min. NF XI (1960)	USP XVI (1960)	NF XII (1965)		Reduction, min.
Acetaminophen	30		30		...
Aluminum hydroxide gel		30	30		...
Aminobenzoic acid			30		
Amodiaquine hydrochloride		30	30		...
Amphetamine phosphate	30		15		15
Amphetamine sulfate	30		15		15
Anileridine hydrochloride			25		
Apomorphine hydrochloride	30		15		15
Arecoline hydrobromide	30		30		...
Aspirin, phenacetin, and caffeine	15		10		5
Azacyclonol hydrochloride	30		20		10
Benzestrol	30		15		15
Benztropine mesylate	15		15		...
Betamethasone			30		
Bismuth subnitrate	60			60	...
Three bromides (NH_4Br, KBr, and NaBr)	30		30		...
Brompheniramine maleate			30		
Citrated caffeine	30		30		...
Calcium aminosalicylate	120		30		90
Calcium carbonate (gastric)	30		30		...
Calcium cyclamate and calcium saccharin					
Calcium cyclobarbital			30		
Calcium lactate	60		30		30
Calomel			30		
Carbinoxamine maleate	30		15		15
Cascara	90			60	30
Chlormerodrin			30		
Chlorothen citrate	30		30		...
Chlorothiazide		30	30		...
Choline dihydrogen citrate	60		30		30
Cocaine hydrochloride	30		15		15
Codeine sulfate	30		15		15
Cortisone acetate		30	30		...
Cyclophosphamide			30		
Cycrimine hydrochloride	30		30		...
Dehydrocholic acid		30	30		...
Dexamethasone			30		
Dexchlorpheniramine maleate			15		
Dextroamphetamine phosphate	30		15		15
Dextromethorphan hydrobromide	120		30		90
Dienestrol		30	30		...
Diethylstilbestrol dipropionate	30		30		...
Dihydroxyaluminum aminoacetate	60		30		30
Diphemanil methylsulfate	15		10		5
Diphenadione	30		30		...
Doxylamine succinate		30	30		...
Ephedrine hydrochloride	60		30		30
Ephedrine sulfate	30		30		...
Estradiol	120		15		105
Ethinamate	30		30		...
Ethisterone		30	30		...
Ethoheptazine citrate			15		
Ethyl biscoumacetate			15		
Ferrous gluconate		60		60	...
Glutethimide	30		30		...
Glycobiarsol		30	30		...
Halazone	15		10		5
Hexavitamin	120		30		90
Homatropine methylbromide		30	30		...
Hydralazine hydrochloride	30		30		...
Hydrocodone bitartrate			30		
Hydromorphone hydrochloride			30		
Hydroxyzine hydrochloride	30		30		...
Imipramine hydrochloride (gastric)			30		
Iodoalphionic acid	60			15	45
Iodochlorhydroxyquin		30	30		...
Lanatoside C	60			15	45
Levorphanol tartrate	45		30		15
Magnesium hydroxide (gastric)	30		30		...
Menadiol sodium diphosphate		60		60	...
Meperidine hydrochloride		30	30		...
Mephenesin	30			15	15
Mephobarbital		30	30		...
Meprobamate		30	30		...
Mercurophylline	60		30		30
Mercury bichloride large poison				15	
Methacholine bromide	30		30		...
Methantheline bromide	30			15	15
Methapyrilene hydrochloride	30		30		...
Metharbital	15			10	5
Methenamine	30		30		...
Methenamine and sodium biphosphate	30		30		...
Methionine (gastric)	30		30		...
Methscopolamine bromide			30		
Methylphenidate hydrochloride			30		
Methylprednisolone			30		
Methyltestosterone		30	30		...
Methylthiouracil		30	30		...
Methyprylon	45		30		15
Morphine and atropine sulfates	30			15	15
Niacin			30		
Nylidrin hydrochloride	30		30		...
Papaverine hydrochloride		30	30		...
Penthienate bromide	30		30		...
Phenindamine tartrate		60		60	...
Pheniramine maleate	60			15	45
Phenmetrazine hydrochloride				10	
Phenolphthalein	60		30		30
Phenoxymethyl penicillin					
Phentolamine hydrochloride	60			60	...
Phenylbutazone				45	
Phthalylsulfacetamide	60			15	45
Pipradrol hydrochloride	30		30		...
Polymyxin B sulfate		30			
Potassium phenethicillin					
Potassium warfarin			30		
Progesterone (buccal)					
Promazine hydrochloride			30		
Pyrilamine maleate		30	30		...
Pyrrobutamine phosphate			30		
Quinine sulfate	60		30		30
Rauwolfia serpentina	60		30		30
Rescinnamine	30		30		...
Salicylamide	30		30		...
Scopolamine hydrobromide	30		30		...
Sodium butabarbital				15	
Sodium carboxymethylcellulose		120		120	...
Sodium chloride and dextrose	60		30		30

(*Continued on next page.*)

TABLE XII—(*Continued.*)

Tablet	Disintegration Time, min. NF XI (1960)	USP XVI (1960)	NF XII (1965)	Reduction, min.
Sodium cyclamate and sodium saccharin				
Sodium pentobarbital			30	
Sodium phenobarbital			30	
Sodium saccharin				
Sulfacetamide	60		15	45
Sulfacetamide, sulfadiazine, and sulfamerazine	60		30	30
Sulfadiazine and sulfamerazine	60		30	30
Sulfadimethoxine			30	
Sulfamerazine	60		30	30
Sulfamethizole	60		30	30
Syrosingopine			30	
Tetracycline hydrochloride	30			
Thenyldiamine hydrochloride	30		30	...
Theophylline	60		30	30
Theophylline sodium acetate	60		30	30
Theophylline sodium glycinate	60		30	30
Thonzylamine hydrochloride	30		30	...
Tridihexethyl chloride (gastric)	30		30	...
Trimethadione	60		30	30
Urethan		30	30	...
Dried yeast	120		60	60

TABLE XIII—COMPARISON OF DISINTEGRATION TIME DATA GIVEN IN USP XVII AND NF XII

	USP XVII	NF XII
Total number of tablets covered in monographs	117	139[a]
Tablets for which disintegration time was reduced	11 (9.4%)	36 (33.1%)
Tablets for which disintegration time was reduced by 50%	9 (7.7%)	29 (20.9%)
Tablets for which disintegration time was reduced by more than 50%	1 (0.8%)	9 (6.5%)
Tablets for which disintegration time was reduced by less than 50%	1 (0.8%)	8 (5.8%)
Tablets for which disintegration time was increased	1[b]	...
Tablets required to disintegrate in 30 min.	97 (82.9%)	91 (65.5%)
Tablets required to disintegrate in less than 30 min.	7 (6.0%)	30 (21.6%)
Tablets (nonenteric coated) permitted to disintegrate in more than 30 min.	9 (7.7%)	9 (6.5%)
Enteric-coated tablets for which disintegration times are specified	2[c]	...
Tablets for which disintegration times are not given in monographs	1	7 (5.0%)[d]

[a] Includes 26 preparations deleted from USP XVI. Disintegration times for 24 of these were left unchanged. [b] Probenecid. [c] Ammonium chloride and sodium sulfoxone. [d] Tablets of calcium cyclamate and calcium saccharin, phenoxymethyl penicillin, polymyxin B sulfate, potassium phenethicillin, sodium cyclamate and sodium saccharin, sodium saccharin, and tetracycline hydrochloride. Disintegration times were specified, however, for tablets of polymyxin B sulfate and sodium saccharin in USP XVI and for tablets of tetracycline hydrochloride in NF XI.

pharmaceutical quality control. Physiological availability data obtained through properly designed objective studies on humans together with *in vitro* disintegration and dissolution parameters reflecting the clinical performance of a dosage form are clearly emerging as prerequisites in the manufacture of drug products justifiably claimed to be safe and therapeutically effective. Any change in product formulation made subsequent to such standardization requires that both sets of data be reassembled so that adequately calibrated *in vitro* data alone may be utilized with confidence as monitors of quality control, gauging not only physicochemical variability within and between lots, but allowing also for interpretation of product safety and efficacy.

For certain dosage forms physiological availability data are already legal requirements. It is mandatory, for example, that in accordance with regulations of the Canadian Food and Drugs Act and Regulations "the manufacturer of a drug in oral dosage form represented as releasing the drug at timed intervals or in sustaining quantities over a period of time shall (a) conduct such investigations, using an acceptable method, as may be necessary to demonstrate that the drug is released

TABLE XIV—SPECIFICATIONS FOR BULK DRUGS OF THE UNITED STATES PHARMACOPEIA XVII

Assay Limits, %	No. of Compd.	Assay Limits, %	No. of Compd.
73–81	1	97.5–100.5	4
88–100.5	1	97.5–101	1
		97.5–102	1
90–100.5	2	97.5–102.5	1
93.5–101.5	1	98–100.5	25
95–100.5	5	98–101	14
95–101	1	98–101.5	7
95–103	1	98–102	17
95–105	7	98.5–100.5	14
96–100.5	5	98.5–101	7
96–101	1	98.5–102	2
96–104	1		
96.5–101	1	99–99.5	1
		99–100.5	16
97–100.5	6	99–101	8
97–101	1		
97–101.5	4	99.5–100.5	2
97–102	10	99.5–101	1
97–103	13	99.5–102.5	1

and is available as represented, and (b) on request submit the record of such investigations to the Director" (*Reference* 7, Section C.01.012).

More and more companies, realizing the need for such studies to ensure the safety and clinical effectiveness of a pharmaceutical formulation, are submitting to the Director-General on their own accord experimental evidence documenting the physiological availability of new drugs as defined in Section C.08.001 of the Food and Drugs Act and Regulations (7). It is merely a question of time until such data will become official requirements.

Great strides are being made in the field of biopharmaceutics and it is essential that they be evaluated periodically in order to determine whether pharmacopeial tests and specifications for drug efficacy can be established that are more meaningful and informative than those upon which we must rely at the present time.

ASSAYS OF BULK DRUGS AND COMPRESSED TABLETS

Bulk Drugs—Assay limits for bulk drugs[9] specified in the United States Pharmacopeia, the National Formulary, and the British Pharmacopoeia are summarized in Tables XIV–XVI. As a rule, limits are given in the form of a statement defining that the drug contains not less than a certain percentage and not more than a certain percentage of the chemical compound specified in the monograph. For many products only lower limits are given. In each such instance, the upper limit is considered be 100.5%.

The distribution of ranges most frequently specified in USP XVII, NF XII, and B.P. 1963 is shown in Table XVII. For all three pharmacopeias, the most popular range extends from 98–100.5%. The second most popular range specified in the USP XVII and NF XII is 98–102% (9.3 and 14.6% of the compounds listed, respectively), while that of the B.P. 1963 extends from 99–100.5% (17.5% of compounds listed).

The Pharmacopoeia of the U.S.S.R. (English edition) designates lower limits for all drugs, but specifies upper limits for only a few.[10] For compliance, 97 drugs out of 150 (64.7%) must assay 99–101%, 34 must assay 98–102%, 7 must assay 97–103%, and 3 must assay 95–105%. These requirements are more stringent than those of the USP, NF, or B.P.

Assay limits specified for bulk drugs included in both the USP and B.P., or B.P. and NF, are shown in Table XVIII. Differences in either upper or lower limits of at least 1% were considered prerequisites for entry of compounds into this table. The data recorded illustrate the magnitude of interpharmacopeial drug content variations.

Thus, phenylephrine hydrochloride B.P. assays 98.5–100.5%, whereas phenylephrine hydrochloride USP may assay 97.5–102.5%. Methylprednisolone NF XII assays from 97–103%, but methylpredni-

TABLE XV—SPECIFICATIONS FOR BULK DRUGS OF THE NATIONAL FORMULARY XII

Assay Limits, %	No. of Compd.	Assay Limits, %	No. of Compd.
91.5–100.5	1	98–100.5	45
		98–101	12
93–107	1	98–101.5	1
		98–102	26
94–100.5	2		
94–106	1	98.2–100.5	1
		98.2–100.7	1
95–100.5	6		
95–102	1		
95–103	3	98.5–100.5	13
		98.5–101	3
96–100.5	4	97.5–101.5	2
96–102	1		
96–104	2	98.8–100.7	1
96.5–100.5	1	99–100.5	14
		99–101	5
97–100.5	7	99–101.8	1
97–101	4	99–102	1
97–102	4	95.5–101	1
97–103	11		
97.5–102.5	1	99.7–100.5	1

TABLE XVI—SPECIFICATIONS FOR BULK DRUGS OF THE BRITISH PHARMACOPOEIA 1963

Assay Limits, %	No. of Compd.	Assay Limits, %	No. of Compd.
87.5–100.5	1	98–100.5	54
		98–101	17
90–100.5	3	98–101.5	4
94–100.5	2	98–102	13
		98–104	1
95–100.5	11		
95–101	1	98.5–100.5	30
95–105	1	98.5–101	9
		98.5–101.5	4
96–100.5	3	98.5–102	1
96–101	1	98.5–102.5	3
96–104	13		
97–100.5	6	99–100.5	50
97–101	1	99–100	18
97–101.5	1	99–101.5	4
97–102	3	99–102	1
97–103	14	99–103	1
97.5–100.5	4	99.5–100.5	6
97.5–101.5	1	99.5–101	1
97.5–102	1	99.5–104.5	1

TABLE XVII—FREQUENCY OF SPECIFIC ASSAY LIMITS FOR BULK DRUGS OF THE USP XVII, NF XII, AND B.P. 1963

	Compd., %		
Drug Content, %	USP XVII	NF XII	B.P. 1963
98–100.5	13.7	25.3	18.9
98.5–100.5	7.7	7.3	10.5
98–102	9.3	14.6	4.6
99–100.5	8.7	7.9	17.5
99–101	4.4	2.8	6.3
98–101	7.7	6.7	6.0

[9] Antibiotics, inorganics, and multicomponent drugs, *e.g.*, aminophylline (theophylline + ethylenediamine) are not included in this survey.

[10] The authors are indebted to Dr. A. N. Klimov, Division of Biology and Pharmacology, World Health Organization, Geneva, Switzerland, for advising that in the absence of specifications for complete tolerance ranges, upper limits of 100.5% apply.

TABLE XVIII—ASSAY LIMITS FOR BULK DRUGS INCLUDED IN THE UNITED STATES PHARMACOPEIA, NATIONAL FORMULARY, AND BRITISH PHARMACOPOEIA

Compd.	USP XVII	NF XII	B.P. 1963
Acetazolamide	98–102		99.0–100.5
Amodiaquine hydrochloride		97–102	98.0–101.5
Amphetamine sulfate		98–100.5	99.0–100.5
Benzocaine		98–101	99.0–100.5
Calcium lactate		98–101	97.0–103.0
Chloral hydrate	99.5–102.5		99.0–100.5
Chloroquine phosphate	98–102		98.0–100.5
Chlorothiazide		97–100.5	98.0–100.5
Chlorpromazine hydrochloride	98–101.5		99.0–101.0
Chlorpropamide	97–103		99.0–101.0
Codeine phosphate	98–101.5		98.0–100.5
Cortisone acetate		97–103	96.0–104.0
Desoxycorticosterone acetate	97–103		96.0–104.0
Dextroamphetamine sulfate	98–100.5		99.0–100.5
Dextromethorphan hydrobromide		98–100.5	99.0–100.5
Dichlorphenamide	97–100.5		98.0–100.5
Dienestrol		98–100.5	98.5–101.5
Diethylstilbestrol	97–100.5		98.5–101.5
Diiodohydroxyquin	96–100.5		97.0–100.5
Epinephrine	97–100.5		99.0–100.5
Epinephrine bitartrate	97–102		99.0–100.5
Ergonovine maleate	98–101		95.0–100.5
Folic acid	98–102		95.0–100.5
Glutethimide		98–100.5	99.0–100.5
Guanethidine sulfate	95–105		99.0–101.0
Hydralazine hydrochloride		98–100.5	98.0–102.0
Hydrochlorothiazide	97–101.5		98.0–100.5
Hydrocortisone	97–103		96.0–104.0
Hydrocortisone acetate	97–103		96.0–104.0
Hydrocortisone sodium succinate	97–103		96.0–104.0
Hydroxychloroquine sulfate	98–102		98.0–100.5
Imipramine hydrochloride		98–102	98.0–100.5
Iopanoic acid	97–101		98.0–101.0
Levarterenol bitartrate	97–102		99.0–101.0
Meclizine hydrochloride	97–100.5		98.0–100.5
Meperidine hydrochloride		98–101	99.0–100.5
Mercaptopurine	97–102		98.0–100.5
Methylene blue	98.5–100.5		96.0–101.0
Methylergonovine maleate	97–103		95.0–105.0
Methylprednisolone		97–103	96.0–104.0
Methylthiouracil		97–100.5	98.0–100.5
Neostigmine bromide	98–102		98.5–100.5
Neostigmine methylsulfate	98–102		98.5–100.5
Phenindamine tartrate		98–101.5	98.5–100.5
Phenolsulfonphthalein	95–105		94.0–100.5
Phentolamine hydrochloride		98–100.5	99.0–100.5
Phentolamine mesylate	98–102		99.0–100.5
Phenylbutazone		98–100.5	99.0–100.5
Phenylephrine hydrochloride	97.5–102.5		98.5–100.5
Prednisolone	97–103		96.0–104.0
Prednisolone acetate	97–103		96.0–104.0
Prednisone	97–103		96.0–104.0
Primaquine phosphate	98–102		97.5–100.5
Primidone	98–102		99.0–100.5
Promethazine hydrochloride	97.0–101.5		98.5–102.0
Quinidine sulfate	98–100.5		99.0–101.5
Reserpine	96–101		97.0–101.5
Riboflavin	98–102		97.0–102.0
Sodium aminosalicylate	98–101		99.0–101.0
Sodium indigotindisulfonate	96–100.5		90–100.5
Stibophen	98.5–102		98.5–101.0
Thiamine hydrochloride	98–102		98.5–100.5
Thimerosal		97–101	98.0–100.5
Tolbutamide	98–101		99.0–101.0
Undecylenic acid		95–100.5	96.0–100.5

solone B.P. may assay 96–104%. Ergonovine maleate USP must contain 98–101% of the alkaloid, but ergometrine maleate B.P. (ergonovine maleate) may assay 95–100.5%. Chlorpropamide assaying 97–103% meets USP specifications, but fails to meet B.P. requirements (99–101%).

Tolerance ranges for some drugs may differ by as much as 8%, *e.g.*, guanethidine sulfate B.P. assays 99–101%; guanethidine sulfate USP assays 95–105%. The following ranges apply to promethazine hydrochloride: USP 97.0–101.5%, B.P. 98.5–102.0%, State Pharmacopoeia of the U.S.S.R. (diprazine) 99.5–101.0%.

Diethylstilbestrol assaying not less than 97% meets USP specifications, samples assaying not less than 98.5% comply with B.P. requirements, and products assaying not less than 99.0% will meet specifications of the State Pharmacopoeia of the U.S.S.R. Sodium suramin (B.P. and USP) must contain not less than 97.5% (volumetric analysis) of the antitrypanosomal drug, but may assay 92.5% (gravimetric analysis) or 93.5% (volumetric analysis) to meet specifications of the State Pharmacopoeia of the U.S.S.R. (naganin).

It should be pointed out that bulk drugs are also covered in pharmacopeial monographs lacking methods of assay, *e.g.*, progesterone NF XII,[11] oestradiol B.P. 1963, cortisone acetate State Pharmacopoeia of the U.S.S.R.,[12] colchicine USP. Yet quantitative methods of analysis and tolerances may be specified for pharmaceutical dosage forms containing such drugs as active ingredients, *e.g.*, progesterone tablets NF XII (90–110%), oestradiol benzoate injection B.P. 1963 (90–110%), cortisone acetate tablets, State Pharmacopoeia of the U.S.S.R. (90–110%), colchicine tablets USP (90–110%). It is essential that reliable assays for bulk drugs used in the manufacture of such products be developed and tested collaboratively in pharmaceutical quality control laboratories.

Variations between assay limits for bulk drugs common to different pharmacopeias are, in part at least, due to differences in methodology. Thus ergonovine maleate USP is determined titrimetrically, but ergometrine maleate B.P. (ergonovine maleate) is assayed colorimetrically. Imipramine B.P. is determined by Kjeldahl analysis, imipramine NF by nonaqueous titration. Acetazolamide USP is assayed by infrared, acetazolamide B.P. by ultraviolet spectrophotometry. Phenylephrine hydrochloride USP is assayed iodometrically, phenylephrine hydrochloride B.P. is determined by nonaqueous titration. Chlorpropamide USP is estimated by ultraviolet spectrophotometry, chlorpropamide B.P. by Kjeldahl analysis. Guanethidine sulfate B.P. is assayed by column chromatography, guanethidine sulfate USP by colorimetry. Promethazine hydrochloride, State Pharmacopoeia of the U.S.S.R. (diprazine), is estimated by aqueous titrimetry, and promethazine hydrochloride USP by nonaqueous titrimetry, and promethazine hydrochloride B.P. by ultraviolet spectrophotometry. Diethylstilbestrol USP is assayed colorimetrically, stilboestrol B.P. (diethylstilbestrol) and diethylstilbestrol State Pharmacopoeia of the U.S.S.R. are determined titrimetrically following acetylation in pyridine.

Compressed Tablets—The drug content of compressed tablets covered in pharmacopeial monographs is usually given in the form of a statement indicating upper and lower limits. The tolerances allow for sampling variations, errors inherent in analytical methodology, variations due to compounding, differences in bulk drug uniformity, and losses in active strength during storage. The general nature and scope of the assays has been described under *Drug Content*.

Relationships between drug content and permitted variation in compressed tablets for which symmetrical tolerances are specified in the USP, NF, B.P., and the State Pharmacopoeia of the U.S.S.R. are depicted schematically in Fig. 1. It can be seen that the USP endorses six and the NF seven different ranges. Only three of these are frequently applied (95–105%, 93–107%, and 90–110%). The B.P. recognizes but three limits of variability (95–105%, 92.5–107.5%, and 90–110%), and the State Pharmacopoeia of the U.S.S.R. allows for just two such ranges (95–105% and 90–110%).

The length of the bars shown in the illustration is proportional to the number of tablets to which the specified relationship between drug content (dosage) and permitted variation applies. For example, only one product is listed in the B.P. containing 500 mg. of active ingredient for which a tolerance of ±7.5% is permitted. Three products to which this tolerance applies contain 300 mg. of active ingredient. Five, nine, and 17 products meeting this specification contain 250 mg., 200 mg., and 100 mg. of active ingredient, respectively. Similarly, only one product is found to contain 100 mg. of active ingredient to which a tolerance of ±10% applies.

For 17 products containing this concentration of active ingredient, tolerances of ±7.5% are permitted and for 11 such products, variations of only ±5% are allowed. Thus, Fig. 1 readily demonstrates the overlap in dosage levels between tablets subject to identical dosage variations as well as the overlap in dosage variation between products of identical drug content. In general, dosage variations are maximal for products containing only small concentrations of active ingredients.

Totals shown in Fig. 1 represent both different drug products as well as different dosage levels of the same drug product. Thus, for phenobarbital tablets USP, dosage levels of 15 mg., 30 mg., 60 mg., and 100 mg. are included in the data, while for phenobarbitone tablets B.P., dosage levels of 15 mg., 30 mg., 60 mg., 100 mg., and 125 mg. have been recorded.

Ranges of 93–107% and 95–105% are most frequently specified in the USP (49 and 48 products, respectively, or about 30%). Variations ranging from 90–110% are most often applied in the NF (52 products or 29.9%). The B.P. recognizes limits of 92.5–107.5% for most medications (115 products or 42.4%), allowing for an extension of these limits if assays are based on less than 20 tablets. While the USP and NF specify, furthermore, unsymmetrical ranges for a few dosage forms containing overages of drugs prone to deterioration on prolonged storage, the B.P. does not follow this practice. The most often used limits of drug content specified in the State Pharmacopoeia of the U.S.S.R. extend from 95–105% (52 products or 70.3%). Thus, as for bulk drugs, limits for solid oral dosage forms are generally also narrower and more exacting in this pharmacopeia than in the USP, NF, or B.P. Formulations composed of antibiotics, inorganics, and mixtures of drugs are not included in these data.

[11] A gravimetric assay for the steroid is given in the State Pharmacopoeia of the U.S.S.R. (98.5–100.5%) and an ultraviolet method described in the B.P. (97.0–103.0%).

[12] A colorimetric assay is given in both the B.P. (96–104%) and NF (97–103%).

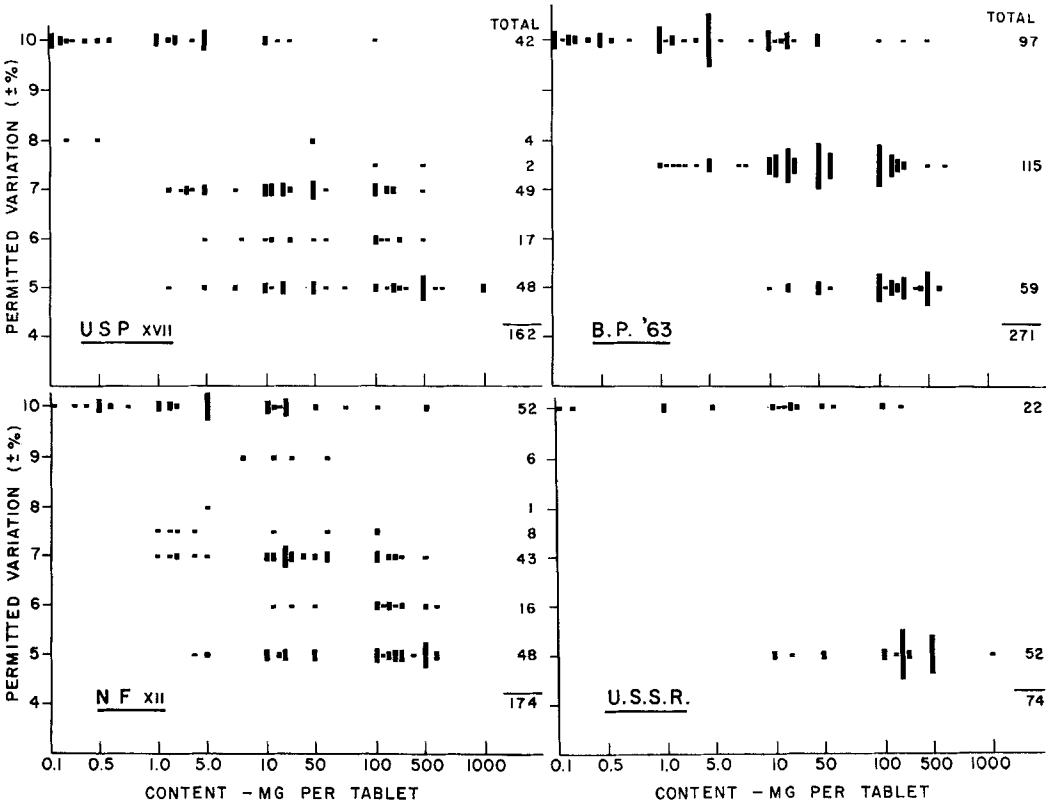

Fig. 1—Drug content tolerances for tablets included in pharmacopeial compendia.

Tablets for which different limits with regard to drug content are specified in different pharmacopeias are listed in Table XIX. Products for which symmetrical limits have been specified are given in part *A* and those for which unsymmetrical limits have been specified in at least one pharmacopeia are shown in part *B* of this table. Deviations of at least 1% in either lower or upper assay limits were considered prerequisites for compilation. Of all products thus screened—112—no less than 61 (54.5%) satisfied this requirement. Comparison of the data reproduced illustrates the extent of variation which exists between pharmacopeial specifications for products subject to identical label claims for drug content.

It is difficult to assess some of these discrepancies. They cannot always and solely be ascribed to different analytical methods. For example, guanethidine tablets USP as well as B.P. are assayed colorimetrically, yet their assay limits range from 95–105% and 90–110%, respectively. Phentolamine hydrochloride tablets B.P. and NF are both assayed gravimetrically, yet pharmacopeial limits vary from 90–110% and 93–107%, respectively. Similarly, diethylcarbamazine citrate tablets USP and B.P. are determined titrimetrically, yet assay tolerances extend from 95–105% to 92.5–107.5%, respectively. On the other hand, products may be examined by different methods yet comply with identical specifications for drug content. Thus, primidone tablets USP, B.P., and State Pharmacopoeia of the U.S.S.R. (hexamidine) (95–105%) are analyzed by either ultraviolet spectrophotometry or gravimetry. Acetylsalicylic acid tablets USP, B.P., and State Pharmacopoeia of the U.S.S.R. (95–105%) are determined by either ultraviolet spectrophotometry or by titrimetry. Similarly cyclizine hydrochloride tablets USP (93–107%) are subjected to spectral analysis, whereas cyclizine hydrochloride tablets B.P. (92.5–107.5%) are assayed by nonaqueous titrimetry.

Examples illustrating different specifications for drug content based on different methods of analysis may also be cited. Tolbutamide tablets USP (95–105%) are assayed by ultraviolet spectrophotometry, but tolbutamide tablets B.P. (92.5–107.5%) are assayed by Kjeldahl analysis. Nitrofurantoin tablets USP (95–105%) are examined by polarography. Nitrofurantoin tablets B.P. (90–110%) are examined spectrophotometrically. Sodium aminosalicylate tablets B.P. (90–110%) are analyzed by titration with nitrous acid, but sodium aminosalicylate tablets, State Pharmacopoeia of the U.S.S.R. (95–105%), are analyzed by iodimetry. Thus, limits may range from 10–20% for pharmacopeial products subject to identical label claims for drug content.

Finally, examples illustrating identical specifications based on identical methods of analysis can also be given. Diethylstilbestrol tablets USP, B.P., and Pharmacopoeia of the U.S.S.R. are all assayed colorimetrically (drug content limit 90–110%). Cortisone acetate tablets NF, B.P., and State Pharmacopoeia of the U.S.S.R. are all analyzed spectrophotometrically (assay limit 90–110%). Similarly, sulfadiazine tablets USP and B.P. are

TABLE XIX—SPECIFICATIONS FOR TABLETS INCLUDED IN TWO OR MORE PHARMACOPEIAS

Tablet	Assay Limits, %			
	USP XVII	NF XII	B.P. 1963	State Pharmacopoeia U.S.S.R. IX
A—Symmetrical				
Acetazolamide	95–105		92.5–107.5	
Amobarbital	94–106		92.5–107.5	
Amodiaquine hydrochloride		93–107	95.0–105.0	
Amphetamine sulfate		90–110	90.0–110.0	95–105
Busulfan	93–107		90.0–110.0	
Calcium cyclobarbital		94–106	95.0–105.0	
Calcium lactate		94–106	95.0–105.0	
Chlorambucil	93–107		90.0–110.0	
Chlorothiazide		93–107	95.0–105.0	
Chlorpromazine hydrochloride	95–105		92.5–107.5	
Chlorpropamide	95–105		92.5–107.5	
Dapsone	92.5–107.5		95.0–105.0	
Dehydrocholic acid		94–106	...	95–105
Dextroamphetamine sulfate	93–107		90.0–110.0	
Dextromethorphan hydrobromide		90–110	92.5–107.5	
Diethylcarbamazine citrate	95–105		92.5–107.5	
Digoxin	92–108		90.0–110.0	
Diiodohydroxyquin	95–105		92.5–107.5	
Ephedrine hydrochloride		93–107	92.5–107.5	95–105
Ferrous gluconate		93–107	90.0–110.0	
Guanethidine	95–105		90.0–110.0	
Hydralazine hydrochloride		95–105	92.5–107.5	
Isoniazid	93–107		95.0–105.0	
Lucanthone hydrochloride	93–107		95.0–105.0	
Mepacrine hydrochloride			95.0–105.0	90–110
Meperidine hydrochloride		95–105	92.5–107.5	
Methamphetamine hydrochloride	93–107		90.0–110.0	
Methylprednisolone		92.5–107.5	90.0–110.0	
Methyprylon		95–105	92.5–107.5	
Nitrofurantoin	95–105		90.0–110.0	
Phenacetin	94–106		...	95–105
Phenobarbital	94–106		92.5–107.5	95–105
Phenolphthalein		92.5–107.5	92.5–107.5	95–105
Phentolamine hydrochloride		93–107	90.0–110.0	
Phenylbutazone		93–107	95.0–105.0	
Phthalylsulfathiazole	94–106		95.0–105.0	95–105
Probenecid	92.5–107.5		95.0–105.0	
Prochlorperazine maleate	95–105		90.0–110.0	
Proguanil hydrochloride			95.0–105.0	90–110
Propantheline bromide	95–105		92.5–107.5	
Pyridostigmine bromide	95–105		92.5–107.5	
Quinidine sulfate	94–106		95.0–105.0	
Quinine sulfate		94–106	95.0–105.0	95–105
Sodium aminosalicylate	95–105		90.0–110.0	95–105
Sodium warfarin	95–105		90.0–110.0	
Tolbutamide	95–105		92.5–107.5	95–105
Trihexyphenidyl hydrochloride	93–107		90.0–110.0	
Tripelennamine hydrochloride	93–107		90.0–110.0	
Sodium barbital			95.0–105.0	95–105
B—Unsymmetrical				
Ascorbic acid	95–115		92.5–107.5	95–105
Ethinyl estradiol	90–115		90.0–110.0	
Folic acid	95–115		90.0–110.0	
Meclizine	95–110		92.5–107.5	
Mepacrine hydrochloride			95.0–105.0	95–110
Mercaptopurine	93–110		90.0–110.0	
Niacin		95–115	92.5–107.5	
Nitroglycerin	80–112		85.0–115.0	86–116
Promazine hydrochloride		95–110	90.0–110.0	
Promethazine hydrochloride	95–110		92.5–107.5	90–110
Sodium amobarbital			92.5–107.5	93–105
Sodium barbital			95.0–105.0	93–105
Sodium pentobarbital		90–105	92.5–107.5	90–105

both examined titrimetrically and, to comply with specifications for drug content, must assay 95–105%.

Further comparisons of pharmacopeial specifications for drug content of compressed tablets are made in Table XX. The compilation illustrates that 45 products for which specifications are found in the B.P. are covered in other compendia as well. Only 11 such products are included in the Russian pharmacopeia. It is also seen that of the four pharmacopeias surveyed, the USP contains the

TABLE XX—PHARMACOPEIAL SPECIFICATIONS FOR DRUG CONTENT OF COMPRESSED TABLETS

	USP XVII	NF XII	B.P. 1963	U.S.S.R. IX
No. of tablets listed in one or more pharmacopeial texts	29	17	45	11
No. of tablets with potency limits narrower than specified in other pharmacopeial texts	20	6	12	6
No. of tablets with potency limits equal to those specified in other pharmacopeial texts	1	0	2	4

highest proportion of products (covered in other compendia as well) with narrowest limits. On the other hand, the State Pharmacopoeia of the U.S.S.R. commands the highest ratio of products (for which monographs are given in other compendia as well) with limits narrower than or equal to those of the USP, NF, and B.P. (10 products out of 11).

Of the four reference compendia, the State Pharmacopoeia of the U.S.S.R. appears to be the most rigorously standardized. It allows for only two ranges and very few dosage levels of the same product. It specifies, moreover, all substances which may be incorporated as excipients into solid oral dosage forms and states their concentrations as normally used. Thus, tablet manufacture and pharmaceutical quality assurance are more strictly controlled.

SUMMARY

Considerable differences exist between pharmacopeial specifications applied in the quality control of bulk drugs and solid oral dosage forms. Often experimental results obtained by different national laboratories in accordance with national standards cannot be readily compared. Methodology and compliance vary significantly for even a test as simple as weight variation. Only a few common features characterize the disintegration test. Products meeting one pharmacopeia's standard may therefore fail to meet specifications, and be rejected, if tested by another pharmacopeia's standard.

Progress in drug evaluation, advances in drug therapy, and improvement of international drug trade would be facilitated immeasurably if a code of pharmaceutical specifications could be sanctioned internationally and adopted universally.

These objectives can and must be reached. They are embodied in the resolution adopted by the World Health Assembly at its recent plenary session and endorsed by the World Health Organization (60, 61). This resolution "requests the Director-General (a) to continue his assistance to Member States for the improvement of the quality control of pharmaceutical preparations, and for the establishment of quality control laboratories for national or regional purposes where such laboratory facilities are insufficient; (b) to implement the proposals made in his report; particularly in regard to the establishment of general principles for the quality of pharmaceutical preparations and the quality control of the products entering into international commerce; and (c) to report on the results to the Executive Board and the Twentieth World Health Assembly."[13]

It should be the solemn duty of all privileged to participate in these programs to do their utmost so that collectively they may be brought to fruition. There are indeed no higher stakes—the health and happiness of mankind.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.
(3) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1963.
(4) "Pharmacopée Française," 8th ed., Les Presses de l'Imprimerie Maisonneuve à Sainte-Ruffine, par Moulins-Lés-Metz (Moselle), France, 1965.
(5) "State Pharmacopoeia of the Union of Soviet Socialist Republics," 9th ed., Ministry of Health, Moscow, U.S.S.R., 1961.
(6) "Pharmacopoea Nordica," editio danica, vol. III, Nyt Nordisk Forlag Arnold Busck, Copenhagen, Denmark, 1963.
(7) Office Consolidation of the Food and Drugs Act and of the Food and Drug Regulations, Queen's Printer, Ottawa, Ontario, Canada, 1954.
(8) Mattocks, A. M., *Am. J. Pharm. Educ.*, **30**, 110(1966).
(9) Tawashi, R., and Speiser, P., *Pharm. Acta Helv.*, **39**, 734(1964).
(10) Banker, G. S., Christian, J. E., and DeKay, H. G., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 628(1958).
(11) Train, D., *ibid.*, **49**, 265(1960).
(12) Train, D., *Pharm. J.*, **185**, 129(1960).
(13) Garrett, E. R., and Olson, E. C., *J. Pharm. Sci.*, **51**, 764(1962).
(14) Garrett, E. R., *ibid.*, **51**, 672(1962).
(15) Breunig, H. L., and King, E. P., *ibid.*, **51**, 1187(1962).
(16) Smith, C. D., Michaels, T. P., Chertkoff, M. J., and Sinotte, L. P., *ibid.*, **52**, 1183(1963).
(17) Lachman, L., and Sylwestrowicz, H. D., *ibid.*, **53**, 1234(1964).
(18) Brochmann-Hanssen, E., and Medina, J. C., *ibid.*, **52**, 630(1963).
(19) Moskalyk, R. E., Chatten, L. G., Cox, C. E., and Pernarowski, M., *ibid.*, **50**, 651(1961).
(20) Airth, J. M., Bray, D. F., and Radecka, C., *ibid.*, **56**, 233(1967).
(21) French, W. N., Cook, D., and Levi, L., Minutes of Proceedings and Evidence, No. 30, House of Commons Special Committee on Drug Costs and Prices, Appendix "D", Queen's Printer, Ottawa, Ontario, Canada, 1967.
(22) Official Method Issued by Food and Drug Directorate, Department of National Health and Welfare, Method DO-25, Ottawa, Ontario, Canada, 1965.
(23) "Pharmacopoeia Helvetica," 5th ed., Bern, Switzerland, 1934.
(24) Lazarus, J., and Cooper, J., *J. Pharm. Pharmacol.*, **11**, 257(1959).
(25) Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961).
(26) Nelson, E., *ibid.*, **50**, 181(1961).
(27) Campbell, J. A., and Morrison, A. B., *J. Am. Med. Assoc.*, **181**, 102(1962).
(28) Morrison, A. B., and Campbell, J. A., *J. Pharm. Sci.*, **54**, 1(1965).
(29) Wood, J. H., Proceedings of the Industrial Pharmacy Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966, pp. 181–200; Wood, J. H., *Pharm. Acta Helv.*, **42**, 129(1967).
(30) Wagner, J. G., Proceedings of the Industrial Pharmacy Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966, pp. 201–232.
(31) Levy, G., *ibid.*, pp. 233–252.
(32) Keller, W., *Pharmazie*, **15**, 56(1960).
(33) Chapman, D. G., Crisafio, R., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 297(1954).
(34) Crisafio, R., Taylor, J., and Chatten, L. G., *Drug Std.*, **23**, 1(1955).
(35) Chapman, D. G., Crisafio, R., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 374(1956).
(36) Chapman, D. G., Chatten, L. G., and Campbell, J. A., *Can. Med. Assoc. J.*, **76**, 102(1957).
(37) Campbell, J. A., Chapman, D. G., and Chatten, L. G., *ibid.*, **77**, 602(1957).

[13] This report has now been published (see WHO Document A20/P and B/10, issued April 27, 1967, Geneva, Switzerland).

(38) Morrison, A. B., Chapman, D. G., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 634(1959).
(39) Campbell, J. A., and Morrison, A. B., *Practitioner*, **183**, 758(1959).
(40) Morrison, A. B., Perusse, C. B., and Campbell, J. A., *New Engl. J. Med.*, **263**, 115(1960).
(41) Morrison, A. B., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 473(1960).
(42) Morrison, A. B., and Campbell, J. A., *Am. J. Clin. Nutr.*, **10**, 212(1962).
(43) Morrison, A. B., Perusse, C. B., and Campbell, J. A., *J. Pharm. Sci.*, **51**, 623(1962).
(44) Morrison, A. B., and Campbell, J. A., *Indian J. Pharm.*, **25**, 209(1963).
(45) Middleton, E. J., Davies, J. M., and Morrison, A. B., *J. Pharm. Sci.*, **53**, 1378(1964).
(46) Middleton, E. J., Nagy, E., and Morrison, A. B., *New Engl. J. Med.*, **274**, 136(1966).
(47) Levy, G., and Hayes, B. A., *ibid.*, **262**, 1053(1960).
(48) Levy, G., *J. Am. Med. Assoc.*, **177**, 689(1961).
(49) Levy, G., Antkowiak, J. M., Procknal, J. A., and White, D. C., *J. Pharm. Sci.*, **52**, 1047(1963).
(50) Levy, G., and Gumtow, R. H., *ibid.*, **52**, 1139(1963).
(51) Levy, G., *ibid.*, **52**, 1039(1963).
(52) Singh, P., Guillory, J. K., Sokoloski, T. D., Benet, L. Z., and Bhatia, V. N., *ibid.*, **55**, 63(1966).
(53) "Guide for Completing Pre-Clinical Submissions on Investigational Drugs," Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada, 1965.
(54) Levi, L., Walker, G. C., and Pugsley, L. I., *Can. Med. Assoc. J.*, **91**, 781(1964).
(55) World Health Organization, WHO/Pharm/66.432, unpublished working document (cited with permission of Secretariat).
(56) Cook, D., Chang, H. S., and Mainville, C. A., *Can. J. Pharm. Sci.*, **1**, 69(1966).
(57) Cook, D., *Med. Serv. J., Canada*, **23**, 323(1967).
(58) Morrison, A. B., *ibid.*, **23**, 349(1967).
(59) Wood, J. H., *Pharm. Acta Helv.*, **42**, 129(1967).
(60) World Health Assembly Document WHO 1947, World Health Organization, Geneva, Switzerland, issued May 20, 1966.
(61) World Health Organization Document A19/P&B/5, Geneva, Switzerland, issued April 4, 1966.

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A method has been developed to determine trace amounts of antimony present in talc. The spectrophotometric method is based upon the reaction of antimony (V) with rhodamine B in isopropyl ether after extraction of the antimony from 1.5 *M* hydrochloric acid.

RECENTLY, the Food and Drug Administration set limits for antimony in certain foodstuffs and dyes at 2 p.p.m. Because of the possible extension of this regulation to talc, a method has been developed for the semiquantitative determination of antimony in talc in the concentration range of 2 p.p.m.

Several methods have been developed to determine micro amounts of antimony. Iodide ion in acid solution (1, 2) or with iodide and pyridine (3) have been used, but not with the sensitivity of some other chromophoric reagents. Busev (4, 5) and his co-workers have developed a sensitive method using antipyrine dyes. However, the unavailability of these noncommercial dyes negated any work with them. Matulis and Guyon (6) have recently developed a sensitive system of analysis based on the enhancement by antimony of a blue hue due to the reduction of the molybdate aggregate near pH 1.4.

The most common technique has employed rhodamine B (7–14). A relatively simple and moderately accurate method for the determination of antimony in talc has been developed with rhodamine B as the chromophoric reagent.

Received January 6, 1967, from the Johnson & Johnson Research Center, New Brunswick, NJ 08903
Accepted for publication August 18, 1967.
The authors express their appreciation to Mrs. A. Bollman for her technical assistance.

EXPERIMENTAL

Apparatus and Reagents—A Zeiss PMQ II spectrophotometer employing 1-cm. silica cells was used.

Hydrochloric Acid, 6 M—Dilute 500 ml. of concentrated hydrochloric acid with sufficient purified water to make 1000 ml. of solution.

Hydrochloric Acid, 1 M—Dilute 20.8 ml. of concentrated hydrochloric acid with sufficient purified water to make 250 ml. of solution.

Sulfuric Acid, 0.5 M—Dilute 2.8 ml. of concentrated sulfuric acid with sufficient purified water to make 100 ml. of solution.

Ceric Sulfate—Dissolve 3.3 Gm. of anhydrous ceric sulfate in 100 ml. of 0.5 *M* sulfuric acid.

Hydroxylamine Hydrochloride—Dissolve 1 Gm. of hydroxylamine hydrochloride in 100 ml. of purified water.

Hydroxylamine Hydrochloride, Acidic—Dissolve 1 Gm. of hydroxylamine hydrochloride in 100 ml. of 1 *M* hydrochloric acid.

Isopropyl Ether (Peroxide Free)—Saturate the isopropyl ether with 1 *M* hydrochloric acid.

Rhodamine B Reagent—Dissolve 0.02 Gm. of rhodamine B in 100 ml. of 1 *M* hydrochloric acid.

Sodium Sulfite—Dissolve 1 Gm. of reagent grade sodium sulfite in 100 ml. of purified water.

Antimony Postassium Tartrate, Reagent Grade—$[K(SbO)C_4H_4O_6 \cdot {}^1/_2H_2O]$.

Preparation of Standard—Weigh to the nearest tenth of a milligram a 274-mg. sample of antimony potassium tartrate into a 100-ml. volumetric flask. Dissolve the sample in and make up to the mark with 6 *M* hydrochloric acid. Pipet 10.00 ml. of this solution into a 100-ml. volumetric flask and dilute

(38) Morrison, A. B., Chapman, D. G., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 634(1959).
(39) Campbell, J. A., and Morrison, A. B., *Practitioner*, **183**, 758(1959).
(40) Morrison, A. B., Perusse, C. B., and Campbell, J. A., *New Engl. J. Med.*, **263**, 115(1960).
(41) Morrison, A. B., and Campbell, J. A., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 473(1960).
(42) Morrison, A. B., and Campbell, J. A., *Am. J. Clin. Nutr.*, **10**, 212(1962).
(43) Morrison, A. B., Perusse, C. B., and Campbell, J. A., *J. Pharm. Sci.*, **51**, 623(1962).
(44) Morrison, A. B., and Campbell, J. A., *Indian J. Pharm.*, **25**, 209(1963).
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(46) Middleton, E. J., Nagy, E., and Morrison, A. B., *New Engl. J. Med.*, **274**, 136(1966).
(47) Levy, G., and Hayes, B. A., *ibid.*, **262**, 1053(1960).
(48) Levy, G., *J. Am. Med. Assoc.*, **177**, 689(1961).
(49) Levy, G., Antkowiak, J. M., Procknal, J. A., and White, D. C., *J. Pharm. Sci.*, **52**, 1047(1963).
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(52) Singh, P., Guillory, J. K., Sokoloski, T. D., Benet, L. Z., and Bhatia, V. N., *ibid.*, **55**, 63(1966).
(53) "Guide for Completing Pre-Clinical Submissions on Investigational Drugs," Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada, 1965.
(54) Levi, L., Walker, G. C., and Pugsley, L. I., *Can. Med. Assoc. J.*, **91**, 781(1964).
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(56) Cook, D., Chang, H. S., and Mainville, C. A., *Can. J. Pharm. Sci.*, **1**, 69(1966).
(57) Cook, D., *Med. Serv. J., Canada*, **23**, 323(1967).
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(59) Wood, J. H., *Pharm. Acta Helv.*, **42**, 129(1967).
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to the mark with 6 *M* hydrochloric acid (solution *A*). Pipet 10.00 ml. of solution *A* into a 500-ml. volumetric flask and dilute to the mark with 6 *M* hydrochloric acid (solution *B*). One milliliter of solution *B* is equivalent to 2 mcg. of antimony.

Add to two 125-ml. separators 27.0 and 30.0 ml. of 6 *M* hydrochloric acid, respectively. Buret or pipet into the first separator 3.00 ml. of solution *B*. The second separator serves as the blank. Add 1 ml. of the sodium sulfite solution to each separator, swirl, add 15 ml. of purified water, and shake each separator gently to mix the contents.

Cool the solution in each separator to 25° or less and add 3 ml. of ceric sulfate solution. Mix thoroughly and allow the funnel contents to stand for 5 min. Add 1 ml. of aqueous hydroxylamine hydrochloride to each separator and mix the contents by gently shaking the funnels. Allow the funnel contents to stand for 1 min. Add 71 ml. of purified water to each separator and cool the contents to 25° or less. Add 5.00 ml. of isopropyl ether to each separator and shake the funnel with moderate vigor for 1 min. Allow the solution to stand for approximately 5 min. and drain off all but 0.5 ml. of the aqueous phase. Add 2 ml. of the acidic hydroxylamine hydrochloride solution to each separator and shake the funnels for approximately 5 sec. Allow the phases to separate and drain off all but 0.5 ml. of the aqueous phase. Add 2 ml. of 1 *M* hydrochloric acid to each separator and shake for approximately 3 sec. Allow the phases to separate and drain off the aqueous phase. Add 2 ml. of the rhodamine B solution to the separators and shake vigorously for 15 sec. Allow the phases to separate, drain off the lower layer, and pour the ether extract (from the top of the separator) into a cell. If so desired, one may pipet the isopropyl ether solution into the cell with the aid of a 3-ml. pipet.

Measure the absorbance of the solution at 550 mμ with the blank in the reference cell.

Procedure—Weigh to the nearest milligram a 3-Gm. sample of talc into a 100-ml. beaker. Add 25 ml. of 6 *M* hydrochloric acid to the beaker and disperse the sample thoroughly with the aid of a stirring rod. Cover the beaker with a watchglass.

Place the beaker on a steam bath for 0.5 hr. with occasional gentle stirring (approximately every 10 min.).

Add 1 ml. of the sodium sulfite solution to the beaker and stir; filter the contents through white ribbon filter paper and collect the filtrate in a 125-ml. separator. Rinse the beaker and residue in the funnel with 5 ml. of 6 *M* hydrochloric acid, followed by three 5-ml. washings with hot water.

Continue the procedure as indicated under *Preparation of the Standard*, starting with the paragraph, "Cool the solution . . . ."

The absorbance reading of the sample should not exceed that of the standard which represents 2 p.p.m.

RESULTS

Recovery studies were performed in duplicate by two operators at 4 different levels of antimony (1.0, 1.5, 2.0, and 2.5 p.p.m.). Antimony standards (ranging from 3.0 to 7.6 mcg.) were first added to the talc and the recovery was calculated (from a standard curve) after subtracting the antimony found in the talc samples. The accuracy was found to be 91.1%. The results of this study are given in Table I.

TABLE I—RECOVERY OF ANTIMONY FROM SPIKED TALC

Antimony Added, mcg.	Antimony Found,[a] mcg.
3.0	2.7
4.6	4.2
6.0	5.5
7.6	6.9

[a] Average of 8 determinations at each level of antimony.

Analyses for the determination of antimony in two types of talc were performed by two operators in duplicate. The range of antimony found in the talcs was 0.04 to 0.15 p.p.m.

DISCUSSION

Originally, antimony trichloride was used to prepare a standard curve for the recovery study. However, antimony potassium tartrate was substituted for antimony trichloride since the latter compound required a titration for standardization, while the former could be used as a standard by simply weighing the salt. Similar results were obtained with both compounds, indicating either salt could be used as an antimony standard with equal reliability.

Ward (13) indicates that the addition of acidic hydroxylamine hydrochloride to the isopropyl ether solution decreases the concentration of iron (III) by reducing it to iron (II) which isopropyl ether does not extract. He also mentions that 1 and 2 *M* hydrochloric acid fails to extract quantitatively some other elements, including iron (III), which also reacts with rhodamine B. It has been found that a very small percentage of iron (III) is extracted from a 1.5 *M* hydrochloric acid solution (8). Hence, with this system, antimony can be detected satisfactorily in the presence of 30,000 mcg. of iron (III). Since talc contains a significant amount of iron, it was felt that 1.5 *M* hydrochloric acid would be a desirable system to reduce any possible error contributed by the iron or any other interfering elements present in the talc. This choice of acid concentration was further reinforced as higher concentrations of acid were found to be unsuitable because of the apparent loss of isopropyl ether during the extraction process. It was found that shaking 8.0, 6.0, 5.0, 3.0, and 1.5 *M* hydrochloric acid solutions with 5.00 ml. of isopropyl ether gave significantly lower recovery volumes at the 8.0, 6.0, and 5.0 *M* hydrochloric acid concentration. This decrease in volume occurred even though the ether was previously saturated with its respective acid concentration.

Van Aman and co-workers (14) have shown that dilution of concentrated hydrochloric acid before the antimony is extracted into the ether phase results in low absorbance values. The ratio of ether to acid phase (e/a = 2.1) and concentration of antimony employed by Van Aman is considerably higher as compared to that used by the authors (e/a = 0.1). However, it was not feasible to employ their system for our samples. Although lower absorbance values would be obtained with this system, it still represents a highly sensitive one.

In order to substantiate the method of preparing the standard curve for the recovery study, the standards were determined according to the exact procedure employed for the talc samples (heating, filtering). There was no significant change in the least square line as compared to a standard curve prepared without the heating and filtering steps. This indicated that any loss of antimony in the spiked samples probably involves the presence of talc.

An experiment, similar in design to the foregoing procedure, was performed with a final concentration of 3 *M* hydrochloric acid instead of the 1.5 *M* hydrochloric acid as in the preceding method. In this procedure, the concentration of acid was kept constant throughout the method. The major advantage of the modified method is the elimination of adding 71 ml. of water before extraction with the isopropyl ether. This particular step is not desirable because of the possible increase in rate of hydrolysis of antimony (V) to a nonreactive form, antimony (IV). In the original procedure, the volume of aqueous phase was 121 ml. up to the ether extraction step as compared to 65 ml. in the modified method. Thus, the latter procedure should create a more favorable environment for a higher partition coefficient.

A recovery study was performed at the 4 levels of antimony previously mentioned. The recoveries were similar to those obtained in the original method. These results seem to indicate that the partition coefficient is the same for both methods even though the ratio of organic to aqueous phase is different.

Several authors have indicated with their systems that the rhodamine B–antimony complex is fairly stable. However, the stability of the complex in isopropyl ether has been found to vary between authors. Ward and Lakin (13) state that the color is stable up to 6.5 hr., while Van Aman (14) indicates the color fades 1.2% in 1.5 hr. or 30% in 18 hr. However, Maren (8) mentions that the color is not stable, and the absorbance readings must be made within 30 min. of the extraction. We have found that there was a decrease in the absorbance readings of the color complex with time when water was used in the reference cell. A study of absorbance *versus* time for three levels of antimony and a blank was performed at 5-min. intervals for 35 min. The results obtained show that the initial absorbance readings decreased continuously for 20 min. The difference between the initial reading and those taken at 20 min. ranged from 0.017–0.027 absorbance units with 1–4 mcg. of antimony, respectively, and in most cases much less for the blank. However, from 25–35 min. the absorbance readings for the antimony complex remained almost constant (0.004 maximum difference). Subtraction of the blank absorbance reading from the antimony complex absorbance reading at the same respective time yielded a nearly constant absorbance for 35 min. In effect, absorbance readings can be made any time during the first 35 min. after the extraction step for the samples as long as the blank is prepared and read at the same time as the sample.

In conclusion, the methods show that the antimony content found in the talcs tested is considerably lower than the 2 p.p.m. level which may be required by the Food and Drug Administration.

REFERENCES

(1) Dym, A., *Analyst*, **88**, 232(1963).
(2) Elkind, A., Gayer, K. H., and Boltz, D. F., *Anal. Chem.*, **25**, 1744(1953).
(3) Clarke, S. G., *Analyst*, **53**, 373(1928).
(4) Busev, A. I., Tiptsova, V. G., Bogdanova, E. S., and Andreichuk, A. M., *J. Anal. Chem. USSR*, **20**, 887(1965).
(5) Busev, A. I., Bogdanova, E. S., and Tiptsova, V. G., *ibid.*, **20**, 542(1965).
(6) Matulis, R. M., and Guyon, J. C., *Anal. Chem.*, **37**, 1391(1965).
(7) Fredrick, W. G., *ibid.*, **13**, 922(1941).
(8) Maren, T. H., *ibid.*, **19**, 487(1947).
(9) Edwards, F. C., and Voigt, A. F., *ibid.*, **21**, 1204 (1949).
(10) Luke, C. L., *ibid.*, **25**, 674(1953).
(11) Luke, C. L., and Campbell, M. E., *ibid.*, **25**, 1592 (1953).
(12) White, C. E., and Rose, H. J., *ibid.*, **25**, 351(1953).
(13) Ward, F. N., and Lakin, H. W., *ibid.*, **26**, 1168(1954).
(14) Van Aman, R. E., Hollibaugh, F. D., and Kanzelmeyer, J. H., *ibid.*, **31**, 1783(1959).

Determination of Meprobamate in Tablets by NMR

By JOHN W. TURCZAN and THEODORE C. KRAM

A rapid NMR procedure was developed for the analysis of meprobamate tablets with malonic acid as internal standard. An average error of 0.9 percent was obtained on seven synthetic mixtures. The NMR spectrum, in addition, provided a very specific means of identification for meprobamate.

THE CURRENT development of more complex drugs and drug mixtures, and the necessity for the analysis of many more samples has led to the need for rapid and simple methods of analysis. We have successfully analyzed many high-dosage-form pharmaceuticals by extracting them with a suitable solvent and obtaining their NMR spectra with the aid of an internal standard. The recorded spectrum from the NMR spectrometer provides an identification of the active ingredient, which contributes to the specificity of the procedure. Meprobamate has been determined by this technique, using acetone as solvent and

Received June 15, 1967, from the New York District Laboratories, Food and Drug Administration, Brooklyn, NY 11232
Accepted for publication August 15, 1967.
The authors acknowledge the assistance of Dr. Robert Ginell, Food and Drug Administration, for technical advice, especially in leading to the selection of a very useful internal standard, and to Mr. George Schwartzman, Food and Drug Administration, for his encouragement in this and related projects.

In order to substantiate the method of preparing the standard curve for the recovery study, the standards were determined according to the exact procedure employed for the talc samples (heating, filtering). There was no significant change in the least square line as compared to a standard curve prepared without the heating and filtering steps. This indicated that any loss of antimony in the spiked samples probably involves the presence of talc.

An experiment, similar in design to the foregoing procedure, was performed with a final concentration of 3 *M* hydrochloric acid instead of the 1.5 *M* hydrochloric acid as in the preceding method. In this procedure, the concentration of acid was kept constant throughout the method. The major advantage of the modified method is the elimination of adding 71 ml. of water before extraction with the isopropyl ether. This particular step is not desirable because of the possible increase in rate of hydrolysis of antimony (V) to a nonreactive form, antimony (IV). In the original procedure, the volume of aqueous phase was 121 ml. up to the ether extraction step as compared to 65 ml. in the modified method. Thus, the latter procedure should create a more favorable environment for a higher partition coefficient.

A recovery study was performed at the 4 levels of antimony previously mentioned. The recoveries were similar to those obtained in the original method. These results seem to indicate that the partition coefficient is the same for both methods even though the ratio of organic to aqueous phase is different.

Several authors have indicated with their systems that the rhodamine B–antimony complex is fairly stable. However, the stability of the complex in isopropyl ether has been found to vary between authors. Ward and Lakin (13) state that the color is stable up to 6.5 hr., while Van Aman (14) indicates the color fades 1.2% in 1.5 hr. or 30% in 18 hr. However, Maren (8) mentions that the color is not stable, and the absorbance readings must be made within 30 min. of the extraction. We have found that there was a decrease in the absorbance readings of the color complex with time when water was used in the reference cell. A study of absorbance *versus* time for three levels of antimony and a blank was performed at 5-min. intervals for 35 min. The results obtained show that the initial absorbance readings decreased continuously for 20 min. The difference between the initial reading and those taken at 20 min. ranged from 0.017–0.027 absorbance units with 1–4 mcg. of antimony, respectively, and in most cases much less for the blank. However, from 25–35 min. the absorbance readings for the antimony complex remained almost constant (0.004 maximum difference). Subtraction of the blank absorbance reading from the antimony complex absorbance reading at the same respective time yielded a nearly constant absorbance for 35 min. In effect, absorbance readings can be made any time during the first 35 min. after the extraction step for the samples as long as the blank is prepared and read at the same time as the sample.

In conclusion, the methods show that the antimony content found in the talcs tested is considerably lower than the 2 p.p.m. level which may be required by the Food and Drug Administration.

REFERENCES

(1) Dym, A., *Analyst*, **88**, 232(1963).
(2) Elkind, A., Gayer, K. H., and Boltz, D. F., *Anal. Chem.*, **25**, 1744(1953).
(3) Clarke, S. G., *Analyst*, **53**, 373(1928).
(4) Busev, A. I., Tiptsova, V. G., Bogdanova, E. S., and Andreichuk, A. M., *J. Anal. Chem. USSR*, **20**, 887(1965).
(5) Busev, A. I., Bogdanova, E. S., and Tiptsova, V. G., *ibid.*, **20**, 542(1965).
(6) Matulis, R. M., and Guyon, J. C., *Anal. Chem.*, **37**, 1391(1965).
(7) Fredrick, W. G., *ibid.*, **13**, 922(1941).
(8) Maren, T. H., *ibid.*, **19**, 487(1947).
(9) Edwards, F. C., and Voigt, A. F., *ibid.*, **21**, 1204 (1949).
(10) Luke, C. L., *ibid.*, **25**, 674(1953).
(11) Luke, C. L., and Campbell, M. E., *ibid.*, **25**, 1592 (1953).
(12) White, C. E., and Rose, H. J., *ibid.*, **25**, 351(1953).
(13) Ward, F. N., and Lakin, H. W., *ibid.*, **26**, 1168(1954).
(14) Van Aman, R. E., Hollibaugh, F. D., and Kanzelmeyer, J. H., *ibid.*, **31**, 1783(1959).

Determination of Meprobamate in Tablets by NMR

By JOHN W. TURCZAN and THEODORE C. KRAM

A rapid NMR procedure was developed for the analysis of meprobamate tablets with malonic acid as internal standard. An average error of 0.9 percent was obtained on seven synthetic mixtures. The NMR spectrum, in addition, provided a very specific means of identification for meprobamate.

THE CURRENT development of more complex drugs and drug mixtures, and the necessity for the analysis of many more samples has led to the need for rapid and simple methods of analysis. We have successfully analyzed many high-dosage-form pharmaceuticals by extracting them with a suitable solvent and obtaining their NMR spectra with the aid of an internal standard. The recorded spectrum from the NMR spectrometer provides an identification of the active ingredient, which contributes to the specificity of the procedure. Meprobamate has been determined by this technique, using acetone as solvent and

Received June 15, 1967, from the New York District Laboratories, Food and Drug Administration, Brooklyn, NY 11232
Accepted for publication August 15, 1967.
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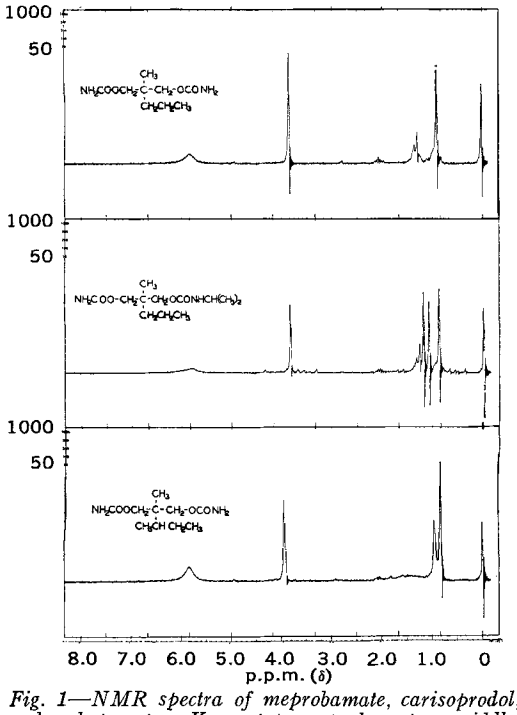


Fig. 1—NMR spectra of meprobamate, carisoprodol, and mebutamate. Key: top, meprobamate; middle, carisoprodol; bottom, mebutamate.

malonic acid as internal standard (Fig. 1). Two drugs closely related in structure and use, carisoprodol[1] and mebutamate, show distinct differences in their spectra from that of meprobamate and also can be analyzed by the same procedure.

Meprobamate is usually determined by the NF XII method (1) which involves an acidic solvolysis followed by a titration with sodium hydroxide in the presence of formaldehyde. This method is more time consuming and less specific than the proposed NMR method. Other published procedures are based on alkaline hydrolysis of the carbamate group followed by steam distillation (2), color reactions (3, 4), and infrared spectroscopy (5, 6). A near infrared method recently developed in this laboratory appears to be promising (7). The chief advantages of the NMR method over these other methods is rapidity and specificity.

PROCEDURE

Weigh and finely powder not less than 20 tablets. Then weigh accurately a portion of the powder, equivalent to about 400 mg. of meprobamate, in a glass-stoppered centrifuge tube and add about 400 mg. of malonic acid. Shake with 5 ml. of acetone for 2 min. and centrifuge. Transfer approximately 0.4 ml. of the clear supernatant to an analytical NMR tube, place in an NMR spectrometer (60 MHz), and obtain the spectrum, adjusting the spin rate so that no spinning sidebands occur between 3.1 and 4.0 p.p.m. The peaks of interest are integrated several times in each direction.

[1] Soma. Wallace Laboratories, Cranbury, N. J.

RESULTS AND DISCUSSION

The 60-MHz spectrum of meprobamate (Fig. 1) shows a peak at 3.8 p.p.m., representing 2 equivalent methylene groups. It was chosen for quantitative analysis and compared with the peak produced by the two methylene protons of malonic acid at 3.3 p.p.m. The clusters appearing at 0.8–1.4 p.p.m. confirm the meprobamate identity. Since acetone's carbon-13 satellite at 3.1 p.p.m. does not interfere with the upfield portion of the meprobamate spectrum, it is unnecessary to use the deuterated solvent for routine work.

The amount of meprobamate per tablet may be calculated as follows:

$$\frac{\text{mg. meprobamate}}{\text{tablet}} = \frac{A_{\text{mep.}}}{A_{\text{mal.}}} \times \frac{EW_{\text{mep.}}}{EW_{\text{mal.}}} \times \text{mg. mal.} \times \frac{\text{wt. 1 tablet}}{\text{wt. sample}}$$

$A_{\text{mep.}}$ = integral value of the singlet at 3.8 p.p.m.
$A_{\text{mal.}}$ = integral value of the singlet at 3.3 p.p.m.
$EW_{\text{mep.}}$ = formula weight of meprobamate/4 = 54.56.
$EW_{\text{mal.}}$ = formula weight of malonic acid/2 = 52.03.

The average deviation obtained for 7 standard preparations was 0.9% (Table I). The proportion of malonic acid to meprobamate appears to have no significant bearing on the accuracy of the determination for the range of proportions shown in the table.

Table I—Determination of Meprobamate in Synthetic Samples by NMR

Synthetic Sample	Malonic Acid Internal Std., mg. Added/ml.	Meprobamate, mg./ml. Added	Found	Deviation, %
1	87.3	99.7	98.0	1.7
2	87.5	98.1	99.9	1.8
3	85.1	100.1	99.0	1.1
4	87.6	100.3	99.7	0.6
5	67.5	73.5	73.3	0.3
6	129.8	74.6	74.4	0.3
7	70.7	91.5	91.7	0.2

Table II—Determination of Meprobamate in Commercial Samples by NMR

Commercial Tablet	Malonic Acid Internal Std., mg. Added/ml.	Declared mg./ Tablet	Found, mg./Tablet NMR	NF XII
1	76.1	400	393	391
2	78.8	400	398	396
3	75.1	400	413	397
4	72.9	400	399	402

Since this method was developed, approximately 30 commercial preparations of meprobamate have been analyzed by NMR. Four of these samples have also been analyzed by the NF XII method as well (1). Results obtained by both methods are shown in Table II.

Tablets have been marketed in which meprobamate was combined with other active ingredients. The following substances have been examined and found to produce no interference in the determination of meprobamate: amphetamine sulfate, hydrochlorothiazide, dexamethasone, pentaerythritol tetranitrate, benactyzine hydrochloride, and aspirin.

Carisoprodol and mebutamate were analyzed by the same procedure. Their NMR spectra are also shown in Fig. 1. Unlike meprobamate, two peaks are produced at 3.8 p.p.m. Further differences may be noted in the area of 0.8 to 1.4 p.p.m. Preliminary work shows the accuracy to be of the same order as for meprobamate.

The speed, accuracy, and specificity of the NMR method make it useful as a rapid procedure that can provide both a quick and conclusive identification and an assay having an accuracy of about 1–2%.

REFERENCES

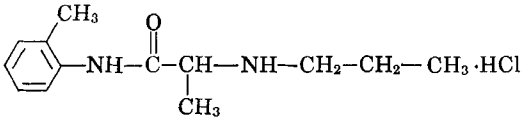
(1) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 238.
(2) Harris, E. S., and Reik, J. J., *Clin. Chem.*, **4**, 241(1958); through *Anal. Abstr.*, **6**, 279(1959).
(3) Sasaki, S., Mori, H., and Ichimura, S., *Yakugaku Zasshi*, **78**, 1185(1958); through *Chem. Abstr.*, **53**, 3602(1959).
(4) Maggiorelli, E., *Farmaco (Pavia), Ed. Prat.*, **13**, 656(1958); through *Chem. Abstr.*, **53**, 8540(1959).
(5) Brisson, E. L., and Schwartzman, G., private communication.
(6) Maynard, W. R., Jr., *J. Assoc. Offic. Agr. Chemists*, **43**, 791(1960).
(7) Sherken, S., paper presented to the Mid-Atlantic Regional Meeting of the American Chemical Society, February 1967.

Qualitative and Quantitative Tests for Prilocaine Hydrochloride

By EDWARD F. SALIM* and BERTIL ÖRTENBLAD†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

2-(PROPYLAMINO) - *o* - PROPIONOTOLUIDIDE HYDROCHLORIDE; $C_{13}H_{20}N_2O \cdot HCl$; mol. wt. 256.78. The structural formula of prilocaine hydrochloride may be represented as:



Physical Properties—Prilocaine hydrochloride occurs as a white, odorless crystalline powder with a bitter taste, m.p. 166–169° (USP class I). It is freely soluble in water and in alcohol, slightly soluble in chloroform, very slightly soluble in acetone, and practically insoluble in ether.

Identity Tests—The infrared spectrum of a 0.5% dispersion of prilocaine hydrochloride in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 1.

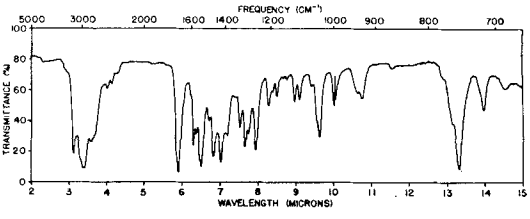


Fig. 1—Infrared spectrum of prilocaine hydrochloride in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Dissolve about 100 mg. of prilocaine hydrochloride in about 3 ml. of water, add ammonia T.S. until basic, and filter. Acidify the filtrate with diluted nitric acid, and add 1 ml. of silver nitrate T.S.: a white precipitate forms, which is insoluble in diluted nitric acid, but soluble in ammonia T.S. (presence of chloride).

Transfer about 300 mg. of prilocaine hydrochloride to a separator and dissolve in 5 ml. of water. Add 4 ml. of ammonia T.S. and extract with four 15-ml. portions of chloroform, filtering the extracts through paper. Evaporate the combined

Received July 3, 1967, from the *Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037
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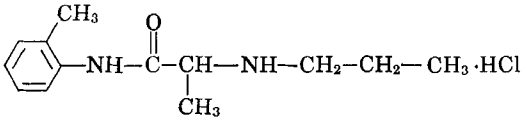

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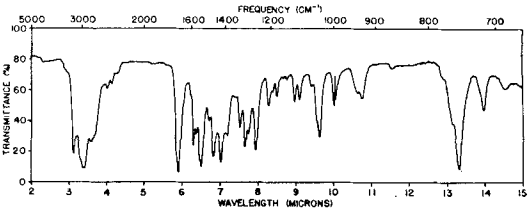

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extracts on a steam bath with the aid of a current of air: the oily residue responds to the following tests.

Dissolve about 100 mg. of the free base in 1 ml. of alcohol, add 10 drops of cobaltous chloride T.S., and shake for 2 min.: a bright green color develops, and a fine precipitate is formed.

Dissolve about 100 mg. in a mixture of 5 ml. of water and 1 ml. of diluted nitric acid, warming if necessary to effect solution. Add 3 ml. of mercuric nitrate T. S., and heat nearly to boiling: the solution turns yellow or yellow-green.

Purity Tests—Dry about 1 Gm. of prilocaine hydrochloride, accurately weighed, at 105° for 4 hr.: it loses not more than 0.3% of its weight.

Char about 1 Gm. of prilocaine hydrochloride, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Dissolve about 200 mg. of prilocaine hydrochloride in 10 ml. of water, add 2 ml. of diluted hydrochloric acid, and 1 ml. of barium chloride T.S.: no turbidity is produced (absence of sulfate).

Dissolve about 200 mg. of prilocaine hydrochloride in 20 ml. of water, add 2 ml. of diluted hydrochloric acid and saturate the solution with hydrogen sulfide: no color or precipitate results (absence of heavy metals).

Assay—*Chloride*—Transfer about 200 mg. of prilocaine hydrochloride accurately weighed, to a 125-ml. conical flask, and dissolve in 10 ml. of water. Add 10 drops of potassium chromate T.S. and titrate with 0.1 *N* silver nitrate to a red-brown end point. Each milliliter of 0.1 *N* silver nitrate is equivalent to 3.546 mg. of chloride (Cl). The chloride content is not less than 13.4% and not more than 14.2%.

Prilocaine Hydrochloride—Transfer about 500 mg. of prilocaine hydrochloride, accurately weighed, to a tall-form 200-ml. beaker, and dissolve in 50 ml. of glacial acetic acid. Add 10 ml. of mercuric acetate T.S. and 2 drops of crystal violet T.S., and titrate with 0.1 *N* acetous perchloric acid to a blue-green end point. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 25.68 mg. of $C_{13}H_{20}N_2O \cdot HCl$. The amount of prilocaine hydrochloride found is not less than 99.0% and not more than 101.0%.

DOSAGE FORMS OF PRILOCAINE HYDROCHLORIDE

Prilocaine Hydrochloride Injection

Physical Properties—Prilocaine hydrochloride injection is a sterile aqueous solution with methylparaben present as a preservative. The pH of the solution is adjusted between 6.0 and 7.0 with sodium hydroxide.

Identity Tests—Transfer to a separator a volume of injection equivalent to about 300 mg. of prilocaine hydrochloride, and proceed as directed under the bulk drug beginning with "Add 4 ml. of ammonia T.S . . . ."

Assay—Transfer to a separator an accurately measured volume of prilocaine hydrochloride injection equivalent to about 200 mg. of prilocaine hydrochloride. Add 2 ml. of sodium hydroxide T.S. and extract with four 25-ml. portions of chloroform, filtering each portion through chloroform moistened paper into a titrating beaker. Concentrate the filtrate to about 10 ml. on a steam bath with the aid of a current of air, add 40 ml. of glacial acetic acid, 2 drops of crystal violet T.S., and titrate with 0.1 *N* acetous perchloric acid to a blue-green end point. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 25.68 mg. of $C_{13}H_{20}N_2O \cdot HCl$. The amount of prilocaine hydrochloride found is not less than 95.0% and not more than 105.0% of the labeled amount.

DISCUSSION

USP and NF terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Prilocaine hydrochloride,[1] synthesized by Lofgren and Tegner (1), is a local anesthetic agent whose potency is comparable to lidocaine but whose toxicity is significantly reduced (2, 3). It is intended for use in infiltration and regional anesthesia, therapeutic nerve blocks such as intercostal and paravertebral blocks, and blockades of major nerve trunks *via* the peridural and caudal routes. Prilocaine hydrochloride is administered by injection and is available in 1, 2, 3, and 4% solutions. Prilocaine hydrochloride was formerly known by the generic name propitocaine hydrochloride.

Identity Tests—The reaction of prilocaine and mercuric nitrate to produce a yellow or yellow-green color differentiates the compound from anesthetics derived from *p*-aminobenzoic acid which turn red or orange. The free base isolated by chloroform extraction from alkaline solution can be reacted with methanesulfonic acid in anhydrous ether for identification. The methanesulfonate (mesylate) salt so obtained recrystallized from dehydrated alcohol–isopropyl ether (1,3) melts between 168–171°.

Quantitative Tests—Determination of chloride content by the Mohr method gave an average value of 13.8 ± 0.1%.[2] The theoretical value for the chloride content is 13.81%. The halide salt can be determined by many conventional methods of analysis for ionizable chloride.

Nonaqueous titration of prilocaine hydrochloride with perchloric acid gave an average value of 100.2 ± 0.3%.[2] The isolation of prilocaine base from the 1% injection and subsequent titration gave an average value of 100.4 ± 0.5%[2] of the labeled amount of prilocaine hydrochloride. Analyses of the 2, 3, and 4% injections yielded recoveries corresponding to 99–100% of label declaration.

REFERENCES

(1) Lofgren, N., and Tegner, C., U. S. pat. 3,160,662 (December 8, 1964); through *Chem. Abstr.*, **63**, 4211(1965).
(2) Wiedling, S., *Acta Pharmacol. Toxicol.*, **17**, 233(1960).
(3) Astrom, A., and Persson, N. H., *Brit. J. Pharmacol.*, **16**, 32(1961).

[1] Marketed as Citanest by Astra Pharmaceutical Products, Inc., Worcester, Mass.
[2] Maximum deviation from the mean value.

Titration Assembly and Experimental Procedure for Accurate pH Stat Measurements of Substrate Hydrolysis Rates in Blood Plasma

By CLIFFORD W. CHONG, LEWIS W. DITTERT*, HARRY B. KOSTENBAUDER†, and JOSEPH V. SWINTOSKY‡

A study was undertaken to establish an experimental procedure whereby accurate rates of enzyme-catalyzed substrate hydrolysis could be measured with a pH stat when solutions of blood plasma are used as the enzyme source. pH stat procedures which neglect the effects of plasma deterioration and loss of CO_2 from plasma solutions yield apparent hydrolysis rates which are faster and slower, respectively, than the true rates. To overcome the problems of anomalous substrate hydrolysis rates and apparent nonstoichiometric titrations, the plasma solutions were prepared aseptically and the titration assembly from a Radiometer pH stat was modified to effect a virtually closed system. Hydrolysis rate data are presented for acetylcholine, benzoylcholine, and phenyl butyrate in 0.5 percent solutions of human blood plasma to demonstrate the utility of the proposed experimental procedure and the modified pH stat titration assembly.

SOLUTIONS of whole human blood plasma are useful and convenient media for studying the *in vitro* enzymic cleavage of a number of drugs (1–7). However, they are seldom used in kinetic studies which utilize the pH stat for the determination of hydrolysis rates. This is apparently due to the labile nature of blood plasma (8). When exposed to air, solutions of blood plasma are prone to deterioration and loss of CO_2. Plasma deterioration results in a spontaneous increase in acidity, whereas loss of CO_2 decreases acidity. Thus, either effect may cause sizable errors in studies where hydrolytic processes occurring in blood plasma solutions are followed potentiometrically. This report describes a pH stat titration assembly and an experimental procedure useful for circumventing these errors.

Most studies which utilize blood plasma solutions as the enzyme media employ spectrophotometric (3, 4, 6, 7, 9, 10) or manometric (11–13) means of following the kinetics of substrate hydrolysis. Although capable of considerable precision, both methods have limitations. The spectrophotometric method is applicable only (*a*) to substrates with convenient absorption spectra which are distinguishable from those of their respective products; (*b*) to reaction mixtures devoid of extraneous substances which interfere with the assay of the substrates or products; and (*c*) to relatively low concentrations of substrate and enzyme. The manometric method, in addition to being laborious and time consuming, requires precise control of the CO_2 content of the reaction mixture. Both methods require the use of added buffers and, because of this, the enzymic reactions occur in an environment which differs from the physiological state of blood plasma.

Continuous potentiometric titration at constant pH without the use of additional buffers provides a convenient method for following enzymic hydrolysis reactions, especially of substrates with indistinguishable ultraviolet or visible absorption spectra. With the recent development of commercial automatic pH stats, this method has become increasingly useful (14–16, 18). Several factors which may influence pH stat results have been mentioned, but these are of little significance when the more recently developed techniques and apparatus are employed (17–21). The main objection to the method has concerned dilution of the reaction mixture by the titrant, but this effect can be overcome by keeping the volume of titrant small with respect to the volume of the reaction mixture or by correcting the kinetic data mathematically (22). When solutions containing blood plasma are used as the enzyme media, two additional effects must be considered. These are (*a*) the spontaneous production of an acid due to deterioration of the plasma, and (*b*) the loss of CO_2 from the plasma solution. Both effects, to our knowledge, are often either overlooked or inadequately considered in studies

Received May 15, 1967, from *Smith Kline & French Laboratories, Philadelphia, PA 19101, and the †School of Pharmacy, Temple University, Philadelphia, PA 19140
Accepted for publication July 19, 1967.
Abstracted in part from a thesis to be submitted by Clifford W. Chong to the Graduate School, Temple University, in partial fulfillment of Master of Science degree requirements.
The authors thank Mr. Allan Cook and Mr. Andrew C. Airey for their help in constructing the apparatus.
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which utilize the pH stat to follow hydrolytic processes occurring in blood plasma solutions.

It has been observed in these laboratories that nonsterile solutions of blood plasma slowly decrease in pH on standing in a closed vessel where the CO_2 content of the solutions is kept constant. This effect is presumably due to bacterial deterioration of some ingredient of plasma, the result of which is a spontaneous production of an acid. Although the identification of the acid as well as its source have not been an objective of this study, the possible effect of the acid on pH stat results is of paramount importance. The pH stat plots the course of a hydrolysis reaction as a function of time by automatically titrating with a standard base solution the hydrogen ions produced as the reaction proceeds at a constant pH. Therefore, the acid produced as a result of plasma deterioration will obviously contribute to the rate as well as to the extent of the hydrolysis reaction. It was found that the acid may be produced in such large amounts that it could completely mask the hydrolytic process of substrates which hydrolyze at relatively slow rates. It is apparent, therefore, that, in order to obtain reliable data with the pH stat, adequate steps should be taken to minimize bacterial contamination during all phases of handling of the blood plasma.

The presence of large amounts of CO_2 in solutions of blood plasma presents another problem in pH stat determinations of hydrolysis rates. If the reaction is carried out in an open vessel, CO_2 will rapidly diffuse from the plasma solution to the atmosphere. The loss of CO_2 results in an increase in the basicity of the system which subsequently contributes to the titration of the hydrogen ions produced by the hydrolysis reaction. The conventional use of a nitrogen flush, while useful in systems completely free of CO_2 and bicarbonates (19), accelerates the loss of CO_2 by expelling it out of solution at a faster rate. Thus, to maintain constant CO_2 content of blood plasma solutions, the hydrolysis reactions should be carried out in a closed system.

In preliminary work with the pH stat using a 0.5% solution of blood plasma as the reaction medium, several attempts were made to completely purge the solution of CO_2. This was done by bubbling nitrogen gas through the plasma solution. In every instance, the pH of the solution first rose quite rapidly to about 9.5 where it remained constant. Since there was no further rise in pH, it was assumed that no more CO_2 could be removed from the solution. However, upon returning the pH of the solution to 7.4 and then continuing with the nitrogen flush, the pH once more rose to about 9.5, this time at a slower rate than previously. The same procedure was repeated several times with similar results, except that it took longer for the pH to reach 9.5 with each succeeding trial. While it may have been possible to remove all of the CO_2 from the solution, the procedure would be considerably difficult and time consuming. In addition, the rise in pH and change in plasma composition resulting from such treatment could affect the activity of the enzymes in blood plasma.

On the basis of the foregoing discussions, it seems obvious that adequate control over plasma deterioration and loss of CO_2 must be exercised to obtain accurate hydrolysis rate data with the pH stat. The manner in which this is achieved, that is, the use of an aseptic procedure for preparing the blood plasma solutions and the design of a virtually closed titration assembly, is described in this report.

EXPERIMENTAL

Materials—One-half percent solutions of whole human blood plasma[1] were prepared by an aseptic procedure using sterile, pyrogen-free, isotonic saline solution (0.15 *M* NaCl)[2] as the dilution medium. Alcoholic stock solutions of acetylcholine iodide,[3] benzoylcholine chloride,[3] and phenyl butyrate[3] in a concentration of 0.04 *M* were used as substrates. The standard base titrant was 0.02 *M* NaOH which was prepared free of CO_2 in the usual manner.

Apparatus—A Radiometer pH stat[4] was used. It consisted of a type TTT1 automatic titrator, SBR2c recorder, SBU1 syringe buret, and TTA3 titration assembly which was modified to effect a virtually closed system. The modified titration assembly is shown in Fig. 1.

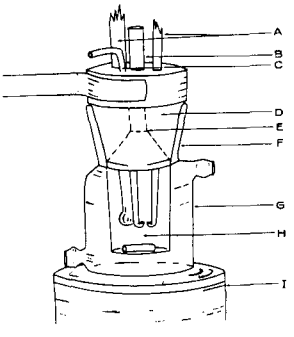

Fig. 1—Diagram of modified pH stat titration assembly described in the text.

The reaction vessel (H) is equipped with a water jacket (G) and a ground-glass joint (F) which pro-

[1] Philadelphia Serum Exchange, Philadelphia, Pa.
[2] Abbott Laboratories, North Chicago, Ill.
[3] Eastman Kodak Co., Rochester, N. Y.
[4] Radiometer, Copenhagen, Denmark.

vides an air-tight fit on the tapered Teflon plug (D). The Teflon plug holds the electrodes (A), the titrant delivery tube (C), and an inlet tube (B) 6 mm. in diameter and concentrically located on the plug. The bottom of the plug is tapered inward to form an inverted cone which peaks at the bottom of the inlet tube. The vessel is so made that when 20 ml. of plasma solution is used and the vessel is placed in position on the plug, the level of the solution just covers the bottom end of the inlet tube. A drop of light liquid petrolatum is then placed in the inlet tube to form a thin layer of oil on the surface of the plasma solution (E). By confining the oil layer only to the small opening of the inlet tube, the tendency is minimized for any oil-soluble component of the reaction mixture to partition into the oil. The inlet tube also serves as a means of introducing the enzyme, substrate, and other additives into the system by the use of a hypodermic syringe and needle. Stirring of the reaction mixture is done by means of magnetic stirrer (I) located beneath the vessel.

Preparation of the Blood Plasma Solutions—Blood plasma is usually supplied frozen in 120- to 150-ml. quantities. When defrosted and exposed to air, it deteriorates quite readily. Since plasma deterioration is apparently due to bacterial contamination, it is expected that antibacterial agents such as a streptomycin and sulfathiazole might be used to alleviate this condition (23). However, it was felt that the addition of such agents might further complicate the system, for example, by reducing the enzyme activity or by interfering with the potentiometric titration, and that the use of an aseptic procedure for preparing the plasma solutions might be more practical.

The procedure is as follows. The plasma is first defrosted, mixed well, and then transferred in a sterile room to serum flint vials in 1–2-ml. portions. The vials are sealed with rubber closures and placed in a freezer at −18°. Dividing the plasma into small portions eliminates the need to defrost and refreeze the bulk plasma every time a sample is removed and, thus, reduces the chances for the plasma to deteriorate. At the time of use, a vial of plasma is removed from the freezer and allowed to thaw at 37°. One milliliter of the plasma is transferred under sterile conditions to a 100-ml. volumetric flask and brought up to volume with sterile, pyrogen-free, isotonic saline solution to make a 0.5% solution of blood plasma. This solution is stable up to 24 hr. with respect to its enzyme activity and relative freedom from deterioration by bacteria.

Cleaning of the Titration Assembly—Immediately prior to the start of a run, the reaction vessel, as well as all parts of the titration assembly which normally come in contact with the plasma solution, are cleaned first by washing with a 70% solution of ethanol, and then thoroughly rinsing with the sterile saline solution. They are then wiped dry with a sterile cotton gauze.

Procedure for a Typical Kinetic Run—Twenty milliliters of the 0.5% blood plasma solution is transferred to the reaction vessel by means of a sterile pipet and the vessel is placed in position on the pH stat. A drop of light liquid petrolatum is placed on the surface of the solution through the inlet tube. The speed of the magnetic stirrer is adjusted so that stirring is just sufficient to cause rapid flow of the solution past the electrodes without creating cavitation and foaming. Constant-temperature water (37 ± 0.2°) is circulated through the jacket of the reaction vessel.

The plasma solution is first adjusted to pH 7.4 with the pH stat. The pH stat is set so that, as the pH of the solution approaches 7.4, the titrant is added at a rate essentially the same as that required by the reaction. This procedure quickly titrates the free acids present, prevents the possibility of the automatic titration falling behind the reaction, and eliminates large "step-effects" due to the addition of excess titrant. The pH stat is left to run until no titrant is consumed for at least 15 min. with the pH remaining constant at 7.4. When this occurs, the system is said to have achieved pH equilibrium. The recording pen is returned to zero and the syringe buret is refilled with titrant.

The kinetic run commences when 0.1 ml. of stock substrate solution is added to the plasma solution. The pH stat is adjusted once more to compensate for any change in reaction rate, and the reaction is allowed to proceed either to completion or until sufficient data are obtained.

Several investigations were made using 0.5% solutions of blood plasma with and without substrates to determine the effects of (*a*) plasma deterioration, (*b*) the loss of CO_2 from the reaction mixture, and (*c*) a virtually sterile and closed reaction system in which the effects of both plasma deterioration and loss of CO_2 are minimized.

The magnitude of the effect of plasma deterioration was determined by the use of two solutions of blood plasma: one which was freshly prepared and the other which was left exposed to air at room temperature for 2 hr. No precautions were taken to maintain the sterility of these solutions. To eliminate the effects due to the loss of CO_2 from the reaction mixture, the reactions were carried out in the modified titration assembly.

The effect due to the loss of CO_2 from the reaction mixture was determined by the use of a sterile solution of blood plasma in the titration assembly supplied with the instrument. This assembly, not being a closed system, allows CO_2 to escape freely from the reaction vessel. A 0.02 *N* HCl titrant was used to follow the rate of base production. After a specified time, a nitrogen flush similar to the one described by Applewhite (20) was introduced to show the in-

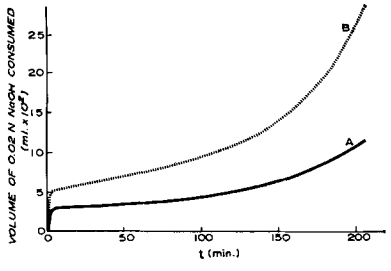

Fig. 2—Plots showing the effect of blood plasma deterioration. The increased rate of base consumed reflects the rate at which acid is spontaneously produced when plasma degrades. Curve A *was obtained using a freshly prepared plasma solution. For curve* B, *a plasma solution which had been aged for 2 hr. at room temperature was used. (Plasma concentration, 0.5%.)*

creased rate of base production due to a faster loss of CO_2.

Finally, the utility of the proposed experimental procedure and the modified pH stat titration assembly was demonstrated. Solutions of 0.5% blood plasma, prepared in the manner described, were used as the enzyme media with acetylcholine, benzoylcholine, and phenyl butyrate as substrates. To check the accuracy of the results obtained with the pH stat, parallel runs were made with the latter two substrates on a Cary model 15 spectrophotometer according to the method of Kalow *et al.* (9).

All experiments were conducted at pH 7.4 and 37°. For purposes of comparison, the hydrolysis rates for benzoylcholine and phenyl butyrate were determined from apparent zero-order rate plots of the initial stages of the hydrolytic processes.

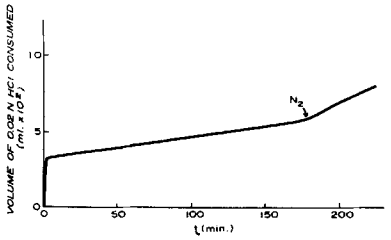

Fig. 3—Plot showing the effect of CO_2 loss. The rate of base production appears linear in the time range studied. At the point N_2 a nitrogen flush was introduced into the system. The increased rate of base production is due to the faster rate at which CO_2 is expelled from solution. (Plasma concentration, 0.5%.)

RESULTS AND DISCUSSION

The effect of the acid produced as the result of plasma deterioration is shown by curves A and B in Fig. 2.

Curve A was obtained from the freshly prepared, but nonsterile solution of blood plasma. During the early stages of the run, the rate of acid production appears linear and the amount of acid produced is relatively insignificant. However, as the run progresses, the acid is produced at an increasing rate. The system behaves as if the acid exerts a catalytic effect which accelerates the production of more acid.

Curve B was obtained using a similar solution of blood plasma except that it was left to stand in an open vessel at room temperature for 2 hr. The higher initial uptake of the base titrant indicates that more free acids were present in this solution at the beginning of the run than in the fresh solution of plasma. The plasma had obviously undergone some deterioration during the 2-hr. period to produce the additional acid. Apparently, due to autocatalytic effects, the rate of acid production is noticeably faster at corresponding time periods than in the previous run with the fresh plasma solution.

During the initial stages, the rate of uptake of the base titrant is 2.0 μm./L./min. and 6.5 μm./L./min. for curves A and B, respectively. After 2 hr., the rates increase markedly to 9.6 μm./L./min. for curve A and 18.8 μm./L./min. for curve B. Thus, it is highly probable that the effect due to plasma deterioration might largely influence or even mask hydrolytic processes especially of substrates which hydrolyze slowly.

Figure 3 shows the effect due to the loss of CO_2 from the reaction mixture. The rate of CO_2 loss is 2.8 μm./L./min. and it is linear in the range studied. It is slightly greater than the initial rate of plasma deterioration for the freshly prepared plasma solution, but less than that for the plasma solution which was aged for 2 hr. At the point N_2 on the curve, a nitrogen flush was introduced into the system. This resulted in a more rapid loss of CO_2 as indicated by the higher rate of uptake of the acid titrant.

The consequences of plasma deterioration and loss of CO_2 are opposite in effect. Usually, the rate of CO_2 loss exceeds the rate of plasma deterioration during the early stages of a run. However, as the run progresses, the rate of plasma deterioration accelerates markedly, presumably by autocatalysis,

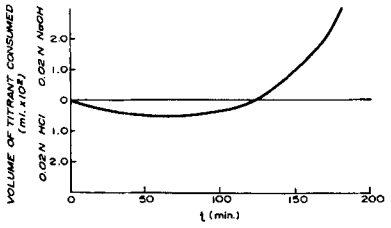

Fig. 4—Data showing the simultaneous effects of plasma deterioration and CO_2 loss with time. Up to about 2 hr., the effect of CO_2 loss predominates, after which time the effect of plasma deterioration becomes more pronounced. (Plasma concentration, 0.5%.)

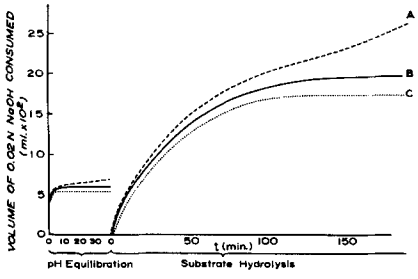

Fig. 5—Plots showing the effects of plasma deterioration and CO_2 loss on the enzymic hydrolysis rate of acetylcholine. Curve A shows the effect of plasma deterioration, curve C the effect of CO_2 loss, and curve B the true hydrolysis rate of the substrate. Curve B was obtained by use of the experimental procedure described in the text. (Plasma concentration, 0.5%.)

and becomes far greater than the rate of CO_2 loss. To demonstrate these effects, which are shown in Fig. 4, two separate pH stat units were used with a common titration assembly. One unit contained a 0.02 *N* NaOH titrant which followed the rate of plasma deterioration while the other unit, with a 0.02 *N* HCl titrant, followed the rate of CO_2 loss.

The data represent the difference between the amounts of base and acid titrants consumed during the run. It can be seen that up to about 2 hr., the rate of CO_2 loss predominates. Beyond this time, the rate of plasma deterioration becomes increasingly faster and gradually exceeds the rate of CO_2 loss. Thus, the effect on the over-all hydrolysis rate of a substrate which cleaves slowly would probably be

TABLE I—INITIAL HYDROLYSIS RATES OF ACETYLCHOLINE, BENZOYLCHOLINE, AND PHENYL BUTYRATE IN 0.5% HUMAN BLOOD PLASMA DETERMINED AT DIFFERENT EXPERIMENTAL CONDITIONS

Substrate →	Hydrolysis Rate, μm./L./min.								
	Acetylcholine			Benzoylcholine			Phenyl Butyrate		
Method →	pH Stat	Spectrophotometer	% Dev.	pH Stat	Spectrophotometer	% Dev.	pH Stat	Spectrophotometer	% Dev.
Experimental Condition									
Closed titration assembly with nonsterile plasma solution. (Effect of plasma deterioration.)	57.3	...	+ 7.5	121	...	+2.5	106	...	+2.9
Closed titration assembly with sterile plasma solution.	53.1	...	...	118	120	...	103	104	...
Open titration assembly with sterile plasma solution. (Effect of CO_2 loss.)	47.6	...	−11.3	108	...	−8.5	93.1	...	−9.7

first a slowing down of the rate up to the 2-hr. period, followed by an acceleration of the rate. The hydrolysis reaction then appears to proceed beyond the stoichiometric level because the acid from plasma deterioration continues to be titrated.

Jensen-Holm (16), who was perhaps the first to note that a nonspecific acid production takes place in most tissue homogenates and blood solutions, reported that: "By keeping the system anaerobic (bubbling nitrogen through it for 20 minutes and then maintaining an atmosphere of nitrogen in a half-closed system) the spontaneous, unspecific acid liberation can be not only greatly reduced, but also often converted into a fairly constant base production." It seems apparent that by bubbling nitrogen through the system, he was driving off CO_2 (resulting in an increased basicity of the system) at a rate which was nearly the same or which exceeded the rate of acid production.

Figure 5 shows the effects of plasma deterioration and CO_2 loss on the hydrolysis rate of acetylcholine in a 0.5% solution of blood plasma. The figure also shows data which attest to the utility of the proposed experimental procedure and the modified pH stat titration assembly for minimizing these effects.

The effect due to plasma deterioration is shown by curve A. It can be seen that a pH equilibration could not be attained due to the slow but continuous titration of the acid produced. Upon the addition of acetylcholine, the hydrolysis reaction proceeds at a rate faster than normal because the acid resulting from the deterioration of plasma is titrated in addition to the acid produced by the hydrolytic process. Curve A also shows that the consumption of base titrant continues far beyond the stoichiometric amount equivalent to complete hydrolysis of the substrate. The excessive uptake of titrant is obviously due to the acid produced as the result of plasma deterioration.

Curve C shows the effect due to the loss of CO_2 from the reaction mixture. Although a pH equilibration seemed to have been attained prior to the addition of acetylcholine, the pH of the plasma solution was actually above 7.4 due to the loss of some CO_2 from the plasma solution. Since the pH stat does not begin to record the amount of titrant delivered until the pH of the solution is returned to 7.4, there is a slight lag between the time the substrate is added and the start of the titration. The hydrolysis reaction then proceeds at a rate slower than normal, and reaches a level below that for complete hydrolysis of the substrate. Obviously, the base formed as the result of CO_2 loss also serves to titrate the acid produced by the hydrolysis reaction. Thus, less of the base titrant is needed for the over-all reaction.

The data obtained using the proposed experimental procedure and the modified pH stat titration assembly are shown by curve B. The pH equilibration of the plasma solution is attained readily and remains stable until the substrate is added. The pH stat, which is now unaffected by errors due to plasma deterioration and loss of CO_2, plots the true course of the hydrolytic process and reaches an equilibrium when the amount of base titrant consumed is nearly the same as that theoretically required by the hydrolysis reaction.

The enzymic hydrolysis rates for benzoylcholine and phenyl butyrate, determined both by the potentiometric titration method (pH stat) and the spectrophotometric method (Cary model 15), are shown in Table I. The pH stat data for acetylcholine are also presented for the purpose of comparison. The "initial" rates were determined from the slopes of the essentially linear portions of the titrator plots in the initial 10-min. periods.

The results obtained by the use of the closed pH stat titration vessel and sterile plasma solutions correlate well with the spectrophotometric results. The results, which were influenced by the effects of plasma deterioration and the loss of CO_2, were higher and lower, respectively, than the results obtained spectrophotometrically. It will be noted that the percent deviation from normal becomes smaller the faster the hydrolysis reaction. For example, in the case of benzoylcholine which hydrolyzes 1.14 and 2.22 times faster than phenyl butyrate and acetylcholine, respectively, the deviation due to plasma deterioration is 2.5% and that due to the loss of CO_2 is 8.5%. On the other hand, in the case of acetylcholine, the slowest hydrolyzing substrate of the three, the deviation is 7.5% for plasma deterioration and 11.3% for CO_2 loss. Thus, while the effects of plasma deterioration and CO_2 loss may not be highly significant in studies of rapidly hydrolyzing substrates, they may have considerable influences on the hydrolysis of substrates which cleave at relatively slow rates.

SUMMARY

The effects of plasma deterioration and CO_2 loss may be significant in studies which utilize the pH stat for the determination of enzymic hydrolysis rates occurring in solutions of blood plasma. To minimize these effects, the plasma must be kept virtually free of bacterial contamination and the

reactions should be carried out in a closed system. A method for the aseptic preparation of blood plasma solutions and the utility of a closed pH stat titration assembly are described. Data are presented to show the utility of the experimental procedure for following the hydrolysis of substrates such as acetylcholine, benzoylcholine, and phenyl butyrate in solutions of blood plasma.

REFERENCES

(1) Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, **133**, 375(1940).
(2) Adams, D. H., and Whittaker, V. P., *Biochim. Biophys. Acta*, **3**, 358(1949).
(3) Kalow, W., *J. Pharmacol. Exptl. Therap.*, **104**, 122 (1952).
(4) Kalow, W., and Maykut, M. O., *ibid.*, **116**, 418(1956).
(5) Levine, R. M., and Clark, B. B., *ibid.*, **113**, 272 (1955).
(6) Netter, K. J., *Arch. Exptl. Pathol. Pharmakol.*, **235**, 498(1959).
(7) Casida, J. E., Augustinsson, K.-B., and Jonsson, G., *J. Econ. Entomol.*, **53**, 205(1960).
(8) Mudd, S., and Thalhimer, W., "Blood Substitutes and Blood Transfusions," Charles C Thomas, Springfield, Ill., 1942.
(9) Kalow, W., Genest, K., and Staron, N., *Can. J. Biochem. Physiol.*, **34**, 637(1956).
(10) Marton, A., and Kalow, W., *ibid.*, **37**, 1367(1959).
(11) Glick, D., *J. Biol. Chem.*, **125**, 729(1938).
(12) Sturge, L. M., and Whittaker, V. P., *Biochem. J.*, **47**, 518(1950).
(13) Aldridge, W. N., and Davison, A. N., *ibid.*, **51**, 62 (1952).
(14) Jensen-Holm, J., Lausen, H. H., Milthers, K., and Moller, K. O., *Acta Pharmacol. Toxicol.*, **15**, 384(1959).
(15) Jorgensen, K., *Scand. J. Clin. Lab. Invest.*, **11**, 282 (1959).
(16) Jensen-Holm, J., *Acta Pharmacol. Toxicol.*, **18**, 379 (1961).
(17) "Methods of Biochemical Analysis," vol. 4, Glick, D., ed., Interscience Publishers, Inc., New York, N. Y., 1957, p. 184.
(18) Nielands, J. B., and Cannon, M. D., *Anal. Chem.*, **27**, 29(1955).
(19) Applewhite, T. H., Martin, R. B., and Niemann, C., *J. Am. Chem. Soc.*, **80**, 1457(1958).
(20) Applewhite, T. H., Ph. D. Thesis, California Institute of Technology, Pasadena, Calif., 1957.
(21) Baines, N. J., Baird, J. B., and Elmore, D. T., *Biochem. J.*, **90**, 470(1964).
(22) Breuer, M. M., and Jenkins, A. D., *Trans. Faraday Soc.*, **59**, 1310 (1963).
(23) Mudd, S., and Thalhimer, W., "Blood Substitutes and Blood Transfusions," Charles C Thomas, Springfield, Ill., 1942, p. 267.

Notes

Radiation and the Detoxication of Chemicals

By THOMAS J. HALEY* and L. KOSTE

The detoxication of benzoate and resorcinol has been investigated in irradiated animals. In both instances detoxication was inhibited from the third to the seventh post-irradiation days. The common denominator for this action of X-irradiation appears to be co-enzyme A and/or its substrates.

EXPOSURE to X-irradiation is known to increase protein catabolism (1) and increase the free and dynamic glycine pools in the animal (2–4) but Schreier *et al.* (5) found no change in hippurate excretion during the first 3 days post-irradiation. However, Gerber and Remy-Defraigne (6) found that the irradiated liver synthesized more hippurate when exogenous glycine was excluded from the perfusion fluid than when it was present. Chiriboga (7) reported that whole body irradiation caused a decreased glucuronide excretion and suggested that further investigation was necessary to elucidate its role in the radiation syndrome. Inasmuch as both hippurate and glucuronide synthesis involve co-enzyme A and through it the detoxication of benzoate and phenolic compounds, we have investigated the detoxication of benzoate and resorcinol in irradiated rats.

METHODS AND MATERIALS

Hippurate excretion was determined in 18 Charles River CD strain female rats weighing 250 to 300 Gm. and glucuronide excretion was obtained with 6 similar rats. Total 24-hr. urine volumes based upon 137 collections was 22.1 ± 10.5 ml. with a range of 6.5 to 55.8 ml. Urinary output depended upon water consumption which also had the same degree of variation. Normal excretion values for both hippurate and glucuronide were obtained in nine separate experiments and control values after administration of either 100 mg./Kg. of sodium benzoate or 50 mg./Kg. of resorcinol intraperitoneally in three separate experiments after which the animals received 600 rads acute whole body X-irradiation. In all cases urine was collected over a 7-day period by the method of Haley *et al.* (8). Urinary hippurate was determined with an Aminco-Bowman spectrophotofluorometer using the method of Ellman *et al.* (9) and glucuronide was determined by the method of Mead *et al.* (10). Quadruplicated samples of each urine specimen were analyzed. The results were analyzed statistically by the Student *t* test for significance. The radiation characteristics of the industrial unit were: 250 KVP; 15 ma.; FOD 55 cm.; filters 0.21 mm. Cu inherent; 0.5 mm. Cu parabolic and 1 mm. Al; HVL 1.95 mm. Cu; dose rate measured in air, 24.97 rads/min. The machine was calibrated prior to and after each experiment with a Victoreen thimble *r*-meter and during exposure with a Radacon unit.

Received June 2, 1967, from the Laboratory of Nuclear Medicine and Radiation Biology, Department of Biophysics and Nuclear Medicine, School of Medicine, University of California, Los Angeles, CA 90007
Accepted for publication July 26, 1967.
This investigation was supported by contract AT(04-1)-GEN-12 between the U. S. Atomic Energy Commission and the University of California.
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REFERENCES

(1) Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, **133**, 375(1940).
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(21) Baines, N. J., Baird, J. B., and Elmore, D. T., *Biochem. J.*, **90**, 470(1964).
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By THOMAS J. HALEY* and L. KOSTE

The detoxication of benzoate and resorcinol has been investigated in irradiated animals. In both instances detoxication was inhibited from the third to the seventh post-irradiation days. The common denominator for this action of X-irradiation appears to be co-enzyme A and/or its substrates.

EXPOSURE to X-irradiation is known to increase protein catabolism (1) and increase the free and dynamic glycine pools in the animal (2–4) but Schreier *et al.* (5) found no change in hippurate excretion during the first 3 days post-irradiation. However, Gerber and Remy-Defraigne (6) found that the irradiated liver synthesized more hippurate when exogenous glycine was excluded from the perfusion fluid than when it was present. Chiriboga (7) reported that whole body irradiation caused a decreased glucuronide excretion and suggested that further investigation was necessary to elucidate its role in the radiation syndrome. Inasmuch as both hippurate and glucuronide synthesis involve co-enzyme A and through it the detoxication of benzoate and phenolic compounds, we have investigated the detoxication of benzoate and resorcinol in irradiated rats.

METHODS AND MATERIALS

Hippurate excretion was determined in 18 Charles River CD strain female rats weighing 250 to 300 Gm. and glucuronide excretion was obtained with 6 similar rats. Total 24-hr. urine volumes based upon 137 collections was 22.1 ± 10.5 ml. with a range of 6.5 to 55.8 ml. Urinary output depended upon water consumption which also had the same degree of variation. Normal excretion values for both hippurate and glucuronide were obtained in nine separate experiments and control values after administration of either 100 mg./Kg. of sodium benzoate or 50 mg./Kg. of resorcinol intraperitoneally in three separate experiments after which the animals received 600 rads acute whole body X-irradiation. In all cases urine was collected over a 7-day period by the method of Haley *et al.* (8). Urinary hippurate was determined with an Aminco-Bowman spectrophotofluorometer using the method of Ellman *et al.* (9) and glucuronide was determined by the method of Mead *et al.* (10). Quadruplicated samples of each urine specimen were analyzed. The results were analyzed statistically by the Student *t* test for significance. The radiation characteristics of the industrial unit were: 250 KVP; 15 ma.; FOD 55 cm.; filters 0.21 mm. Cu inherent; 0.5 mm. Cu parabolic and 1 mm. Al; HVL 1.95 mm. Cu; dose rate measured in air, 24.97 rads/min. The machine was calibrated prior to and after each experiment with a Victoreen thimble *r*-meter and during exposure with a Radacon unit.

Received June 2, 1967, from the Laboratory of Nuclear Medicine and Radiation Biology, Department of Biophysics and Nuclear Medicine, School of Medicine, University of California, Los Angeles, CA 90007
Accepted for publication July 26, 1967.
This investigation was supported by contract AT(04-1)-GEN-12 between the U. S. Atomic Energy Commission and the University of California.
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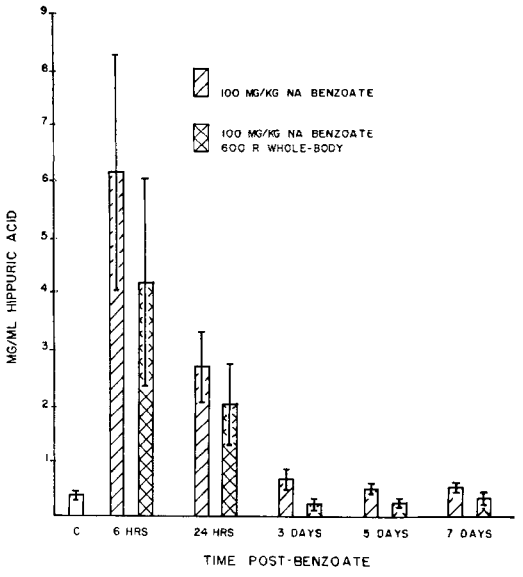

Fig. 1—Hippurate excretion in nonirradiated and irradiated rats. Note nonsignificant decrease in hippurate excretion during the first 24 hr. and significant decrease (P = 0.001) at the third through the seventh days post-irradiation.

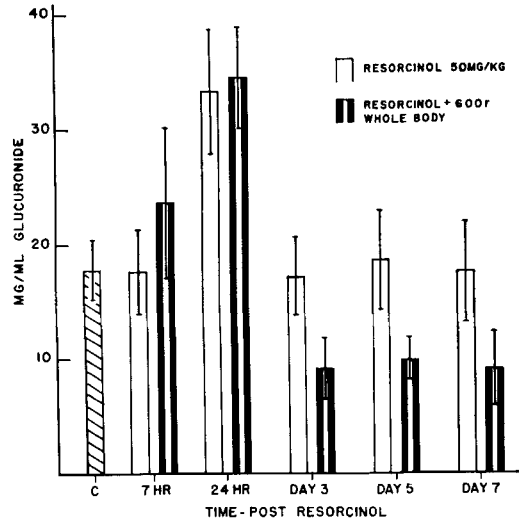

Fig. 2—Effect of 600 rads whole body X-irradiation on glucuronide excretion following 50 mg./Kg. of resorcinol. Compared to the normal excretion all irradiated values from the third to seventh days are significant at P = 0.001. The increased excretion at 24 hr. is significant at P = 0.01.

RESULTS AND DISCUSSION

The results obtained are illustrated in Figs. 1 and 2. In confirmation of Schreier *et al.* (5), it was found that hippuric acid was a normally occurring metabolite in rat urine, but contrary to their report it was observed that X-irradiation did affect hippurate excretion on the third day post-irradiation. Furthermore, there was no significant difference in hippurate excretion between nonirradiated rats until that time. From the third to the seventh post-irradiation days inclusive, the irradiated group's hippurate excretion was significantly depressed ($P = 0.001$), although both groups were again approaching the normal hippurate excretion level by the seventh day.

In confirmation of Chiriboga (7), it was observed that urinary glucuronide excretion was significantly depressed ($P = 0.001$) from the third through the seventh post-irradiation days and had not as yet begun to approach the normal level. Of interest is the observation that both hippurate and glucuronide excretion were unaffected until the third post-irradiation day, indicating that the detoxication pathways for both benzoate and resorcinol seem to be functional in the early post-irradiation period. Perhaps the failure occurring on the third day is related to the intestinal malabsorption of glucose and B-complex vitamins previously reported (11–14) or to exhaustion of the enzyme system(s) involved in the detoxication processes, or both. A clue to one aspect of the problem was the report of Hanel and Wilian-Ulrich (15) that acetylation of sulfanilamide was also depressed on the third day. In every instance the common mechanism of detoxication involved co-enzyme A. Moreover, decreased fatty acid synthesis on the third day also involves this co-enzyme (15). Perhaps other metabolic pathways are activated by irradiation, thus gradually decreasing the co-enzyme's activity or the availability of the substrate required for active detoxication. On the other hand, it is possible that ionizing radiation or the products it produces in cell cytoplasm through free radical formation may have a direct effect on co-enzyme A and/or its substrate. Both are distinct possibilities because ionizing radiation affects sulfhydryl groups and one of the function entities in co-enzyme A is a sulfhydryl group. All of these points remain to be elucidated, if we are to understand why radiation depresses detoxication mechanisms.

REFERENCES

(1) White, J., Burr, B. E., Cool, H. T., David, P. W., and Ally, M. S., *J. Natl. Cancer Inst.*, **15**, 1145(1955).
(2) Schreier, K., Di Ferrante, N., Gaffney, G. W., Hempelman, L. H., and Altman, K., *Arch. Biochem. Biophys.*, **50**, 417(1954).
(3) Haberland, G. L., Schreier, K., Altman, K., and Hempelman, L. H., *Biochim. Biophys. Acta*, **25**, 237(1957).
(4) Jouanovic, M., and Suecenski, N., *Acta Med. Jugoslav.*, **17**, 46(1963).
(5) Schreier, K., Altman, K., and Hempelman, L. H. *Proc. Soc. Expil. Biol. Med.*, **87**, 61(1954).
(6) Gerber, G. B., and Remy-Defraigne, J., *Intern. J. Radiation Biol.*, **57**, 53(1963).
(7) Chiriboga, J., *Nature*, **198**, 803(1963).
(8) Haley, T. J., Koste, L., and Duncan, F., *J. Pharm. Sci.*, **54**, 1391(1965).
(9) Ellman, G. L., Burkhalter, A., and Dorr, J., *J. Lab. Clin. Med.*, **7**, 813(1961).
(10) Mead, J. A. R., Smith, J. N., and Williams, R. T., *Biochem. J.*, **68**, 61(1958).
(11) Detrick, L. E., Upham, H. C., Highby, D., Debley, V., and Haley, T. J., *Radiation Res.*, **2**, 483(1955).
(12) Detrick, L. E., Upham, H. C., Dunlap, A. K., and Haley, T. J., *ibid.*, **15**, 520(1961).
(13) Detrick, L. E., Upham, H. C., Springsteen, R. W., and Haley, T. J., *Intern. J. Radiation Biol.*, **7**, 161(1963).
(14) Detrick, L. E., Upham, H. C., Springsteen, R. W., McCandless, R. G., and Haley, T. J., *Radiation Res.*, **21**, 186 (1964).
(15) Hanel, H. K., and Wilian-Ulrich, L., *Intern. J. Radiation Biol.*, **4**, 366(1959).

Metabolism of 2-Methyl-2-(4-acetaminophenoxy)propane

By A. KLUTCH* and M. BORDUN†

The metabolism of the *tert*-butyl analog of acetophenetidin (phenacetin USP XVII) has been investigated. A metabolite which is hydroxylated in the alkyl side chain was isolated.

THE MAJOR metabolic pathway of acetophenetidin (phenacetin USP XVII) involves *O*-dealkylation to *N*-acetyl-*p*-aminophenol (acetaminophen, NF XII) (1). Studies in these laboratories indicated that the *tert*-butyl homolog of acetophenetidin undergoes very little *O*-dealkylation to *N*-acetyl-*p*-aminophenol but that it possesses analgesic and antipyretic activity (2, 3). Although toxicity studies have ruled out the use of this compound as a substitute for acetophenetidin, the authors have investigated its metabolism because these studies could provide interesting data as to structure–metabolism relationships. The identification of a metabolite which is hydroxylated in the side chain is reported.

EXPERIMENTAL

Chemicals—2 - Methyl - 2 - (4 - acetaminophenoxy)propane [*p-tert*-butoxy acetanilid] (I) and 2-methyl-2-(4-acetaminophenoxy) propanol (II) were synthesized in these laboratories.[1] *N*-Acetyl-*p*-aminophenol was supplied by Eastman's Distillation Products, Inc., Rochester, N. Y.

Animal Experiments—2-Methyl-2-(4-acetaminophenoxy)propane was administered orally to three adult dogs (50 mg./Kg./day) for 2 days, a total of 3.29 Gm. The urine collected (1270 ml.) during the 2 days was combined. Urine was also collected for the day prior to drug administration.

Chemomatographic Analysis of the Urine—Urine was hydrolyzed and extracted according to the method described earlier in connection with urine obtained from animals treated with acetophenetidin (4). For the preliminary studies, the residue obtained after evaporation of the organic solvent was chromatographed on paper with system *1* previously employed for the separation of acetophenetidin metabolites (4). More recent studies were carried out with thin-layer chromatography as previously described (4). The solvent systems used for thin-layer chromatography are shown in Table I. Visualization of compounds was achieved with ultraviolet light.

Isolation of the Metabolite—A mixture of 800 ml. of urine, 800 ml. of pH 5, 0.2 *N* acetate buffer (5), and 800 ml. of β-glucuronidase[2] was incubated for 18 hr. at 37.5°. The urine was then mixed with 60 ml. of 4 *N* NaOH and 800 ml. of 1.0 *M* phosphate buffer (pH 7.4),[3] and extracted 3 times with 3 L. of $CHCl_3$. The organic extracts were combined and evaporated at room temperature under vacuum. The residue (1.3 Gm.) was dissolved in 5 ml. $CHCl_3$ and chromatographed on an aluminum oxide (10 Gm.) column (acid alumina, DG 4 grade, 100–200 mesh as supplied by Bio Rad Co., Los Angeles, Calif.). The column was washed with 1 ml. of chloroform, 15 ml. of benzene, 150 ml. of benzene–chloroform (35:65 v/v), and 400 ml. of chloroform–ethyl acetate (85:15 v/v). Each fraction was examined chromatographically for the presence of metabolite. The solvent was evaporated from the chloroform–ethyl acetate fraction and the residue (200 mg.) was treated 6 times with 100 ml. of boiling cyclohexane. An insoluble black residue was discarded and the cyclohexane extracts were combined. The cyclohexane was evaporated and the residue was recrystallized 6 times from benzene. More metabolite was obtained from the mother liquors, and a total of 90 mg. of pure product was obtained.

TABLE I—ANALYSIS OF TEST COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

Compd.	System *1*[a]	System *2*[b]	System *3*[c]
2-Methyl-2-(4-acetaminophenoxy)propane	0.87	0.78	0.94
2-Methyl-2-(4-acetaminophenoxy) propanol	0.23	0.29	0.52
N-Acetyl-*p*-aminophenol	0.08	0.10	0.35

[a] System *1*, previously described (4). [b] The upper phase of a toluene–benzene–water–acetic acid (2:2:1:2) mixture for thin-layer chromatography. [c] The lower phase of a chloroform–methanol–water–acetic acid (20:10:20:1) mixture for thin-layer chromatography.

Acidic Cleavage of Compounds—The acidic cleavage of compounds I and II was done by treating 200 mcg. of each substance with 1 ml. of 2 *N* HCl at room temperature. After a time lapse of 10 min. to 24 hr., portions of the solution were extracted with 10 ml. of a methylene chloride–isopropanol–water (75:25:2) mixture. The solvent was evaporated and the resulting residues were chromatographed on thin-layer plates.

Gas Chromatography—For gas chromatographic analysis of 2-methyl-2-(4-acetylaminophenoxy)propane and the hydroxylated metabolite, urine was enzyme hydrolyzed and extracted as described above. For the analyses of *N*-acetyl-*p*-aminophenol, hydrolyzed urine was extracted with the methylene chloride–isopropanol mixture described above. The analyses were carried out on a Barber-Coleman model 10 gas chromatograph equipped with a Sr-90 detector. The column packing [SE-30, 6% coated on Gas Chrom S, 80–100 mesh supplied by Applied Science Labs., Inc., College Point, Pa., or a mixture of SE-30 (1%)–Carbowax 20 M (1%) coated on Anakrom AS, 80–90 mesh, supplied by Analabs, Inc. Hamden, Conn.] was packed in a U-shaped, glass column, 120 cm. in length and 3.5 mm. i.d.

Received April 28, 1967, from the Wellcome Research Laboratories, Burroughs Wellcome and Co., Inc., Tuckahoe, NY 10707

Accepted for publication July 5, 1967.

The authors wish to acknowledge the advice, aid, and encouragement of Dr. John J. Burns during the development of this work.

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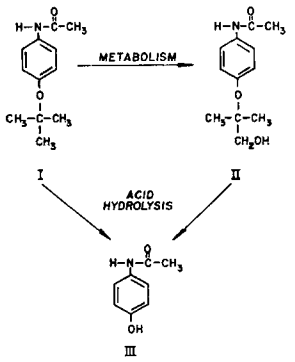
[1] Harfenst, M., to be published.

[2] Ketodase, Warner-Chilcott, Morris Plains, N. J.

[3] KH_2PO_4 (272 Gm.) and 5 *N* KOH (320 ml.) diluted to 2 L. with water.



The transformations of 2-methyl-2-(4-acetaminophenoxy) propane and 2-methyl-2-(4-acetaminophenoxy) propanol

Scheme I

TABLE II—ANALYSIS OF URINE BY GAS CHROMATOGRAPHY

Compd.	Retention Time, min.	Urinary Concn., mcg./ml.	Dose % Recovery in Dogs
2-Methyl-2-(4-acetaminophenoxy)propane	2.1	23	0.9
2-Methyl-2-(4-acetaminophenoxy)propanol[a]	6.1	295	11.4
N-Acetyl-*p*-aminophenol[b]	12.5[c]	101	3.9

[a] The SE-30 column. [b] The SE-30 Carbowax 20 M column. [c] *N*-Acetyl-*p*-aminophenol (retention time = 3 min.) has too broad a peak for accurate quantitation using column a.

The inlet pressure was 25 psig and the outlet pressure was atmospheric. The instrument was operated isothermally at 150° for the SE-30 column and at 200° for the SE-30–Carbowax column. The samples, dissolved in 2–5 μl. of ethyl acetate varied from 0.5–1.5 mcg. The concentration of compound was determined from the proportion of peak area of unknown to peak area of standard (appropriate reference compound added to control urine). Peak areas were calculated from the product of peak height and width at one-half the peak height.

RESULTS AND DISCUSSION

Initial chromatographic studies on extracts of urine obtained from dogs which had received oral doses of 2-methyl-2-(4-acetaminophenoxy)propane, indicated the presence of a metabolite. The chromatographic mobility of the substance was different from that of drug or *N*-acetyl-*p*-aminophenol. Since the compound was freed by treatment with β-glucuronidase it appeared likely that it was hydroxylated.

In order to identify the metabolite, it was isolated as described under *Experimental*. The crystals obtained melted at 120.5–121.5°. The elemental analysis for carbon-hydrogen was in agreement with a hydroxylated derivative of the *tert*-butoxy compound administered.

Anal.—Calcd. for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.68. Found: C, 64.90; H, 7.70.

The location of the hydroxyl group was now considered. Since the metabolite did not react with the Pauly reagent (6), it appeared that the compound was hydroxylated in the side chain.

The metabolite and the drug administered were subjected to mild acid hydrolysis and the products were examined chromatographically. The drug was hydrolyzed completely to *N*-acetyl-*p*-aminophenol in about 15 min. and the metabolite gave about an 80% yield of the same phenol after 24 hr. Since the same product was formed from both, it was established that the hydroxylation was associated with the alkyl moiety.

Authentic 2-methyl-2-(4-acetaminophenoxy) propanol (II) was now synthesized[1] and the metabolite and the authentic synthetic sample were shown to be identical in the following way. A mixed melting point determination carried out on the metabolite and the synthetic compound showed no depression of the melting point. The infrared spectra of the metabolite and of II were identical.

The chromatographic mobilities of the three compounds are shown in Table I.

The structures of 2-methyl-2-(4-acetaminophenoxy)propane (I), the metabolite (II), and *N*-acetyl-*p*-aminophenol (III) are shown in Scheme I.

During the studies on the metabolism of 2-methyl-2-(4-acetaminophenoxy) propane, small amounts of a substance with the chromatographic mobility of *N*-acetyl-*p*-aminophenol were observed. Gas chromatography was utilized to measure the amounts of unchanged drug, the hydroxylated metabolite, and *N*-acetyl-*p*-aminophenol excreted in the urine. The results are shown in Table II.

The results for the small amount of *N*-acetyl-*p*-aminophenol in the urine of animals treated with the *tert*-butoxy compound contrasts with larger amounts of the dealkylated compound excreted by animals treated with acetophenetidin (1). These results, however, were not investigated further.

The substitution of a *tert*-butyl group in the acetophenetidin molecule inhibited the *O*-dealkylation of this compound. These observations can be applied in the synthesis of potential substitutes for drugs when the slowing down of the metabolic process would help in providing a longer duration of therapeutic action. This approach to the development of new drugs has already been discussed in connection with another group of compounds (7).

REFERENCES

(1) Brodie, B. B., and Axelrod, J., *J. Pharmacol. Exptl. Therap.*, **97**, 58(1949).
(2) Conney, A. H., Sansur, M., Soroko, F., Koster, R., and Burns, J. J., *ibid.*, **151**, 133(1966).
(3) Conney, A. H., Sansur, M., and Harfenist, M., *Pharmacologist*, **7**, 160(1965).
(4) Klutch, A., Harfenist, M., and Conney, A. H., *J. Med. Chem.*, **9**, 63(1966).
(5) "Biochemists Handbook," Van Nostrand and Co., Inc., Princeton, N. J., 1961, p. 31.
(6) "Chromatographic and Electrophoretic Techniques," Smith, I., ed., 2nd ed., vol. I, Interscience Publishers, Inc., New York, N. Y., 1960, pp. 296–297.
(7) Klutch, A., and Bordun, M., *J. Med. Chem.*, to be published.

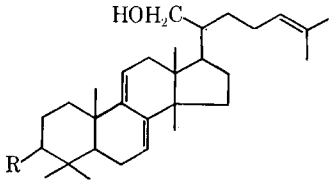
Isolation and Identification of Eburicoic Acid from *Fomes pinicola*

By KIRTI SHETH*, PHILIP CATALFOMO, and LEO A. SCIUCHETTI†

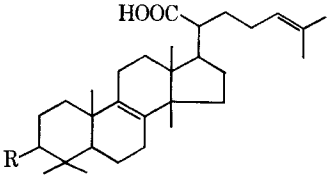
The petroleum ether extract of *Fomes pinicola*, upon chromatography on an alumina column, yielded ergosterol and a crystalline compound which was identified as eburicoic acid by means of IR and NMR spectroscopy. Ultraviolet spectrum analysis indicated that eburicoic acid was contaminated with trace impurities of dehydroeburicoic acid.

BASIDIOMYCETES belonging to family *Polyporaceae* have been found to be rich sources of triterpene acids. The genera *Polyporus* and *Fomes* are noteworthy members among this group. Kariyone and Kurono (1), in 1940, isolated eburicoic acid, a triterpenic acid, from *Fomes officinalis*. Since then it has been isolated from a number of other species such as *P. sulphureus, P. anthracophilus, P. eucalyptorum, etc.*, when these species were grown on a synthetic medium (2). Besides eburicoic acid, its hydroxylated derivatives such as sulphurenic acid and tumulosic acid have also been encountered (3, 4). When grown on a synthetic medium, the mycelia of *P. sulphureus* produced eburicoic acid in huge yields (as much as 50% or more of the dried mycelial weight). This ready availability of eburicoic acid has led to extensive investigation of its biogenesis on the one hand (5) and degradation to steroids of various structures for potential medicinal usage on the other (6).

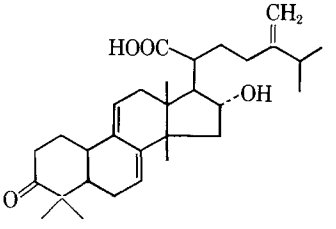
Polyporus pinicola is now recognized as the same species as *Fomes pinicola* (7). From this species, Halsall and co-workers (8–11) have isolated, among other steroidal derivatives, five closely related triterpenes. They are 21-hydroxylanosta-7,9(11)-24-triene-3-one (I*a*), lanosta-7,9(11)-24-triene-3β,21-diol (I*b*), 3α-oxylanosta-8,24-diene-21-oic acid (II*a*), pinicolic acid A (II*b*), and polyporenic acid C (III). Eburicoic acid (IV*a*), which can be viewed as a reduced form of polyporenic acid C (III), has not been reported to occur in *F. pinicola*. This fact, coupled with the widespread occurrence of eburicoic acid in species of *Fomes* and *Polyporus*, led to the investigation of this species. This paper reports that eburicoic acid indeed occurs in *F. pinicola*.



I*a*, R = O=
I*b*, R = HO, H



II*a*, R = HO, H
II*b*, R = O=



III

RESULTS AND DISCUSSION

The petroleum ether extract of *F. pinicola*, upon thin-layer chromatography, revealed a complex mixture of sterols–triterpenes. Hence the mixture was chromatographed over a column of alumina. Elution with chloroform removed a sterol which after several crystallizations from diluted ethanol gave a homogeneous compound melting at 159–161°. It was identified as ergosterol from its characteristic UV spectrum with $\lambda_{max.}^{CHCl_3}$ at 264, 273, 283, and 294 mμ. Undepressed mixed melting point with an authentic specimen of ergosterol and their superimposable IR spectra further verified the identity of the isolate as ergosterol.

Continued elution of the column with methanol yielded a highly polar compound which after repeated crystallizations from dilute ethanol gave fine needles of a compound, m.p. 280–283°. It gave a positive Liebermann-Burchard color reaction and it was identified as eburicoic acid (IV*a*) from the following evidence.

The IR spectrum (KBr pellet) of the isolate (IV*a*) showed strong absorption bands at 3430 (—OH), a group of small bands at 2700, a strong absorption band at 1700 (—COOH), and bands at 1640 and 887 cm.$^{-1}$ (>=CH$_2$). In the IR spectrum of the acetyl derivative (IV*b*), the absorption band in the 3500 cm.$^{-1}$ region was eliminated while the bands due to free carboxyl and terminal methylene groups were maintained. An additional band in the carbonyl region at 1725 cm.$^{-1}$ indicated the esterification of the hydroxyl group in IV*b*. On the other hand, the IR spectrum of the methyl ester (IV*c*) showed the absorption bands due to free hydroxyl (3420 cm.$^{-1}$) and terminal methylene (1640 and 885 cm.$^{-1}$) groups but it lacked absorptions due to a free carboxyl group. Instead, a single strong absorption band at 1730 cm.$^{-1}$ indicated the esterification of the free

Received June 19, 1967, from the Department of Pharmacognosy, School of Pharmacy, Oregon State University, Corvallis, OR 97331

Accepted for publication August 16, 1967.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by grant GM-10963-03 from the U. S. Public Health Service, Bethesda, Md.

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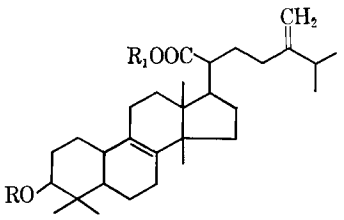
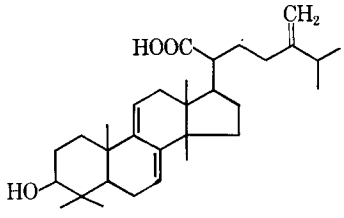


IV



V

IV*a*, eburicoic acid; R = R_1 = H
IV*b*, acetyl eburicoic acid; R = CH_3CO, R_1 = H
IV*c*, methyl eburicoate; R = H, R_1 = CH_3

carboxyl group of IV*a*. The NMR spectra of methyl eburicoate (IV*c*) and acetyl eburicoic acid (IV*b*) were in complete agreement with those recorded by Fried *et al.* (3).

Finally, the IR spectra of the isolate (IV*a*) and its methyl ester (IV*c*) were superimposable over those of eburicoic acid and its methyl ester,[1] thus confirming the identity of the isolate as eburicoic acid.

The UV spectrum of the isolate (IV*a*) showed a trio of absorption bands of low intensity but characteristic $\lambda_{max.}^{MeOH}$ at 234, 243, and 251 mμ. These absorption bands are ascribed to the presence of multicyclic terpenes with a $\Delta^{7,9(11)}$ conjugated diene system. These dienes, often called dehydro compounds, occur frequently as adjuncts to the more common terpenes with a $\Delta^{8,9}$ double bond. Holker *et al.* (12) have commented upon the co-occurrence of dehydroeburicoic acid (V) along with eburicoic acid (IV*a*) and also on the very great difficulty of separating these compounds from each other. Hence, the characteristic but low intensity UV absorption bands in the sample of the isolate must be ascribed to the traces of dehydroeburicoic acid in it.

The melting points of eburicoic acid isolated from different sources and its derivatives prepared by different workers vary considerably (*cf.* under *Experimental*). These variations can be ascribed possibly to the differing amounts of dehydroeburicoic acid which may be present in the samples of eburicoic acid.

EXPERIMENTAL

Melting points were determined using a Kofler micromelting point apparatus. Ultraviolet spectra were recorded on a Beckman spectrophotometer, model DB. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer. NMR spectra were determined in $CDCl_3$ on a Varian Associates A-60 instrument through the courtesy of Dr. F. T. Bond, Oregon State University.

[1] Eburicoic acid and methyl eburicoate were obtained through the courtesy of the Squibb Institute for Medical Research, New Brunswick, N. J., and Professor Josef Fried, Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill., respectively.

Material—Naturally occurring carpophores of *Fomes pinicola*, growing on larch trees (*Larix occidentalis*) in Linn County, Oreg., were collected during the summer of 1966. The collection was identified by Dr. Lewis F. Roth, Oregon State University.

Extraction—Approximately 250 Gm. of fungus was cut up in small bits and pulverized in an Abbé mill. The powdered material was exhaustively extracted with petroleum ether in a Soxhlet extractor for 48 hr. The extract was concentrated under reduced pressure at 40° to a small volume of syrupy residue.

Isolation of Ergosterol—About one-half the quantity of the concentrated petroleum ether extract was dissolved in 75 ml. of benzene and the solution was chromatographed on an alumina[2] column (500 Gm., 35 × 5 cm.). Careful elution with chloroform yielded a fraction which deposited crystals upon evaporation. The crystals were collected and crystallized once from ethyl acetate and then thrice from dilute ethanol. Colorless plates were obtained m.p. 159–161°, $[\alpha]_D$ = −128° ($CHCl_3$). [Reported m.p. 163°, $[\alpha]_D$ = −133° ($CHCl_3$) (13).]

Isolation of Eburicoic acid—After ergosterol was eluted, the column was developed with solvents of increasing polarity. Methanol eluted a chalky component which was found to contain inorganic impurities. These were removed by treating the compound with boiling water for 10 min. and then collecting it on a Büchner funnel. The compound thus collected was crystallized four times from dilute ethanol. Colorless needles of eburicoic acid (IV*a*) were obtained, m.p. 280–283°, $[\alpha]_D$ = +31° (pyridine). [Reported m.p. 283° (1); m.p. 280° (14); m.p. 292–293°, $[\alpha]_D$ = +34° (pyridine) (15).] Mixed melting point with an authentic sample of eburicoic acid (m.p. 275–277°) did not show any depression.

Preparation of Acetyl Eburicoic Acid—Eburicoic acid (48 mg.) was dissolved in 1.0 ml. of pyridine, and 1.0 ml. of acetic anhydride was added to the solution. After refluxing the mixture for 30 min., it was poured onto crushed ice. The precipitate was collected, washed thoroughly with water, and crystallized thrice from ethanol. Acetyl eburicoic acid was obtained, m.p. 248–253°, $[\alpha]_D$ = +40° ($CHCl_3$). [Reported m.p. 240° (1); m.p. 259° $[\alpha]_D$ = +42° ($CHCl_3$) (15).]

Preparation of Methyl Eburicoate—A chilled solution of diazomethane (prepared from 5 Gm. of *N*-nitrosomethylurea) in ether was added to a chilled solution of eburicoic acid (150 mg.) in methanol (100 ml.). The mixture was allowed to evaporate spontaneously and the residue was crystallized repeatedly from *n*-hexane. Fine needles of methyl eburicoate were obtained, m.p. 138–141°, $[\alpha]_D$ = +42° ($CHCl_3$). [Reported m.p. 141° (1); m.p. 146–147°, $[\alpha]_D$ = +45° ($CHCl_3$) (15).] An admixture with an authentic sample of methyl eburicoate (m.p. 132–137°) did not show depression in melting point.

REFERENCES

(1) Kariyone, T., and Kurono, G., *J. Pharm. Soc. Japan*, **60**, 318(1940).
(2) Gascoigne, R. M., Holker, J. S. E., Ralph, B. J., and Robertson, A., *J. Chem. Soc.*, **1951**, 2346.

[2] Alumina activated, chromatographic grade, Matheson, Coleman, and Bell, East Rutherford, N. J.

(3) Fried, J., Grabowich, P., Sabo, E. F., and Cohen, A. I., *Tetrahedron*, **20**, 2297(1964).
(4) Cort, L. A., Gascoigne, R. M., Holker, J. S. E., Ralph, B. J., Robertson, A., and Simes, J. J. H., *J. Chem. Soc.*, **1954**, 3713.
(5) Dauben, W. G., Ban, Y., and Richards, J. H., *J. Am. Chem. Soc.*, **79**, 968(1957).
(6) Rosenthal, D., Grabowich, P., Sabo, E. F., and Fried, J., *ibid.*, **85**, 3971(1963).
(7) Overholts, L. O., "*Polyporaceae* of the United States, Alaska and Canada, Scientific Series XIX," University of Michigan Press, Ann Arbor, Mich., 1953, p. 42.
(8) Halsall, T. G., and Sayer, G. C., *J. Chem. Soc.*, **1959**, 2031.
(9) Beereboom, J. J., Fazakerley, H., and Halsall, T. G., *ibid.*, **1957**, 3437.
(10) Guider, J. M., Halsall, T. G., and Jones, E. R. H., *ibid.*, **1954**, 4471.
(11) Bowers, A., Halsall, T. G., and Sayer, G. C., *ibid.*, **1954**, 3070.
(12) Gascoigne, R. M., Robertson, A., and Simes, J. J. H., *ibid.*, **1953**, 1830.
(13) "Dictionary of Organic Compounds," Oxford University Press, New York, N. Y., vol. 3, 1965, p. 1350.
(14) Yamamoto, I., and Yamamoto, Y., *Bull. Agr. Chem. Soc. Japan*, **24**, 628(1960).
(15) Lahey, F. N., and Strasser, P. H. A., *J. Chem. Soc.*, **1951**, 873.

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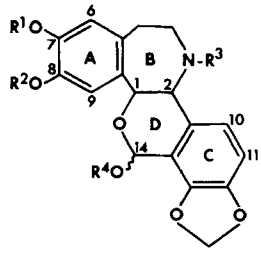
In 1837, MERCK (1) discovered a minor opium alkaloid which he named porphyroxine because it produced a red color when heated with dilute mineral acids. He suggested that this reaction might be used as a test for opium in forensic cases (2). The concentration of the alkaloid varies with the source of opium, and the color reaction is useful for determining the geographic origin of opium seized in illicit traffic (3, 4). Although there has been a great deal of interest in porphyroxine for more than 100 years and many investigators claim to have isolated it, it is only recently that it has been obtained in a form sufficiently pure for structure studies (5–7). In 1962 the empirical formula was determined by Pfeifer and Teige (6) as $C_{20}H_{21}NO_6$. In a series of investigations of the alkaloids of the genus *Papaver*, Pfeifer *et al.* (8, 9) described a total of six alkaloids which gave the characteristic red color with mineral acids. They coined the name "papaverrubines" for these compounds. All papaverrubines are secondary amines. The *N*-methyl derivatives, several of which have been found in the *Papaveraceae* family, do not give the red color reaction with acids. The gross structures of some of these alkaloids are illustrated in I–V.

Papaverrubine A (I), glaudine (III), isorhoeadine (IV), and glaucamine (V) have a *trans*-configuration at the junction of ring B and ring D, whereas papaverrubine E (I), oreodine (III), and rhoeadine (IV) have the *cis*-configuration (10–12).

Porphyroxine (II) has been established as a member of the B/D *trans*-series by methylation to glaudine (13). The only structural features of porphyroxine which have not yet been reported are the exact position of the hydroxyl group in ring A and the stereochemistry at position-14. During studies



I, $R^1 + R^2 = CH_2$, $R^3 = H$, $R^4 = CH_3$; papaverrubine A and E
II, $R^1 = H$ or CH_3, $R^2 = CH_3$ or H, $R^3 = H$, $R^4 = CH_3$; papaverrubine D (porphyroxine)
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(3) Fried, J., Grabowich, P., Sabo, E. F., and Cohen, A. I., *Tetrahedron*, **20**, 2297(1964).
(4) Cort, L. A., Gascoigne, R. M., Holker, J. S. E., Ralph, B. J., Robertson, A., and Simes, J. J. H., *J. Chem. Soc.*, **1954**, 3713.
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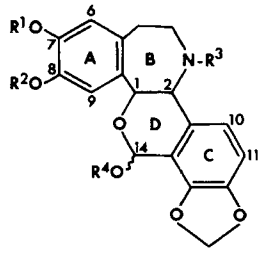

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TABLE I—CHEMICAL SHIFTS (τ) AND COUPLING CONSTANTS (c.p.s.) FOR THE PROTON RESONANCES OF PORPHYROXINE, GLAUDINE, EPIGLAUDINE, AND *N*-METHYLPORPHYROXINE IN $CDCl_3$ AT 60 Mc./sec.

Compd.	N—CH_3	14-OCH_3	Ar-OCH_3	14-H OCH-OCH_3	—OCH_2O—[a]	6-H	9-H	10- and 11-H	2-H[a]	1-H[a]
Porphyroxine	...	6.33	6.12	4.17	4.08[d], 3.93[d] J = 1.2	3.37	2.60	3.31, 3.17, 2.98, 2.84 AB quartet J = 8.5	6.13[d] J = 9	5.31[d] J = 9
Glaudine (10)	7.76	6.31	6.12 (6H)	4.23	4.08[d], 3.96[d] J = 1.5	3.33	2.63	3.28, 3.14, 3.01, 2.87 AB quartet J = 8.5	5.92[d] J = 9	4.81[d] J = 9
Epiglaudine	7.70	6.44	6.10 (6H)	4.25	4.02[d], 3.95[d] J = 1.7	3.32	2.68	3.28, 3.14, 3.02, 2.88 AB quartet J = 8.5	5.98[d] J = 9	4.43[d] J = 9
N-Methyl-porphyroxine	7.77	6.32	6.12	4.23	4.05[d], 3.93[d] J = 1.5	3.33	2.63	3.27, 3.13, 3.00, 2.86 AB quartet J = 8.5	5.93[d] J = 9	4.83[d] J = 9

[a] d, Doublet.

sprayed with dilute hydrochloric acid and heated. The IR and UV spectra were identical with those reported for porphyroxine (6). The NMR spectrum[1] in $CDCl_3$ (internal TMS standard) was consistent with the structure assigned to porphyroxine (II).

RESULTS AND DISCUSSION

The chemical shifts and coupling constants are listed in Table I. The C-1 and C-2 protons resonated as an AX quartet, $J_{1,2}$ 9 c.p.s., indicative of a B/D *trans*-configuration and in good agreement with the values reported for glaudine (10) (*cf.* Table I), glaucamine (11), and isorhoeadine (12). A smaller coupling constant ($J_{1,2}$ 2–2.5 c.p.s.) is observed for the B/D *cis*-series (10, 12).

Recently, the position of phenolic hydroxyl groups of alkaloids has been determined by deuteration of the *ortho* and/or *para* aromatic protons, followed by NMR spectroscopy (16–19). Attempts to apply this technique to porphyroxine were unsuccessful because of the instability of the alkaloid. Instead, a comparison of the chemical shifts of the aromatic protons in the phenolate ion and the undissociated phenol was used (20–22). In hexadeutero DMSO, the signals of the C-6 and C-9 protons appeared at 3.27 and 2.83 τ, respectively. Small amounts of sodium deuteroxide were added successively until no further changes took place in the chemical shifts. The results are listed in Table II. Porphyroxine

TABLE II—CHANGES IN CHEMICAL SHIFTS OF C-6 AND C-9 PROTONS OF PORPHYROXINE ON ADDITION OF NaOD IN $(CD_3)_2SO$

τ	1	2	3	4	$\Delta\tau$
C-6 proton	3.27	3.33	3.43	3.57	0.30
C-9 proton	2.83	2.99	3.03	3.25	0.42
	phenol ——→ phenolate				

could be recovered from the solution, proving that no structural changes occurred in the alkaloid as a result of the alkali treatment. The C-6 proton was shifted upfield 0.30 p.p.m. by conversion to the phenolate, and the C-9 proton was shifted 0.42 p.p.m. in the same direction. The following ranges have been reported (20) for anion shifts in alkyl and alkoxyl substituted phenols: *ortho*, 0.42–0.59 p.p.m.; *meta*, 0.19–0.39 p.p.m.; and *para*, 0.71–0.79 p.p.m. Therefore, it may be concluded that the C-6 proton in porphyroxine is *meta* to the phenolic hydroxyl group, and C-9 proton is *ortho*. Thus, the hydroxyl group in ring A was assigned to position-8.

Most of the naturally occurring papaverrubine alkaloids and their *N*-methyl analogs have a thermodynamically unstable configuration at position-14 and are readily epimerized by heating with methanol containing catalytic amounts of acid (23, 24). Porphyroxine has the same configuration at position-14 as glaudine, which can be epimerized to epiglaudine (13, 23).

The C-1 proton resonance of porphyroxine appears at a much higher τ-value than that of glaudine (Table I) due to the *N*-methyl group. *N*-Methylporphyroxine, prepared by reacting porphyroxine with formaldehyde and sodium borohydride (18, 25), gave essentially the same NMR spectrum as glaudine, except for the *Ar*-OMe group in position-8 (Table I).

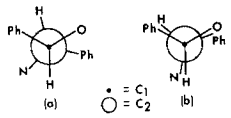


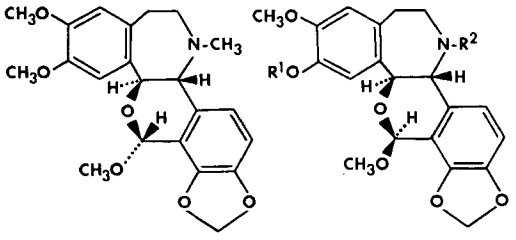
Fig. 1—Possible conformations and configurations of ring D.

Epiglaudine was prepared from glaudine[2] as described by Pfeifer *et al.* (23) and its NMR spectrum determined in $CDCl_3$ (Table I). There was no change in the coupling constants for the C-1 and C-2 proton signals, indicating that the epimerization did not involve a change in the conformation of ring D, the two possible conformations of which are shown in Fig. 1. Conformation (a) is close to the staggered form and is the more stable, while conformation (b) approaches the eclipsed form. The large coupling constants (9 c.p.s.) of the C-1 and C-2 protons (Table I) tend to indicate an approximately diaxial orientation corresponding to Fig. 1 (a) (26) and illustrated in structures VI and VII. The conversion of glau-

[1] Varian A-60A NMR spectrometer.

[2] The authors are indebted to Professor S. Pfeifer for a sample of glaudine.

dine to epiglaudine caused the 14-OMe signal to be shifted upfield 0.13 p.p.m. while a strong downfield shift of 0.38 p.p.m. was observed for the C-1 proton. This downfield shift may be explained on the basis of a 1,3-diaxial orientation of the C-1 proton with respect to the O—CH_3 group. Thus, it may be attributed to anisotropy effects of the C—OMe single bond, to effects due to C—OMe electric dipoles (27) or, perhaps equally well, to van der Waals interactions deshielding the C-1 proton (28). Therefore, one may conclude that the C-1 proton and the OMe group of epiglaudine are on the same side of the acetal oxygen bridge, with the OMe group in the axial position, as illustrated in VI. It follows that



VI VII

glaudine and porphyroxine may be represented by structure VII or its mirror image (glaudine: $R^1 = R^2 = CH_3$; porphyroxine: $R^1 = R^2 = H$).

There was no significant difference in the ORD and CD spectra[3] of glaudine, epiglaudine, and *N*-methylporphyroxine {glaudine ORD: $[\phi]_{299}23325$ (pk), $[\phi]_{282}0$ (tr.); CD: $[\theta]_{309}0$, $[\theta]_{293}15640$, $[\theta]_{269}0$, $[\theta]_{251}-5536$, $[\theta]_{244}0$}. These compounds have three asymmetric centers adjacent to benzene chromophores, and the weak effect of the configurational change at C-14 appears to be covered by strong active absorption due to the C-1 and C-2 centers. Only porphyroxine showed a slightly different spectrum in the short wavelength region {ORD: $[\phi]_{333}6010$, $[\phi]_{298}15025$ (pk), $[\phi]_{281}2394$ (tr.), $[\phi]_{242}19240$ (pk), $[\phi]_{237}18028$ (tr.), $[\phi]_{249}60092$; CD: $[\theta]_{313}0$, $[\theta]_{292}10314$, $[\theta]_{267}0$, $[\theta]_{255}0$, $[\theta]_{238}8727$}.

SUMMARY AND CONCLUSIONS

Porphyroxine has been isolated from the phenolic alkaloid fraction of opium. It belongs to the "papaverrubine" group of alkaloids which contain a 7-membered heterocyclic nitrogen ring (ring B) fused to a cyclic lactol ether (ring D). The nitrogen is a secondary amine, and the B/D ring junction of porphyroxine (II) has a *trans*-configuration. Based on NMR spectroscopy at different pH values, the phenolic hydroxyl group has been assigned to position-8. The steric configuration at position-14 of porphyroxine is the same as that of glaudine (III) and is thermodynamically unstable. Epimerization of glaudine results in a strong downfield shift of the C-1 proton signal, while the coupling constants for the C-1 and C-2 protons are unchanged. This indicates that the conversion of glaudine to epiglaudine has not changed the conformation of ring D, but only the stereochemistry at the asymmetric center at position-14. From model considerations and the large coupling constants for the C-1 and C-2 protons, it may be concluded that these protons have an approximately diaxial orientation as illustrated in Fig. 1 (a) and in structures VI and VII. The downfield shift of the C-1 proton signal on epimerization may be explained by anisotropy effects of the C—OMe single bond or by a deshielding caused by van der Waals forces, thereby indicating a 1,3-diaxial orientation of the C-1 proton and the O—Me group in epiglaudine (VI). Consequently, glaudine and porphyroxine must have the configuration at position-14 illustrated in structure VII.

The ORD and CD spectra were not useful for elucidating the configuration of ring D because the effect of the configuration change at position-14 was masked by the stronger absorption due to the asymmetric centers at C-1 and C-2.

[3] Jasco ORD/CD-5 instrument.

REFERENCES

(1) Merck, E., *Ann. Pharm.*, **21**, 201 (1837).
(2) Merck, E., in "Jahres-Ber. über die Fortschritte der Chemie und Mineralogie," Berzelius, J., vol. 24, Tübingen, Germany, 1845, p. 399.
(3) Anon., *Bull. Narcotics*, **4**, 15(1952).
(4) United Nations Secretariat, United Nations Document, ST/SOA/SER.K/65.
(5) Klayman, D. L., Dissertation, Rutgers—The State University, New Brunswick, N.J., 1956; through *Reference 7*.
(6) Pfeifer, S., and Teige, J., *Pharmazie*, **17**, 692(1962).
(7) Genest, K., and Farmilo, C. G., *J. Pharm. Pharmacol.*, **15**, 197(1963).
(8) Pfeifer, S., and Banerjee, S. K., *Pharmazie*, **19**, 286(1964).
(9) Pfeifer, S., and Banerjee, S. K., *Arch. Pharm.*, **298**, 385(1965).
(10) Cross, A. D., Mann, D., and Pfeifer, S., *Pharmazie*, **21**, 181(1966).
(11) Cross, A. D., and Slavik, J., *Coll. Czech. Chem. Commun.*, **31**, 1425(1966).
(12) Šantavý, F., Kaul, J. L., Hruban, L., Doleyš, L., Hanuš, V., Blaha, K., and Cross, A. D., *ibid.*, **30**, 3479 (1965).
(13) Pfeifer, S., *J. Pharm. Pharmacol.*, **18**, 133(1966).
(14) Brochmann-Hanssen, E., Nielsen, B., and Hirai, K., *J. Pharm. Sci.*, **56**, 754(1967).
(15) Mary, N. Y., and Brochmann-Hanssen, E., *Lloydia*, **26**, 223(1963).
(16) Haynes, L. J., Stuart, K. L., Barton, D. H. R., and Kirby, G. W., *Proc. Chem. Soc.*, **1964**, 261.
(17) Haynes, L. J., and Stuart, K. L., *Chem. Commun.*, **1965**, 141.
(18) Tomita, M., Lu, S. T., and Wang, S. J., *Yakugaku Zasshi*, **85**, 827(1965).
(19) Tomita, M., Shingu, T., and Furukawa, H., *ibid.*, **86**, 373(1966).
(20) Highet, R. J., and Highet, P. F., *J. Org. Chem.*, **30** 902(1965).
(21) Brown, J. M., *Tetrahedron Letters*, **1964**, 2215.
(22) Pachler, K. G. R., Arndt, R. R., and Baarschers, W. H., *Tetrahedron*, **21**, 2159(1965).
(23) Mann, I., Döhnert, H., and Pfeifer, S., *Pharmazie*, **21**, 494(1966).
(24) Pfeifer, S., *Pharm. Ztg.*, **111**, 463(1966).
(25) Tomita, M., Lu, S. T., and Lan, P. K., *Yakugaku Zasshi*, **85**, 588(1965).
(26) Bhacca, N. S., and Williams, D. H., "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, p. 69.
(27) Baarschers, W. H., Arndt, R. R., Pachler, K., Weisbach, J. A., and Douglas, B., *J. Chem. Soc.*, **1964**, 4778.
(28) Bhacca, N. S., and Williams, D. H., "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, p. 188.

Ability of Certain Additives to Influence the Absorption of Salicylic Acid from Solutions in an *In Vivo* Study: Preliminary Report

By CLYDE W. WHITWORTH* and LOUDON D. YANTIS

The influence of polysorbates, sucrose, polyethylene glycols (PEG), propylene glycol, and dimethylsulfoxide (DMSO) on the absorption of salicylic acid has been studied. An immersion technique utilizing the frog was used. Results were analyzed by the Student *t* test. Certain concentrations of additives were seen to enhance absorption.

In recent years interest has been growing in the area of pharmaceutical research involving factors that influence drug absorption. Certain theories or concepts have been advanced concerning drug absorption. Shore *et al.* (1) reported certain drugs cross the intestinal epithelium chiefly in the unionized form and these workers advanced the pH-partition theory to explain the passive absorption of drugs. Various investigators (2–5) have found that oil-soluble drugs penetrate the skin more readily than water-soluble substances.

It was found that the frog, *Rana pipiens*, absorbed salicylic acid from solutions (6). The object of this study was to determine the effect of certain additives on the absorption of salicylic acid utilizing this immersion technique.

EXPERIMENTAL

Ten frogs were used in each determination. Each frog was placed in 500 ml. of the drug-additive solution and at 20-min. intervals the solutions were assayed for salicylic acid content using a Beckman DU spectrophotometer at a wavelength of 297 mμ. Samples were quickly returned to the solutions in order to maintain volume. It was determined that none of the additives interfered with the assay procedure. The solutions were 2.5×10^{-4} *M* with respect to salicylic acid.

It has been pointed out (6) that the absorption of salicylic acid by the frog in the manner described appears to conform to the pH-partition theory of passive absorption of drug in that the molecular form of drug is favored over the ionic. Proper maintenance of pH during such a study is therefore recognized to be of importance. It was found that most of the solutions except those containing the polysorbates exhibited a slight increase in pH (averaging about 0.15 pH units) during the second hour of the measurements. Part of this increase can be accounted for by the reduction in salicylic acid concentration as the drug is absorbed. Several buffer systems were tried. All were rejected because they either were deleterious to the frogs, or they greatly changed the rate of absorption possibly indicating an effect on the permeability of the skin of the frog. It appeared that the slight increase in pH observed did not appreciably influence the results of the study.

The aqueous solubility of salicylic acid in the presence of the additives was determined in an effort to correlate this property to the rates of absorption.

RESULTS AND DISCUSSION

Additives studied included polysorbate 80 USP, dimethylsulfoxide, sucrose, propylene glycol USP, polyethylene glycol 300 NF, and polyethylene glycol 4000 USP. The concentration of each additive was varied to observe this influence.

Figure 1 shows the effect of various concentrations of polysorbate 80 on the absorption of salicylic acid from solutions. This surfactant is seen to alter the rate of absorption of salicylic acid. The 0.1% concentration of additive has the greater effect in increasing absorption whereas the 1.0% polysorbate 80 is seen to have little effect for the first hour and then to inhibit absorption thereafter. Statistical analyses of the results by the Student *t* test at the 2-hr. mark show significant differences between control and the solutions containing 0.1% polysorbate 80 but not with the 0.5% additive solution. Levy *et al.* (7) found low concentrations of polysorbate 80 increased the absorption rate of certain barbiturates by goldfish, but higher concentrations of the surfactant inhibited absorption. Table I shows the aqueous solubility of salicylic acid to be greater in the presence of polysorbate 80. Each increase in surfactant concentration produced an increase in solubility. The surface tension lowering properties of polysorbate 80 prob-

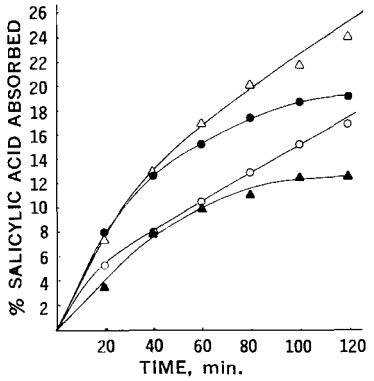


Fig. 1—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5×10^{-4} M and salicylic acid, 2.5×10^{-4} M in the presence of: △, 0.1% polysorbate 80; ●, 0.5% polysorbate 80; ▲, 1.0% polysorbate 80.

Received March 6, 1967, from the School of Pharmacy, Northeast Louisiana State University, Monroe, LA 71201
Accepted for publication August 10, 1967.
This study was supported by a Mead Johnson undergraduate research grant for 1965–1966.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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TABLE I—SOLUBILITY OF SALICYLIC ACID AT 27° IN THE PRESENCE OF ADDITIVES

Solvent, %	Solubility, Gm./100 ml.
Water	0.275
Polysorbate 80, 0.1	0.275
Polysorbate 80, 0.5	0.367
Polysorbate 80, 1.0	0.458
DMSO, 0.5	0.275
DMSO, 1.0	0.275
DMSO, 5.0	0.321
Sucrose, 0.5	0.275
Sucrose, 1.0	0.275
Sucrose, 5.0	0.275
Propylene glycol, 0.5	0.275
Propylene glycol, 1.0	0.275
Propylene glycol, 5.0	0.275
PEG 300, 0.5	0.275
PEG 300, 1.0	0.275
PEG 300, 5.0	0.343
PEG 4000, 0.5	0.275
PEG 4000, 1.0	0.275
PEG 4000, 5.0	0.390

ably permit better wetting action by the solution, thereby enhancing absorption of the salicylic acid.

Dimethylsulfoxide has received wide acclaim recently for its alleged ability to penetrate the skin. The use of this solvent as a vehicle for enhancing absorption of drugs remains a possibility. DMSO was found in this study to be more effective than any other additive observed in increasing the rate of absorption of salicylic acid by the live frog. All concentrations of DMSO significantly increased the amount of salicylic acid absorbed at the end of 2 hr. (Fig. 2). There appears to be an optimum concentration of DMSO, however, which will increase absorption just as was true with the polysorbate 80. Morain and his co-workers (8) observed DMSO to increase the permeability of frog skin to mannitol, urea, and other substances. The solubility of salicylic acid is seen to be increased by DMSO only by the 5.0% concentration.

The presence of sucrose (Fig. 3) increases the rate of absorption of salicylic acid by the frog but does not influence the solubility. All differences between control and solutions containing additives were significant at the 2-hr. limit. Again there appears to be an optimum concentration of additive for increasing absorption with 1% sucrose having the greatest effect.

As seen in Fig. 4 propylene glycol significantly increases the absorption of salicylic acid. As the concentration of additive is increased, however, the effect is less pronounced. No change in solubility was noted.

Figure 5 shows the 1.0% concentration of polyethylene glycol 300 to be more effective in increasing absorption than the 0.5% (not significant) or the

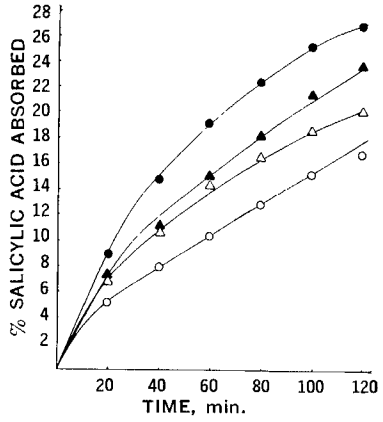

Fig. 3—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5 × 10^{-4} M and salicylic acid, 2.5 × 10^{-4} M in the presence of: △, 0.5% sucrose; ●, 1.0% sucrose; ▲, 5.0% sucrose.

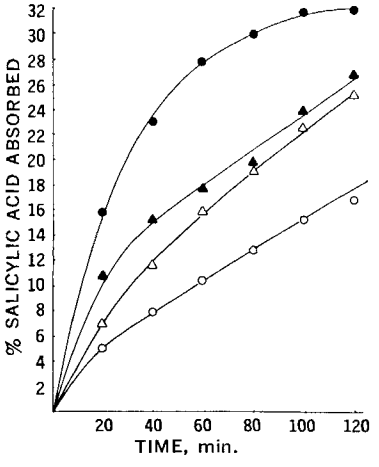

Fig. 2—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5 × 10^{-4} M and salicylic acid, 2.5 × 10^{-4} M in presence of: △, 0.5% DMSO; ●, 1.0% DMSO; ▲, 5.0% DMSO.

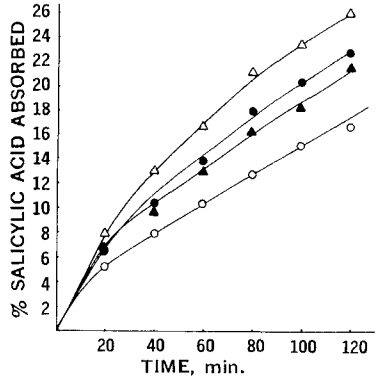

Fig. 4—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5 × 10^{-4} M and salicylic acid, 2.5 × 10^{-4} M in presence of: △, 0.5% propylene glycol; ●, 1.0% propylene glycol; ▲, 5.0% propylene glycol.

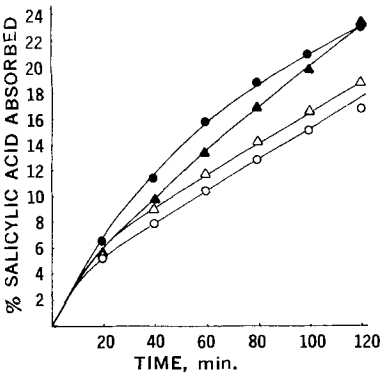

Fig. 5—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5 × 10^{-4} M and salicylic acid, 2.5 × 10^{-4} M in presence of: △, 0.5% polyethylene glycol 300; ●, 1.0% polyethylene glycol 300; ▲, 5.0% polyethylene glycol 300.

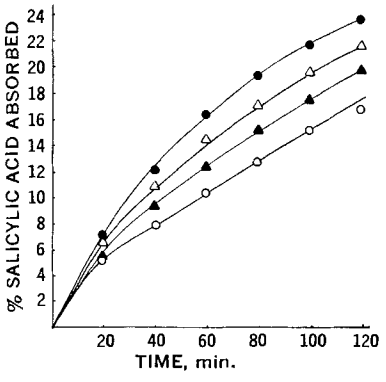

Fig. 6—Percent of salicylic acid absorbed over 2-hr. period when ten frogs each were placed in 500 ml. of aqueous drug solution. Key: ○, salicylic acid, 2.5 × 10^{-4} M and salicylic acid, 2.5 × 10^{-4} M in presence of: △, 0.5% polyethylene glycol 4000; ●, 1.0% polyethylene glycol 4000; ▲, 5.0% polyethylene glycol 4000.

5.0%. Only the 5.0% concentration of the additive influenced solubility which was slightly increased.

Polyethylene glycol 4000 significantly increased the absorption of salicylic acid (Fig. 6) with the 1.0% concentration again being the most effective. The solubility was increased only by the 5.0% additive solution.

Efforts at linearizing all the curves in the same manner were not successful. It was previously shown (6) that the absorption rate for salicylic acid from solutions using this technique appeared to be first order. This did not prove to be true, however, for most of the solutions containing the additives especially in the instances in which the absorption rate was greatly increased. Throughout the experiments the concentration of drug in the immersion media greatly exceeded the concentration of drug in the frogs. Considering this and the fact that a large volume of drug solution was used indicates that a fairly constant concentration gradient of drug was maintained after the initial 20-min. period of absorption. This explains the apparent zero-order absorption process seen for some of the solutions after the first 20 min. Several of the plots, namely for propylene glycol and polyethylene glycol 4000 additives, show similar slopes for all concentrations of additive indicating similar absorption rates after the initial absorption period.

The failure of certain of the data to show zero-order or first-order characteristics might be due to excretion of the drug by the frog toward the end of the experiments. This explanation is perhaps plausible for those additives that showed the greatest ability to enhance absorption after which the drug may have been partially excreted.

Current studies are being conducted to determine what part complexation, pH, concentration of drug, and other parameters are playing in the absorption of salicylic acid by the frog.

SUMMARY AND CONCLUSIONS

1. The effect of six additives at various concentrations on the absorption of salicylic acid from solutions has been studied using the frog as the test animal. The solubility of salicylic acid in each solvent was determined.

2. The additives were seen to enhance absorption of salicylic acid significantly in most cases, however, there appeared to be an optimum concentration for most of the additives.

3. The influence of various adjuvants on the absorption of the active ingredient of a pharmaceutical formulation should be carefully considered.

4. The frog may be useful for studying drug absorption.

REFERENCES

(1) Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **119**, 361(1957).
(2) Treherne, J. E., *J. Physiol. (London)*, **133**, 171(1956).
(3) Rothman, J., *J. Lab. Clin. Med.*, **28**, 1305(1943).
(4) Stolar, M. E., Rossi, G. V., and Barr, M., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 148(1960).
(5) Nogami, H., Hasegawa, J., and Hanano, M., *Pharm. Bull. (Tokyo)*, **1956**, 347.
(6) Whitworth, C. W., *J. Pharm. Sci.*, **54**, 463(1965).
(7) Levy, G., Miller, K. E., and Reuning, R. H., *ibid.*, **55**, 394(1966).
(8) Morain, W. D., Replogle, C. A., and Curran, P. F. *J. Pharmacol. Exptl. Therap.*, **154**, 298(1966).

Biotransformation Studies on 1-Chloro-2,3-propanediol Dinitrate

By ALBERT J. DIETZ, JR.*

Metabolic studies on the coronary vasodilator, 1-chloro-2,3-propanediol dinitrate (CPD), show that two metabolites can be isolated from the urine of dogs receiving the compound orally. The data suggest that the biotransformation of CPD commences with the splitting of the nitrate ester forming a free hydroxyl group which is then conjugated with glucuronic acid. The probable metabolites are 1-chloropropyl-3-glucuronide-2-nitrate and 1-chloropropyl-2-glucuronide-3-nitrate. Upon treatment with β-glucuronidase, these metabolites correspond to the products from the partial hydrolysis of CPD, which could only be the mono nitrate esters.

IN THE TREATMENT of coronary insufficiency, glyceryl trinitrate is the standard coronary vasodilator with which others have been compared. Krantz *et al.* (1) have shown that the number of nitrate groups in the molecule is not the major factor in determining vasodilating potency. Activity was found to be due to the intact molecule and not to the hydrolysis products of the ester. Likewise, the oil/water coefficient was found to be a factor in determining potency.

In recent years the search has been directed to related compounds that show a greater specificity for the coronary arteries and provide a longer duration of action with less side effects. Toward this end, 1-chloro-2,3-propanediol dinitrate[1] (CPD) was investigated. Burgison *et al.* (2) have shown that CPD and glyceryl trinitrate are equipotent in increasing coronary flow in the Langendorff rabbit heart preparation, and the latter appears to evoke a lesser degree of depressor response in the dog. Further, they have shown that the chlorinated ester manifests more persistent activity, and clinical trials (3) have confirmed these findings.

Previous metabolic studies on coronary vasodilators in this laboratory have shown that no CPD could be found in the urine of dogs which had received the compound orally. The logical step in metabolism would be the hydrolysis of the nitrate ester to the free hydroxyl group and conjugation with glucuronic acid. It was also of interest to see if the chlorine atom was cleaved during biotransformation.

METHODS

Three female mongrel dogs were fasted for 24 hr., and the urine was then collected for an additional 24 hr. as a control. On the next day, the dogs were again fasted for 24 hr., then 6 mg. of CPD was administered orally, and the urine was collected for 24 hr. after administration of the compound. The test urines were evaporated to dryness at 40° *in vacuo*. The residues were dissolved in buffer (pH 4.5) consisting of 5.79 Gm. $CH_3COONa \cdot 3H_2O$ and 3.25 ml. of glacial acetic acid in 1 L. of distilled water. Each test urine was then divided into two parts. To one part β-D-glucuronide glucuronohydrolase (β-glucuronidase) from *Helix pomatia*[2] was added, after which each part was incubated at 37° for 2 hr. Each part was then evaporated to dryness at 40° *in vacuo* and the residues were extracted three times with 5-ml. portions of anhydrous ethyl ether and the ether extracts were combined. The volume was reduced to 5 ml. by spontaneous air evaporation. The control urines were treated similarly but without the enzyme β-glucuronidase. A 10-μl. portion of the control, of the enzyme-treated sample, and of the untreated urines of each dog were spotted on thin-layer chromatography plates coated with silica gel + fluorescein (0.25 mm.) which had been activated at 100° for 30 min. The plates were developed by ascending techniques with two solvent systems, one a 2-propanol–ammonium hydroxide (4:1) mixture, the second a benzene–ethyl acetate (4:1) mixture. One plate from each solvent system was sprayed with a 1,3-dihydroxynaphthalene (naphthoresorcinol)–trichloroacetic acid mixture[3]

TABLE I—COMPARISON OF URINARY METABOLITES OF CPD TO SELECTED REFERENCE COMPOUNDS

Material	Spray	Solvent System: Benzene–Ethyl Acetate (4:1)	Solvent System: 2-Propanol–Ammonium Hydroxide (4:1)
Control urine	[a]	No spots appeared	No spots appeared
Control urine	[b]	No spots appeared	No spots appeared
Control urine	[c]	No spots appeared	No spots appeared
Untreated urine	[a]	0.07, 0.10	0.30, 0.43
Untreated urine	[b]	0.07, 0.10	0.30, 0.43
Untreated urine	[c]	0.07, 0.10	0.30, 0.43
Treated urine[d]	[a]	No spots appeared	No spots appeared
Treated urine	[b]	0.17, 0.27	0.38, 0.53
Treated urine	[c]	0.17, 0.27	0.38, 0.53
CPD	[a]	No spots appeared	No spots appeared
CPD	[b]	0.67	0.87
CPD	[c]	0.67	0.87
CPD (partial alkaline hydrolysis)	[a]	No spots appeared	No spots appeared
	[b]	0.17, 0.27, 1.00	0.38, 0.53, 0.77
	[c]	0.17, 0.27	0.38, 0.53
Chloroacetic acid	[a]	No spots appeared	No spots appeared
Chloroacetic acid	[b]	0.23	0.47
Chloroacetic acid	[c]	No spots appeared	No spots appeared
3-Chloropropionic acid	[a]	No spots appeared	No spots appeared
	[b]	0.30	0.50
	[c]	No spots appeared	No spots appeared
3-Chloro-1,2-propanediol	[a]	No spots appeared	No spots appeared
	[b]	1.00	0.77
	[c]	No spots appeared	No spots appeared
3-Chloro-1-propanol	[a]	No spots appeared	No spots appeared
	[b]	1.00	0.40
	[c]	No spots appeared	No spots appeared

[a] Naphthoresorcinol–trichloroacetic acid. [b] Silver nitrate–ammonium hydroxide. [c] Diphenylamine–methanol. [d] Urine incubated with β-glucuronidase.

Received July 10, 1967, from Huntingdon Research Center, Towson, MD 21204

Accepted for publication August 21, 1967.

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[1] Supplied as Bangina by Organon, Inc.

[2] Boehringer Mannheim Corporation, GRD/ARS 15427.

[3] One part by volume of an alcoholic naphthoresorcinol solution (0.2 Gm. naphthoresorcinol dissolved in 100 ml. ethanol) is mixed with 1 part by volume of an aqueous 20% solution of trichloroacetic acid (4).

and incubated in a humidified oven at 75° for 15 min. An additional plate from each solvent system was sprayed with a silver nitrate–ammonium hydroxide mixture[4] and exposed to an intense source of ultraviolet light for 5 min. A third plate was sprayed with a 1% solution of diphenylamine in methanol and exposed to ultraviolet light (4).

To help identify the metabolites, 1 mmole of CPD and 1.25 mmoles of NaOH were placed in 100 ml. of water and shaken for 2 hr. at 37°. The aqueous solution was concentrated to 25 ml. and extracted three times with anhydrous ethyl ether. The ether extracts were combined and evaporated to dryness. The residue was dissolved in 5 ml. of ether and 10-μl. portions spotted on each plate.

RESULTS

Table I gives the R_f values of the urine samples and reference compounds in a benzene–ethyl acetate solvent system and a 2-propanol–ammonium hydroxide solvent system sprayed with various identifying reagents. Two spots appeared on each plate from each solvent system sprayed with the naphthoresorcinol reagent which visualizes glucuronides. Both spots appeared in the test urines not treated with β-glucuronidase. No spots appeared in either the control urines or the enzyme-treated test urines, suggesting that the metabolites of CPD are glucuronide conjugates. On plates sprayed with silver nitrate–ammonium hydroxide mixture which visualizes organic chlorides, spots appeared in both the untreated and treated test urines, indicating that organically bound chlorine is present.

However, the spots in the untreated urine did not correspond to those in the enzyme-treated urines. Finally, one plate from each solvent system was sprayed with diphenylamine which colors organic nitrate esters. Spots appeared in both the treated and untreated test urines but they did not correspond to each other.

The partial alkaline hydrolysis of CPD was used to produce a mixture of the two mono nitrate esters and the completely hydrolyzed 1-chloro-2,3-propanediol. The conclusive identification of which spot corresponds to which mono nitrate ester could not be made without very sophisticated instrumentation, and the introduction by synthesis of only one nitrate group into the molecule without two or a mixture of the mono nitrates is nearly impossible. The spots from the partially hydrolyzed CPD, which could only be a mixture of the mono nitrates, do indeed correspond to the spots from the urine hydrolyzed by β-glucuronidase.

[4] One part by volume of a 1% solution of silver nitrate in ammonium hydroxide is mixed with 1 part by volume of ethanol (4).

REFERENCES

(1) Krantz, J. C., Carr, C. J., Forman, S. E., and Ellis, F. W., *J. Pharmacol. Exptl. Therap.*, **67**, 191(1939).
(2) Burgison, R. M., Lu, G., Cowley, R. A., and Krantz, J. C., *Angiology*, **13**, 412(1964).
(3) Krasno, L. R., and Kidera, G. J., *ibid.*, **16**, 233(1965).
(4) "Thin-Layer Chromatography, A Laboratory Handbook," Stahl, E., ed., Academic Press Inc., New York, N. Y., 1965.

Smooth Muscle and Cardiovascular Pharmacology of α-Elaterin-2-D-glucopyranoside Glycoside of *Citrullus colocynthis*

By S. P. BANERJEE and P. C. DANDIYA

The active principle present in the pulp of *Citrullus colocynthis* which exhibits cathartic abortifacient activity is not yet well established. The cardiovascular and smooth musculature activity of a recently identified glycoside, α-elaterin-2-D-glucopyranoside (coloside A) was therefore carried out to discover if it was the purgative or the ecbolic principle. Coloside A failed to stimulate the uterus, but elicited purgative properties. It demonstrated antihistaminic and antiacetylcholine-like activity on the intestinal musculature and exhibited negative chronotropic and negative inotropic activity in isolated mammalian and amphibian heart.

IN THE Ayurvedic system of medicine, the pulp of *Citrullus colocynthis* is recommended as a purgative (1) and is known to cause miscarriage when administered to pregnant women. The abortive activity of *C. colocynthis* is considered to be an indirect action, a manifestation of the cathartic activity of the drug produced by the congestion in the pelvic region (2). However, the possibility of some direct action on the uterus cannot be completely ruled out, since Stimpson (3) had shown that alcohol-free tincture of *C. colocynthis* produced increased tone and amplitude of contraction of isolated rabbit uterus. It is therefore likely that besides causing congestion in the pelvic region *C. colocynthis* possesses some principle which directly stimulates the uterus.

There is a controversy regarding the constituent responsible for its purgative action. From the water extract of the pulp, Walz (4) isolated a glycoside, colocynthin, which was believed to be the purgative principle. Subsequent workers, however, failed to isolate the glycoside from the water-soluble fraction. Power and Moore (5) after detailed chemical and pharmacological studies concluded that *C. colocynthis* does not contain any glycoside, and the purgative principle resided in the chloroform and ether-soluble resins and amorphous alkaloid (6–8). Lavie *et al.* (9) isolated a glycoside from the chloroform extract of the pulp of *C. colocynthis* which was identified by Khadem and Rahman (10, 11) to be α-elaterin-2-D-glucopyranoside. Since no systemic

Received June 1, 1967, from the Department of Pharmacology, S.M.S. Medical College, Jaipur, India.
Accepted for publication August 14, 1967.

and incubated in a humidified oven at 75° for 15 min. An additional plate from each solvent system was sprayed with a silver nitrate–ammonium hydroxide mixture[4] and exposed to an intense source of ultraviolet light for 5 min. A third plate was sprayed with a 1% solution of diphenylamine in methanol and exposed to ultraviolet light (4).

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(2) Burgison, R. M., Lu, G., Cowley, R. A., and Krantz, J. C., *Angiology*, **13**, 412(1964).
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Smooth Muscle and Cardiovascular Pharmacology of α-Elaterin-2-D-glucopyranoside Glycoside of *Citrullus colocynthis*

By S. P. BANERJEE and P. C. DANDIYA

The active principle present in the pulp of *Citrullus colocynthis* which exhibits cathartic abortifacient activity is not yet well established. The cardiovascular and smooth musculature activity of a recently identified glycoside, α-elaterin-2-D-glucopyranoside (coloside A) was therefore carried out to discover if it was the purgative or the ecbolic principle. Coloside A failed to stimulate the uterus, but elicited purgative properties. It demonstrated antihistaminic and antiacetylcholine-like activity on the intestinal musculature and exhibited negative chronotropic and negative inotropic activity in isolated mammalian and amphibian heart.

IN THE Ayurvedic system of medicine, the pulp of *Citrullus colocynthis* is recommended as a purgative (1) and is known to cause miscarriage when administered to pregnant women. The abortive activity of *C. colocynthis* is considered to be an indirect action, a manifestation of the cathartic activity of the drug produced by the congestion in the pelvic region (2). However, the possibility of some direct action on the uterus cannot be completely ruled out, since Stimpson (3) had shown that alcohol-free tincture of *C. colocynthis* produced increased tone and amplitude of contraction of isolated rabbit uterus. It is therefore likely that besides causing congestion in the pelvic region *C. colocynthis* possesses some principle which directly stimulates the uterus.

There is a controversy regarding the constituent responsible for its purgative action. From the water extract of the pulp, Walz (4) isolated a glycoside, colocynthin, which was believed to be the purgative principle. Subsequent workers, however, failed to isolate the glycoside from the water-soluble fraction. Power and Moore (5) after detailed chemical and pharmacological studies concluded that *C. colocynthis* does not contain any glycoside, and the purgative principle resided in the chloroform and ether-soluble resins and amorphous alkaloid (6–8). Lavie *et al.* (9) isolated a glycoside from the chloroform extract of the pulp of *C. colocynthis* which was identified by Khadem and Rahman (10, 11) to be α-elaterin-2-D-glucopyranoside. Since no systemic

Received June 1, 1967, from the Department of Pharmacology, S.M.S. Medical College, Jaipur, India.
Accepted for publication August 14, 1967.

pharmacological studies have yet been made with this compound, the smooth musculature and cardiovascular pharmacology of α-elaterin-2-D-glucopyranoside was undertaken. The glycoside α-elaterin-2-D-glucopyranoside will be referred to as coloside A in the text.

EXPERIMENTAL

Coloside A (m.p. 156°) was isolated from the Indian variety of the pulp of *C. colocynthis* by the method described by Khadem and Rahman (11). The solution of the drug was made by dissolving a weighed quantity of the compound in a known minimal quantity of warm ethanol, and the volume was made up with warm water so that solution did not contain more than 10% of ethanol. Experiments were run simultaneously with the solution of coloside A and the solvent containing 10% ethanol. Since the control experiments with the solvents, *i.e.*, 10% ethanol, did not exhibit any pharmacological action, only the activity of coloside A is described here.

Smooth Muscle—The isolated uterus of rat, the intestine of rabbit, and the ileum of guinea pig were set up by the methods described by Smith (12) and Turner (13).

The interaction between coloside A and uterine stimulants, such as oxytocin 0.002 units/ml. and ergometrine 0.01 mg./ml., were studied on the isolated rat uterus suspended in oxygenated de Jalons solution as described by Burn (14).

Antiacetylcholine, antihistaminic, and antibarium chloride activity of the glycoside was determined both on rabbit intestine and guinea pig ileum. The doses employed and preparations used are shown in Table I.

TABLE I—DOSES AND PREPARATIONS

Drug	Rabbit Intestine, mcg./ml.	Guinea Pig Ileum, mcg./ml.
Acetylcholine	0.5	5
Histamine	5	0.1
Barium chloride	0.2	0.2

Cardiovascular System—The experiment of frog heart perfusion was carried out by Bulbring's method as described by Burn (15). For the Straub heart preparation the excised frog heart was prepared according to the Straub procedure as quoted by Gaddum (16). Perfusion of the isolated rabbit heart was performed by the modified method of Langendorff as described by Burn (17).

To study the effect of coloside A on the blood pressure and heart *in situ*, mongrel dogs of either sex, between 7–12 Kg. weight, were employed and anesthetized with morphine, 5 mg./Kg., followed 15 min. later by urethan, 1.4 Gm./Kg., both given intramuscularly. The left common carotid artery was cannulated and blood pressure was recorded on a slowly moving kymograph. The drug was administered through the cannulated right femoral vein. The chest was then opened under artificial respiration. A bent entomological pin passed near the tip of the ventricle and was attached to a Starling lever after passing through a pulley. Similar arrangements were made to record auricular beats.

RESULTS

Results described below are the average of three experiments.

Smooth Muscle—*Isolated Rat Uterus*—Coloside A did not produce any contractions of the rat uterus in concentration up to 0.1 mg./ml. On the other hand, it exhibited a uterine depressant property. Though the drug did not elicit any relaxation of the uterus in concentrations up to 0.1 mg./ml., it decreased the rate and amplitude of contraction. Coloside A also failed to alter the response produced by uterine stimulants such as oxytocin and ergometrine.

Isolated Rabbit Intestine and Guinea Pig Ileum—Coloside A did not produce any spasmogenic activity on the isolated intestine. Neither did it affect the rhythmic movements of rabbit intestine, nor did it produce any contraction of the guinea pig ileum suspended in Tyrode's solution up to 0.1 mg./ml. However, it exhibited antihistaminic and antiacetylcholine-like action which is described in Table II. Coloside A did not counteract the action of barium chloride on intestine.

TABLE II—ANTIACETYLCHOLINE AND ANTIHISTAMINIC ACTIVITY OF COLOSIDE A[a]

Dose, mg./ml.	Prepn.	Stimulant	Dose, mcg./ml.	% Inhibition
0.1	Rabbit intestine	Acetylcholine	0.5	43
0.1	Rabbit intestine	Histamine	5.0	92
0.05	Guinea pig ileum	Acetylcholine	5.0	27
0.1	Guinea pig ileum	Acetylcholine	5.0	49
0.05	Guinea pig ileum	Histamine	0.1	67
0.1	Guinea pig ileum	Histamine	0.1	90

[a] Three experiments were performed in each case.

Cardiovascular System—*Frog Heart Perfusion*—Coloside A diminished the force of contraction of the ventricle and reduced the heart rate. A dose of 1 mg. decreased the amplitude of contraction and heart rate by 35 and 15%, respectively. Results are shown in Table III.

TABLE III—EFFECT OF COLOSIDE A ON THE FROG HEART PERFUSION[a]

Dose, mg.	% Decrease in Amplitude	% Decrease in Heart Rate	Recovery, Time, min.
0.25	0	0	0
0.50	12	7	12
1.00	36	16	22

[a] Three experiments were performed in each case.

Straub Heart Preparation—When a dose of 1:10,000 of the drug was administered to a Straub heart preparation, the amplitude of contraction was decreased by 70%, and the heart rate was reduced by 25%. At 1:10,000 concentration, it produced a mid-diastolic arrest (Table IV).

TABLE IV—EFFECT OF COLOSIDE A ON THE STRAUB HEART PREPARATION[a]

Dose	% Decrease in Amplitude	% Decrease in Heart Rate
1:100,000	11	0
1:10,000	70	23
1:1000	Mid-diastolic arrest	Mid-diastolic arrest

[a] Three experiments were performed in each case.

Isolated Rabbit Heart—The glycoside exhibited a negative chronotropic and a negative inotropic effect and reduced the coronary flow. At a 3-mg. dose it produced a mid-diastolic arrest (Table V).

TABLE V—EFFECTS OF COLOSIDE A ON THE ISOLATED RABBIT HEART[a]

Dose, mg.	% Decrease in Amplitude	% Decrease in Coronary Flow	% Decrease in Heart Rate
0.05	37	14	23
0.3	Mid-diastolic arrest	Mid-diastolic arrest	Mid-diastolic arrest

[a] Three experiments were performed in each case.

Dog Heart In Situ—Coloside A had no effect on this preparation up to 5 mg./Kg. dose level.

Dog Blood Pressure—A dose of 5 mg./Kg. of the drug failed to produce any significant hypotensive action.

DISCUSSION

The pulp of *C. colocynthis* is well-known for its cathartic and abortifacient activity. But the chemical constituents responsible for the purgative and ecbolic activity have not yet been definitely identified. A controversy exists regarding the nature of the cathartic principle. No compound has so far been isolated which exhibits uterine stimulant properties. An attempt was therefore made to distinguish and to isolate in pure form the cathartic and oxytocic principles of the drug.

Coloside A has been found to possess purgative properties.[1] However, it did not produce any contraction of the rat isolated uterus. Thus, coloside A is the cathartic principle without any oxytocic activity. It exhibited a uterine depressant property and decreased the rate and amplitude of contraction, and demonstrated antihistaminic and antiacetylcholine activity to confirm the finding of Hollander (18), who reported that a solution of colocynth blocked the parasympathetic nerve endings. It does not possess a direct depressant activity on the intestine, as it failed to counteract the response of barium chloride.

The drug produced a negative chronotropic and a negative inotropic effect on isolated mammalian and amphibian hearts. Similar activity was reported on the rabbit heart by Stimpson (3) in the alcoholic extract of the pulp of *C. colocynthis*. Since coloside A is a steroidal glycoside and shows cardiac depressant properties, this drug may be a cardiotonic. Further work on this aspect would be fruitful.

Coloside A did not have cathartic activity when administered intraperitoneally, although orally it produced drastic purgation. Since the drug is believed to bring about purgation by irritation of the intestine, one possibility is that when it is given by a route other than oral, the concentration of the glycoside in the lumen of the intestine is not sufficiently high to exhibit any activity. However, Gaddum (19) has suggested that bile is necessary for the activity of the pulp of *C. colocynthis*. It is plausible to assume that the active principle of *C. colocynthis* forms some complex with the bile to manifest its activity. Erspamer and Paolini (20) have shown that antihistaminics slow down the purgative response of *C. colocynthis*. Surprisingly enough coloside A has been found to be an antihistaminic. It is probable that coloside A is an antagonist at the histaminic receptors with affinity and no intrinsic activity, while the complex is an agonist and possesses both affinity as well as intrinsic activity on the same receptors.

[1] Unpublished observations.

REFERENCES

(1) Chopra, R. N., "Indigenous Drugs of India," U. N. Dhur and Sons, Ltd., Calcutta, India, 1958, p. 129.

(2) Grollman, A., and Slaughter, D., "Pharmacology and Therapeutics," Lea & Febiger, Philadelphia, Pa., 1947, p. 238.

(3) Stimpson, H. S., *J. Am. Inst. Homeopathy*, **19,** 50 (1926).

(4) Walz, N., *Jahrb. Pharm.*, 9, **16,** 225(1858); **16,** 18 (1861).

(5) Power, F. P., and Moore, C. W., *J. Chem. Soc.*, **97,** 99(1910).

(6) Youngken, H. W., "Text Book of Pharmacognosy," The Blakiston Co., Philadelphia, Pa., and Toronto, Canada, 1950, p. 859.

(7) Wallis, T. E., "Text Book of Pharmacognosy," J. and A. Churchill Ltd., London, England, 1955, p. 267.

(8) Trease, G. E., "A Text Book of Pharmacognosy," Bailliere Tindall and Cox, London, England, 1957, p. 307.

(9) Lavie, D., Willner, D., Belkin, M., and Hardy, W. G., *Acta Unio. Intern. Contra. Cancerum.*, **15,** 177(1959).

(10) Khadem, H. El., and Rahman, M. M. A., *Tetrahedron Letters*, **24,** 1137(1962).

(11) Khadem, H. El., and Rahman, M. M. A., *J. Chem. Soc.*, **1963,** 4991.

(12) Smith, W. G., "Progress in Medicinal Chemistry," Ellis, G. P., and West, G. B., eds., vol. I, Butterworth, Washington, D. C., 1961, pp. 12–18.

(13) Turner, R. A., "Screening Methods in Pharmacology," Academic Press Inc., New York, N. Y., and London, England, 1965, pp. 42–47.

(14) Burn, J. H., "Practical Pharmacology," Blackwell Scientific Publication Ltd., Oxford, England, 1952, p. 13.

(15) *Ibid.*, p. 30.

(16) Gaddum, J. H., "Pharmacology," Oxford University Press, New York, N. Y., and London, England, 1959, p. 296.

(17) Burn, J. H., "Practical Pharmacology," Blackwell Scientific Publication Ltd., Oxford, England, 1952, p. 25.

(18) Hollander, N., *Compt. Rend. Soc. Biol.*, **93,** 1175 (1925).

(19) Gaddum, J. H., "Pharmacology," Oxford University Press, New York, N. Y., and London, England, 1953, p. 252.

(20) Erspamer, V., and Paolini, A., *Experientia*, **2,** 445 (1946).

New Derivatives of Quinuclidine as Potential Antihypertensive Agents

By LANCE L. COLLINS, CHARLES A. PAPACOSTAS*, and SAMUEL ELKIN

Five new 3-aminoalkoxy derivatives of quinuclidine have been synthesized. All compounds reduced blood pressure in dogs to varying degrees, the most effective being 3-(3-dimethylaminopropoxy) quinuclidine. The evidence points to ganglionic blockade as the principal mechanism of action with the possibility that it may also be due to some direct depressant action on the cardiovascular system

QUINUCLIDINE, 1-azabicyclo[2.2.2]octane, is an integral part of the quinine molecule. Because of the many and varied responses produced by quinine and the other cinchona alkaloids, there has been a great interest in the quinuclidine nucleus as a possible therapeutic agent. Quinuclidine itself, as the hydrochloride salt, has been found to be a potent hypotensive and smooth muscle relaxant (1).

Therapeutically active quinuclidine derivatives have been synthesized and pharmacological activity has been found in both mono- and disubstituted products. The range in activity is quite varied.

The greatest volume of synthetic work involved the preparation of 3-substituted quinuclidines. Series of esters of bicyclic aminoalcohols, including 3-quinuclidinol have been prepared by Martell and Soine (2), by Sternbach and Kaiser (3), and by Mikhlina and Rubtsov (4). These derivatives have marked spasmolytic activity, one of the most active being 3-diphenylacetoxyquinuclidine.

Several 3-substituted derivatives of quinuclidine have been reported by Grob (5) to stimulate the central nervous system.

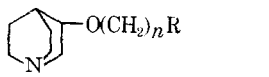
A series of 2-aminomethyl quinuclidines have been prepared and tested by Nikitskaya *et al.* (6). Some members of the series were found to have strong ganglionic blocking activity. Diethylaminoethyl-quinuclidine-2-carboxylate was found to be a good hypotensive. Ganglionic blocking was also noticed with 2,3-disubstituted products (7), the greatest activity being shown by 2-*N*-phenylaminomethyl-3-(2-*N*-phenylaminoethyl) quinuclidine. Perrine (8) synthesized a series of 4-phenyl substituted compounds for investigation as possible analgesics.

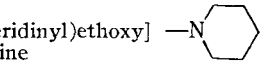
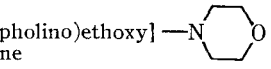
This investigation is involved with the synthesis of a series of 3-aminoalkoxy derivatives of quinuclidine and the pharmacological testing of their dimethiodide salts. All of the compounds showed antihypertensive activity, the most effective being 3-(3-dimethylaminopropoxy) quinuclidine.

DISCUSSION

Five new compounds were synthesized and are represented by the general formula shown. All products were prepared as the dimethiodide salts.

All compounds were prepared by the Williamson synthesis. A mixture of sodium and 3-quinuclidinol was refluxed in dry benzene to give sodium 3-quinuclidinoxide. This was reacted with the various aminoalkyl chlorides to give the corresponding ethers. These compounds were high boiling, colorless liquids which darkened upon standing.



	R	*n*
I, 3-(2-diethylaminoethoxy) quinuclidine	$-N(C_2H_5)_2$	2
II, 3-(2-dimethylaminoethoxy) quinuclidine	$-N(CH_3)_2$	2
III, 3-(3-dimethylaminopropoxy) quinuclidine	$-N(CH_3)_2$	3
IV, 3-[2-(1-piperidinyl)ethoxy] quinuclidine		2
V, 3-[2-(1-morpholino)ethoxy] quinuclidine		2

An attempt was made to isolate the ethers as their hydrochloride salts. This was done by bubbling dry HCl into a cold benzene solution of the ethers. The oily residues which formed were extremely hygroscopic and could not be crystallized.

The products were ultimately isolated as their dimethiodide salts which were stable in air and very soluble in water.

The infrared spectra showed characteristic alkyl ether bands between 8.9–9.4 mμ. Elemental analysis further confirmed the preparation of the desired products (Table I).

EXPERIMENTAL

All melting points are uncorrected. Analyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

Sodium 3-Quinuclidinoxide—Into a 300-ml. 3-necked flask equipped with stirrer, dropping funnel, and condenser (drying tube) were placed 10.0 Gm. (0.08 mole) of 3-quinuclidinol[1] and 100 ml. of dry benzene. This mixture was heated until the 3-quinuclidinol was completely dissolved. Then 1.0 Gm. (0.08 mole) of sodium, cut into small strips, was added over a period of 1 hr. while heating and stirring. The mixture was refluxed until all the sodium had reacted (about 48 hr.) whereupon a light yellow slurry resulted.

Aminoalkyl Chlorides—These were prepared by the neutralization of the hydrochloride salts with sodium hydroxide according to the method of

Received July 12, 1967, from the School of Pharmacy and the * School of Medicine, Temple University, Philadelphia, PA 19140

Accepted for publication August 18, 1967.

This investigation was supported by research grant MH-07702 from the National Institute of Mental Health, U. S. Public Health Service, Bethesda, Md.

Abstracted from a thesis submitted by Lance L. Collins in partial fulfillment of Master of Science degree requirements.

[1] Supplied by U. S. Army Chemical Center, Edgewood, Md. Can be prepared by the method described by Aaron, H. S., Elkin, S., Owens, O. O., Rosenstock, P. D., Leonard, S., and Miller, J. I., *J. Org. Chem.*, **30**, 1331(1965).

TABLE I—ANALYSIS OF THE 3-AMINOALKOXY DERIVATIVES OF QUINUCLIDINE

	Compd.	Yield, %	B.p.	Yield, % (Dimethiodide)	M.p., °C.	Anal. (Dimethiodide) Calcd.	Found
I	3-(2-Diethylaminoethoxy) quinuclidine	79	129–132°/6 mm.	63.3	214–216°	$C_{15}H_{32}I_2N_2O$	
						C, 35.31	35.77
						H, 6.32	6.46
						N, 5.49	5.14
						I, 49.47	49.57
II	3-(2-Dimethylaminoethoxy) quinuclidine	55	110/112°/4 mm.	57.3	282–283°	$C_{13}H_{28}I_2N_2O$	
						C, 32.38	32.33
						H, 5.85	5.86
						N, 5.81	5.40
						I, 52.64	51.66
III	3-(Dimethylaminopropoxy) quinuclidine	40	102–103°/2 mm.	62.2	258–259°	$C_{14}H_{30}I_2N_2O$	
						C, 33.89	33.59
						H, 6.09	6.06
						N, 5.65	5.43
						I, 51.15	50.18
IV	3-[2-(1-Piperidinyl)ethoxy] quinuclidine	35	128–132°/1 mm.	24	233–235°	$C_{16}H_{32}I_2N_2O$	
						C, 36.79	36.73
						H, 6.18	6.22
						N, 5.35	5.22
						I, 48.60	47.92
V	3-[2-(1-Morpholino)ethoxy] quinuclidine	12	137–138°/1 mm.	19	241–243°	$C_{15}H_{30}I_2N_2O$	
						C, 34.36	35.13
						H, 5.34	5.87
						N, 5.51	5.32
						I, 48.41	47.55

Shirley (9). *N*-(β-Chloroethyl) piperidine could not be isolated in pure form as heating produced a viscous nondistillable substance. An ether solution of the crude material was used in the reaction.

Preparation of Ethers—The aminoalkyl chloride (0.08 mole) was added dropwise, over a period of 1 hr., to a refluxing suspension of sodium 3-quinuclidinoxide (0.08 mole) in dry benzene. The mixture was refluxed for 48 hr., cooled, filtered, and the residue washed with 10–20 ml. of dry benzene. After removing the benzene the residue was distilled under reduced pressure to give the ether and unreacted 3-quinuclidinol. The distillate, cooled in a dry ice–acetone bath, was treated with 10–20 ml. of anhydrous ether, and the precipitated 3-quinuclidinol was removed by filtration. The filtrate, after removal of the ether, was distilled under reduced pressure.

Preparation of Dimethiodides—Excess methyl iodide was added dropwise, with stirring, to a cold benzene solution of the ether whereupon a white precipitate formed. The mixture was filtered and the residue recrystallized from hot absolute ethanol.

PHARMACOLOGY

Preliminary pharmacological evaluation showed that all of the compounds evoked some degree of antihypertensive activity, the most effective being the dimethiodide of 3-3-dimethylaminopropoxy quinuclidine. The pharmacological study of the latter is described.

Method—Mongrel dogs weighing 8 to 13 Kg. were anesthetized with sodium pentobarbital, 30 mg./Kg., given intravenously. Mean arterial blood pressure was monitored from an exposed cannulated femoral artery, using a Statham pressure transducer attached to a multichannel recorder (Electronics for Medicine); heart rate was measured with a lead 2 to electrocardiogram and cardiac output using a standard indocyanine green[2] dye dilution technique. Injections of drugs were made into a cannulated femoral vein.

TABLE II—CARDIOVASCULAR DYNAMICS OF 3-(3-DIMETHYLAMINOPROPOXY) QUINUCLIDINE

	Normal	After Drug Adm. 5 min.	25 min.
B.P.[a]	131	99	90
H.R.[b]	184	144	128
C.O.[c]	2.715	1.564	1.546
S.V.[d]	14.7	10.7	12.0
T.P.R.[e]	3.13	3.97	3.72
No. animals	5	4	5

[a] Mean blood pressure in mm. Hg. [b] Mean heart rate in beats per minute. [c] Mean cardiac output in liters per minute. [d] Mean stroke volume in ml. [e] Mean calculated total peripheral resistance in arbitrary units.

Following control measurements of the various parameters described, the compound was injected intravenously at a dose of 3.0 mg./Kg. in one animal and at 5.0 mg./Kg. in 9 other animals since the latter dose produced greater and more prolonged effects.

Results—Blood pressure usually fell precipitously reaching a nadir within 30 to 40 sec. following injection, then rose slightly but generally stabilized within 3 to 4 min. at a level significantly lower than the control. Along with the hypotension there occurred a significantly reduced heart rate and cardiac output and some depression in stroke volume. The calculated total peripheral resistance showed a slight increase (Table II). Pressure gradually rose over a period of 2 hr. but was still significantly reduced at the end of this time interval.

[2] Cardio-green.

In an attempt to delineate the mechanism of action of this compound a number of pharmacologic techniques were employed. Atropine sulfate, 0.5 mg./Kg., did not alter the action of the compound. It was subsequently determined that the compound could effectively block the following procedures: electrical stimulation of the peripheral end of the right vagus, the carotid occlusion pressor reflex, and the pressor effect of nicotine salicylate. In addition, potentiation of the pressor response to epinephrine was noted as well as elimination of the reflex vagal effect that usually develops at the height of the epinephrine response. These findings were observed in 3 animals 20 to 30 min. following administration of the compound.

CONCLUSIONS

The evidence points to ganglionic blockade as the principal mechanism of action of this agent, however, the possibility that it may also exert some direct depressant action on the cardiovascular system has not been eliminated.

SUMMARY

Five new derivatives of quinuclidine have been prepared by reacting 3-quinuclidinol with various aminoalkyl halides *via* the Williamson synthesis. The resulting ethers, as the dimethiodide salts, were tested for antihypertensive activity. All showed varying degrees of activity, the most effective being 3-(3-dimethylaminopropoxy) quinuclidine.

REFERENCES

(1) Stern, P., *Arch. Expil. Pathol. Pharmakol.*, **197,** 377 (1941).

(2) Martell, M., and Soine, T., *J. Pharm. Sci.*, **52,** 331 (1963).

(3) Sternbach, L. H., and Kaiser, S., *J. Am. Chem. Soc.*, **74,** 2215(1952).

(4) Mikhlina, E. E., and Rubtsov, M. V., *Zh. Obshchei. Khim.*, **30,** 163(1960); through *Chem. Abstr.*, **54,** 22632(1960).

(5) Grob, C. A., U. S. pat. 2,917,515 (December 15, 1959).

(6) Nikitskaya, E. S., Usovskaya, V., and Rubtsov, M. V., *Zh. Obshchei. Khim.*, **28,** 161(1958); through *Chem. Abstr.*, **52,** 12863e(1958).

(7) Rubtsov, M. V., Mikhlina, E. E., and Yakhontov, L. N., *Russ. Chem. Rev.*, **29,** 44(1960).

(8) Perrine, T. D., *J. Org. Chem.*, **22,** 1484(1957).

(9) Shirley, D. A., "Preparation of Organic Intermediates," John Wiley & Sons, Inc., New York, N. Y., 1951, pp. 115–117.

Determination of Moisture in Crude Drugs by Gas–Liquid Chromatography

By NOURI Y. MARY

The use of gas–liquid chromatography was investigated as a means of determining the moisture content of crude drugs. Sixteen crude drugs representing a variety of plant parts and products were analyzed using this technique. Water was extracted from the crude drugs by disintegration with anhydrous methanol in a Waring blender and analyzed with a column of Teflon-6 coated with 10 percent polyethylene glycol 1500, using *n*-propanol as an internal standard. Peak height ratios were used to calculate the moisture content. The results obtained with the gas–liquid chromatographic procedure were quantitatively in accord with the results obtained by oven drying at 105° and toluene distillation methods. In addition, the new chromatographic procedure is simpler, more rapid, and has a very good precision (the maximum standard deviation was 0.36).

THE USE of gas–liquid chromatography for the direct analysis of moisture in a number of materials has been reported by many investigators. This technique was found to be reliable and convenient. Most of these studies utilized the method for the analysis of aqueous solutions of organic compounds (1–8). In only a few instances has this method been applied to the determination of water in natural products. The National Bureau of Standards (9) has adopted gas–liquid chromatography for moisture determination of grains. Schwecke and Nelson (10) used gas–liquid chromatography in determining the moisture content of cereal pellets, dried raisins, flour, and other food, and Brekke and Conrad (11) measured the water content of fruits and fruit products employing the same technique.

The simplicity, rapidity, and accuracy of the gas chromatographic process should also make the method well suited to the estimation of moisture in crude drugs. The methods currently recognized in the USP and NF for moisture content measurement of crude drugs include oven drying and toluene distillation. Both of these methods are time-consuming, and in the case of toluene distillation, the use of a comparatively large sample of the crude drug is a further disadvantage. The present investigation was, therefore, undertaken in order to develop an alternate procedure for the rapid measurement of moisture content in crude drugs utilizing a direct gas–liquid chromatographic technique.

EXPERIMENTAL

The crude drugs used in this work were obtained from S. B. Penick and Co., New York, N.Y. All drugs, except digitalis and peppermint leaves, were in powdered form. The leaf samples of digitalis and peppermint were powdered, before analysis, according to the NF XII (12) specification.

Moisture Determination by Oven Drying—The moisture content of all of the crude drugs, except

Received May 24, 1967, from the Department of Biological Sciences, Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216

Accepted for publication August 7, 1967.

Presented to the American Society of Pharmacognosy, Ann Arbor meeting, June 1967.

In an attempt to delineate the mechanism of action of this compound a number of pharmacologic techniques were employed. Atropine sulfate, 0.5 mg./Kg., did not alter the action of the compound. It was subsequently determined that the compound could effectively block the following procedures: electrical stimulation of the peripheral end of the right vagus, the carotid occlusion pressor reflex, and the pressor effect of nicotine salicylate. In addition, potentiation of the pressor response to epinephrine was noted as well as elimination of the reflex vagal effect that usually develops at the height of the epinephrine response. These findings were observed in 3 animals 20 to 30 min. following administration of the compound.

CONCLUSIONS

The evidence points to ganglionic blockade as the principal mechanism of action of this agent, however, the possibility that it may also exert some direct depressant action on the cardiovascular system has not been eliminated.

SUMMARY

Five new derivatives of quinuclidine have been prepared by reacting 3-quinuclidinol with various aminoalkyl halides *via* the Williamson synthesis. The resulting ethers, as the dimethiodide salts, were tested for antihypertensive activity. All showed varying degrees of activity, the most effective being 3-(3-dimethylaminopropoxy) quinuclidine.

REFERENCES

(1) Stern, P., *Arch. Expil. Pathol. Pharmakol.*, **197,** 377 (1941).

(2) Martell, M., and Soine, T., *J. Pharm. Sci.*, **52,** 331 (1963).

(3) Sternbach, L. H., and Kaiser, S., *J. Am. Chem. Soc.*, **74,** 2215(1952).

(4) Mikhlina, E. E., and Rubtsov, M. V., *Zh. Obshchei. Khim.*, **30,** 163(1960); through *Chem. Abstr.*, **54,** 22632(1960).

(5) Grob, C. A., U. S. pat. 2,917,515 (December 15, 1959).

(6) Nikitskaya, E. S., Usovskaya, V., and Rubtsov, M. V., *Zh. Obshchei. Khim.*, **28,** 161(1958); through *Chem. Abstr.*, **52,** 12863e(1958).

(7) Rubtsov, M. V., Mikhlina, E. E., and Yakhontov, L. N., *Russ. Chem. Rev.*, **29,** 44(1960).

(8) Perrine, T. D., *J. Org. Chem.*, **22,** 1484(1957).

(9) Shirley, D. A., "Preparation of Organic Intermediates," John Wiley & Sons, Inc., New York, N. Y., 1951, pp. 115–117.

Determination of Moisture in Crude Drugs by Gas–Liquid Chromatography

By NOURI Y. MARY

The use of gas–liquid chromatography was investigated as a means of determining the moisture content of crude drugs. Sixteen crude drugs representing a variety of plant parts and products were analyzed using this technique. Water was extracted from the crude drugs by disintegration with anhydrous methanol in a Waring blender and analyzed with a column of Teflon-6 coated with 10 percent polyethylene glycol 1500, using *n*-propanol as an internal standard. Peak height ratios were used to calculate the moisture content. The results obtained with the gas–liquid chromatographic procedure were quantitatively in accord with the results obtained by oven drying at 105° and toluene distillation methods. In addition, the new chromatographic procedure is simpler, more rapid, and has a very good precision (the maximum standard deviation was 0.36).

THE USE of gas–liquid chromatography for the direct analysis of moisture in a number of materials has been reported by many investigators. This technique was found to be reliable and convenient. Most of these studies utilized the method for the analysis of aqueous solutions of organic compounds (1–8). In only a few instances has this method been applied to the determination of water in natural products. The National Bureau of Standards (9) has adopted gas–liquid chromatography for moisture determination of grains. Schwecke and Nelson (10) used gas–liquid chromatography in determining the moisture content of cereal pellets, dried raisins, flour, and other food, and Brekke and Conrad (11) measured the water content of fruits and fruit products employing the same technique.

The simplicity, rapidity, and accuracy of the gas chromatographic process should also make the method well suited to the estimation of moisture in crude drugs. The methods currently recognized in the USP and NF for moisture content measurement of crude drugs include oven drying and toluene distillation. Both of these methods are time-consuming, and in the case of toluene distillation, the use of a comparatively large sample of the crude drug is a further disadvantage. The present investigation was, therefore, undertaken in order to develop an alternate procedure for the rapid measurement of moisture content in crude drugs utilizing a direct gas–liquid chromatographic technique.

EXPERIMENTAL

The crude drugs used in this work were obtained from S. B. Penick and Co., New York, N.Y. All drugs, except digitalis and peppermint leaves, were in powdered form. The leaf samples of digitalis and peppermint were powdered, before analysis, according to the NF XII (12) specification.

Moisture Determination by Oven Drying—The moisture content of all of the crude drugs, except

Received May 24, 1967, from the Department of Biological Sciences, Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216

Accepted for publication August 7, 1967.

Presented to the American Society of Pharmacognosy, Ann Arbor meeting, June 1967.

cinnamon, clove, ginger, and peppermint was determined by the NF XII method (12) for drugs containing no constituents volatile at 105°. The moisture content of cinnamon, clove, ginger, and peppermint was determined by the NF XII method (12) for drugs containing ether-soluble constituents volatile at 105°.

Moisture Method by Toluene Distillation—The azeotropic procedure described in the USP XVII (13) was followed in determining the moisture content of all of the crude drugs.

Moisture Determination by Gas–Liquid Chromatography—*Apparatus*—The chromatograph used in this study was a Perkin-Elmer model 154 vapor fractometer equipped with a dual chamber thermistor thermal conductivity cell.

Column Packing—The column used was a stainless steel tubing, 2 m. long and 0.625 cm. in o.d. packed with Teflon-6, 35-mesh, coated with 10% polyethylene glycol (PEG) 1500.[1] The packed column was purchased from Perkin-Elmer, Norwalk, Conn., and was conditioned at 100° for 24 hr. before use.

Chromatography Conditions—The operating temperature was 100°, the detector voltage was 8 v., and the helium carrier gas flow rate was 65 ml./min. The sample size injected was 4 μl.

Standard Curve—The internal standard used in this work was *n*-propanol, which is eluted before the water. Elvidge and Proctor (2) used *n*-propanol and obtained good separation from water in their analysis of aqueous pharmaceutical preparations.

A standard curve was prepared by chromatographing samples containing various amounts of water in mixtures of 3 ml. *n*-propanol and 100 ml. anhydrous methanol. The ratio of peak height of water to *n*-propanol was calculated from the chromatogram and plotted against the corresponding weight[2] ratio of the two compounds; a linear relation was obtained (Fig. 1). Although the standard curve appeared to be very reproducible (the standard deviation of peak height ratios for the standard curve noted at four different runs was 0.047), new standard solutions were prepared every day that unknown samples were chromatographed.

Calculations—Samples of unknowns were chromatographed under the same conditions as the standards. The amount of moisture in the unknown was determined by computing the water–*n*-propanol peak height ratio from the chromatogram, obtaining the corresponding weight ratio from the standard curve, and multiplying by the weight of *n*-propanol. The value so obtained was then converted to percent moisture in the original drug sample.

Extraction Procedure—The procedure used to remove water from the crude drugs was a simple disintegration of the drug with anhydrous methanol in a Waring blender. This procedure has been utilized, with good quantitative results, in the extraction of water from a variety of food products (10, 11).

A 15-Gm. sample of the crude drug to be analyzed was transferred to a stainless steel Waring blender fitted with air-tight screw cap and threaded sampling plug. One hundred milliliters of anhydrous methanol and 3.00 ml. of *n*-propanol were added and

[1] Carbowax 1500, Union Carbide Corp., New York, N.Y.
[2] Water and *n*-propanol were measured by volume and converted to weight using specific gravity calculations.

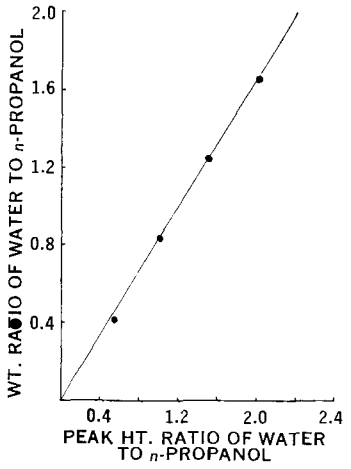


Fig. 1—Weight/peak height ratios relation of water to n-propanol.

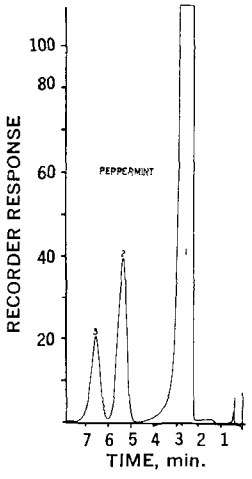


Fig. 2—Typical gas chromatogram of the methanol extract from crude drug (peppermint.) Key: 1, methanol; 2, n-propanol; 3, water.

the sample was disintegrated for 5 min. After blending, the mixture was allowed to settle for 1 min., a sample of the supernatant was transferred with a pipet into a 10-ml. vial, and 4 μl. was injected with a Hamilton microliter syringe into the gas chromatograph.

RESULTS AND DISCUSSION

Water exhibits tailing during elution, particularly on nonpolar liquid phases or on those of intermediate polarity, and on solid supports with appreciable adsorptive properties (14). The separation and quantitative analysis of water has been markedly improved by the use of highly polar liquid phases and inert supports of fluorinated hydrocarbon polymers. Recent work on the analysis of various aqueous systems (5, 8, 10, 11) has shown that the use of columns of Teflon powder coated with 5–20% PEG produced water peaks with greatly reduced tailing and low retention time. The chromatographic column used in this work was Teflon-6 powder coated with 10% PEG 1500. It afforded good resolution and symmetrical peaks. In addition, the retention time of water was low; this allowed its rapid analysis in the extracts. Peak heights were used in the calculation, since standard operating conditions could be well reproduced,

TABLE I—PERCENT MOISTURE IN CRUDE DRUGS AS DETERMINED BY OVEN DRYING (OD), TOLUENE DISTILLATION (TD), AND GAS–LIQUID CHROMATOGRAPHY (GLC) METHODS

Crude Drug	OD[a]	TD[a]	GLC[b]
Agar	15.13	14.00	14.72
Aloe (Cape)	5.00	4.00	5.47
Belladonna root	6.66	6.32	6.43
Cascara sagrada	7.09	6.32	7.11
Colchicum corm	6.22	6.63	5.74
Powdered digitalis (*D. purpurea*)	4.21	4.00	3.89
D. purpurea leaf	5.80	5.49	5.92
D. lanata leaf	7.01	6.00	7.07
Ergot	5.64	5.03	5.55
Gentian	6.07	5.99	6.08
Glycyrrhiza	7.49	6.32	6.87
Starch (corn)	8.69	8.66	9.00
Cinnamon	6.56	6.65	7.38
Clove	5.42	5.44	4.60
Ginger	6.84	6.50	5.95
Peppermint	7.12	6.74	6.91

[a] The values are the average of two determinations each. [b] The results are the means of five chromatographic injections, representing two or more extractions of each crude drug sample.

TABLE II—REPRODUCIBILITY OF GLC PROCEDURE[a]

	% Moisture	
	3-min. Blending	5-min. Blending
Cascara sagrada	6.91, 6.99	6.99, 7.23
D. purpurea leaf	5.78, 5.95	5.78, 6.08
Ergot	5.47, 5.79	5.47, 5.63
Gentian	5.95, 6.11	6.08, 6.08
Starch (corn)	9.08, 9.32	8.92, 9.08

[a] Each value recorded represents the mean of five chromatographic injections from an extract of the crude drug.

the peaks were sharp and symmetrical, and the half-peak widths of water and the standard were essentially equal. A typical chromatogram of the methanol extract from peppermint appears in Fig. 2.

The moisture content of the crude drugs analyzed by gas–liquid chromatography are given in Table I. The results are compared with those obtained by oven drying at 105° and toluene distillation methods. The values obtained with the gas–liquid chromatographic procedure in each case are quantitatively in accord with the values given by the other two methods. The reproducibility of the chromatographic analysis is satisfactory. The maximum standard deviation found was 0.36. The reproducibility of the method for five crude drugs can be readily seen from an examination of the figures shown in Table II.

In preparing the extracts for chromatographic analysis, the crude drugs were disintegrated with anhydrous methanol in a Waring blender for 5 min. Although it seemed that 3 min. of blending were adequate (Table II), the drugs were disintegrated for 5 min. to insure the complete extraction of their water content. The results show that the increased blending time did not increase the values for moisture content. While the gas–liquid chromatographic method furnishes a reliable value for water extractable by methanol, there is a good reason to believe that this water consists of all but that which is chemically combined with the sample of the crude drug (9). This chemically combined water represents a negligible amount of the total moisture present in the drug.

It was possible in this work to evaluate some of the technical problems encountered with each of the three methods. Where it is certain that the loss on drying of a drug is actually water, moisture determination by oven drying is a fairly quantitative procedure. Since a large number of vegetable drugs contain variable amounts of volatile substances other than water, oven drying is not generally applicable except for rough estimations, and the toluene method is to be preferred. However, some disadvantages are also inherent in the toluene distillation method. Moisture determination by this method requires considerable care and attention during operation, particularly when frothing or similar difficulties occur; previous cleaning of the condenser and collecting tubes with dichromate–sulfuric acid solutions does not always prevent the collection of droplets of water on the internal surface, particularly if the material contains traces of volatile fatty acids. Moreover, large amounts of a drug, in some cases more than 50 Gm., must be used, and the direct use of a solvent like toluene without dispersion of the material may fail to obtain the release of all the moisture from the crude drug, a fact which was especially noticeable in this work in the case of aloe. Gas–liquid chromatography, on the other hand, is simple, requires a relatively small sample size, has very good precision, and is more selective than oven drying or toluene distillation methods. When the speed of the three methods is compared, it is found that the chromatographic method is much more rapid. Once the standard curve has been prepared, a determination by gas–liquid chromatography can be completed in a maximum of 15–20 min. Oven drying requires more than 5 hr. for drugs containing no volatile constituents and more than 48 hr. for drugs containing ether-soluble constituents which volatilize at the oven temperature used, while a determination by toluene distillation takes from 3–4 hr.

When all these factors are considered, it can be readily seen that the new chromatographic method is distinctly superior. It surpasses oven drying and toluene distillation in many ways and should be easily adaptable to moisture estimation in a variety of crude drugs and similar natural products.

REFERENCES

(1) Smith, B., *Acta Chem. Scand.*, **13**, 480(1959).
(2) Elvidge, D. A., and Proctor, K. A., *Analyst*, **84**, 461(1959).
(3) Carlstrom, A. A., Spencer, C. F., and Johnson, J. F., *Anal. Chem.*, **32**, 1056(1960).
(4) Stein, H. H., and Ambrose, J. M., *ibid.*, **35**, 550 (1963).
(5) Bennett, O. F., *ibid.*, **36**, 684(1964).
(6) Jones, R. M., *ibid.*, **38**, 338(1966).
(7) Bluestein, C., and Posmanter, H. N., *ibid.*, **38**, 1865(1966).
(8) Casazza, W. T., and Steltenkamp, R. J., *J. Gas Chromatog.*, **3**, 253(1965).
(9) U. S. National Bureau of Standards, *Tech. News Bull.*, **47** (No. 7), 116(1963).
(10) Schwecke, W. M., and Nelson, J. H., *Anal. Chem.*, **36**, 689(1964).
(11) Brekke, J., and Conrad, R., *J. Agr. Food Chem.*, **13**, 591(1965).
(12) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 507.
(13) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 925.
(14) Giddings, J. C., and Keller, R. A., "Advances in Chromatography," vol. 1, Marcel Dekker, Inc., New York, N. Y., 1965, p. 277.

Percutaneous Toxicity of Pyridinethiones in a Dimethylsulfoxide Vehicle

By WELLON D. COLLOM and CHARLES L. WINEK

The percutaneous toxicity of three salts of pyridinethione in water and dimethylsulfoxide is described. Sodium and zinc pyridinethione penetrated the skin of rabbits when applied in a dimethylsulfoxide vehicle and caused hind quarter paralysis. Cadmium pyridinethione did not penetrate the skin in amounts comparable to the other two salts regardless of the vehicle.

THE SALTS of 1-hydroxypyridine-2-thiol have been shown to be effective antifungal and antibacterial agents. The zinc salt (ZnPT) is in current use as an antidandruff agent. Snyder *et al.* (1) have reported on the toxicity of ZnPT in a shampoo base and found no percutaneous effects in rabbits. The sodium salt (NaPT) was shown to penetrate the skin of rabbits causing hind quarter paralysis. Winek and Buehler (2) reported on the intravenous toxicity of the pyridinethiones. Following the recent usage of dimethylsulfoxide (DMSO) by Jacob *et al.* (3, 4) and others as a topical drug for arthritic conditions, considerable work on the enhancement of percutaneous absorption of various compounds by DMSO has been reported. Kligman (5) studied the percutaneous absorption of a large number of different types of compounds in different concentrations of DMSO and other solvents. With most, he found absorption greatly enhanced over other solvents.

The present study concerns the effects of the sodium, zinc, and cadmium salts of pyridinethione in a DMSO vehicle after application to the skin of rabbits.

MATERIALS AND METHODS

Albino rabbits of both sexes with a weight range of 2 to 3 Kg. were used in the study.

Nuded areas on the backs of rabbits, each approximately 100 sq. cm. in size, were dosed for 14 consecutive days. The material was applied using a dermal applicator. Some concentration occurred at the hairline edging the nuded area. The rabbits were fitted with the Newmann (6) restraining harnesses. A commercial depilatory was used to denude the skin. The dose levels were 300 mg./Kg. with sodium-1-hydroxypyridine-2-thiol (NaPT) as a 10% solution in either water or DMSO, 400 mg./Kg. of cadmium-1-hydroxypyridine-2-thiol (CdPT) as a 20% suspension in a 2% solution of methylcellulose[1] in either water or DMSO, and 400 mg./Kg. of zinc-1-hydroxypyridine-2-thiol (ZnPT) as a 10% suspension in a 2% solution of methylcellulose in either water or DMSO. The difference in dose is based on preliminary data concerning toxicity. The number of animals treated is indicated in the tables. A water control without PT salt and two DMSO control animals without PT salt were used for each group.

The pyridinethione salts used were technical, new drug grade obtained from the R. T. Vanderbilt Co. The dimethylsulfoxide was a technical grade obtained from Fisher Scientific. The animals were individually housed with food and water *ad libitum*. Both abraded and nonabraded skin were studied. The head of a small animal clipper was used to abrade the skin.

Animals were observed during the dosing period and for 1 week after treatment. Incoordination, paralysis, and death were the major points of observation. Minor points of observation were diarrhea, weight, and effects on the eyes of the animals.

RESULTS

Results are summarized in Table I; observations on individual animals are presented in Tables II–V. All animals treated with NaPT in DMSO showed signs of incoordination[2] by day 3, while those treated with aqueous NaPT showed these signs on day 5. The NaPT–DMSO treated animals were unable to rise[2] by the sixth day while the two NaPT–water treated animals were unable to rise on the sixth and eighth days, respectively. Seventy percent of the animals dosed with NaPT–DMSO showed sluggish pupillary response by the fourth day and 80% developed diarrhea by the third day. One animal treated with NaPT–water developed a sluggish pupillary response on day 11 and had cloudy drainage of the eye on day 12; it also developed diarrhea.

The animals treated with NaPT–DMSO all died by day 7 with a mean value of 4.3 days, while the two animals treated with NaPT–water died on day 8 and day 13, respectively.

One animal treated with CdPT–water and two treated with CdPT–DMSO showed changes. The remainder of the animals were grossly comparable with the water control. One treated with CdPT–water was unable to rise and showed weight loss. One treated with CdPT–DMSO developed incoordination and weight loss, while the other on the same treatment showed only weight loss.

The animals which were treated with Zn PT–DMSO all developed incoordination from day 2 to day 7, and were unable to rise from times beginning between day 8 and day 13. Twelve animals developed eye drainage, three developed sluggish pupillary responses, and two developed corneal opacities. Thirteen animals died between days 9 and 17. This represents 72% of the animals on this treatment. The other three were sacrificed after 21 days, when they appeared to be recovering. Of the animals dosed with ZnPT–water two died, one on day 2 and one on day 9. These two also developed eye diffi-

Received March 31, 1967, from the School of Pharmacy, Toxicology Laboratories, Duquesne University, Pittsburgh, PA 15219, and Allegheny County Coroner's Office, Pittsburgh, PA 15219

Accepted for publication August 7, 1967.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

[1] Trademarked as Methocel by the Dow Chemical Co., Midland, Mich.

[2] Struggle to right itself when placed on side: no paralysis. Hind limb paralysis.

TABLE I—SUMMARY OF RESULTS

Material Dosed	Onset of Incoordination: No. Affected / No. Dosed	Onset of Incoordination: Days Av.	Onset of Incoordination: Days Range	Onset of Paralysis: No. Affected / No. Dosed	Onset of Paralysis: Days Av.	Onset of Paralysis: Days Range	Death: No. Affected / No. Dosed	Death: Days Av.	Death: Days Range
DMSO–NaPT	10/10	1.7	1–3	10/10	3.7	2–6	10/10	4.3	2–7
H_2O–NaPT	2/2	5	5–5	2/2	7	6–8	2/2	10.5	8–13
DMSO–CdPT	1/10	2	2	0/10	...	...	0/10	...	...
H_2O–CdPT	1/2	5	5	1/2	5	5	0/2	...	...
DMSO–ZnPT	16/16	5.1	2–7	16/16	9.4	7–13	13/16	10.4	9–17
H_2O–ZnPT	1/7	6	6	1/7	7	7	2/7	5.5	2–9
DMSO	0/6	...		0/6	...		0/6	...	...

TABLE II—RABBITS DOSED WITH 10% NaPT AT A LEVEL OF 300 mg./Kg.[a]

Vehicle	Dose Vol., ml.	Onset of Incoordination, Days	Onset of Paralysis, Days	Death, Days	Wt. Loss, Gm.
DMSO[b]	7.0 N[c]	3	6	7	320
DMSO	6.6 N	2	4	5	510
DMSO	7.2 N	3	3	4	320
DMSO	6.5 N	2	5	6	585
DMSO	6.5 N	2	3	3	335
Water	6.1 N	5	6	8	320
DMSO	7.1 A[d]	2	4	4	500
DMSO	7.1 A	2	4	5	440
DMSO	6.6 A	2	3	4	285
DMSO	6.5 A	2	3	3	335
DMSO	6.6 A	1	2	2	435
Water	6.9 A	5	8	13	410

[a] Daily topical application. [b] Three grams per kilogram. [c] Nonabraded skin. [d] Abraded skin.

TABLE III—RABBITS DOSED WITH 20% CdPT AT A LEVEL OF 400 mg./Kg.[a] (NO DEATHS OCCURRED)

Vehicle[b]	Dose Vol., ml.	Onset of Incoordination, Days	Onset of Paralysis, Days	Wt. Loss, Gm.
DMSO[c]	5.4 N[d]	...	...	+95
DMSO	5.9 N	...	...	+40
DMSO	5.2 N	...	...	+45
DMSO	4.8 N	...	...	−410
DMSO	4.8 N	...	...	+60
Water	5.1 N	5	5	−730
DMSO	4.5 A[e]	...	...	+110
DMSO	4.6 A	2	...	−970
DMSO	5.5 A	...	...	+215
DMSO	5.7 A	...	...	+270
DMSO	3.7 A	...	...	+280
Water	4.8 A	...	...	+120

[a] Daily topical application. [b] With 2% methylcellulose. [c] Two grams per kilogram. [d] Nonabraded skin. [e] Abraded skin.

culties; both had drainage and one developed corneal opacity. Weight change data varied, ranging from the loss of 1035 Gm. to a gain of 360 Gm. The two deaths represent 29% of the animals treated. Figure 1 shows the hind limb paralysis produced by treatment and Fig. 2 shows a control animal.

Lung necrosis was present in seven of the 12 animals which died after dosing with NaPT, and in 14 of the 17 which died after ZnPT treatment.

The control animals which were dosed with DMSO–methylcellulose did not develop any of the outward signs of intoxication, and gained from 30 Gm. to 445 Gm. during the course of the dosing. None of the animals developed corneal opacities or irritations of the eye.

TABLE IV—RABBITS DOSED WITH 10% ZnPT IN DMSO AT A LEVEL OF 400 mg./Kg.[a]

Vehicle[b]	Dose Vol., ml.	Onset of Incoordination, Days	Onset of Paralysis, Days	Death, Days	Wt. Loss, Gm.
DMSO[c]	11.2 N[d]	6	8	15	−760
DMSO	10.2 N	4	12	...	+130
DMSO	10.8 N	6	9	...	−740
DMSO	9.5 N	7	11	15	−610
DMSO	10.4 N	5	12	16	−765
DMSO	12.4 N	5	8	9	−635
DMSO	13.2 N	2	9	...	−530
DMSO	11.2 N	5	8	10	−295
Water	9.6 N	6	7	9[f]	−310
Water	12.8 N	...	...	...	−160
Water	14.4 N	...	...	...	−290
Water	15.6 N	...	...	...	−510
DMSO	10.2 A[e]	6	10	13	−270
DMSO	10.3 A	7	13	15	−480
DMSO	9.8 A	6	7	10	−310
DMSO	11.2 A	6	8	12	−1035
DMSO	10.2 A	5	9	17	−995
DMSO	12.8 A	3	9	10	−360
DMSO	12.4 A	3	9	14	−655
DMSO	13.2 A	6	9	10	−525
Water	10.0 A	...	...	...	+360
Water	10.4 A	...	...	2[f]	−200
Water	12.4 A	...	...	...	+10

[a] Daily topical application. [b] With 2% methylcellulose. [c] Four grams per kilogram. [d] Nonabraded skin. [e] Abraded skin. [f] Animals were observed to lick material from sides of cages.

TABLE V—ANIMALS DOSED WITH DMSO CONTAINING 2% METHYLCELLULOSE[a]

Dose Vol.,[b] ml.	Onset of Incoordination, Days	Onset of Paralysis, Days	Death, Days	Wt. Gain, Gm.
4.8 N[c]	...	...	...	30
9.8 N	...	...	...	250
6.6 N	...	...	...	330
4.5 A[d]	...	...	...	140
11.6 A	...	...	...	445
6.9 A	...	...	...	360

[a] The amount used is based on the volume of DMSO used with NaPt, CdPT, and ZnPT. [b] Daily topical application. [c] Nonabraded skin. [d] Abraded skin.

Fig. 1—Hind limb paralysis produced by treatment with ZnPT in DMSO. Animal is in a Newmann harness.

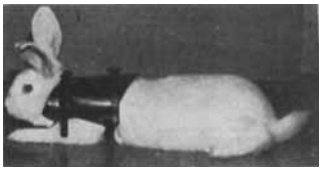


Fig. 2—Control animal in Newmann harness.

All of the animals treated with a pyridinethione salt, either in DMSO or water, showed skin irritation at the site of application which cleared when the treatment was terminated.

DISCUSSION

Dimethylsulfoxide apparently aided in the penetration of both NaPT and ZnPT in comparison with water. The sodium salt readily penetrated the skin when applied in an aqueous solution, but when applied in DMSO, the onset of toxic signs was earlier and paralysis and death resulted earlier in the treatment regimen. The animals all lost weight which is attributed to their inability to eat because of the hind quarter paralysis.

In the ZnPT treated group, DMSO apparently facilitated percutaneous absorption with the production of toxic signs and, in most cases, death. ZnPT in water did not produce paralysis or death except in two cases where the animals were observed to lick applied material from the sides of their cages. A precaution that must be taken in percutaneous studies is to avoid the transfer of applied material from the backs of animals to the sides of the cages. If this occurs, the study becomes an oral ingestion study and not a percutaneous study. In all other cases the material remained on the backs of the animals and was not observed on the sides of the cages. Adequate-size cages can prevent the transfer in most cases.

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The concurrent use by the public of DMSO and other drugs, cosmetics, and soap products can be a potential hazard, as this study and others indicate. Substances that do not penetrate the skin and have been shown to be safe may become hazardous if applied concurrently with DMSO. This may be the strongest indication for restrictions on the use of DMSO regardless of the safety of DMSO itself.

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REFERENCES

(1) Snyder, F. H., Buehler, E. V., and Winek, C. L., *Toxicol. Appl. Pharmacol.*, **7**, 425(1965).
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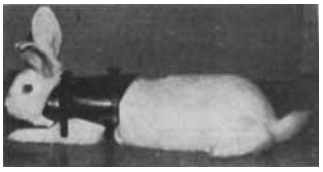

Fig. 2—Control animal in Newmann harness.

All of the animals treated with a pyridinethione salt, either in DMSO or water, showed skin irritation at the site of application which cleared when the treatment was terminated.

DISCUSSION

Dimethylsulfoxide apparently aided in the penetration of both NaPT and ZnPT in comparison with water. The sodium salt readily penetrated the skin when applied in an aqueous solution, but when applied in DMSO, the onset of toxic signs was earlier and paralysis and death resulted earlier in the treatment regimen. The animals all lost weight which is attributed to their inability to eat because of the hind quarter paralysis.

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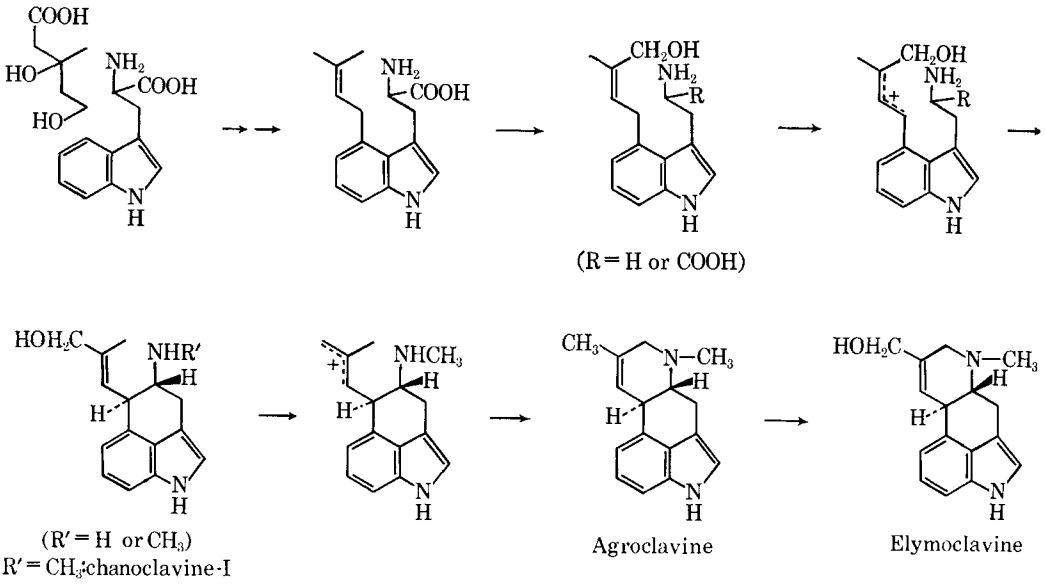

Hypothetical Scheme of Ergot Alkaloid Formation

Scheme I

shown to involve an initial attack at position 10 and rearrangement of the product to give the 8-hydroxylated compound (5). It is also known that these conversions cannot only be carried out by the ergot fungus but also by a whole variety of other organisms (6–10). However, agroclavine was not converted to elymoclavine by either the peroxidase system or by the organisms that do not produce ergot alkaloids. These findings suggest that the mechanism of 8-hydroxylation differs from that of the other oxidations involved in ergot alkaloid formation.

It seems to be a reasonable assumption that the oxidative cyclizations involve formation of allylic carbonium ions as the reactive species. Evidence for this, for instance, is the fact that the hydroxymethyl group of chanoclavine-I reacts with the amino nitrogen to form ring D (11, 3). Several mechanisms can, however, be discussed for the formation of these carbonium ions. The above-mentioned conversion of chanoclavine-I to agroclavine would suggest that initially a hydroxyl group is introduced at an allylic position, followed by phosphorylation and phosphate elimination. In this case the oxygen of the hydroxyl groups would originate from molecular oxygen. It has, however, not been assessed with certainty that chanoclavine-I is a natural intermediate in ergot alkaloid formation. It may only be in equilibrium with an intermediate, the corresponding allylic carbonium ion. In this case, the latter could be formed by hydride abstraction from the allylic position. Reaction of the carbonium ion with the nitrogen would lead to agroclavine, whereas its reaction with water would lead to chanoclavine-I. The oxygen of the hydroxyl group would then originate from the oxygen of the water. The same considerations hold for the formation of elymoclavine from agroclavine, except that cyclization is not possible here.

In order to distinguish between these possibilities the authors have conducted experiments in ^{18}O enriched water and determined the incorporation of ^{18}O into chanoclavine-I and elymoclavine.

METHODS

Claviceps strain SD-58 was grown for 3 days in the medium given previously (12). The mycelium from three 100-ml. shake cultures was then transferred under sterile conditions into a flask containing 110 ml. of the same medium made up with water containing about 1.3% excess ^{18}O. After 14 days of fermentation the culture was harvested and found to contain 40 mg. of alkaloid. The culture filtrate was lyophilized to recover the ^{18}O water for analysis. The residue was dissolved in normal water, and elymoclavine was isolated and purified from this solution (12).

Two fermentations were carried out to produce chanoclavine-I using *Claviceps paspalum* strain Li 342/SE 156. The fungus was grown for 8 days in medium NL 563 (13). In the first experiment the mycelium was then transferred to a 500-ml. flask containing 100 ml. of the following medium (NL 619), made up with water containing about 0.6% excess ^{18}O: ammonium succinate, 3 Gm.; sorbitol, 5 Gm.; propylene glycol, 2 ml.; KH_2PO_4, 0.1 Gm.; $MgSO_4$, 0.03 Gm.; water to 100 ml.; pH adjusted to 5.4 with HCl. After 5 days of fermentation in this medium the alkaloid yield was 44.6 mg. In the second experiment the my-

TABLE I—INCORPORATION OF ^{18}O FROM $H_2{}^{18}O$ INTO ELYMOCLAVINE AND CHANOCLAVINE-I BY *Claviceps*

Claviceps Strain Used	Alkaloid Isolated	Atom % ^{18}O Excess in Water of Medium	Atom % ^{18}O Excess in Isolated Alkaloid	Atoms of Oxygen Incorporated
SD 58	Elymoclavine	1.111	0.006	0.005
Li 342/SE 156	Chanoclavine-I	0.496	0	0
Li 342/SE 156	Chanoclavine-I	3.397	0.021	0.006

celium was transferred to two 500-ml. flasks each containing 50 ml. of NL 500 (13) made up with water containing about 3.5% ^{18}O excess. After 7 days of fermentation the alkaloid yield was 65 mg. In both experiments an aliquot of the culture filtrate was distilled to recover water for ^{18}O analysis. From the remainder, chanoclavine-I was isolated as described previously (13).

^{18}O analyses were carried out by pyrolysis of the organic compounds at 650° in a sealed glass tube with break tip (14). For the analysis of water, naphthalene was added as a carbon source. The tubes were opened in a vacuum system connected to the inlet of an Atlas M 86 mass spectrometer. CO_2 was condensed with liquid nitrogen and the noncondensible gases were pumped off. The CO_2 was then introduced into the mass spectrometer and analyzed for its ^{18}O content. As a reference for the natural ^{18}O content, the same chemical compounds containing no ^{18}O excess were analyzed.

RESULTS AND DISCUSSION

The results given in Table I show that the incorporation of oxygen from water into the hydroxyl groups of both chanoclavine-I and elymoclavine is negligible. Since the ^{18}O enrichment of the water was determined after the fermentation, the possibility can be excluded that extensive dilution of the ^{18}O by oxygen of other components of the medium is responsible for this result. The oxygen of the hydroxyl groups of these two alkaloids must therefore originate from molecular oxygen. Attempts to confirm this directly in the case of elymoclavine have been made using *Claviceps* strain SD-58. However, on incubation under an artificial atmosphere containing ^{18}O enriched oxygen gas, the metabolism of the fungus changed drastically, resulting in a consistently low alkaloid production. Nevertheless, the conclusion seems to be valid that the introduction of the hydroxyl groups into these alkaloids does not involve reaction of OH^- with a carbonium ion generated by hydride abstraction from an allyl position. The results suggest that the oxidation reactions involved in ergot alkaloid formation occur by an initial hydroxylation, possibly by a mixed function oxygenase. In the case of the oxidative cyclizations, these hydroxylations may be followed by phosphorylation and phosphate elimination to generate the reactive carbonium ion.

REFERENCES

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(3) Floss, H. G., Hornemann, U., Schilling, N., Groeger, D., and Erge, D., *Chem. Commun.*, **1967**, 105.
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(6) Brack, A., Brunner, R., and Kobel, H., *Helv. Chim. Acta*, **45**, 276(1962).
(7) Yamano, S., Kozu, Y., Yamada, S., and Abe, M., *Ann. Repl. Takeda Res. Lab.*, **21**, 88(1962).
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(9) Beliveau, J., and Ramstad, E., *Lloydia*, **29**, 234(1966).
(10) Taylor, E. H., Goldner, K. J., Pong, S. F., and Shough, H. R., *ibid.*, **29**, 239(1966).
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A major alkaloid has been isolated from the leaves of *Lobelia portoricensis* Urban. Alkaloids are extracted with ether in alkaline ammoniacal solution, an average of 1.25 percent in the dried leaves. The crystalline alkaloid has a melting point of 115–116°, the hydrochloride, m.p. 187–188°, perchlorate, m.p. 156–158°, and the picrate, m.p. 175–176°. The new alkaloid showed the same sensitivity to reagents as lobeline. Paper chromatography, ascending process, was employed. Stationary phase consisted of formamide, ammonium formate, and formic acid; the mobile phase, equal parts of benzene and chloroform; R_f 75. Infrared absorption spectra showed a R—CO—aromatic organization and the presence of a —NH group; no CH_3 or OH group as in lobeline. The empirical formula is $C_{21}H_{23}NO_{23}$. The formula is shown in structure I. The new alkaloid was found to stimulate the respiratory center.

AMONG THE various members of the *Lobeliaceae* in Puerto Rico, there grows in the wet mountain forests a medium size handsome endemic plant, commonly named tupa and tibey tupa, the *Lobelia portoricensis* Urban, *Tupa portoricensis* Vatke or *Tupa assurgens* A.DC. (1).

The plant is described by Britton (2) and also in *Linnaea* (3) and by Stahl (4). A complete study of the anatomy and histology of the plant is being conducted by the author for publication.

Leaves were collected while the plants were blooming and dried in an air-drier at 45° and powdered in a ball grinder. The material was sifted through a No. 40 sieve.

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until the ether in the Soxhlet became almost colorless and the last extractions gave a negative reaction with the usual alkaloidal reagents.

The liquid extract was evaporated on a water bath and the residue shaken with 50 ml. of ether and 150 ml. of 0.5 *N* sulfuric acid and evaporated again to remove the ether. The acid solution was filtered through a dry filter and the filtrate transferred to a separator. The residue was dissolved again in ether and 0.5 *N* sulfuric acid, shaken, and filtered. This procedure was repeated until a small portion of the last filtrate gave a negative test for alkaloids. Silicotungstic acid reagent was used.

The combined filtered portions in the separator were made slightly alkaline with ammonia T.S. and extracted with 40-ml. portions of ether.

The alkaloids present in the combined ether extracts were purified by the addition of 20-ml. portions of 0.5 *N* sulfuric acid, the separation of the aqueous layer, and extraction with ether in slightly alkaline solution.

The combined ether extracts were evaporated *in vacuo* at a temperature of 40° until the volume was markedly reduced. The temperature was changed to about 20° and the evaporation continued. Abundant crystallization was observed. The last liquid residue, about one-tenth the original volume, was also collected. The crystals in the flask were carefully washed with a small portion of ether and the washings added to the liquid residue collected. The crystals were dried *in vacuo* over phosphoric acid. The melting point of the dried crystalline alkaloidal base was 115–116°. The determination was repeated after the material was recrystallized.

Preparation of Various Alkaloidal Salts—Crystalline hydrochloride, picrate, and perchlorate were prepared and carefully purified, m.p. 187–188°, 175–176°, and 156–158°, respectively.

Quantitative Alkaloidal Determination—The quantitative determination of the alkaloids was performed using the principles of Lynch and Evers (5), who recommended a procedure involving their careful isolation and titration with 0.02 *N* sulfuric acid. The official method of the "Pharmacopoeia of Japan" (6) for lobelia using a Soxhlet extractor was followed. It was found that the leaves contain an average of 1.25% alkaloids.

Sensitivity of the New Alkaloid to Several Common Alkaloid Precipitating Reagents—Martello and Farnsworth (7) published a detailed study on the sensitivity of several common alkaloid precipitating reagents. Their observations included alkaloids of broad spectrum of the various groups.

Seven different alkaloidal reagents for the pyridine–piperidine group of alkaloids were selected for testing the new alkaloid. Five drops of the alkaloidal reagent was added to 1 ml. of a 1 mg. in 100 ml. aqueous solution of the alkaloid dissolved in tartaric acid solution. The amount and nature of the precipitate was observed. Mercuric chloride 1% solution gave a negative result. The rest of the reagents gave a positive reaction in the following order: phosphomolybdic acid reagent, only slightly positive reaction; picric acid, silicotungstic acid, Dragendorff, Mayer, and Wagner reagent.

The same reagents were respectively added to a 1-ml. solution of lobeline sulfate of the same concentration. The same results were obtained.

Chromatography—Several studies in chromatography with the lobelias and their alkaloids are reported in the literature. Steinegger and Ochsner determined the alkaloids in *Lobelia salicifolia* (8) and in *L. inflata* (9) using chromatographic procedures. They also described a method for the determination of lobeline in small quantities in alkaloidal extracts followed by spectrometric work (10). Schmidt and Steinegger (11) reported the separation of the alkaloids of *L. syphilitica* with the aid of paper and column chromatography. In 1959 Steinegger and Ochsner (12) reported new alkaloids in *L. salicifolia* also by means of paper chromatography.

Paper chromatography, ascending process, was employed for the separation of the alkaloids in the extract of *L. portoricensis*. Scheibler and Schull paper No. 204-B was used.

The stationary phase consisted of a solution of 9 vol. formamide saturated with ammonium formate, the excess salt precipitated with 20 vol. of acetone, and the mixture filtered and 1 vol. of formic acid added to the filtrate. The mobile phase best adapted for the resolution of the alkaloids consisted of a mixture of equal parts of benzene and chloroform.

Microburets with ether–formamide solution of the total alkaloidal extract of the leaves of *L. portoricensis*, crystals of the main alkaloid, and residue of the extract after the main alkaloid was separated were used.

A run of about 6 hr. in the solution at a room temperature of 24° sufficed. Then it was placed in the drying oven at a temperature of about 85° for about 1 hr.; it was then examined with ultraviolet light. Alkaloidal spots were clearly seen. Dragendorff reagent with Muniere's modification (13) was used for the development of the chromatogram. Orange-colored precipitates were obtained.

In the first column where the total alkaloidal extract was placed, good resolution of the alkaloids was obtained. A distinct spot of the main alkaloid was observed in the upper part away from the rest of the alkaloids. Six other small size spots were observed.

In the second column where the main alkaloid was placed, a large spot was observed just at the same level as the main spot in the upper part of the first column. Both spots consisted of the same alkaloid. Repeated procedures revealed an R_f of 75.

In the third column, where the rest of the alkaloidal extracts were placed, successive spots were observed at the same level as those of the total alkaloidal extract. No spot was observed in the upper part of the paper in this case, a confirmation that the main alkaloid was already separated and observed in the second column.

Chromatographic studies were conducted under the same conditions with the total alkaloidal extract of *L. inflata*, lobeline alkaloid, lobelanine, lobelanidine, lobelidine, and the total alkaloids of *L. portoricensis*.

Upon examination of the chromatogram of these alkaloidal materials, it was observed that none of the alkaloidal spots were located in a position near or at the same level as the main alkaloid of *L. portoricensis*. Their R_f's were less than 50.

Preliminary Chemical Tests—Chemical tests were

performed with the new alkaloid and with lobeline to test for the formation of acetophenone in alkaline solution. Nitroprussate 5% solution and glacial acetic acid were employed. A positive reaction was obtained; it suggested a lobeline type of alkaloid.

The presence of a carbonyl nucleus was confirmed by the formation of an oxime, which was prepared with various mg. amounts of the crystalline alkaloid. The crystalline compound obtained has a melting point of 237°.

Spectrometric Studies—Infrared absorption spectra analysis was conducted with the pure alkaloid using also pure lobeline for comparison (Fig. 1.). A Perkin-Elmer infrared instrument, model 125, was employed as well as a Perkin-Elmer nuclear magnetic resonance, 60 Mc. apparatus.

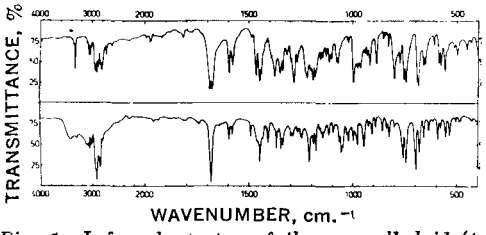
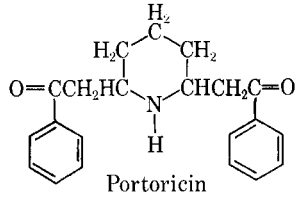


Fig. 1—Infrared spectra of the new alkaloid (top) and lobeline (bottom).

The spectra showed the presence of benzene rings at 1601, 1587, 1495, and 1452 cm.$^{-1}$ confirmed by the presence of small bands at 3085 and 3005 cm.$^{-1}$.

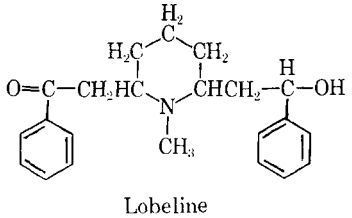
The band at 1690 cm.$^{-1}$ showed valence vibration of —CO groups, in R—CO—Ar organization. The band at 3260 cm.$^{-1}$ is due to the presence of a —NH group. No CH_3 group as in lobeline was indicated nor any —OH group. The suggested empirical formula for the new alkaloid of *L. portoricensis* is $C_{21}H_{23}NO_2$.

The formula suggested is shown in structure I and for lobeline in II.



Portoricin

I



Lobeline

II

Pharmacologic Studies—Though lobelia was introduced into medicine as an emetic by the Rev. Mannasse Gutler in 1775, the first important pharmacologic work was done by Von Ott in 1875. The separation of the alkaloids was done by Heinrich Wieland and Mayer in 1922 (13). Alpha lobeline, lobelidine, and the base B were found. It was determined that α-lobeline has a marked effect on the respiration and at the same time is free from the emetic action which characterizes the administration of the crude lobelia preparations.

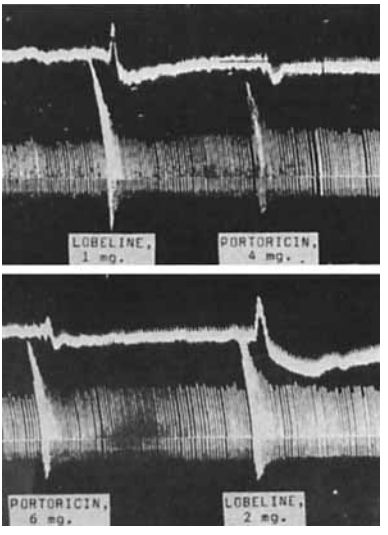


Fig. 2—Action of the new alkaloid on the respiration and blood pressure in dogs.

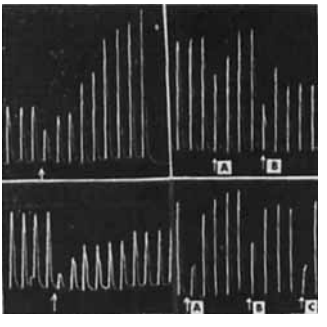


Fig. 3—Action on parasympathetic nervous system of the guinea pig intestine. Contractions with acetylcholine, 0.4 ml. solution, 2:1,000 each, 4 min. Top left: 0.8 mg. portoricin. Top r ght: A, *0.4 mg. lobeline;* B, *0.2 mg. portoricin. Bottom left: 0.8 mg. lobeline. Bottom right:* A, *0.002 mg. atropine sulfate;* B, *0.200 mg. portoricin:* C, *0.001 mg. atropine sulfate.*

Action of the New Alkaloid on the Respiration and Blood Pressure in the Dog—Dogs were anesthetized with pentobarbital using 50 mg./Kg. intravenously and the animal positioned for respiration and blood pressure records. Twenty milligrams of the alkaloid was dissolved in 20 ml. of distilled water with the aid of an equivalent quantity of tartaric acid. Different doses were injected intravenously. A solution of lobeline of the same concentration was prepared and injected periodically to compare the action in the anesthetized dog.

It was clearly shown that the alkaloid stimulates the respiratory center, though the effect is not so marked as with the same concentration of lobeline.

However, the effect in lowering the blood pressure was very slightly demonstrated. (Fig. 2.)

Action in the Cat—Cats were anesthetized with chloralose, 150 mg./Kg. Respiration and arterial pressure were recorded when the alkaloidal solution was injected through the femoral vein. Intravenous injection of the alkaloid stimulates the respiration, though not so marked as when administered to dogs. The action is stronger in the case of lobeline.

Action on the Isolated Intestine of the Guinea Pig—It was demonstrated that the alkaloid blocks the action of acetylcholine in a way similar to atropine, though in a lesser degree, approximately 1/200. Lobeline exerts less activity than the alkaloid (Fig. 3).

Action on the Isolated Intestine of the Rabbit—Trendelenburg technique was employed. When 30 mcg. of the alkaloid was used, inhibition of intestinal motility was observed. With an increased dosage of 100 mcg., the effects were stronger and persisted even after several washings.

Action on the Bronchi of the Isolated Lungs of the Guinea Pig—Konsett technique on the isolated lungs of the guinea pig was used, except that for the registration of the bronchial tonus a water manometer was employed instead of the Piston-Rekorder. Part of the experiments were done on the opened thorax and the rest on the intact organ. Results did not vary, especially when the animal received adequate anesthesia. The experiment was repeated many times.

Results showed that neither the alkaloid nor lobeline inhibited the bronchiospasms produced by the intravenous injection of 12.5 and 25 mcg. of histamine. These effects were neutralized by some synthetic antihistaminics in low concentration.

LD_{50} Determination—White mice in sets of six were used, each of uniform size and weighing 20 to 25 Gm. The determination was carefully performed. The LD_{50} was found to be 95 mg.

SUMMARY

A major alkaloid has been isolated from the leaves of *Lobelia portoricensis* Urban, an endemic woody plant of the wet mountain forests of Puerto Rico.

The alkaloids are extracted with ether in an alkaline ammoniacal medium, an average of 1.25% in the dried leaves. The main alkaloid is separated in monoclinic prisms upon the partial vacuum evaporation of the ether extract at low temperature. The crystalline alkaloid has a melting point of 115–116°. The crystalline hydrochloride has a m.p. of 187–188°; the crystalline alkaloidal perchlorate, m.p. of 156–158°, and the crystalline alkaloidal picrate, m.p. of 175–176°.

The new alkaloid exhibits the same sensitivity as lobeline to the alkaloidal reagents applied to the alkaloids with a pyridine or piperidine structure. This alkaloid was used to compare results and showed the same degree of sensitivity as the new alkaloid in the following order: Wagner, Mayer, Dragendorff, silicotungstic acid, picric acid, and phosphomolybdic acid reagents.

The new alkaloid is separated with paper chromatography, ascending process, using a liquid phase which consisted of formamide, ammonium formate, and formic acid. The mobile phase consisted of a mixture of equal parts of benzene and chloroform. The alkaloid separates in the uppermost portion with an R_f of 75 and quite far from the next six visible much smaller spots.

Infrared absorption spectra showed the presence of benzene rings, a carbonyl structure in a R—CO— aromatic organization, and the presence of a —NH group. No CH_3 or OH group as in lobeline is indicated.

The name portoricin is suggested for the new alkaloid. The suggested empirical formula is $C_{21}H_{23}NO_2$ and the developed formula is shown in structure I.

It stimulates the respiratory center in various animals, particularly in the dog, though the effect is not so marked as with lobeline in the same concentration. However, it has only a very slight effect in lowering the blood pressure.

It inhibits the action of acetylcholine on the isolated intestine of the guinea pig and the rabbit.

Neither the new alkaloid nor lobeline inhibited the bronchiospasms produced by the intravenous injections of small amounts of histamine.

The LD_{50} was determined with white mice and was found to be 95 mg.

REFERENCES

(1) Otero, J. I., Toro, R. A., and Pagán de Otero, L., "Catálogo de los Nombres Vulgares y Científicos de Algunas Plantas Puertorriqueñas," Bulletin No. 37, Agricultural Station, University of Puerto Rico, 1945, p. 164.

(2) Britton, N. L., "Scientific Survey of Puerto Rico and the Virgin Islands," vol. 6, New York Academy of Science, New York, N. Y., p. 272.

(3) *Linnaea*, **38**, 727(1874).

(4) Stahl, A., "Estudios Sobre la Flora de Puerto Rico," Publicaciones de la Federal Emergency Relief Administration, Imprenta Venezuela, San Juan, P.R., 1937, vol. III, p. 148.

(5) Lynch, G. R., and Evers, N., *Analyst*, **64**, 581(1939).

(6) "The Pharmacopoeia of Japan," 7th ed., part II, 1962, Section of the Japanese Pharmacopoeia, Hirokawa Publishing Co., Inc., Tokyo, Japan, 1964, p. 146.

(7) Martello, R., and Farnsworth, N. R., *Lloydia*, **25**, 176(1952).

(8) Steinegger, E., and Ochsner F., *Pharm. Acta Helv.*, **30**, 345(1955).

(9) *Ibid.*, **31**, 89(1956).

(10) *Ibid.*, **31**, 65(1956).

(11) Schmidt, H., and Steinegger, E., *ibid.*, **32**, 205(1955).

(12) Steinegger, E., and Ochsner, F., *ibid.*, **34**, 97(1959).

(13) Randerath, K., "Thin Layer Chromatography," Academic Press Inc., New York, N.Y., 1963, p. 71.

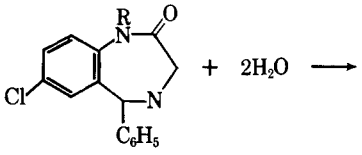
(14) *J. Pharmacol. Exptl. Therap.*, **31**, 145(1927).

Stability Assay for Prazepam and Related Drugs

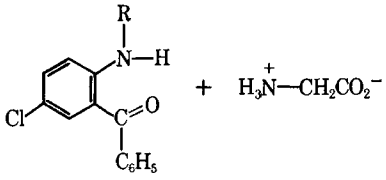
By LESTER CHAFETZ and CHARLES A. GAGLIA, JR.*

The observation that a pronounced difference in spectra of prazepam and its hydrolysis products can be produced by protonation was exploited in the development of an ultraviolet spectrometric stability assay for the drug in tablet and capsule dosage forms. The modified spectrophotometric assay provides sensitivity equivalent to that of a polarographic assay, and its precision and convenience are superior. The procedure applies equally to diazepam dosage forms.

PRAZEPAM (1) is a new psychotropic drug structurally related to diazepam. The compounds differ chemically in the alkyl substituent at the 1-position of the diazepin ring, and their analytical chemistry is closely similar. The only route of degradation observed with these benzodiazepine drugs has been hydrolysis to a benzophenone derivative and glycine. The reaction is illustrated below, where I is prazepam when R is cyclopropylmethyl; diazepam when R is methyl. A suitable assay for use in stability studies should thus discriminate between I and the 2-alkylamino-5-chlorobenzophenone (II).



I



II

Oelschläger, Volke, and Kurek (2) reported that diazepam and its hydrolysis products exhibit closely similar ultraviolet spectra in methanol solution, with absorption maxima at 230 mμ and 236 mμ, respectively, and similar absorptivities. On the basis of this limitation, they developed an oscillographic polarographic determination for the drug in the presence of its degradation products. Polarographic methods have also been reported for diazepam by Senkowski *et al.* (3) and Cimbura and Gupta (4).

Examination of the spectra of prazepam and its benzophenone hydrolysis product showed that one could measure the latter selectively in methanol solution at 408 mμ in the violet; however, it was deemed preferable to combine the stability determination with a content assay by measuring the intact drug instead of the decomposition product. Use of the 365 mμ absorbance maximum in acid–methanol was found to provide a direct measure of unhydrolyzed prazepam in dosage forms. The method was compared with conventional polarography and found to be superior in precision and convenience.

Received June 29, 1967, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication August 10, 1967.

The authors are grateful to F. H. McMillan for preparation of 2-cyclopropylmethylamino-5-chlorobenzophenone and to Allen I. Kay for providing the data used in Table II.

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EXPERIMENTAL

Equipment and Supplies—A Beckman DU spectrophotometer and a Beckman DK-2 recording instrument were employed with 1-cm. silica cells. A Sargent model XXI polarograph equipped with a dropping mercury electrode (DME) and a saturated calomel reference electrode (SCE) was used with the variable instrumental parameters set as follows: span, −1.0 v.; percent EMF applied, 0–50; initial, −0.6 v.; DME (−); d.c. EMF, 1.5 v. span; initial EMF, additive; sensitivity, 0.010 μamp./mm.; drop rate, 1/3–4 sec. damping, off. $E_{1/2}$-values and diffusion currents were determined by the method described by Meites (5).

A 4.08% potassium biphthalate solution was used as buffer, polysorbate 80[1] as maximum suppressor, and 2 *M* potassium chloride as the supporting electrolyte in the polarographic work. Methanol and a mixture of 3 vol. of absolute methanol diluted to 100 vol. with concentrated sulfuric acid (acid–methanol reagent) were the spectrophotometric solvents.

Spectrometric Procedure—Accurately weigh a sample corresponding to about 5 mg. of prazepam into a 100-ml. volumetric flask. Add about 50 ml. of acid–methanol reagent, mix, and dilute to the mark with acid–methanol. Filter the mixture, and determine the absorbance at the maximum, about 365 mμ. Concomitantly determine the absorbance of a dilution of reference prazepam containing about 5 mg./100 ml., accurately known, in the same solvent and at the same wavelength. Designating the absorbance of the sample A_u, the absorbance of the standard, A_s, and the concentration of the standard in mg./100.0 ml. as *C:*

$$\text{prazepam, mg./cap. (tab.)} = A_u/A_s \times C \times \frac{\text{mg. av. cap. (tab.) wt.}}{\text{mg. sample wt.}}$$

Polarographic Method—Accurately weigh an amount of sample equivalent to about 5 mg. of prazepam in a 100-ml. volumetric flask. Add exactly 20.0 ml. of absolute methanol and shake the flask mechanically for 30 min. Add exactly 25.0 ml. of biphthalate, 1 drop of polysorbate 80, and 5.0 ml. of 2 *M* potassium chloride, and dilute the solution to the mark with distilled water. Filter the mixture through paper, discarding the first 10–15 ml. of filtrate. Rinse the polarographic cell with portions of filtrate three times, then fill the cell and deaerate with nitrogen. Insert the DME, passing nitrogen over the surface of the solution, and

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

allow the cell to equilibrate 3 min. Record the polarogram. Prazepam exhibits an $E^1/_2$ of −0.83 to −0.84 v.; the $E^1/_2$ of the decomposition product is about −0.94 v.

Accurately weigh about 100 mg. of reference standard prazepam into a 100-ml. volumetric flask, add 50–60 ml. of absolute methanol, shake the mixture to dissolve the drug, and dilute to the mark with methanol. Pipet 4.0-ml. and 6.0-ml. volumes of the standard into appropriately labeled 100-ml. volumetric flasks, add 16.0 ml. and 14.0 ml. of absolute methanol to the two flasks to bring the volume in each to 20 ml., and proceed as described above for the sample, omitting filtration. Designate the concentration of the standard solution in mg./100 ml. as C, and perform the calculation using both values of C. Designating the diffusion current value of the sample as i_u and the diffusion current of the standard, i_s:

$$\text{prazepam, mg./cap. (tab.)} = C \times i_u/i_s \times \frac{\text{mg. av. cap. (tab.) wt.}}{\text{mg. sample wt.}}$$

RESULTS AND DISCUSSION

Validity of the Spectral Method—Ultraviolet spectral data obtained in methanol and in acid–methanol for prazepam, diazepam, and 2-cyclopropylmethylamino-5-chlorobenzophenone are displayed in Table I. Measurement of the absorbance at 365 mμ in acid–methanol provides a close approximation of the concentration of intact prazepam (or diazepam), since the hydrolysis product has less than 5% its absorptivity. A concomitant of the use of this wavelength is relative freedom from interference by dosage form excipients or many other drugs. The absorbance of prazepam solutions in the acid–methanol solvent was found to be stable for at least 2 hr. This is consistent with the observation of Cimbura and Gupta (4) on the relative stability of diazepam in acid solution. No degradation of prazepam was apparent by either the spectrometric or polarographic techniques in dosage forms stored up to 35 months at room temperature. In order to assess the validity of the method with degraded samples, mixtures of prazepam and molar equivalents of its degradation product were prepared to simulate various degrees of hydrolysis, and the mixtures were assayed for intact prazepam by spectrometry in acid–methanol. The results are presented in Table II.

TABLE I—ULTRAVIOLET SPECTRAL DATA FOR PRAZEPAM, DIAZEPAM, AND 2-CYCLOPROPYLMETHYLAMINO-5-CHLOROBENZOPHENONE

Compd.	λmax., mμ	ε, L./mole cm.	a, L./Gm. cm.
In Methanol			
Prazepam	227	33,350	102.5
	250i[a]	17,550	54.1
	313	2,160	6.7
Diazepam	227	32,500	114.1
	250i	16,700	58.5
	315	2,400	8.5
2-Cyclopropylmethylamino-5-chlorobenzophenone	235.5	24,800	86.7
	365	2,700	9.5
	408	7,100	24.8
In Acid–Methanol			
Prazepam	241	31,200	96.5
	285	16,300	50.2
	365	3,800	11.6
Diazepam	241	28,900	101.5
	283	13,000	45.6
	364	3,900	13.9
2-Cyclopropylmethylamino-5-chlorobenzophenone	257	12,700	44.2
	365	170	0.6

[a] Inflection.

TABLE II—RECOVERY OF PRAZEPAM FROM MIXTURES WITH ITS HYDROLYSIS PRODUCT

Prazepam	mg. Taken "Hydrolyzed Prazepam"	mg. Found Intact Prazepam
5.00	0	5.00, 5.00
4.75	0.25	4.77, 4.77
4.50	0.50	4.51, 4.50
4.00	1.00	3.97, 3.97
3.75	1.25	3.78, 3.77

The inherent advantage in precision of spectrometry over conventional polarography is demonstrated in Table III.

TABLE III—PRECISION OF ANALYTICAL METHODS FOR PRAZEPAM

Trial	Polarography $i_d \times 10^2$, μamp.[a]	Spectrometry $a_{365\ m\mu}$
1	77.04	11.51
2	79.09	11.51
3	79.27	11.50
4	81.09	11.48
5	74.60	11.75
6	73.12	11.51
Mean	77.37	11.54
Rel. S.D.	3.83%	0.90%

[a] Normalized to a concentration of 5.0 mg./100.0 ml.

SUMMARY

A simple spectrophotometric assay for the stability assessment of prazepam dosage forms has been described which uses the absorption maximum at 365 mμ of the protonated form of the drug. Recovery and precision data indicate the validity of the method in routine stability testing of prazepam formulations, and the superiority of the procedure to polarographic assay has been demonstrated. The method would be expected to be equally applicable to diazepam dosage forms because of the close structural relationship of the drugs.

REFERENCES

(1) McMillan, F. H., and Pattison, I., French pat. 1,394,287(April 2, 1965); through *Chem. Abstr.*, **63**, 8387*b* (1965); Wuest, H. M., U. S. pat. 3,192,200(June 29, 1965); through *Chem. Abstr.*, **63**, 11592*c*(1965).

(2) Oelschläger, H., Volke, J., and Kurek, E., *Arch. Pharm.*, **297**, 431(1964).

(3) Senkowski, B. Z., Levin, M. S., Urbigkit, J. R., and Wollish, E. G., *Anal. Chem.*, **36**, 1991(1964).

(4) Cimbura, C., and Gupta, R. C., *J. Forensic Sci.*, **10**, 282(1965).

(5) Meites, L., "Polarographic Techniques," Interscience Publishers, Inc., New York, N. Y., 1955, p. 60.

New Compounds: *N*-Arylglyoxylohydroxamyl Derivatives of Nitrogen Mustard

By JOSEPH P. LAROCCA and COY AVERY GIBSON

The synthesis and physical properties of some *N,N*-bis(2-chloroethyl)arylglyoxylohydroxamamides are described.

COMPOUNDS having antineoplastic activity have been classified in the following types (1, 2): alkylating agents, antimetabolites, hormones and steroids, antibiotics, and miscellaneous. Of these types, the alkylating agents and the antimetabolites are represented by the greatest number of clinically useful antineoplastic drugs. The common alkylating agents are derivatives of bis(2-chloroethyl)-amine (nitrogen mustard), ethylenimines, methane sulfonates, and epoxides. These compounds react spontaneously with electron-rich centers of biologically important material. The first alkylating agent to be used was methyl bis(2-chloroethyl)amine which was also the first drug to be used systemically against cancer (2). Its high toxicity led to the development of other nitrogen mustards (1) of the general type, $R—N(CH_2CH_2Cl)_2$, in an effort to reduce the toxicity. In many cases the nitrogen mustard has been attached to a "carrier moiety" in an effort to permit the nitrogen mustard to be more specific or localized in its effect (3). Recently, the work of Popp (4, 5), Wiley and Irick (6), and Sen and Shirley (7) has shown that less toxic and more effective compounds can be developed through this concept. This "carrier moiety" theory led to the synthesis and testing of benzimidazole, quinacrine, chloroquine, and amino acid mustards (3).

The synthesis and reduction of various arylglyoxylohydroxamides and chlorides have been studied (8). The unreduced chlorides have slight antineoplastic activity (9) probably due to the chemical reactivity of the chlorine (10) resulting in a monoalkylating-like action.

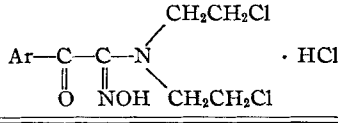
EXPERIMENTAL

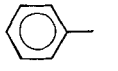
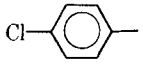
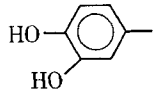
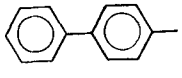
Preparation of Intermediates—*Arylglyoxylohydroxyamyl Chlorides*—These compounds were prepared by previously described procedures (8, 10).

Bis(2-chloroethyl)amine—Bis(2-chloroethyl)amine hydrochloride was prepared according to the method of Ward (11). The free amine was obtained by the method employed by Friedmann and Seligman (12).

Synthesis of Arylglyoxylohydroxamamides—A solution of 0.04 mole of arylglyoxylohydroxamyl chloride in 50 ml. of anhydrous ether was treated dropwise with 0.071 mole of bis(2-chloroethyl)amine in a benzene–chloroform mixture (4:1). The solution became warm and bis(2-chloroethyl)-amine hydrochloride precipitated as the addition was continued. After the addition of the amine was completed, the flask was stoppered tightly and allowed to stand overnight in the refrigerator.

TABLE I—*N,N*-BIS(2-CHLOROETHYL)ARYLGLYOXYLOHYDROXAMAMIDE HYDROCHLORIDES



No.	Ar	M.p.,[a] °C.	% Yield	Anal.—Cl Calcd.	Cl Found[c]	N Calcd.	N Found[c]
1		101–103.5	47.3	32.40	31.30 32.00	8.64	7.86 7.64
2		124–126	50.5	39.38	38.24 39.00	7.79	7.30 7.55
3		125–127	43.4[b]	29.78	27.87 26.99	7.85	7.10 7.14
4		145–147	24.0	26.44	25.84 25.79	6.99	6.55 6.55

[a] All melting points were uncorrected and taken with a Thomas-Hoover capillary melting point apparatus. [b] Allowed to stand under refrigeration for 6 days before crystallization occurred. [c] Analyses performed in this laboratory. Chlorine determined by the Parr peroxide bomb method and nitrogen by the Henegar method of Kjeldahl's analysis.

Received May 29, 1967, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication July 28, 1967.

Presented to the Walter H. Hartung Memorial Symposium.

Abstracted in part from a thesis presented by Coy Avery Gibson to the Graduate School, University of Georgia, Athens, Ga., in partial fulfillment of the Master of Science degree requirements.

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REFERENCES

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New Compounds: Preparation and Hydrogenation of Azomethines Derived from 2,4-Dihydroxyphenyl Benzyl Ketones

By O. LEROY SALERNI, A. POST, F. BAIOCCHI, B. E. SMART, and C. C. CHENG

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The synthetic method is shown in Scheme I. Data on the compounds with structures II and III are presented in Tables I and II, respectively. All the compounds of structure III were isolated and characterized as the hydrochlorides.

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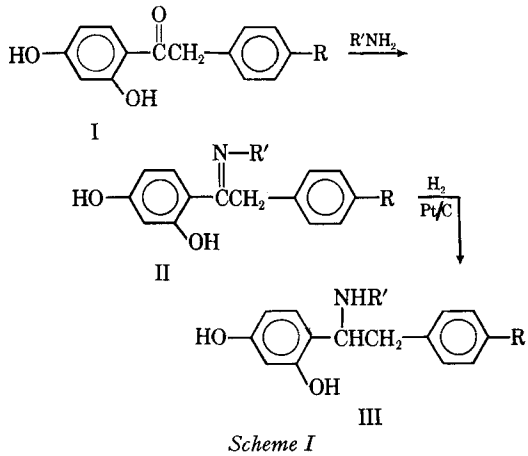

Scheme I

N - (2,4 - Dihydroxyphenyl - p - methoxybenzylmethylene)benzylamine—A solution of 10.3 Gm. (0.04 mole) of 2,4-dihydroxyphenyl-*p*-methoxybenzyl ketone and 4.3 Gm. (0.04 mole) of benzylamine in 350 ml. of dry toluene was refluxed for 24 hr. until the theoretical amount of water was collected in a Dean-Stark apparatus. The solution was concentrated *in vacuo* and the residue on recrystallization from toluene gave 14.0 Gm. (quantitative yield) of yellow crystalline product, m.p. 177–179°. An analytical sample was obtained after an additional recrystallization from toluene, m.p. 180–181°. (See Table I.)

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Received June 7, 1967, from the Midwest Research Institute, Kansas City, MO 64110
Accepted for publication August 8, 1967.
The authors express their appreciation to Mrs. Margaret L. Rounds, Mr. John R. Gravatt, and Mr. Leland R. Lewis for the analytical and instrumental measurements.
This investigation was supported by contract DA-47-193-MD-2953, U. S. Army Medical Research and Development Command, Office of the Surgeon General, Washington, D. C.
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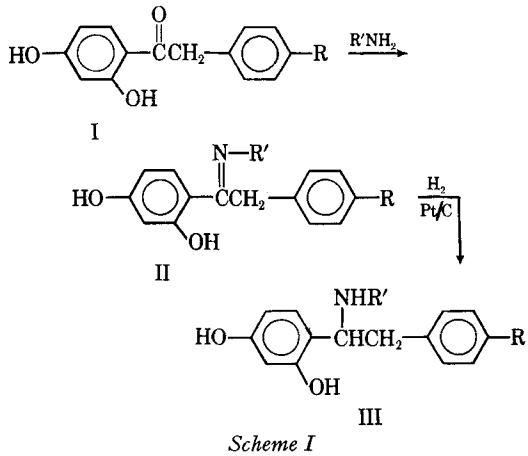

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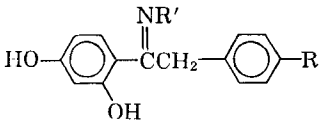
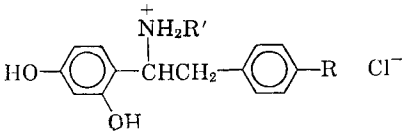
TABLE I—AZOMETHINES



R	R′	M.p., °C.	Yield, %	Recrystn. Solvents	Anal., % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
H	Benzyl	165–167	45	Methanol	79.47	6.03	4.41	79.20	6.34	4.26
Cl	Benzyl	209–210	85	Methanol	71.69	5.16	3.98	71.86	5.29	4.11
OCH_3	Benzyl	180–181	100	Toluene	76.06	6.09	4.03	75.89	6.08	4.01
Cl	Piperonyl	178–180	93	Toluene	66.75	4.58	3.54	66.95	4.68	3.64
Cl	Veratryl	102 dec.	80	Benzene	67.07	5.38	3.40	67.32	5.70	3.24
OCH_3	Piperonyl	169–170.5	89	Ethanol	70.58	5.41	3.58	70.51	5.41	3.51
OCH_3	Veratryl	147–149	80	Benzene	70.75	6.18	3.44	70.76	6.36	3.45
OCH_3	2,4-Dichlorobenzyl	197–198	64	Methanol	63.47	4.60	3.36	63.58	4.56	3.47
OCH_3	3,4-Dichlorobenzyl	179–180	90	Xylene	63.47	4.60	3.36	63.80	4.59	3.29

TABLE II—SECONDARY AMINE SALTS



R	R′	M.p., °C.	Yield, %	Recrystn. Solvents	Anal., % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
H	Benzyl[a]	170–172	66	Propanol–hexane	70.88	6.23	3.94	71.13	6.50	3.77
OCH_3	Benzyl	180–181	100	Ethanol	68.48	6.27	3.63	68.32	6.36	3.50
Cl	Piperonyl[b]	181–183	74	Propanol–ether	60.84	4.87	3.23	61.13	5.17	3.49[d]
Cl	Veratryl[c]	185–187	67	Isopropyl alcohol	61.34	5.60	3.11	61.70	5.83	3.22
OCH_3	Piperonyl	229–231	42	Ethanol	64.26	5.63	3.26	64.03	5.43	3.10
OCH_3	Veratryl	174–175.5	69	Ethanol	64.64	6.33	3.14	64.76	6.27	3.28
Cl	Benzyl	178–180	38	Propanol ether	63.17	5.55	3.51	62.92	5.46	3.38[e]

[a] Compounds (R′ = benzyl) were hydrogenated in methanol. [b] Compounds (R′ = piperonyl) were hydrogenated in methanol–acetonitrile (1:4). [c] Compounds (R′ = veratryl) were hydrogenated in acetone. [d] Calcd. for Cl: 16.33. Found: Cl, 16.28. [e] Analyzed for 0.5 mole water.

methanol and saturated in the cold with dry hydrogen chloride which gave 3.0 Gm. (67% yield) of hydrochloride salt, m.p. 184–186°. Recrystallization from isopropyl alcohol gave an analytical sample, m.p. 185–187°. (See Table II.)

REFERENCES

(1) Bovarnick, M. R., Lindsay, A., and Hellerman, L., *J. Biol. Chem.*, **163**, 535(1946).
(2) Speck, J. F., and Evans, E. A., Jr., *ibid.*, **159**, 71, 83 (1945).
(3) Keller, D. M., and Lotspeich, W. D., *ibid.*, **234**, 987 (1959).
(4) Dickens, F., and Salmony, D., *Biochem. J.*, **64**, 645 (1956).
(5) Smith, M. J. H., and Jeffrey, S. W., *ibid.*, **64**, 589 (1956).
(6) Lieberman, M., and Biale, J. B., *Plant Physiol.*, **31**, 420(1956).
(7) Chapman, E., and Stephen, H., *J. Chem. Soc.*, **1923**, 404.
(8) Klarmann, E., *J. Am. Chem. Soc.*, **48**, 2358(1926).

Some Biological Properties of 2-(4-Isobutylphenyl)-propionic Acid

Sir:

We have previously described some of the biological properties of ibufenac (4-isobutylphenylacetic acid) (1), an anti-inflammatory, analgesic antipyretic compound, which subsequently has proved to be effective in the treatment of rheumatoid arthritis and other rheumatic diseases.

Since then we have prepared and investigated the biological properties of a large number of other substituted phenylalkanoic and alkenoic acids and derivatives. The highest pharmacological activity is found in substituted 2-phenylpropionic acids, and 2-(4-isobutylphenyl)-propionic acid, ibuprofen (British Pharmacopoeia Commission approved name), has been selected for further studies. Some of the properties of this compound are given below.

Ibuprofen, a colorless crystalline stable solid, m.p. 75–77°, is relatively insoluble in water, but readily soluble in most organic solvents.

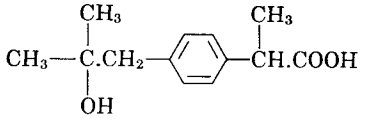
The anti-inflammatory, analgesic, and antipyretic potencies of this compound compared with ibufenac and acetylsalicylic acid are shown in Table I. Three of the experimental methods used have been described previously (1), the other is a modification of a mouse-writhing technique in which intraperitoneal acetylcholine was used as the writhing agent. In all tests the compounds were administered orally in 10% mucilage of acacia in graded doses. From the results in Table I it will be noted that ibuprofen is approximately 4 to 8 times more active than ibufenac.

TABLE I—ANTI-INFLAMMATORY, ANALGESIC, AND ANTIPYRETIC ACTIVITIES OF IBUPROFEN AND IBUFENAC AFTER ORAL ADMINISTRATION (ACETYLSALICYLIC ACID = 1)

	Anti-Inflammatory Activity UV Erythema	Analgesic Activity—Mouse Writhing	Analgesic Activity—Inflamed Rat's Paw	Anti-pyretic Activity Yeast-Fevered Rat
Ibufenac	2–4	2	2–4	4
Ibuprofen	16–32	16–32	8–16	20

The resolution of ibuprofen has been effected with (−)-1-phenylethylamine, and no difference has been detected in the anti-inflammatory potency of the two enantiomorphs in the guinea pig ultraviolet erythema test. Shen and his colleagues (2), have however found in related compounds, *e.g.*, 2-(3-chloro-4-cyclohexylphenyl) propionic acid, that a large part of the anti-inflammatory activity resides in the *dextro* isomer.

The principal urinary metabolites of ibuprofen in man have been isolated and characterized by infrared and nuclear magnetic resonance spectra as I and II. It may be significant that both of these metabolites were found to be *dextro*-rotatory.



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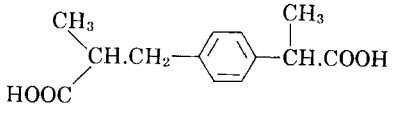

II

No appreciable species differences have been found in the blood level/time curves after single oral doses of ibuprofen. In the dog, rat, and guinea pig, peak levels of the drug occurred at or before 1.5 hr. When six healthy men took a dose of 200 mg. after overnight fasting, the mean plasma concentrations at 0.75, 1.5, 3, and 6 hr. were 15.0, 14.0, 7.4, and 2.3 mcg./ml., respectively. The amount of drug in the serum or plasma of animals was measured by the method previously described (3). The same method was used for human plasma, except that the amount of drug was assayed by thin-layer chromatography. Experiments in which the ^{14}C-labeled drug was administered orally to dogs indicated that ibuprofen is well absorbed. Clinical studies are in progress and will be reported later.

(1) Adams, S. S., Cliffe, E. E., Lessel, B., and Nicholson, J. S., *Nature*, **200**, 271(1963).
(2) Shen, T. Y., reported in *Chem. Eng. News*, **45** (7), 10(1967).
(3) Adams, S. S., and Cliffe, E. E., *J. Pharm. Pharmacol.*, **17**, 173(1965).

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Received August 17, 1967.
Accepted for publication September 14, 1967.

Kinetics of Pharmacologic Activity of Succinylcholine in Man

Sir:

Studies of the kinetics of pharmacologic effects of drugs in man are only in their beginning. The limited knowledge of this aspect of pharmacokinetics restricts considerably the utility of mathematical analyses of the kinetics of drug absorption, distribution, and elimination. This author has recently developed a number of mathematical expressions which relate the time course of pharmacologic activity of a drug to its apparent first-order rate constant for elimination, to the nature of its dose-response relationship, and to the dose given (1–5). While they have been found to describe satisfactorily the time course of pharmacologic activity of a number of drugs (5), a relatively rigorous evaluation of these mathematical expressions has been difficult due to the paucity of quantitative clinical data. A very extensive quantitative clinical study of the effects of the skeletal muscle relaxant succinylcholine in man by Walts and Dillon (6) has now provided an opportunity for a thorough evaluation of the pharmacokinetic formulations developed in previous reports from this laboratory (1–5).

Walts and Dillon (6) administered succinylcholine chloride in doses of 0.5, 1.0, 2.0, and 4.0 mg./Kg. body weight to a total of 120 patients under general anesthesia and determined the effect of this drug on the contraction of a skeletal muscle (thumb adduction) in response to stimulation of its motor nerve (ulnar nerve at the wrist). The results of their study form the basis of the pharmacokinetic analyses which follow.

It has been proposed that the duration (t) of a pharmacologic effect elicited by a drug given intravenously may be related to its dose (A_0) by the expression:

$$t = \frac{2.3}{k} \log A_0 - \frac{2.3}{k} \log A_{\min.} \qquad \text{(Eq. 1)}$$

where k is the apparent first-order rate constant for elimination, and $A_{\min.}$ is the minimum effective dose. The derivation of the equation and the assumptions upon which it is based have been outlined previously, and the linear relationship between duration of effect and logarithm of dose predicted by the equation has been shown to occur with several drugs (5). It has been pointed out that the equation should hold for any arbitrary end point (for example, 20, 80, or 100% of a normal value for grip strength) (5). A rigorous test of the applicability of Eq. 1 would be to demonstrate that the slope of the line obtained by plotting t *versus* log A_0 is the same regardless of the end point used. Such a test is now possible with the data of Walts and Dillon (6). Figure 1 shows that (*a*) there is a linear relationship between the duration of a given degree of muscular paralysis and the logarithm of the dose of succinylcholine, and (*b*) the slopes of the respective lines for 3 different end points are essentially the same. The apparent first-order elimination rate constant for succinylcholine determined from these slopes is 0.20 reciprocal minutes. $A_{\min.}$ values for 10, 50, and 90% muscle paralysis were calculated by use of Eq. 1; a plot of the logarithm of these values against the intensity of the pharmacologic effect yields a straight line (inset of Fig. 1).

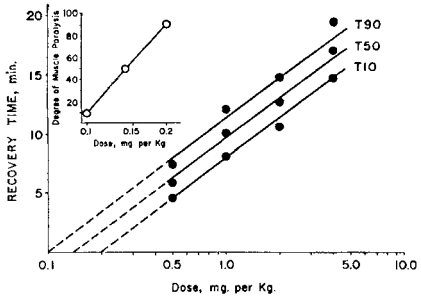

Fig. 1—Relationship between intravenous dose of succinylcholine chloride and duration of various degrees of neuromuscular inhibition in human subjects. T10, T50, *and* T90 *are the times required to recover 10, 50, and 90% of normal muscle contraction force. The data are from* Reference 6 *and represent average values from 13–16 subjects for each dose. Inset: intensity of effect* vs. *log dose, based on dose values obtained by extrapolation of duration of effect* vs. *log dose plots.*

The rate of decline of a pharmacologic effect with time should be constant (rather than exponential) if (*a*) the drug is eliminated by apparent first-order kinetics, (*b*) there is a linear relationship between the logarithm of the dose and the intensity of the elicited pharmacologic effect, and (*c*) certain other requirements are met (5). Specifically,

$$E = E_0 - \frac{km}{2.3} t \qquad \text{(Eq. 2)}$$

where E is the intensity of the pharmacologic effect at time, t, k is the apparent first-order rate constant for drug elimination, m is the slope of the linear portion of a plot of E *versus* log dose, and E_0 is the intercept at zero time of the extrapolated linear portion of a plot of E against t (5). Since the requirements listed above are met by succinylcholine, the intensity of its effect should

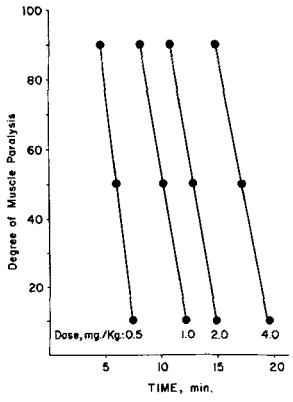

Fig. 2—Degree of muscle paralysis as a function of time after intravenous administration of 0.5 to 4.0 mg./Kg. succinylcholine chloride to groups of 13–15 human subjects. Data from Reference 6.

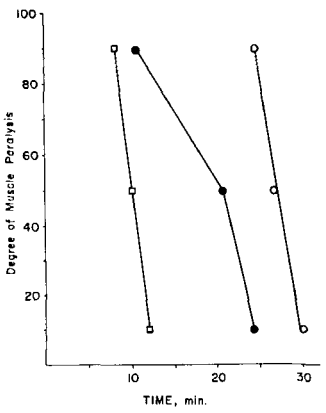

Fig. 3—Degree of muscle paralysis as a function of time after intravenous and intramuscular administration of succinylcholine chloride to human subjects. Key: □, 1 mg./Kg. i.v., 15 subjects; ●, 1 mg./Kg. i.m., 7 subjects; ○, 2 mg./Kg. i.m., 9 subjects. Data from Reference 6.

decline linearly with time, the rate of decline being independent of dose. This is indeed the case with doses ranging from 0.5 to 4 mg./Kg. (Fig. 2). The apparent first-order rate constant for elimination of succinylcholine, as determined from the slopes in Fig. 2 (which yield values of *km*) and the response-log dose plot shown as the inset in Fig. 1 (which yields the value of *m*) is 0.18 reciprocal minutes. This value, obtained from *terminal* pharmacologic activity data (*i.e.*, from the last 0.2 mg./Kg. of each dose), is in very good agreement with the 0.20 $min.^{-1}$ value obtained from *total* pharmacologic activity data (*i.e.*, duration of effect from doses up to 4 mg./Kg.).[1] These results demonstrate therefore the successful application of both equations to the data and provide further support for the contention that the kinetics of many pharmacologic effects of drugs in man can be described adequately by means of appropriate mathematical expressions.

An interesting example of the use of pharmacologic activity data for biopharmaceutic analyses is provided by the data shown in Fig. 3. It is evident that the intensity of pharmacologic activity after intramuscular administration of 1 mg./Kg. succinylcholine declines more slowly than after the same dose given intravenously. This shows that the drug is still being absorbed from its intramuscular injection site. The activity decline slope following the 1 mg./Kg. intramuscular (i.m.) dose approaches that obtained with the i.v. dose when 10–15% of the i.m. dose remains in the body (as evident by reference to the log dose-response plot in Fig. 1). In the case of the 2 mg./Kg. i.m. dose, the activity decline 25 min. after injection is similar to that following an i.v. dose, showing that absorption is essentially complete when about 10% of the i.m. dose remains in the body. This consistency in the data from the 1 mg./Kg. and 2 mg./Kg. doses suggests, as a first approximation, that absorption was by first-order kinetics. Additional information for doses between 1 mg./Kg. and 2 mg./Kg. would permit more definitive conclusions. Thus, a kinetic analysis of pharmacologic activity data is useful not only in clinical pharmacology where it can be employed to establish the most effective dosage regimen for a drug, but also in biopharmaceutics where it can be applied for the evaluation of dosage form effects (5).

[1] This rate constant reflects the rate of decline of drug concentration at the neuromuscular junction; the rate-limiting step in this process is probably diffusion of succinylcholine from that site to the blood rather than enzymic hydrolysis in the blood (7).

(1) Levy, G., *J. Pharm. Sci.*, **53**, 342(1964).
(2) Levy, B., *Brit. J. Anaesth.*, **36**, 694(1964).
(3) Levy, G., *Nature*, **206**, 517(1965).
(4) Levy, G., and Nelson, E., *J. Pharm. Sci.*, **54**, 812 (1965).
(5) Levy, G., *Clin. Pharmacol. Therap.*, **7**, 362(1966).
(6) Walts, L. F., and Dillon, J. B., *Anesthesiology*, **28**, 372 (1967).
(7) Foldes, F. F., *Acta Anaesth. (Scand.)*, **1**, 63(1957).

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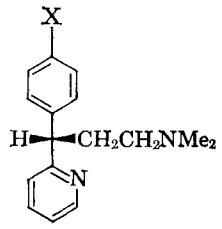
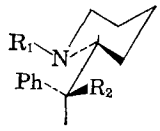
Received August 23, 1967.
Accepted for publication September 29, 1967.

Absolute Configurations of the Enantiomeric Pheniramines, Methylphenidates, and Pipradrols

Sir:

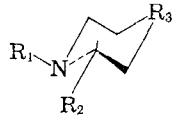
The configurations of biologically active compounds are of interest in studies on the modes of drug action and on the interactions of drugs with receptors (1, 2). Antipodes of the title compounds exhibit significant differences in biological activity (2–6) and speculation regarding the absolute configuration of chlorpheniramine (I*a*) and its receptor exists in the literature (*cf. References 7* and *8*). Since the title compounds are congenerically related, it was possible to develop and exploit a single sequence of reactions leading to the determination of the absolute configurations of all 16 optical isomers of the seven compounds (I*a*, *b*, *c*, threo-VII, XVIII, XIX, XX) of biological interest. Accordingly, the pheniramines (I) were converted to the methylphenidate series (*cf.* VII) in which the relative configuration of the two asymmetric centers is known (6, 9). The endocyclic center of asymmetry introduced in the process was maintained intact while the original exocyclic center was destroyed in the sequence leading to XIII and the pipradrols (XVIII, XIX, XX) the absolute configuration of which was determined by an aufbau sequence[1] involving pipradrol (XVIII) prepared from XIV, (+)-(R)-piperidine-2-carboxylic acid.





I*a* (X = —Cl)
I*b* (X = —Br)
I*c* (X = —H)

II (R₁ = —COPh, R₂ = —$CH_2CH_2NMe_2$)
III (R₁ = —COPh, R₂ = —$CH{=}CH_2$)
IV (R₁ = —COPh, R₂ = —COOH)
V (R₁ = —H, HCl, R₂ = —COOH)
VI (R₁ = —H, R₂ = —$CONH_2$)
VII (R₁ = —H, HCl, R₂ = —CO_2Me)
VIII (R₁ = —SO_2Ph, R₂ = —CO_2Me)
IX (R₁ = —SO_2Ph, R₂ = —CH_2OH)
X (R₁ = —SO_2Ph, R₂ = —CH_2Cl)



XI (R₁ = —SO_2Ph, R₂ = —$CPh{=}CH_2$, R₃ = —CH_2—)
XII (R₁ = —SO_2Ph, R₂ = —COPh, R₃ = —CH_2—)
XIII (R₁ = —SO_2Ph, R₂ = —$COHPh_2$, R₃ = —CH_2—)
XIV (R₁ = —H, R₂ = —COOH, R₃ = —CH_2—)
XV (R₁ = —SO_2Ph, R₂ = —COOH, R₃ = —CH_2—)
XVI (R₁ = —SO_2Ph, R₂ = —CO_2Me, R₃ = —CH_2—)
XVII (R₁ = —H, R₂ = —CO_2Me, R₃ = —CH_2—)
XVIII (R₁ = —H, R₂ = —$COHPh_2$, R₃ = —CH_2—)
XIX (R₁ = —H, R₂ = —$CHPh_2$, R₃ = —CH_2—)
XX (R₁ = —H, R₂ = —$COHPh_2$, R₃ = —S—)

Palladium–hydrogen dehalogenation of both (+) - 3 - (*p* - chlorophenyl) - 3 - (2′ - pyridyl)-1-dimethylaminopropane (I*a*) hydrogen maleate, m.p. 113–114°, $[\alpha]_D^{22}$ + 19°, (c 1.15, water) and the corresponding bromine analog (I*b*), m.p. 106–108°, $[\alpha]_D^{25}$ +34° (c 10.40, dimethylformamide) afforded (+)-3-phenyl-3-(2′-pyridyl)-1-dimethylaminopropane (I*c*) identified as the monohydrobromide salt, m.p. 176–177°. Hydrogenation of I*a*, I*b*, and I*c* with platinum oxide yielded, in each case, an uncrystallizable, dextrorotatory (ethanol) mixture of diastereoisomeric amines. The mixtures were subjected to Schotten-Baumann conditions to convert them to the dextrorotatory *N*-benzoyl derivatives which gave (+)-erythro-3-phenyl-3-(2′-[1′-benzoyl]-piperidyl)-1-dimethylaminopropane (II), m.p. 134–136°, $[\alpha]_D^{30}$ +50.5° (c 6.95, ethanol), by fractional crystallization. Subjection of II to the conditions of the Hofmann elimination yielded (+) - erythro - 3 - phenyl - 3 - (2′ - [1′ - benzoyl]-piperidyl)-1-propene (III), m.p. 125–126°, $[\alpha]_D^{26}$ +121° (c 4.66, carbon tetrachloride). Ozonolysis of III provided (−)-erythro-2-phenyl-2-(2′-[1′-benzoyl]piperidyl)acetic acid (IV), m.p. 221–222°, $[\alpha]_D^{27}$ −51.4° (c 4.71, ethanol), from which (−) - erythro - 2 - phenyl - 2 - (2′ - piperidyl)-acetic acid hydrochloride (V), m.p. 233–235°, $[\alpha]_D^{26}$ −84° (c 1.00, water), was obtained by acid hydrolysis.

An authentic sample of V was prepared from racemic erythro-2-phenyl-2-(2′-piperidyl)acetamide (*cf.* VI), m.p. 174–175°. The (−)-erythro-

[1] We recently learned that Portoghese and Pazdernik also completed the aufbau sequence and reported this at the Fourth Annual Medicinal Chemistry Meeting in Miniature, University of Kansas, Lawrence, Kan., April 4, 1966; (*cf. Reference 2*).

isomer (VI), m.p. 168–169°, $[\alpha]_D^{26}$ −64° (c 1.00, 6:4-ethanol-water), obtained by resolution with (−)-tartaric acid (6) afforded V, m.p. 233–235°, $[\alpha]_D^{26}$ − 81°, (c 1.00, water) upon acid hydrolysis. The mixed melting point of both samples of V was not depressed. Esterification of V yielded the crude levorotatory (water) erythro-ester hydrochloride (VII) which was converted to VIII, (−)-erythro-2-phenyl-2-(2′-[1′-benzenesulfonyl]piperidyl)acetic acid methyl ester, m.p. 154–155°, $[\alpha]_D^{26}$ −49°, (c 1.13, methanol) with benzenesulfonyl chloride in pyridine. Lithium aluminum hydride reduction of VIII yielded (−)-erythro-2-phenyl-2-(2′-[1′-benzenesulfonyl]piperidyl)ethanol (IX), m.p. 105–105.5°, $[\alpha]_D^{20}$ −4.5° (c 4.88, methanol). This was treated with *p*-toluenesulfonyl chloride in pyridine to obtain (+)-erythro-2-phenyl-2-(2′-[1′-benzenesulfonyl]piperidyl)ethyl chloride (X), m.p. 157–158°, $[\alpha]_D^{26}$ +25° (c 2.00, benzene) which was converted to 1-phenyl-1-(2′-[1′-benzenesulfonyl]piperidyl)ethylene (XI), m.p. 87–88°, $[\alpha]_D^{23}$ −66.8°, (c 1.80, benzene), with potassium amide in liquid ammonia. Ozonolysis of XI afforded (−)-1-benzenesulfonyl-2-benzoylpiperidine (XII), m.p. 103–104°, $[\alpha]_D^{26}$ −20° (c 7.00, tetrahydrofuran). Treatment of this ketone with phenyl magnesium bromide yielded XIII, (−)-α-(2′-[1′-benzenesulfonyl]piperidyl)-benzhydrol, m.p. 167–169°, $[\alpha]_D^{20}$ −67°, (c 1.19, tetrahydrofuran), which was also obtained, m.p. and mixed m.p. 167–169°, $[\alpha]_D^{20}$ −64° (c 1.21, tetrahydrofuran), from (R)-(+)-piperidine-2-carboxylic acid (XIV), m.p. 276–277°, $[\alpha]_D^{21}$ +25.5° (c 3.00, water). Following platinum oxide reduction of picolinic acid in the presence of, and resolution with (+)-tartaric acid, XIV was obtained and was treated with benzenesulfonyl chloride in base to give a crude dextrorotatory (benzene) product (XV), 1-benzenesulfonylpiperidine-2-carboxylic acid. Esterification afforded (+)-1-benzenesulfonylpiperidene-2-carboxylic acid methyl ester (XVI), m.p. 61–63°, $[\alpha]_D^{24}$ +54° (c 3.03, benzene). Treatment with phenyl magnesium bromide converted XVI to XIII. Esterification of XIV provided crude, dextrorotatory (ethyl ether) piperidine-2-carboxylic acid methyl ester (XVII) which was converted with phenyl magnesium bromide to (+)-α-(2-piperidyl)-benzhydrol (XVIII), m.p. 98–99°, $[\alpha]_D^{26}$ +58° (c 4.00, methanol), and thence, with benzenesulfonyl chloride in pyridine, to XIII, identical in all respects to that obtained by the other methods described. Birch reduction of XVIII afforded (+)-2-benzhydrylpiperidine (XIX) isolated as the levorotatory hydrochloride salt. The rotatory dispersion curves of the levorotatory hydrochlorides of XVIII, XIX, and XX justify assignment of the same configuration to these compounds.

Accordingly, the absolute configuration of the the more active (2, 5) levorotatory pipradrol hydrochlorides and their dextrorotatory free bases is (R). The absolute configuration of V, VI, and VII is (2S:2′R) while the base-catalyzed epimerization (6) of VI to the (+)-threo-amide and conversion of the latter to the corresponding (+)-threo-acid (*cf.* V) and (+)-threo-ester hydrochloride (*cf.* VII) establishes the latter, the more active analeptic antipode (6) as (2R:2′R). The antihistaminically more active, dextrorotatory pheniramine maleates have the (S) configuration.

The significance of these findings will be discussed in definitive reports to follow (*cf. References 2, 8*).

(1) Belleau, B., and Puranen, J., *J. Med. Chem.*, **6**, 325(1963); Belleau, B., and Lacasse, G., *ibid.*, **7**, 768(1964); Belleau, B., *ibid.*, **7**, 776(1964); Portoghese, P., *ibid.*, **8**, 609(1965); Portoghese, P., *J. Pharm. Sci.*, **55**, 865(1966).
(2) Portoghese, P., Pazdernik, T. L., Kuhn, W. L., Hite, G., and Shafi'ee, A., *J. Med. Chem.*, to be published; and previous papers in this series.
(3) Brittain, R. T., D'Arcy, P. F., and Hunt, J. H., *Nature*, **183**, 734(1959).
(4) Roth, F. E., and Govier, W. M., *J. Pharmacol. Exptl. Therap.*, **124**, 347(1958).
(5) Belleau, B., *J. Med. Chem.*, **2**, 553(1960).
(6) Roemetsch, R., U. S. pat. 2,838,519 (1958) p. 2.
(7) Barlow, R. B., "Introduction to Chemical Pharmacology," John Wiley & Sons, Inc., New York, N. Y., 1965, p. 373; see also *Reference 8*.
(8) Hite, G., and Shafi'ee, A., *J. Pharm. Sci.*, **56**, 1041 (1967).
(9) Weisz, I., and Dudas, A., *Acta Pharm. Hung.*, **31/B**, Suppl. 116(1961); through *Chem. Abstr.*, **57**, 11154*d*(1962); Weisz, I., and Dudas, A., *Monatsh.*, **91**, 840(1960).

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Received July 21, 1967.
Accepted for publication September 14, 1967.

This investigation was supported in part by grant NB-3593 from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service, Bethesda, Md.

The authors gratefully acknowledge partial financial support from Schering and Ciba Laboratories and gifts of generous samples of (+)-chlorpheniramine supplied by Drs. H. Wolkoff, B. Jatul, and H. Leitzow, Schering Laboratories, of (+)-brompheniramine supplied by Dr. W. Schlesinger, White Laboratories, of racemic erythro-2-phenyl-2-(2′-piperidyl)acetamide supplied by Drs. L. Lachman and J. Cooper, Ciba Laboratories, and of (+)- and (−)-α-(3-[1,4-thiamorpholinyl])benzhydrol hydrochlorides made available by Dr. B. Belleau, University of Ottawa, and Dr. H. Dickison, Bristol Laboratories.

The authors thank Dr. P. Portoghese, University of Minnesota, for running the ORD curves. Satisfactory elemental analyses were obtained for all compounds for which melting points are reported.

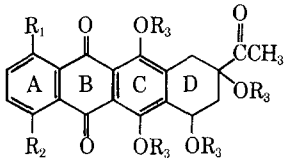
* To whom inquiries should be addressed.

Identity of Rubidomycin and Daunomycin

Sir:

Rubidomycin (1), an antibiotic isolated from the culture of *Streptomyces coeruleorubidus*, has been reported (2) to be chemically identical to daunomycin, an antibiotic isolated from the mycelium of *Streptomyces peucetius*. This identity was based on comparisons of ultraviolet and infrared data, chromatographic behavior, and certain color reactions. Similar biological activities have also been reported (3) for these two antibiotics. The structures of daunomycinone and daunosamine, the aglycone and the sugar moiety, respectively, of daunomycin, were elucidated by Arcamone and co-workers (4). In view of the current interest in rubidomycin and daunomycin as antitumor agents, it was deemed essential to place this identity on a more rigorous chemical basis.

Commercial samples[1] of the two antibiotics showed diffuse and indistinct X-ray diffraction patterns; the recrystallized[2] samples yielded dim, but definitely nonequivalent, patterns. The commercial samples showed similar, but definitely not identical, spectra (infrared, ultraviolet, and NMR), although samples which were exhaustively exchanged with D_2O yielded identical NMR spectra. By thin-layer chromatography, commercial daunomycin was shown to be homogeneous and commercial rubidomycin was shown to be heterogeneous, consisting of one major component (chromatographically identical with daunomycin) contaminated by a minor component possessing the same color and R_f value as the aglycone.[3] Because these antibiotics are hygroscopic and labile, they are difficult to characterize *per se;* therefore a common crystalline derivative for these samples was sought. Since the objective of this investigation was to show the identity of these two antibiotics, and since their elemental data indicated different amounts of water, it was necessary to purify these samples by a common procedure. To remove the aglycone from the rubidomycin sample and to start with materials of the same degree of hydration in the characterization studies, samples of both antibiotics were partitioned between chloroform and water. The aqueous solutions were lyophilized to yield solids that were chromatographically homogeneous. Benzoylation of these materials was conducted under identical conditions with identical stoichiometry. The use of excess benzoyl chloride in pyridine at room temperature for 20 hr. gave mixtures of benzoates consisting primarily of a pentabenzoate. This major component was isolated by preparative thin-layer chromatography and then was recrystallized from ethanol. The NMR of the crystalline solid showed that it was a pentabenzoate, and the infrared spectrum identified it as the tetra-*O*-mono-*N*-benzoate (I),[4] m.p. 236–238°. The equivalence of the two pentabenzoates from the two antibiotics was demonstrated by experimentally identical infrared, ultraviolet, NMR, chromatographic, melting point, and elemental data, as well as by X-ray diffraction patterns, which were identical in spacings and intensities.[5] The crude benzoylation products from the respective antibiotics showed very similar chromatographic patterns. When the major components were isolated from preparative plates, the mixture from rubidomycin yielded 58% pentabenzoate, 11% tetrabenzoates,[6] and 15% of an unknown benzoate(s), and the mixture from daunomycin yielded 56% pentabenzoate, 13% tetrabenzoates,[6] and 16% of the same unknown benzoate(s) isolated from the rubidomycin mixture. The essential identity of the benzoylation yield data and the identity of the pentabenzoates from the two antibiotics provide proof of the identity of these two materials.



I

R_1R_2 = H, OCH_3
R_3 = three of the R_3 are Bz and the fourth is daunosamine, *N,O*-dibenzoate

(1) Dubost, M., Ganter, P., Maral, R., Ninet, L., Pinnert, S., Preud'homme, J., and Werner, G.-H., *Compt. Rend.*, **257**, 1813(1963).
(2) Cassinelli, G., and Orezzi, P., *Giorn. Microbiol.*, **11**, 167(1963).
(3) Price, K. E., Buck, R. E., and Lein, J., in "Antimicrobial Agents and Chemotherapy—1964," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1965, p. 511.

[1] Samples of daunomycin, a product of Farmitalia, Milan, Italy, and rubidomycin, a product of Rhone-Poulenc Co., Vitry sur Seine, France, were generously furnished by Cancer Chemotherapy National Service Center.

[2] The authors thank Dr. W. Yanko, Monsanto Chemical Co., for the recrystallization procedure.

[3] Mild acid hydrolysis of the antibiotics afforded the same aglycone, daunomycinone, in good yields. The equivalence of these aglycones was established by X-ray diffraction patterns, which were identical in spacings and intensities.

[4] The locations of the methoxyl in ring A and the glycosidic linkage in the molecule have not been established. The latter problem is presently under investigation in this laboratory.

[5] The samples which yielded these X-ray patterns were recrystallized from a chloroform–ethanol mixture.

[6] More than one tetrabenzoate is theoretically possible. Chromatography of the isolated tetrabenzoate fraction showed one main component and 2 trace contaminants.

(4) Arcamone, F., Franceschi, G., Orezzi, P., Cassinelli, G., Barbieri, W., and Mondelli, R., *J. Am. Chem. Soc.*, **86**, 5334, 5335(1964).

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Received August 11, 1967.
Accepted for publication October 4, 1967.

This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., under contract PH-43-64-500.

Errata

In the article titled "New Compounds: Synthesis of Some Sulfazopyridines for Pharmacological Study" (1), the first author's name, page 1358, should read:

Hassan Y. Aboul-Enein

(1) Aboul-Enein, H. Y., Khalifa, M., and Abou-Zeid, Y. M., *J. Pharm. Sci.*, **56**, 1358(1967).

In the article titled "Irreversible Enzyme Inhibitors LXXXII. Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase VII. Derivatives of 2,4-Diaminopyrimidine I" (1), the following correction should be made:

In Table I on page 568 under "% Inactivation" the number opposite compound XII should read "0."

(1) Baker, B. R., Jackson, G. D. F., and Meyer, R. B., Jr., *J. Pharm. Sci.*, **56**, 566(1967).

In the Book Review titled "Nonionic Surfactants" (1), the following correction should be made on page 664:

Reviewed by James Swarbrick
School of Pharmacy
University of Connecticut
Storrs, Conn.

(1) Swarbrick, J., *J. Pharm. Sci.*, **56**, 663(1967).

In the article titled "Acetylation of Acetaminophen in Tablet Formulations Containing Aspirin" (1), the following correction should be made in Table II, page 1120:

Under "DAPAP, mg./Tab.," between 4.13 and 36, insert "DAPAP, mcg./Tab."

(1) Koshy, K. T., Troup, A. E., Duvall, R. N., Conwell, R. C., and Shankle, L. L., *J. Pharm. Sci.*, **56**, 1117(1967).

In the article titled "Diffusion of Drugs Across the Isolated Mesentery" (1), *References 8* and *11*, page 469, should read:

(8) Engle, D., and Kerekes, A., *Klin. Wochschr.*, **5**, 1709(1926); through *Chem. Abstr.*, **21**, 133(1927).
(11) Brodie, B. B., Burns, J. J., Mark, L. C., Lief, P. A., Bernstein, E., and Papper, E. M., *J. Pharmacol. Exptl. Therap.*, **109**, 26(1953).

(1) Shenouda, L. S., and Mattocks, A. M., *J. Pharm. Sci.*, **56**, 464(1967).

In the article titled "Radiopharmaceuticals" (1), the following corrections should be made:

1. On page 1, column 1, the first footnote paragraph should read:

Received from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Southern California, Los Angeles, CA 90007, and the *Radioisotope Research Laboratory, Veterans Administration Center and Department of Radiology, School of Medicine, University of California at Los Angeles, Los Angeles, CA 90024

2. On page 5, *Footnotes 1* and *2* should read:

[1] Marketed as Hippuran-131 by Abbott Laboratories, North Chicago, Ill., as Hipputope by E. R. Squibb & Sons, New York, N. Y., and as Hippuran I 131 by Nuclear Consultants, Chicago, Ill.
[2] Marketed as Ethiodol-131 by Abbott Laboratories, North Chicago, Ill.

3. On page 14, column 2, line 32, ^{99m}Te should read ^{99m}Tc.

(1) Wolf, W., and Tubis, M., *J. Pharm. Sci.*, **56**, 1(1967).

In the article titled "Pemoline and Magnesium Hydroxide *Versus* Pemoline: Enhancement of Learning and Memory of a Conditioned Avoidance Response in Rats" (1), the following corrections should be made:

1. On page 290, *Footnote 1* should read:

[1] Tradenamed as Cylert by Abbott Laboratories, North Chicago, Ill. Abbott-30400; an equimolar combination of 2-imino-5-phenyl-4-oxazolidinone (Abbott-13397) and magnesium hydroxide.

2. On page 291, *Footnote b* in Table II should read:

[b] Mean jump-out time significantly different from controls p 0.05 (10).

(1) Plotnikoff, N., and Meekma, P., Jr., *J. Pharm. Sci.*, **56**, 290(1967).

(4) Arcamone, F., Franceschi, G., Orezzi, P., Cassinelli, G., Barbieri, W., and Mondelli, R., *J. Am. Chem. Soc.*, **86**, 5334, 5335(1964).

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Accepted for publication October 4, 1967.

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In the article titled "Acetylation of Acetaminophen in Tablet Formulations Containing Aspirin" (1), the following correction should be made in Table II, page 1120:

Under "DAPAP, mg./Tab.," between 4.13 and 36, insert "DAPAP, mcg./Tab."

(1) Koshy, K. T., Troup, A. E., Duvall, R. N., Conwell, R. C., and Shankle, L. L., *J. Pharm. Sci.*, **56**, 1117(1967).

In the article titled "Diffusion of Drugs Across the Isolated Mesentery" (1), *References 8* and *11*, page 469, should read:

(8) Engle, D., and Kerekes, A., *Klin. Wochschr.*, **5**, 1709(1926); through *Chem. Abstr.*, **21**, 133(1927).
(11) Brodie, B. B., Burns, J. J., Mark, L. C., Lief, P. A., Bernstein, E., and Papper, E. M., *J. Pharmacol. Exptl. Therap.*, **109**, 26(1953).

(1) Shenouda, L. S., and Mattocks, A. M., *J. Pharm. Sci.*, **56**, 464(1967).

In the article titled "Radiopharmaceuticals" (1), the following corrections should be made:

1. On page 1, column 1, the first footnote paragraph should read:

Received from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Southern California, Los Angeles, CA 90007, and the *Radioisotope Research Laboratory, Veterans Administration Center and Department of Radiology, School of Medicine, University of California at Los Angeles, Los Angeles, CA 90024

2. On page 5, *Footnotes 1* and *2* should read:

[1] Marketed as Hippuran-131 by Abbott Laboratories, North Chicago, Ill., as Hipputope by E. R. Squibb & Sons, New York, N. Y., and as Hippuran I 131 by Nuclear Consultants, Chicago, Ill.
[2] Marketed as Ethiodol-131 by Abbott Laboratories, North Chicago, Ill.

3. On page 14, column 2, line 32, ^{99m}Te should read ^{99m}Tc.

(1) Wolf, W., and Tubis, M., *J. Pharm. Sci.*, **56**, 1(1967).

In the article titled "Pemoline and Magnesium Hydroxide *Versus* Pemoline: Enhancement of Learning and Memory of a Conditioned Avoidance Response in Rats" (1), the following corrections should be made:

1. On page 290, *Footnote 1* should read:

[1] Tradenamed as Cylert by Abbott Laboratories, North Chicago, Ill. Abbott-30400; an equimolar combination of 2-imino-5-phenyl-4-oxazolidinone (Abbott-13397) and magnesium hydroxide.

2. On page 291, *Footnote b* in Table II should read:

[b] Mean jump-out time significantly different from controls p 0.05 (10).

(1) Plotnikoff, N., and Meekma, P., Jr., *J. Pharm. Sci.*, **56**, 290(1967).